Cancer Immunology

A Translational Medicine Context

Nima Rezaei
Editor

Second Edition

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This book would not have been possible without the continuous encouragement by my parents and my wife, Maryam.

I wish to dedicate it to my daughters, Ariana and Arnika, with the hope that progress in diagnosis and treatment of these diseases may result in improved survival and quality of life for the next generations, and at the same time that international collaboration in research will happen without barriers.

Whatever I have learnt, comes from my mentors. This book is therefore dedicated also to all of them, but most importantly to the patients and their families whose continuous support has guided me during the years.

Preface

The rapid flow of studies in the field of cancer immunology during the last decade has increased our understanding of the interactions between the immune system and cancerous cells. In particular, it is now well-known that such interactions result in the induction of epigenetic changes in cancerous cells and the selection of less immunogenic clones as well as alterations in immune responses. Understanding the cross-talk between nascent transformed cells and cells of the immune system has led to the development of combinatorial immunotherapeutic strategies to combat cancer.

Cancer Immunology series, a three-volume book series, is intended as an up-to-date, clinically relevant review of cancer immunology and immunotherapy. The first edition of the book was published 4 years ago, which was very welcomed by the readers, made us to work on the second edition of the book in such a short period of time.

Volume I, *Cancer Immunology: A Translational Medicine Context,* is focused on the immunopathology of cancers. Volume II, *Cancer Immunology: Bench to Bedside Immunotherapy of Cancers,* is a translation text explaining novel approaches in the immunotherapy of cancers; and finally, Volume III, *Cancer Immunology: Cancer Immunotherapy for Organ-Specific Tumors,* thoroughly addresses the immunopathology and immunotherapy of organ-specific cancers.

In Volume I, interactions between cancerous cells and various components of the innate and adaptive immune system are fully described. Notably, the principal focus is very much on clinical aspects, the aim being to educate clinicians on the clinical implications of the most recent findings and novel developments in the field. To meet this purpose, this volume was extended from 26 chapters in the first edition to 33 chapters in the second edition. After

an overview on cancer immunology in Chap. 1, the role of innate immunity in cancers is explained in Chaps. 2 and 3, followed by the adaptive immunity, including B-cells, T-cells, and T regulatory and Th17 cells in Chaps. 4–8. NK cells, plasmacytoid dendritic cells, CD95/CD95L signaling pathway, and MHC class I molecules are separately described in Chaps. 9–12, respectively. Cytokines and chemokine receptors are explained in Chaps. 13 and 14, respectively. Chapter 15 focuses on inflammasome in cancer. Cancer immunoediting is a subject that is explained in Chap. 16. Meanwhile, Chaps. 17 and 18 explain apoptosis and autophagy in cancers. Subsequently, Chap. 19 presents the prognostic value of innate and adaptive immunity in cancers. Immunogenetics and epigenetics are explicated in Chaps. 20–22. In addition, immunosenescence (Chap. 23), nutrition (Chap. 24), immunodeficiencies (Chap. 25), and allergies (Chap. 26) are individually described in the following chapters. Chapter 27 enlightens systems biology in cancer immunology, while immunological diagnostic tests, including immunohistochemistry, fluorescent in situ hybridization, molecular and functional imaging as well as imaging with radiolabeled monoclonal antibodies are mentioned in Chaps. 28–32. Finally, by allocating the final chapter to flow cytometry in cancer immunotherapy, Volume I comes to its end.

The *Cancer Immunology* series is the result of valuable contribution of more than 300 scientists from more than 100 well-known universities/institutes worldwide. I would like to hereby acknowledge the expertise of all contributors, for generously devoting their time and considerable effort in preparing their respective chapters. I would also like to express my gratitude to the Springer Nature publication for providing me the opportunity to publish the book.

Finally, I hope that this translational book will be comprehensible, cogent, and of special value for researchers and clinicians who wish to extend their knowledge on cancer immunology.

Tehran, Iran Nima Rezaei

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I would like to express my gratitude to the editorial assistants of this book, Dr. Farnaz Delavari and Dr. Mahsa Keshavarz-Fathi. With no doubt, the book would not have been completed without their contribution.

Contents

Abbreviations

γδ T-cells	Gamma delta T-cells
ACT	Adoptive cell transfer
AICL	Activation-induced C-type lectin
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APCs	Antigen presenting cells
BAT3	HLA-B-associated transcript 3
BCL-6	Transcription factor B-cell lymphoma 6
CAR	Chimeric antigen receptor
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CXCR5	CXC chemokine receptor 5
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HA	Hemagglutinins
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HIV	Human immunodeficiency virus
HMGB1	High-mobility group box 1
HN	Hemagglutinin neuraminidases
HVEM	Herpesvirus entry mediator
ICOS	Inducible co-stimulator
IFN I	Type I interferon
IFN-γ	Interferon-gamma
LIGHT	Homologous to lymphotoxin exhibits inducible expression and competes with HSV glycoprotein D for binding to herpesvirus entry mediator, a receptor expressed on T lymphocytes
MM	Multiple myeloma
NKp44L	NKp44 ligand
NKT	Natural killer T
NLR	Nod-like receptors
*n*Tregs	Natural Tregs
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PCNA	Proliferating cell nuclear antigen

PfEMP1	Plasmodium falciparum erythrocyte membrane protein-1
RCC	Renal cell carcinoma
Tfh	Follicular helper T
Th	T-helper
TILs	Tumor-infiltrating lymphocytes
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
TNM	Tumor, node, and metastasis
Tr1 cells	IL-10-producing type 1 Tregs
Treg	Regulatory T-cells

1 Introduction on Cancer Immunology and Immunotherapy

Nima Rezaei, Seyed Hossein Aalaei-Andabili, Neda Amini, Farnaz Delavari, Mahsa Keshavarz-Fathi, and Howard L. Kaufman

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1.1 Introduction

Cancer is a life-threatening disease, which can involve all human organs and tissues. It is the second leading cause of death and is responsible for 25% of all deaths in the USA. It is estimated that around 1.7 million of new cases of cancer of any site will be diagnosed in 2018 in the USA, and an estimated 609,640 people will die of this disease [1]. The major cancers in adults include lung, breast, prostate, and colorectal cancer. In addition, 4613 adolescents and young adults aged 15–19 years old were diagnosed with invasive cancers. Among all invasive cancers, lymphoma was the most common cancer (20%), followed by invasive skin cancer (15%), male genital system cancer (11%), and endocrine system cancer (11%) [2]. The overall incidence of all type of cancers has been falling on average 1.1% each year over the last 10 years. In addition, death related to cancer has been decreased on average 1.5% each year over 2006–2015 [3].

Many cancer predisposing factors have been recognized; it has been found that cancer incidence is significantly associated with age from 10 to 60 years. Additionally, male gender is at higher risk of developing cancer compared to females [2]. Race is another important factor for cancer development; before 40 years of age, non-Hispanic whites and, after 40 years of age, African-Americans/blacks have the highest incidence [4]. Other risk factors include life style choices, such as tobacco use, obesity, and lack of exercise, and environmental factors, such as exposure to excessive sun, radiation during childhood, human papilloma virus (HPV), human immunodeficiency virus, and Epstein–Barr virus (EBV) infection [4].

Cancer can be a life-threatening health problem, especially when the tumor has metastasized to other organs. Its estimated number of deaths was 163.5 per 100,000 men and women per year based on 2011–2015 database of the USA. Lung and bronchus, colorectal, pancreatic, and breast cancers are responsible for approximately 50% of cancer-related deaths. Fortunately, the overall cancer-related mortality has been decreasing in recent years. Between 2011 and 2015, the death rate decreased on an average of 1.8% per year for men and 1.4% for women. Liver and intrahepatic bile duct cancer showed the greatest increase in mortality among both men and women [3].

Cancer survival significantly impacts patients' quality of life. Five-year mortality rates depend on several factors; survival is worse among males over 30 years of age, and the survival gets worse for patients over 45 years in both males and females. Non-Hispanic whites have the best survival rate and African-Americans have the worst survival with survival differences as great as 20% at 5 years after cancer diagnosis [5]. Furthermore, the type of cancer is another risk factor for patient survival. Total mortality rates vary from 6% in thyroid cancer to 97% in pancreatic cancer [6].

1.2 Cancer Immunity

Cancer immunology has been studied for a long time; however, the molecular and cellular basis of tumor immunity is not completely understood. Advances in understanding the basis of immunosurveillance and progress in the treatment of infectious disease have had a major impact on the development of tumor immunotherapy. The modern era of tumor immunology began in the 1950s when the role of T-cell responses in tissue allograft rejection was initially identified. Since then, it has been confirmed that tumors occur in association with impaired function of T-cells, indicating the importance of the immune system in the development and progression of cancer [7]. The identification of tumor-associated antigens, knowledge of effector T-cell responses, and the role of regulatory and suppressor T-cell populations are now shaping the use of the immune system to treat cancer.

In addition to an improved understanding of the immune system, significant advances in understanding the molecular basis of neoplasia have occurred. Precise control of cellular activity and metabolism is crucial for proper physiologic function. Notably, cell division is an important process that requires precise regulation. The main difference between tumor cells and normal cells is lack of growth control during the cell division process. This uncontrolled cell division can originate from various factors, such as chemical agents, viral infections, and mutations, that lead to escape of cells from the checkpoints which properly control cell division. According to the type of tumor and proliferation rate, cancers can be benign or malignant [8]. It has been found that some tumors are caused by oncogenic viruses that induce malignant transformation. These oncogenic viruses can be both RNA and DNA viruses. Also, viral infection may lead to leukopenia and immunodeficiency, increasing the risk of malignancy. Therefore, prophylactic immunization against oncogenic viruses (such as EBV, HPV, and HBV) might be a logical strategy for prevention of malignancy [9]. Indeed, a vaccine against the human papilloma virus has shown significant impact on preventing cervical intraepithelial neoplasia and may prevent development of cervical carcinoma.

1.3 Cancer and Immune System Impairment

It has been reported that impaired immune response can induce tumor growth and prevent effective antitumor suppression, possibly through a process of "sneaking through" which allows improved growth of small tumors rather than large tumors [10]. Tumors may also produce immunosuppressive factors, such as interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and alpha-fetoprotein, which suppress innate immune responses against cancer. This has led to investigations using neutralizing antibodies against these immunosuppressive factors [7]. In contrast, tumor-specific cytotoxic T lymphocytes (CTLs) can be genetically altered to become resistant to the TGF-β inhibitory effect by transgene expression of a mutant-dominant-negative TGF-β type II receptor (DNR). In addition, specific T-cells genetically manipulated to produce IL-12 can overcome the inhibitory effects of IL-10. On the other hand, tumors may express FasL and stimulate apoptosis of tumor-infiltrating effector T-cells. Small interfering RNA (siRNA) can be used to knock down the Fas receptor in tumor-specific CTL, leading to a significant decrease in their susceptibility to Fas-/FasL-mediated apoptosis [11].

The interaction between the immune system and established cancers is complex, because in addition to increasing carcinogenesis by various carcinogens among compromised subjects, cancer cells themselves can lead to severe immunosuppression. It has been reported that patients involved with primary immunodeficiency syndromes have higher risk of cancer development. In a report by Kersey et al., subjects that had an inherited abnormal lymphoid system were susceptible to malignant transformation and impairment of tumor immunosurveillance [12]. In addition, tumors produce soluble factors which downregulate the interleukin-2 receptor-α (IL-2Rα), leading to suppression of T-cell function. Furthermore, established tumors may result in severe protein expenditures in hosts, contributing to impairment of immune system function [13].

1.4 Immune System Reaction to Cancer

A critical question is whether cancer cells are sufficiently different from their normal cellular counterparts and can thus be recognized by the immune system. The immune system also produces a group of complementary markers with protective effects against cancer and other immunologic or inflammatory stresses. These markers include proteins released by T-cells and are generally classified as "cytokines." Cytokines include interleukins, interferons, tumor-necrosis factors (TNF), and lymphocyte-derived growth factors. The production of tumor-specific antibodies and/or activation of tumor antigen-specific T-cells target tumor-associated antigens typically found on the cell

membrane. Studies have suggested that vaccination in the presence of complements can lead to tumor lysis. While incompletely defined, several soluble and cellular mediators of tumor rejection have been described, including complement factors, active macrophages, T-cells, and NK cells. While T-cells require antigen specificity, the soluble and cellular mechanisms of the innate immune response can recognize the malignant phenotype in the absence of antigen specificity [14].

Since most tumor-associated antigens are self-proteins, the immune response is largely weak and patients may develop immune tolerance to tumor-associated antigens. Furthermore, the cells of the immune system may not adequately penetrate to the internal tumor microenvironment, resulting in slower immune-mediated tumor elimination. However, it is possible that the immune system may be more effective in controlling tumor growth rate rather than tumor regression [10]. Recently, it has been found that nutrition also plays a crucial role in protection against human cancer, and normal levels of zinc are required for protection against the detrimental effects of various immunosuppressive cytokines [15].

1.5 Genetic and Environmental Carcinogenesis

It has been found that genetic factors are as important as environmental carcinogens. Trials have tested carcinogenesis of retrovirus infection between different breeds of animals. A unique carcinogen resulted in disparate outcomes among different breeds, indicating the importance of genetic background in the progression of cancer. Environmental factors may also suppress immune responses and dysregulate immunosurveillance mechanisms [16].

1.5.1 Cancer Cells Escape from Host Immunosurveillance

Antigens that distinguish tumor cells from normal cells depend on the histologic origin of the tumor. Tumor-associated antigens may be viral in origin, represent mutated self-antigens, be cancer-testis antigens which are expressed only by tumor cells and normal testes, or be normal differentiation antigens. Thus, tumor cells may express similar antigens to normal cells, allowing tumor cells to escape immune system attack through induction of innate and/or peripheral tolerance. A corollary to this is that immunotherapy or stimulation of immune responses to some tumor-associated antigens may lead to damage of normal tissues and organs, as exemplified by the development of autoimmunity induced by anti-CTLA-4 or anti-PD-1 monoclonal antibody (mAb) treatment [17].

A number of complex mechanisms have been suggested for the escape of cancer cells from host immunosurveillance. Tumors alter their characteristics by decreased expression of immunogenic tumor-associated antigens, MHC class I molecules, beta2-microglobulin, and costimulatory molecules, which mediate the activation of T-cells. Another strategy resulting in failure of tumor immunosurveillance could be the expression of very low levels of antigens, unable to stimulate an immune response. Under some circumstances, such as failure of the immune response to induce a rapid response, cancer cells may proliferate rapidly. Further strategies for escape of tumor cells from immunosurveillance are based on inhibitory tumor-mediated signaling by CTLs, as occurs through changes in cell death receptor signaling. Other strategies which allow tumor cells to evade the immune system are the secretion immunosuppressive molecules dampening tumor-reactive effector T-cells and the induction of regulatory and/or suppressor cells [18].

To date, most direct evidence on tumor immunosurveillance originates from experimental studies in animal models. These models have supported the potential for antitumor immunity via vaccination, as, for example, by administration of inactivated cancer cells or through removal of a primary tumor. In addition, antitumor immunity can be adoptively transferred through administration of tumor-reactive T lymphocytes. The complexities of immunotherapy are evident as nearly all immune system components can influence tumor growth and progression. Although there is evidence for antitumor immunity in humans, and

several new agents have gained regulatory approval for cancer therapy, further investigation is warranted to increase the impact of tumor immunotherapy for more cancer patients [19].

1.5.2 Cancer Immunodiagnosis

Nowadays, new immunomolecular diagnostic approaches have been suggested for tumor detection. Monoclonal antibodies marked with radioisotopes have been used for in vivo diagnosis of small tumor foci. In addition, monoclonal antibodies have been used for in vitro recognition of the cell of origin for tumors with poor differentiation. Immunodiagnostics have also been used to determine the extent of metastatic disease, especially metastasis to the bone marrow [20].

1.6 Cancer Treatment

Systemic cancer treatment is based on four general therapeutic approaches: (1) chemotherapy, which contains a wide group of cytotoxic drugs that interfere with cell division and DNA synthesis; (2) hormonal therapy, which contains drugs that interfere with growth signaling via tumor cell hormone receptors; (3) targeted therapy, which involves a novel group of antibodies and small-molecule kinase suppressors that principally target proteins crucial in cancer cell growth signaling pathways; and (4) immunotherapy, which targets the induction or expansion of antitumor immune responses [21].

1.6.1 Cancer Immunotherapy

Tumor immunotherapy is a novel therapeutic approach for cancer treatment, with increasing clinical benefits. Tumor immunotherapy is based on strategies which improve the cancer-related immune response through either promoting components of the immune system that mediate an effective immune response or via suppressing components that inhibit the immune response. Two current approaches commonly used for immunotherapy are allogeneic bone marrow transplantation and mAbs targeting cancer cells or T-cell checkpoints [22]. Recently, various other approaches have been tested such as injection of cytokines. FDA recently approved injection of PEG-IFN-a2b in high-risk melanoma [23].

Initially, anticancer vaccines were considered for prevention and treatment of various tumors [22]. It is estimated that more than 15% of human cancers are caused by viral infection [24]. Vaccine-based immunotherapy may, thus, be most useful for virus-induced cancers. Consistent with this hypothesis, a 50% complete remission (CR) of HPV-associated vulvar intraepithelial neoplasia grade III (VINIII) has been reported [25]. An attenuated, oncolytic herpes simplex type 1, which is genetically engineered to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF), has been developed for cancer therapy. This oncolytic immunotherapeutic agent has been injected to the tumor mass and has had beneficial effects in the treatment of melanoma and head and neck squamous cell carcinoma [26]. Although vaccine-based therapy has not been effective in some types of cancer, there are studies that have shown an overall survival benefit compared to placebo therapy [27]. FDA recently approved a vaccination therapy using dendritic cells for prostate cancer [28].

Another immune-targeted approach is mAbs which blocks T-cell checkpoints functioning to suppress T-cell responses. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is a member of a large family of molecules regulating T-cell immune responses. CTLA-4 is expressed on CD4+ and CD8+ T-cells, as well as on FOXP3+ regulatory T-cells [29]. Administration of mAbs targeting human CTLA-4 leads to the rejection of established tumors in a small cohort of patients with metastatic melanoma and demonstrated improved overall survival in patients with metastatic melanoma, resulting in US FDA approval for the treatment of metastatic melanoma [30]. Recent trial showed survival benefit of ipilimumab, a CTLA-4 inhibitor, in setting of metastatic melanoma and also after resection of stage III melanoma [31, 32].

Monoclonal antibodies which block other T-cell checkpoints, such as the programmed cell death protein 1 (PDCD1/PD-1), programmed cell

death ligand 1 (PD-L1/CD274), CD276 (B7H3) antigen, V-set domain-containing T-cell function inhibitor 1 (B7x), and B and T lymphocyte attenuator, have also entered clinical trials. In addition, recent trials have demonstrated significant therapeutic activity in several types of cancer, including melanoma, metastatic urothelial carcinoma, gastric cancer, hepatocellular cancer, colorectal cancer, renal cell carcinoma, non-small cell lung carcinoma, and ovarian cancer [33–38]. It has been reported that PD-L1 expression by tumor cells is associated with poor clinical outcome and may be associated with clinical response to anti-PD-1 and anti-PD-L1 therapy. Also, ligation of PD-L1 leads to inactivation of tumor-infiltrating cells [39]. On the other hand, regulatory T-cells have an immunosuppressive role in the tumor microenvironment. Studies of anti-PD-1 and anti-PD-L1 are in progress. Moreover, the combination of these agents with anti-CTLA-4 and other immunotherapy strategies has yielded promising results.

The combination of antitumor vaccines with agents targeting the IL-12 receptor resulted in conflicting results. This may be due to the upregulation of IL-12 receptor by both activated T effector cells and regulatory T-cells [40]. Thus, new approaches focused on more specific targeting of regulatory T-cells which reduce their suppressive effects on the immune system are necessary. Adoptive T-cell therapy (ACT) has been described as an effective therapeutic approach for cancer immunotherapy in early phase clinical trials. In this method, a large number of tumor-specific T-cells derived from peripheral blood, or preferably from the tumor microenvironment (with or without genetic manipulation to express a high-affinity antigen-specific T-cell receptor, or TCR), are adoptively transferred to patients with established tumors [41]. ACT mostly relies on endogenous T-cell repertoire; recent advancements allow induction chimeric antigen receptors (CARs). In CAR T-cell (CAR-T) therapy, T-cells of patients with B-cell tumors are transfected with anti-CD19 and in result, T-cells will gain the capacity to recognize B-cells in all stages of development. The first CAR-T was recently approved by FDA based on phase 2 trial which showed a dramatic complete response in 83% of patients within 3 months of infusion [42, 43]. Chemotherapy-mediated cell death leads to immune responses in a drug-induced biochemical cell death cascade-dependent manner, suggesting beneficial effects of chemotherapy and immunotherapy, in combination [44]. It seems that future goals of tumor immunotherapy are headed towards chemoimmunotherapy. Potential candidates for this combination approach include antitumor vaccines, Toll-like receptor (TLR) signaling pathway agonists/antagonists, cytokines, and mAbs targeting T-cell checkpoints, such as CTLA-4, PD-1, or PD-L1/2 [45]. Also, it seems that radiation and radiofrequency ablation are future candidates for combination therapy with immunotherapy [46]. Although immunotherapy and its combination with other therapeutic approaches such as radioimmunotherapy may be beneficial for tumor treatment, there are several limitations that need to be addressed; defining the optimal target patient, optimal biological dose, and schedule, the need for better trial designs incorporating appropriate clinical endpoints, and the identification and validation of predictive biomarkers are just a few points to note [22].

1.6.2 Cancer Cell "Switch"

Cancer cells can switch on genes mostly related to the earlier embryonic stages of development. During rapid proliferation of cancer cells, precise orchestrated enzyme formation needed for suitable metabolism of its different components might get unbalanced, and products which are not observed in normal dividing cells are produced [47]. Recently, it has been reported that these biochemical "switches" lead to uncontrolled multiplication of cancer cells. One switch has been found for a type of leukemia. It has been suggested that targeting tumor switches can make treatment of cancers very simple [19]. Nonetheless, it is unclear how this may be used to optimize tumor immunotherapy.

Since cancer immunology is a highly complex process, further research is needed to more completely understand how the immune system recognizes and eradicates cancer. In this book, we will describe a variety of novel mechanisms cur-

rently under investigation for mediating aspects of tumor immunology with a particular focus on promising therapeutic approaches, producing a complete comprehensive up-to-date textbook.

1.7 Concluding Remarks

Cancer is a life-threatening health problem which is related to several genetic and environmental risk factors that manipulate immune system function. Cancers themselves produce immunosuppressor factors to impair cells division check points, leading to uncontrolled proliferation of cancer cells. Importantly, tumor cells have learned how to escape from immune system attack via presenting of similar antigens to normal cells and expression of very low levels of antigens. Therefore, diagnosis of tumors and their progression is not easy. Recently, immunodiagnostic methods are shown to be helpful in the diagnosis of cancers and determining the extent of metastasis. On the other hand, classic treatment of cancers led to unsatisfactory results, and intelligent immunological approaches, such as regulatory T-cell targeting, adoptive T-cell administration, and combination of immunotherapy and chemotherapy are addressed. Results of antitumor vaccines, Toll-like receptor (TLR) signaling pathway agonists/antagonists, cytokines, and mAbs targeting T-cell checkpoints, such as CTLA-4, PD-1, or PDL-1/2 are promising. However, due to the high complexity of the cancer immunology, still a lot of gaps exist in this field that indicate the necessity of further research for complete understanding of cancers' immunological behaviors and emerging of more novel immunotherapeutic strategies.

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Role of Innate Immunity in Cancers and Antitumor Response

2

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2.1 Introduction

Cellular components of the innate immune system serve as a "first line of defense" against tumorigenic cells. Recognition of transformed cells by pattern-recognition receptor (PRRs) on the innate immune cells activates specialized

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inflammatory signaling cascades, including transcription factor nuclear factor-kappa B (NF-κB) and interferon regulatory transcription factor (IRF), which lead to the release of various cytokines and chemokines attracting and activating effector lymphocytes at the tumor site. In addition, effector cells kill transformed cells through the activation of perforin or death receptor-mediated pathways, as well as secretion of cytokines necessary for the initiation of immune responses against transformed cells [1, 2]. However, some tumor cells escape from the innate immune machinery, which leads to the dysfunction of innate immune compartment, signaling pathways, and effector functions. This manipulation of innate immune systems by tumor microenvironments includes impairment of antigen processing and presentation by antigen-presenting cells (APCs) [3], inhibition of innate immune signaling pathways [4, 5], and anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) [6, 7]. Moreover, tumors manipulate innate immune systems to create protumorigenic environments, which lead to further tumor progression and metastasis. Therefore, it is critical to clarify the molecular mechanisms through which the interaction between tumors and innate immune systems is modified during different phases of tumorigenesis.

In this chapter, we describe the general functions of innate immunity in cancer and antitumor host response. In addition, an overview is provided on the mechanism through which coordinated actions of innate immune signals and their downstream effectors have an impact on the immunosurveillance and immune subversion within the tumor microenvironment.

2.2 Role of Innate Immune Cells in Cancer and Antitumor Immunity

2.2.1 Natural Killer (NK) Cells

NK cells are important effector cells for protection against viruses and some tumors, since NK-cell-depleted mice were more susceptible to 3-methylcholanthrene (MCA)-induced tumors [8]. Chemokines, such as CXCL12 and CXCL3L1, are key factors for NK migration to tumor sites [9], where they play an important role in the tumor immunosurveillance [10]. NK cells recognize and eliminate transformed cells by releasing perforin or death signal-associated receptors such as FAS and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) [11–13]. NK cells secrete interferon gamma (IFN-γ) which helps to activate T-cell-mediated immunity and suppress tumor angiogenesis [14, 15]. Moreover, various innate immune networks such as cytokines and PRR recognition systems play an important role in stimulating effector functions of NK cells as discussed later.

NK cells have the ability to distinguish transformed cells from normal cells by recognizing a variety of cell surface receptors, including killer activation receptors (KARs), killer inhibitory receptors (KIRs), natural killer group two member D (NKG2D), DNAX-accessory molecule (DNAM), etc., which will be discussed later in this chapter. For example, KIRs on NK cells have a high affinity to the specific alleles in HLA class I molecules, transducing an inhibitory signal to the NK cells and preventing it from eliminating nontransformed cells. However, deletion of a single allele in HLA class I and/or induction of activating receptors, such as NKG2D ligands, which frequently occurs on transformed cells, triggers effector functions of NK cells against tumor cells [10, 16]. Recent studies have focused on "licensing" NK cells to become functionally competent through the interaction with self-MHC molecules. Ly49C is an inhibitory receptor expressed on a subset of NK cells, which interact with self-MHC molecules on target cells, and plays an unexpected role in enabling immature NK cells to develop into functioning, mature cells. On the other hand, Ly49C-negative NK cells are considered as "non-licensed" and remain at an immature stage [17]. These evolutionary processes of NK cell development and activation may help explain why donor NK cells administrated to leukemia patients during bone marrow transplantation do not always show antitumor

effects [18]. The NK cell-mediated cytotoxic activities mediate the release of granule contents (perforin and granzyme) onto the surface of the tumor cell [19].

The interaction between NK cells and dendritic cells (DCs) is crucial for the amplification of innate responses and the induction of potent adaptive immunity. Immature DCs are susceptible to NK-cell-mediated cytolysis [20], while mature DCs are activated by NK cells through cytokines (TNF-α and IFN-γ) and receptor (NKp30 and NKG2D)-mediated mechanisms [21, 22]. On the other hand, activated DCs trigger effector activities of NK cells, such as IFN-γ production, proliferation, and cytotoxic activities [23]. In addition, treatment with TLR3 agonist polyinosinic-polycytidylic acid (Poly (I: C)) triggers DCs to activate antitumor activities of NK cells [24, 25]. Thus, the reciprocal interaction between NK and DC regulates the direction and quality of antitumor immunity, which is important for the development of effective cancer immunotherapy.

2.2.2 Natural Killer T (NKT) Cells

NKT cells are innate lymphocytes which share features of both NK cells and T-cells. NKT cells express particular NK cell markers such as CD161 or NKR-P1, in addition to an invariant T-cell receptor alpha chain (Vα14-Jα18 in mice and Vα24-Jα18 in humans) [26]. The invariant T-cell receptor alpha chain is specific for glycolipid antigens presented by CD1d, which is an MHC class I-related molecule expressed on antigen-presenting cells and also found in some tumor cells. NKT cells were shown to play a role in the tumor immunosurveillance, since *Jα18*$^{-/-}$ mice showed increased susceptibility to chemically induced tumors and experimentally induced metastases [27]. Moreover, the administration of α-galactosylceramide, a natural lipid isolated from marine sponges which efficiently binds to CD1d and thus activates NKT cells, induces antitumor immune responses against established murine tumors [28]. The antitumor activities of NKT cells are mediated by IFN-γ production, which also activates NK and CD8^{+} T-cells. NKT cell activities are also important for granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-12-based cytokine strategies [29, 30]. Recent reports have identified subpopulations of NKT cells which secrete TH1 or TH2 cytokines and thus play different roles in the pathogenesis of many diseases. For example, CD4^{-} NKT cells serve as potent effectors for triggering tumor rejection in various murine tumor models, while CD4^{+} NKT cells contribute to the pathogenesis of allergic diseases and tumors by promoting the release of IL-4, IL-5, and IL-13 [31, 32]. Indeed, IL-13 released from NKT cells antagonizes tumor immunosurveillance by promoting TGF-β secretion from Gr-1^{+} myeloid suppressor cells [33, 34]. Thus, the identification of factors influencing the differentiation of specific NKT cell subsets during tumor development is important in order to optimize the therapeutic interventions which utilize NKT cell functions against tumors.

2.2.3 γδ-T Cells

Although γδ-T cells represent a small population among T lymphocytes, they share several features with innate immune cells. γδ-T cells show high frequencies in intraepithelial lymphocytes (IELs) in the skin and gut mucosa and possess a distinct T-cell receptor on their surface with limited diversity, which may serve as a pattern-recognition receptor [35]. Moreover, γδ-T cells lack CD4 and CD8 expressed by αβ-T cells and express a number of molecules shared with NK cells or APCs, such as Fc gamma RIII/CD16 and PRRs. γδ-T cells also recognize lipid-derived antigens and function as professional phagocytes which recognize and ingest apoptotic tumor cells and may influence antitumor immune responses [36, 37].

Mice lacking γδ-T cells showed increased incidence of chemically induced sarcoma and spindle cell carcinoma, indicating the importance of these cells in tumor immunosurveillance [38]. In addition, γδ-T cells express NKG2D receptors and interact with their ligands on transformed cells, leading to enhanced cytotoxic activities and

effector cytokine production [39, 40]. The activated γδ-T cells then serve as the major early source of IFN-γ, which contribute to maturation of APCs and prime αβ-T cells, and mediate cytotoxicity against tumor cells [40, 41].

2.2.4 Macrophages

Macrophages serve as a first line of defense against tumorigenesis by directly killing tumor cells and producing various antitumor mediators [42]. On the other hand, macrophages render tumor cells with the ability to acquire invasive and metastatic activities [43]. Macrophages are differentiated from immature myeloid precursors or circulating monocytes released from the bone marrow [44]. In particular, the inflammatory monocytes expressing *Ly6C* are preferentially attracted from the circulation into the tumor site by tumor-derived chemokines, such as CCL2 (MCP1-1) and CCL5 (RANTES) and CXCL12 (SDF1) [45–47]. Immature monocytes are then differentiated into either M1 or M2 macrophages by distinct sets of cytokines when entered into distinct tumor microenvironments [48]. M1 macrophages may induce antitumor response by producing IFN-γ and IL-12 and triggering cytotoxic activities [49, 50]. In contrast, tumor microenvironments adopt multiple strategies to tip a balance in the favor of differentiating M2-type macrophages through complex network of cytokines, chemokines, and growth factors [43, 51].

Taken together, macrophages have a dual role in modulating tumorigenesis and antitumor host responses. Thus, detailed characterization of molecular machineries which govern macrophage polarization in tumors seems necessary for a thorough understanding of pharmacological targeting of macrophages and their derivatives.

2.2.5 Dendritic Cells

DCs are professional APCs contributing to the induction of both innate and adaptive immune responses against pathogens as well as tumors. DCs express Toll-like receptors (TLRs) and costimulatory molecules necessary for the activation of various effectors [52]. Due to the potent immunogenicity of DC, tumor microenvironments adopt multiple tactics to subvert DC functions. In addition, tumor-infiltrating DCs can both induce tumor growth and metastasis by regulating angiogenesis, host immunity, and tumor metastasis [53–56]. Moreover, indoleamine 2, 3-dioxygenase (IDO)-producing DCs cause poor tumor immunogenicity via generating Foxp3-positive regulatory T-cells [57] and interacting with other innate lymphocytes such as γδ-T cells [58] and NKT cells [59].

In summary, tumor-infiltrating DCs represent a double-edged sword which can induce an immune response against tumors or tolerize the immune system against tumors and contribute to tumor growth and metastasis. Thus, a deep understanding about DC biology at tumor microenvironment is critical to optimize anticancer therapies and improve the clinical output of DC vaccines.

2.2.6 Granulocytes

Granulocytes, the key mediators of inflammation, have a potential role in the initiation of immune response cascades against tumors [60]. Granulocytes induce tumor destruction through the release of cathepsin G, azurocidin, reactive oxygen species, and inflammatory cytokines. Moreover, granulocytes, along with macrophages and T-cells, are main effectors that elicit antitumor responses by DNA vaccines in murine tumor models [61]. In addition, dense infiltration of granulocytes in tumor tissues is associated with clinical responses of GM-CSF-secreting cancer cells and Bacillus Calmette-Guérin (BCG) in patients with advanced melanoma and bladder carcinoma, respectively [62, 63]. On the other hand, granulocytes contribute to tumor angiogenesis and metastasis by promoting secretion of proteinases, ROS, and cytokines that may acts as antitumor effectors in different conditions [64]. Therefore, granulocytes have both pro- and antitumor activities depending on distinct environments.

2.3 The Role of Innate Immune Receptors on Innate Immune Cells in Cancer and Antitumor Immunity

2.3.1 Toll-like Receptors (TLRs)

Toll-like receptors (TLRs) are innate immune receptors mainly expressed on APCs, such as macrophages and dendritic cells. They play an important role in host defense against pathogens by recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern molecules (DAMPs). The recognition of PAMPs and DAMPs by PRRs activates inflammatory pathways, such as NF-κB and IRF-mediated signals, leading to antitumor mediators like type I interferons, as well as cell survival and proliferation [65].

Various sets of TLR ligands induce the upregulation of co-stimulatory molecules and proinflammatory cytokine production by APCs, thus breaking the tolerogenic status to various tumor antigens and inducing antigen-specific antitumor immune responses [66–68]. In addition, TLR4 on DCs could interact with high mobility group box 1 (HMGB1) and facilitate antigen cross-presentation to antitumor T lymphocytes [69]. Thus, TLRs agonists serve as effective adjuvants in harnessing potent antitumor immune response and clinical responses.

In contrast, tumor cells license TLRs on myeloid cells to acquire invasive and metastatic activities by promoting the secretion of various protumorigenic mediators, such as TNF-α and S100A8 [70, 71]. Thus, the careful optimization of suitable TLRs ligands for cancer immunotherapy is critical in order to avoid protumorigenic inflammation caused by the TLRs expressed on innate immune cells in tumor microenvironments.

2.3.2 RIG-I-Like Helicases (RLHs)

RIG-I-like helicases (RLHs) are specific families of pattern-recognition receptors bearing caspase-recruitment domain (CARD) at N-terminus and helicase domains, which are responsible for detecting intracellular double-strand RNA and inducing innate immune responses. RLHs include retinoic acid-inducible gene-I (RIG-I), myeloid differentiation antigen-5 (MDA5), and laboratory of genetics and physiology-2 (LGP2 or DHX58), which are expressed constitutively in both immune and nonimmune cells. RLHs recruit specific intracellular adaptors to initiate NF-κB- and IRF-mediated inflammatory signaling pathways that lead to the synthesis of type I interferons (IFNs) and other proinflammatory cytokines [72, 73]. The utilization of RLHs ligands as adjuvants to trigger antitumor immune responses has been validated by several studies. Its administration with retinoic acid-inducible gene-I (RIG-I) ligand triphosphate RNA triggers antitumor immune response by inducing the production of IFN-α/IFN-β and various immunogenic cytokines, as well as activating antitumor immune response cells [74, 75].

Taken together, RLHs ligands may be utilized as adjuvants with other immunotherapies in order to overcome immunosuppressive tumor microenvironments.

2.3.3 NOD-like Receptors (NLRs)

NOD-like receptors (NLRs) are especially important for the recognition of sterile inflammation such as uric acids and silica [76, 77]. NLR-mediated innate immune systems play an important role in both antitumor immunity and tumorigenicity. For example, nucleotide-binding oligomerization domain-containing protein 1 (NOD1) has a protective role against tumors, and the knockdown of NOD1 promotes tumor growth in breast cancer model in vivo [78, 79]. NOD-like receptor family pyrin domain containing 3 (NLRP3) serves as a sensor for activating the inflammasome pathway which regulates procaspase-1 cleavage and subsequent IL-1β activation [80]. NLRP3 is a negative regulator of chemical colon carcinogenesis. In a dextran sulfate sodium (DSS) and azoxymethane-induced colon cancer model, *NLRP3* $^{-/-}$ mice showed increased colitis and colitis-associated cancer, which was correlated with attenuated levels of

IL-1β and IL-18 at the tumor site [81]. However, in other models, NLRP3 may also have a role in the promotion of tumors as in inflammation-induced skin cancers through the enhancement of inflammatory environment [82], which suggests a dual role for NLLRP3 in the regulation of host immunity for pro- or antitumor responses. ATP released by dying tumor cells serves as a "find-me" signal and recruits phagocytes to facilitate the engulfment of apoptotic cells [83]. Thus, ATP serves as an agonist for NLRP3 whose activation triggers IL-1β production and cross-priming of antitumor $CD8^+$ T-cells [84].

2.3.4 Phagocytosis Receptors

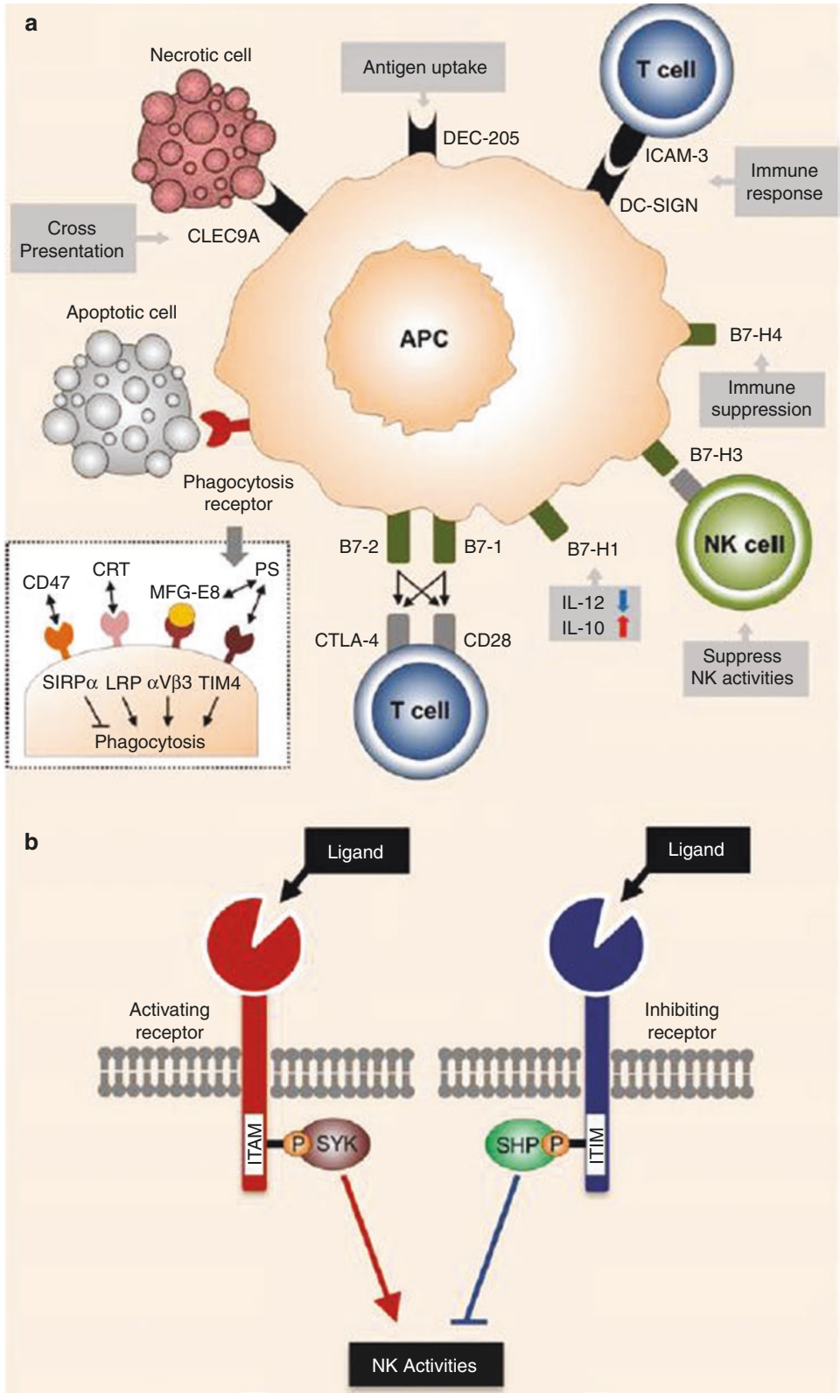
Phagocytes are specialized eating cells responsible for removing apoptotic cells in the body through a function of ligand–receptor interaction. Dying tumor cells attacked by immune cells or targeted by cytotoxic chemotherapeutic reagents are subject to recognition and removal by phagocytic myeloid cells [85, 86]. Molecules responsible for delivering "eat me" signals, including milk-fat globule-EGF factor 8 (MFG-E8), growth arrest-specific 6 (Gas-6), T-cell immunoglobulin-mucin domain protein-4 (TIM-4), and calreticulin (CRT), recognize the phosphatidylserine (PS) on apoptotic cells by integrin αvβ3 on phagocytes [87–90]. On the other hand, the "do not eat me" signal serves as negative regulators for phagocytes. One example includes the interaction between CD47 and signal-regulatory protein-α (SIRP-α), which provides inhibitory signals that block phagocytosis [91] (Fig. 2.1a).

Manipulation of phagocytic systems has emerged as one of the tumor immune evasion machineries, and pharmacological targeting of these pathways provides a feasible option to augment host immune responses and eradicate tumors. For example, blocking CD47 with a monoclonal antibody triggers tumor destruction by inducing phagocytosis of malignant cells [90, 92], and the treatment with anti-MFG-E8 antibodies elicits potent antitumor responses in combination with conventional anticancer drugs [93].

2.3.5 C-Type Lectin-like Receptors (CLRs)

Carbohydrate-binding C-type lectin and lectin-like receptors (CLRs) are a large family of molecules expressed in innate immune cells and play an important role in the regulation of antitumor immunity. For example, the interaction between DC-SIGN (dendritic cell-specific ICAM-3 grabbing nonintegrin) and ICAM-3 (intercellular adhesion molecule 3) facilitates the cross-talk between DCs and T lymphocytes, hence influences immunogenic responses against pathogens and tumors [94]. DEC-205 is highly expressed on DCs and promotes cross-presentation of tumor

Fig. 2.1 Role of innate immune receptors in the regulation of antitumor immunity. (**a**) The functions of the innate immune system are regulated by various receptors expressed in immune cells. C-type lectin-like receptors (CLRs) regulate recognition and uptake of antigens (such as DEC-205), the interactions between immune cells (such as the interaction between DC-SIGN on APCs and ICAM-3 on T-cells), and the recognition of dead cells, such as CLEC-9A, which recognizes necrotic cells and enhances cross-presentation of antigens derived from necrotic cells to $CD8^+$ T-cells. Members of B7 family regulate the functions of APCs, such as B7-H1 and B7-H4, which have immune suppressive effects, while other members regulate the interaction with immune cells, such as B7-H3, which interacts with NK cells and suppresses its functions, and B7-1/B7-2, which regulates APCs-T-cell interactions. Phagocytosis receptors expressed on APCs interact with ligands on apoptotic cells and mediate its removal by APCs. In some cases, ligand-phagocytosis receptor interactions (such as CD47-SIRP-α) provide an inhibitory signal which blocks phagocytosis, a system utilized by tumors to evade immune machineries. (**b**) The balance between activating and inhibiting signals is critical for NK cell activities. Upon interaction with corresponsive ligands, activating and inhibitory receptors deliver a signal which is mediated by ITAM and ITIM in their cytoplasmic domain. Phosphorylated ITAM motifs in activating receptors recruit adaptor proteins which activate downstream signaling pathways, while phosphorylated ITIM motifs in inhibitor receptors recruit proteins, such as SHP-1, which dephosphorylate downstream signal molecules and inhibit NK activities


a
Necrotic cell
Antigen uptake
T cell
DEC-205
ICAM-3
Immune response
DC-SIGN
Cross Presentation
CLEC9A
Apoptotic cell
APC
B7-H4
Immune suppression
B7-H3
Phagocytosis receptor
NK cell
B7-2
B7-1
B7-H1
CD47
CRT
PS
MFG-E8
IL-12
IL-10
CTLA-4
CD28
SIRPα LRP αVβ3 TIM4
Phagocytosis
T cell
Suppress NK activities
b
Ligand
Ligand
Activating receptor
Inhibiting receptor
ITAM
P SYK
SHP P
ITIM
NK Activities

antigens to cytotoxic T lymphocytes [95]. Indeed, agonistic antibody targeting DEC-205 elicits potent antitumor immunity and durable tumor regression in various murine tumor models [96]. In addition, C-type lectin domain family 9A (CLEC9A) utilizes necrotic cells for uptake, antigen presentation, and immune response, hence raising the possibility that CLEC9A-mediated recognition of immunogenic antigens may enhance antitumor immunity and clinical responses [97] (Fig. 2.1a). Therefore, CLRs serve as promising candidates for improving therapeutic responses to cancer immunotherapy. Moreover, deep understanding of the mechanism through which CLRs regulate innate immune response will lead to improvement in cancer vaccines.

2.3.6 NK Cell Receptors

NK cells possess various sets of pattern-recognition receptors which activate or suppress immune responses upon encountering their target cells. The balance between activation and inhibition signals is carefully mediated by signals triggered by both activation and inhibition receptors in combination with cytokines. Signals delivered from NK receptors mainly mediate through immunoreceptor tyrosine-based activation motif (ITAM) and immunoreceptor tyrosine-based inhibition motif (ITIM). ITAM and ITIM bear conserved sequences of four amino acids repeated twice in the cytoplasmic tails of NK cell receptors. Phosphorylation of tyrosine within ITAM motifs recruits adaptor proteins such as DNAX-activating protein-12 (DAP12) and DNAX-activating protein-10 (DAP10) involved in activating downstream signaling pathways. On the other hand, phosphorylation of tyrosine within ITIM motifs recruits proteins, such as SHP, which dephosphorylate downstream signal molecules to inhibit NK stimulation [98] (Fig. 2.1b).

Tumor cells evolve multiple strategies to evade NK cells by modulating ligand expression, ligand shedding, and upregulation of MHC molecules, in addition to the production of immunosuppressive cytokines. Thus, it is important to understand the underlying mechanism of NK cell activation and inhibition by their receptors, which eventually regulate immunosurveillance. NKG2D is a homodimeric C-type lectin-activating receptor expressed on NK, NKT, and activated $CD8^+$ T-cells [16, 99]. Ligands for NKG2D include stress-induced proteins, such as MHC class I chain-related A and B (MICA and MICB) as well as unique long 16 binding proteins (ULBPs) in human [99] and RAE1, H60, and Mult1 in mice. NKG2D ligands are upregulated in stress conditions, such as viral infection and transformation [99–102]. Several signaling pathways are involved in the induction of NKG2D ligands, including HSP70-mediated cellular stress [101] and ATM/ATR-mediated DNA damage pathways [103]. Importantly, blocking of NKG2D pathways increases the susceptibility of mice to chemically induced carcinogenesis [104], indicating the importance of NKG2D in tumor immunosurveillance. Natural cytotoxicity receptor (NCR) family consists of three activating receptors: NKp30, NKp44, and NKp46, which are able to induce a strong cytotoxic reaction by NK cells. Expression levels of NCRs are correlated with cytotoxic ability of NK cells. MHC class I molecules counteract with NCR-mediated activation signals; in addition, the loss of MHC-I molecules, frequently observed in transformed cells, activates NCRs on NK cells [105–107].

Killer cell immunoglobulin-like receptors (KIRs) are a family of cell surface molecules expressed on NK cells. KIRs have many members divided into two groups depending on the number of extracellular Ig domains (2D or 3D) or the length of their cytoplasmic tail, long vs. short (L or S). L-forms are shown to have inhibitory functions, while S-forms enhance cytotoxic activities of NK cells in DAP12-mediated signal pathways. KIRs regulate NK cells' killing function through the interaction with MHC class I molecules [100, 108].

The interaction between inhibitory KIRs and normal MHC-I molecules inhibits NK cell stimulation. Correspondingly, NK cell stimulation can occur due to an interaction between activating KIRs and polymorphic self-MHC class I mole-

cules. Inhibitory KIRs were shown to be involved in the escape mechanism of acute myeloid leukemia (AML) from NK cell immune surveillance, mechanism of which includes a mismatch between donor KIRs and recipient human leukocyte antigen ligands [109]. Thus, the understanding of KIR-mediated recognition of the missing self is important in the treatment of AML [110].

Ly49 family is a large group of receptors expressed in mice but not in humans [111]. Functionally, Ly49 is similar to human KIRs, containing both activating and inhibitory receptors. Inhibitory Ly94 receptors possess ITIM motifs which recruit SHP-1 to trigger an inhibitory signal, while activation receptors interact with DAP12 to activate lytic machinery in NK cells [112]. Ly49H is an activating NK receptor which recognizes m157 glycoprotein encoded by mouse cytomegalovirus (MCMV). Upon interaction with m157, Ly49H associates with DAP12 and DAP10 to stimulate NK cell-mediated cytotoxic activities against infected cells [113], suggesting a role for Ly49H in the protection against viral infection-associated tumors [114].

DNAM-1 (CD226) is an adhesion molecule expressed on the surface of NK cells, monocytes, and a subset of T-cells. DNAM-1 belongs to the immunoglobulin superfamily containing two Ig-like domains of the V-set. DNAM-1 is reported to bind to two ligands: CD112 and CD155 [115]. CD112 and CD155 are highly expressed in some tumors like melanoma and neuroblastoma. Importantly, neuroblastoma cells that do not express CD112 and CD155 are resistant to NK cells, indicating that NK lysis of this neuroblastoma cells requires DNAM-1 interaction with its ligands on tumor cells [116].

2.3.7 B7 Family

B7 family consists of co-stimulatory and co-inhibitory receptors found on activated APC and T-cells, which regulate the interaction between APCs and T-cells. B7-1 and B7-2 are expressed on APCs and are involved in the stimulation of T-cell response. B7-1 and B7-2 on APCs serve as co-stimulatory molecules and play a critical role in regulating antitumor immune responses through reciprocal interaction of their receptor CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) on T lymphocytes [117, 118]. B7-H1 (PD-L1) expression in DCs is induced by IL-10 and VEGF at ovarian tumors [119]. B7-H1 on DCs suppresses IL-12 and promotes IL-10 secretion, creating an immunosuppressive tumor environment. Moreover, the blockade of B7-H1 enhances antitumor immunity by DC-mediated T-cell activation [119, 120]. In addition, treatment with PD-1 neutralizing antibodies has been found to decrease tumor growth and metastasis in B16 melanoma and colon cancer models [121, 122]. B7-H3 on APCs binds to an unidentified receptor on NK cells and transduces an inhibitory signal which suppresses cytotoxic activities of NK cell. In addition, blocking of B7-H3 could restore the antitumor effects of NK cells [116]. Finally, B7-H4 promotes protumorigenic and immunosuppressive phenotypes of macrophages; for example, the blockade of B7-H4 normalized immunogenicity of macrophages and augmented antitumor immunity in ovarian tumor tissues [123] (Fig. 2.1a).

2.4 The Role of Effectors Produced from Innate Immune Cells in Cancer and Antitumor Immunity

2.4.1 Interferons (IFNs)

Type I IFNs are produced by many different cells in response to viral or bacterial infections. Type I IFNs (IFN-α/IFN-β) enhance proliferation and activation of innate immune cells such as DCs, macrophages, and NK cells [124]. In addition, they stimulate antigen processing and presentation to antigen-specific lymphocytes, which greatly contribute to tumor immunosurveillance [125]. The importance of type I IFNs in tumor immunosurveillance also validated enhanced susceptibility to tumorigenesis by treatment with anti-IFN-α/IFN-β neutralizing antibodies or in mice with targeted mutations of type I IFN receptor [126, 127].

Type II IFN (IFN-γ) is a cytokine involved in the activation of adaptive immune cells. IFN-γ is

primarily produced by various innate immune lymphocytes such as NK, NKT, and γδ-T cells and plays a critical role in the induction of Th1 immune responses and the production of NO and ROS by macrophages, leading to enhanced cytotoxic activities against transformed cells [128]. IFN-γ has an important role in the protection against transplanted tumors or chemically induced tumors by increasing intrinsic immunogenicity of tumor cells [129, 130]. IFNGR$^{-/-}$ mice or mice deficient in IFN-γ-downstream signaling molecule Stat-1 developed tumors more rapidly and in greater frequencies compared to wild-type mice [131, 132]. Thus, IFN-γ-mediated regulation of tumor immunogenicity has a great impact on innate immunity and tumor immunosurveillance.

2.4.2 Other Cytokines

Interleukins have an important role in regulating innate immune functions in tumor microenvironments. Several cytokines, such as IL-2, IL-12, IL-18, IL-15, and IL-21, serve as NK cell-stimulants, competent in targeting transformed cells. Mice deficient for IL-12p40 are susceptible to carcinogen-induced tumorigenesis; in addition, IL-21$^{-/-}$ mice showed reduced colitis-associated cancers [133], indicating the role of these cytokine in protecting hosts from arising tumors. With respect to the mechanisms of action, NKG2D systems are involved in the enhancement of NK cell cytotoxic activities by all cytokines suggested above, and perforin-granzyme pathways play an important role in exerting NK cell cytolysis by IL-18. Moreover, IL-21 induces NK cell effector functions by increasing sensitivities to IFN-γ, and IL-15 regulates survival, activation, and proliferation of NK cells [134]. Cytokines produced from innate immune cells serve as feasible adjuvants in activating antitumor responses in patients with advanced cancer. For example, the systemic administration of high doses of recombinant IL-2 or the adaptive transfer of IL-2-stimulated NK cell can trigger potent antitumor responses and mediate durable tumor regressions in patients with advanced melanoma and renal cell carcinoma [135]. The clinical efficacy of IL-12 has been evaluated as a monotherapy or in combination with other immunotherapies in patients with cancer; however, they did not induce durable clinical responses [136, 137].

Several cytokines antagonize immunogenic potential of tumors and innate lymphocytes. IL-10 downregulates the expression of immunogenic cytokines, such as IFN-γ, IL-2, TNF-α, and GM-CSF, and also suppresses antigen presentation by APCs. On the other hand, the carcinogen-mediated tumor incidence was increased in IL-10-knockout mice, whereas IL-10 overexpression protects mice from arising tumors [138]. Thus, IL-10 has a complex role in tumorigenesis, and the pro- and antitumor effects of IL-10 may depend on the different experimental models. TGF-β is a regulatory cytokine which has important roles in the regulation of immune responses and immune tolerance as well as carcinogenesis [139, 140]. TGF-β can inhibit the activities of NK cells through the suppression of IFN-γ production [141], as well as the downregulation of activating receptors such as NKp30 and NKG2D [142]. On the other hand, TGF-β negatively regulates recruitment and differentiation of myeloid-derived suppressor cells (MDSCs) in tumor tissues derived from mammary carcinomas, contributing to enhanced host immunity and tumor rejection [143]. Therefore, TGF-β has different roles in antitumor immunity and tumorigenicity, which are in part dependent on the phase of tumor progression and different cellular components in tumor microenvironments [144]. Vascular endothelial growth factor-A (VEGF-A) also plays a critical role in suppressing DC maturation and differentiation, therefore impacting tumor immunogenicity and host immunosurveillance [145]. Thus, various cytokines are responsible for attenuating immunogenic potentials of innate immune systems in tumors.

Several cytokines derived from innate lymphocytes contribute to smoldering inflammation and tumor progression. IL-23-IL-17 pathway operated in endogenous tumor microenvironments represents prototypical mediators which promote tumor-associate inflammation. IL-23 promotes tumor cell growth and invasion through upregulation of proteins of the matrix metalloproteinase-9

(MMP9), COX-2, and angiogenesis. In contrast, IL-23$^{-/-}$ mice showed reduced inflammation and thus attenuated tumor formation [146]. IL-17 is elevated in various tumors, where it plays an important role in tumor growth. IL-17 can enhance tumor growth by direct effects on tumor cells and tumor-associated stromal cells by activating IL-6-Stat3 pathways [147]. Furthermore, the altered composition of commensal microbes and disruption of epithelial barrier functions facilitate differentiation of IL-17-producing T lymphocytes by IL-23 from myeloid cells in intestine, leading to increased colon tumorigenesis [148, 149].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is produced in vivo by many cells including mast cells, macrophages, T-cells, fibroblasts, and endothelial cells in response to immune activation and proinflammatory cytokines. GM-CSF creates an immunosuppressive tumor microenvironment by differentiating immature myeloid-derived suppressor cells (MDSCs) into tumor tissues [150]. On the other hand, the therapeutic administration of GM-CSF has been emerged as a potent immunogenic adjuvant to stimulate antitumor immunity by enhancing APC functions [151].

Macrophage colony-stimulating factor, M-CSF (also known as CSF-1) is a dimeric polypeptide growth factor which regulates the proliferation, differentiation, and survival of macrophages and their bone marrow progenitors. CSF-1 expression is elevated in different tumors and is found to be accompanied by high grade and poor prognosis [152]. Targeting of CSF-1 has been evaluated in preclinical and clinical studies [153]. The administration of anti-CSF1R-neutralizing antibody (AFS98) or a CSF-1R inhibitor (Ki20227) resulted in reduced numbers of tumor-infiltrated macrophages in an implanted osteosarcoma model and reduced vascularization, angiogenesis, and tumor growth [154, 155].

2.4.3 Chemokines

Chemokines are small cytokines secreted by many cell types in response to pathological conditions, in order to activate and attract effector cells which express appropriate chemokine receptors. Two types of chemokines have been identified: CC chemokines that are chemotactic for monocytes and CXC chemokines which attract polymorphonuclear leukocytes (PMNs). Chemokines have a central role in tumor progression through the recruitment of innate immune cells into tumor site. Most studies have focused on CCL2 and CCL5 as the major chemokines in tumor microenvironment.

CCL2 (MCP-1) is produced by tumor cells and tumor-associated stromal cells and attracts CCR2^{+} inflammatory monocytes to the tumor microenvironment, which differentiate into tumor-associated macrophages and promote tumor aggressiveness, and the blockade of CCL2-CCR2 signaling by neutralizing antibodies suppresses metastasis and prolongs overall survival of tumor-bearing mice [156]. The levels of CCL2 expression and macrophage infiltration into tumors are correlated with poor prognosis and metastases in human breast cancer, suggesting significance of CCL2-mediated immune regulation in cancer patients [157].

CCL5, another important chemokine, plays an important role in the recruitment of monocytes into the tumor microenvironment [158]. CCL5 induces expression of CCL2, CCL3 (MIP-α), CCL4 (MIP-β), and CXCL8 (IL-8) by monocytes, which leads to the recruitment of myeloid cells into tumor site [159]. CCL5 also induces CCR1 expression on monocytes [160]. Hence, chemokines lead to the recruitment of monocytes, which produce more chemokines to further attract more monocytes as well as other leukocytes into the tumor site. CCL5 enhances antitumor immune responses against tumors [161], while it promotes tumorigenesis and metastases in some conditions [162, 163]. These findings suggest dual function of CCL5 in cancer and antitumor immunity.

Taken together, the dynamic interactions between tumor cells and innate immune cells governed by chemokine networks play a pivotal role in the regulation of tumor immunosurveillance and tumorigenicity (Fig. 2.2).

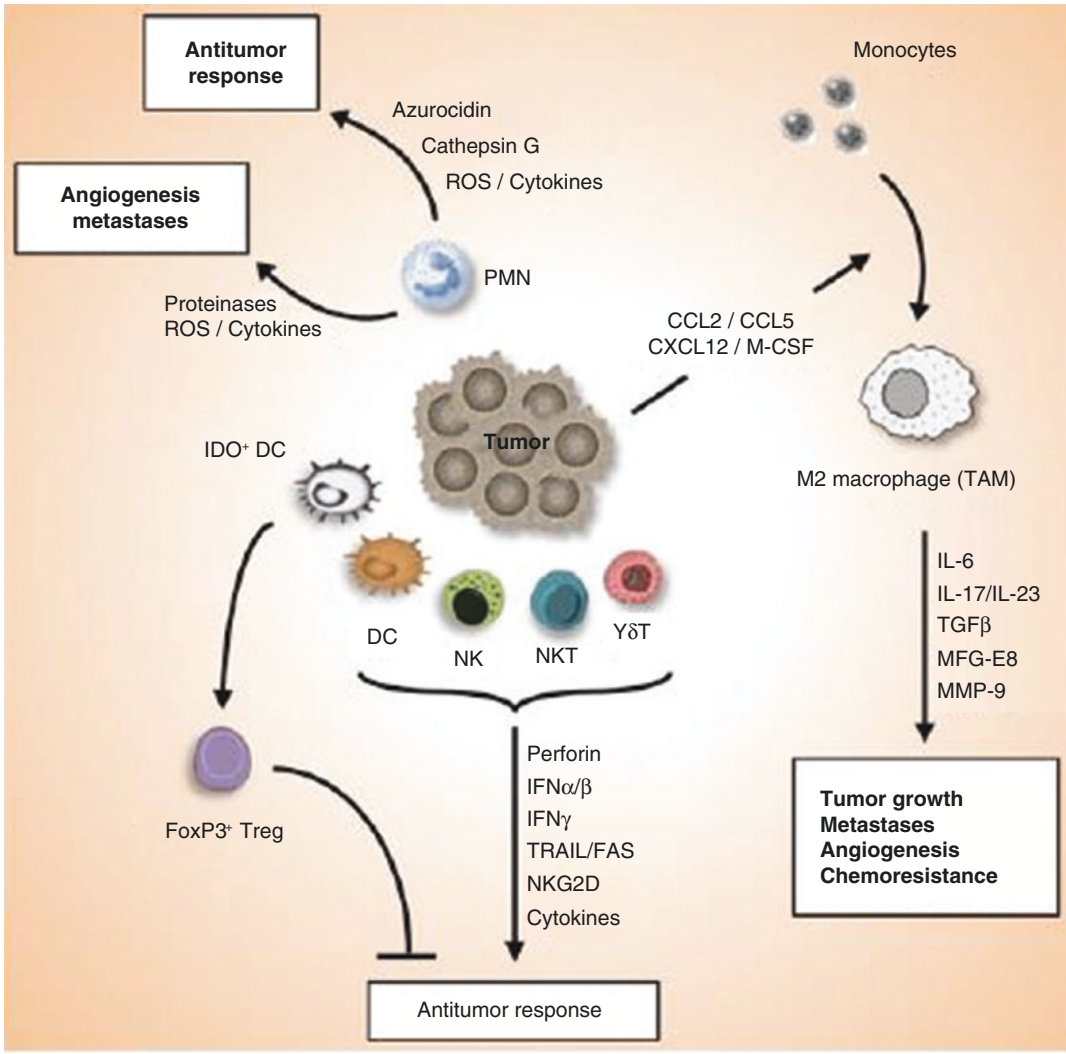


Fig. 2.2 Role of the innate immune system in cancer and antitumor immunity. Innate immune system serves as the first defense line against cancers. Innate immune cells such as DC, NK, NKT, γδ-T cells are attracted into the tumor site, where they recognize the transformed cells and release multiple factors which initiate an antitumor immune response. On the other hand, other innate immune cells may also involve in the promotion of tumor growth, angiogenesis, and metastasis. For example, IDO$^+$ DC induces differentiation of FoxP3$^+$Treg cells which suppress antitumor immunity, and molecules released by PMNs may have protumorigenic or antitumor effects. Furthermore, tumors secrete chemokines and cytokines that attract inflammatory monocytes into the tumor microenvironment and induce its differentiation into M2 macrophages, which play important roles in tumor progression, metastases, angiogenesis, and chemoresistance

2.5 Concluding Remarks

Innate immune system serves as the first line of defense against pathogens and cancers. In tumors, innate immune cells are attracted into the tumor site. Factors released from stressed cells at the tumor microenvironment, such as PAMPs and DAMPs, are recognized by another set of receptors, including TLRs, RLRs, and NLRs, which trigger distinct innate signaling pathways; these pathways lead to maturation, activation, as well as production of cytokines and chemokines from

immune cells, to attract more immune cells into the tumor site and initiate an immune response against tumor cells. Thus, a deep knowledge of the role of innate immune system in tumor immunity and tumorigenesis is critical to develop new strategies for the immunotherapy of cancer.

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Tumor-Associated Myeloid Cells in Cancer Progression

3

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3.1 Introduction

A common characteristic in all cancers is the presence of a host cell infiltrate that includes fibroblasts, endothelial cells, and immune cells [1–3]. Infiltrating host cells, together with soluble factors, signaling molecules, and extracellular matrix components, constitute the tumor microenvironment (TME). It is well-established that TME orchestrates tumor initiation, progression, and spreading, and growing evidences indicate that it also plays a pivotal role in the response to therapy [4].

Solid tumors are infiltrated by several and distinct populations of lymphoid and myeloid immune cells [1–3]. Tumor-infiltrating lymphocytes (TILs) are recognized as major determinants of the host immune response to tumor cells. The therapeutic improvement following the development of immune checkpoint inhibitors (ICI), contributing to restore T-cell cytotoxicity eliminating negative signals blocking T-cell functions, demonstrates the pivotal role of TILs. On the other side, cells of the myeloid lineage, that commonly represent the highest proportion of immune cells in TME, can exert pro- or anti-tumoral functions.

Tumor-associated myeloid cells mainly derive from the correspondent blood cells which in turn originate from hematopoietic stem cells (Fig. 3.1). Among myeloid cells, tumor-associ-

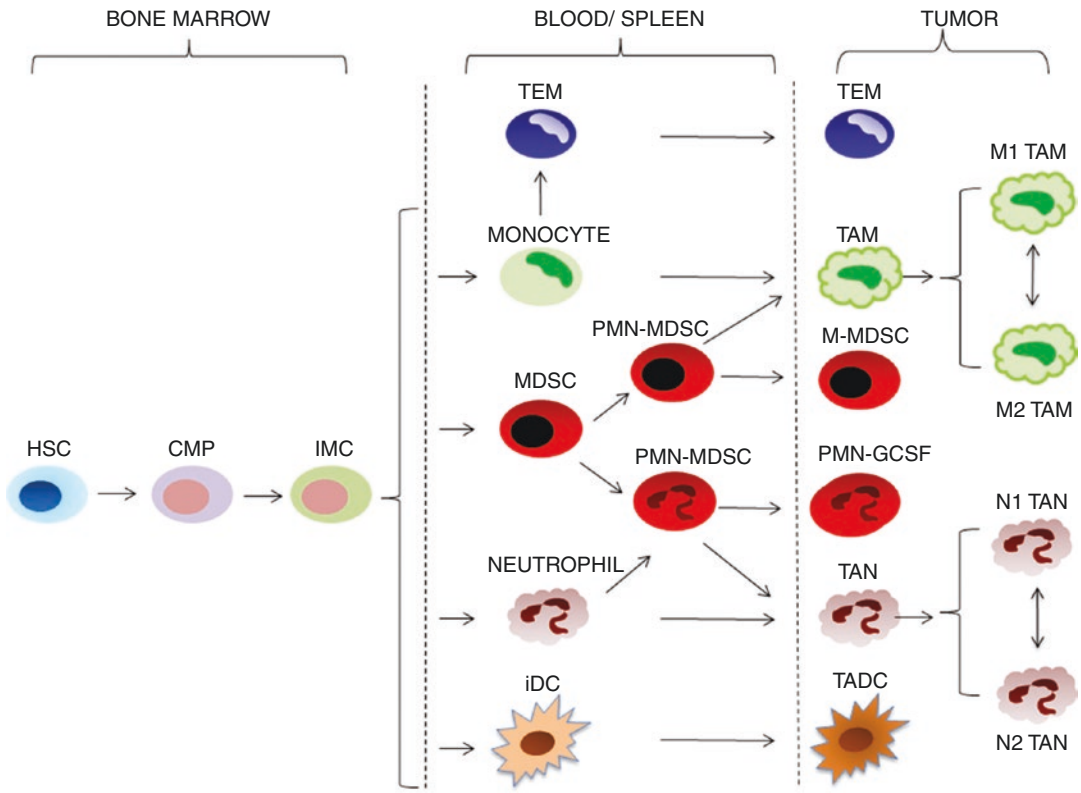

Fig. 3.1 Differentiation pathways of tumor-associated myeloid cells. Myeloid cells originate from hematopoietic precursor in the bone marrow. Here are indicated the precursors and the networks giving rise to the various myeloid cells in the different compartments (bone marrow, blood/spleen, and tumors). Tissue macrophages and neutrophils display a gradient of polarized phenotypes whose extremes are M1 and M2 for macrophages, N1 and N2 for neutrophils. HSC: hematopoietic stem cells; CMP: common myeloid progenitors; IMC: immature myeloid cells; MDSC: myeloid-derived suppressor cells; M-MDSC: monocytic MDSC; PMN-MDSC: polymorphonuclear/granulocytic MDSC; TAM: tumor-associated macrophages; TAN: tumor-associated neutrophils; iDC: immature dendritic cells; TADC: tumor-associated dendritic cells; TEM: Tie-2-expressing monocytes

ated macrophages (TAMs) are the most abundant leukocytes infiltrating tumors. Chemokines, cytokines, and complement components [C-C motif chemokine 2 (CCL2); colony stimulating factor-1 (CSF-1); C5a] are major determinants of macrophage recruitment in tumors [5–8]; however, in situ proliferation has been also reported [9, 10]. TAMs can engage complex and bidirectional interactions with the other cells of the TME, as well as with cancer cells, and are key orchestrators of cancer-related inflammation (CRI) [3, 7, 11]. The set of myeloid cells infiltrating tumors also includes neutrophils, the predominant leukocyte subset in the blood, and a heterogeneous population of immunosuppressive cells defined as myeloid-derived suppressor cells (MDSCs) [12]. Tumor-associated myeloid cells are recognized as major players in the connection between inflammation and cancer, considered the seventh hallmark of cancer [7, 11, 13–18]. Given their role in tumor-promotion (Fig. 3.2), growing efforts are devoted to target tumor-associated myeloid cells and/or to skew their properties toward antitumoral effects.

In this chapter, we focus essentially on tumor-associated macrophages, neutrophils, and MDSCs, providing a summary of the current knowledge centered on these cells and a rationale for their therapeutic targeting.

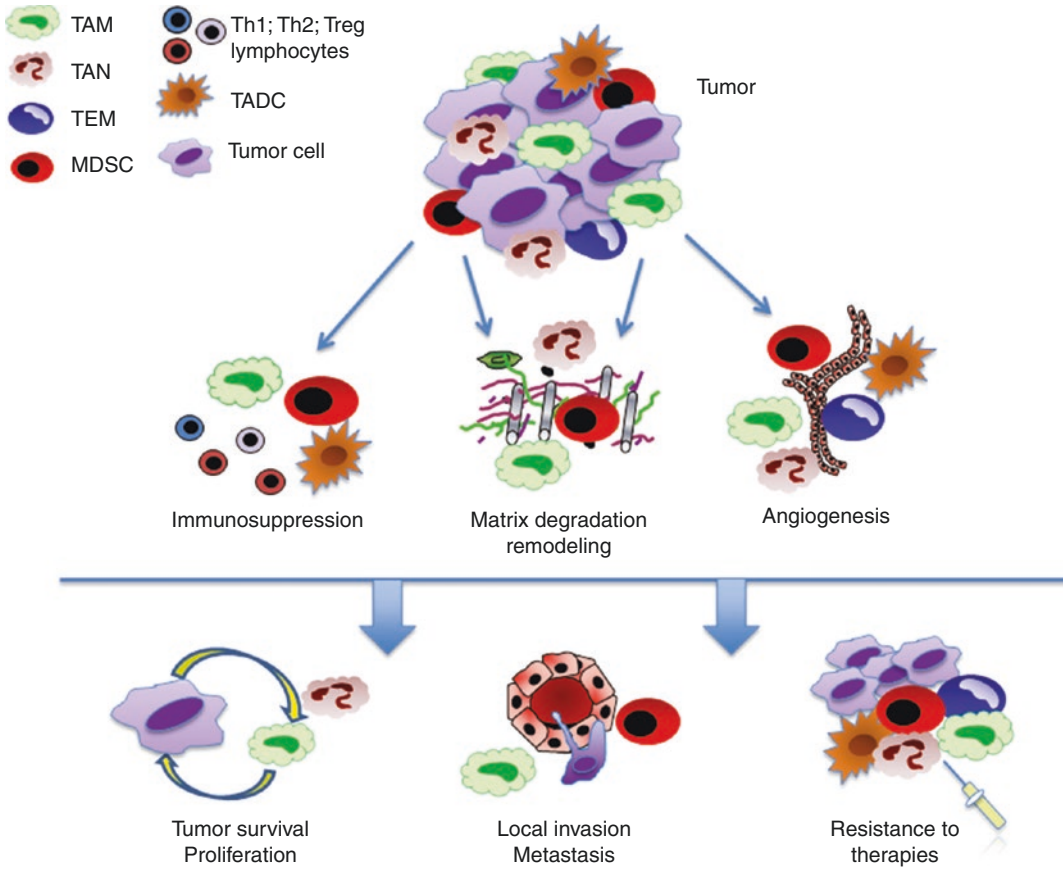

Fig. 3.2 Protumoral functions of tumor-associated myeloid cells. Tumor-associated myeloid cells, once exposed to the tumor microenvironment, acquire the capability to exert several pro-tumoral functions, including promotion of tumor cell survival, angiogenesis, matrix degradation and suppression of adaptive immunity. Soluble factors (cytokines, chemokines, growth factors and proteolytic enzymes) are involved in the regulation of these pro-tumoral effects

3.2 Tumor-Associated Macrophages

3.2.1 General Characteristics

Macrophages (Mφ) commonly represent the highest proportion of myeloid cells in the TME, and they are described to have mostly pro-tumoral activity [3].

The monocyte-Mφ lineage is characterized by its high plasticity and variety [19], influenced by the environment in which they are inserted. Mφ polarization and activation is described as a continuum of different cells, which differ in morphology, function, and metabolism [19, 20]. In the two extremes of this continuum are the M1 (classically activated) Mφ and the M2 (alternatively activated) Mφ [19, 20]. While M1-Mφ are classically pro-inflammatory, being involved in the killing of invading intracellular pathogens and in the activation of anti-tumoral Th1 adaptive immune responses, M2-Mφ mediate the control of parasitic infections, secrete anti-inflammatory, and immunomodulatory mediators, being involved in tissue remodeling and repair and dampening of anti-tumor adaptive immune responses [19, 20]. The M1 and M2 activation states also present very distinct glucose, amino acid, iron, and lipid metabolism [20, 21], which impact their immune functions. M1-Mφ metabolic profile is characterized by enhanced glycolysis, a continuous flux through the pentose phosphatase pathway, fatty acid synthesis, a truncated tricarboxylic acid cycle [20], and an "iron-sequestering" phenotype [21]. Paradoxically, M2-Mφ metabolism is described to be mainly dependent on oxidative phosphorylation and fatty acid oxidation [20], presenting an "iron release" phenotype [21].

Although CRI is highly variable and dependent on the tissue where the tumor develops, being composed of different inflammatory cells and inflammatory mediators, TAMs are always the major coordinators of CRI [2, 3]. In fact, both in the primary tumor and in the metastasis, TAMs are involved in a complex interplay with all the cells composing the TME, from cancer cells to fibroblasts, endothelial cells, stromal cells, and other inflammatory cells [19]. During this interplay, TAMs usually adopt a M2-like phenotype and orchestrate an inflammatory environment that promotes survival and proliferation of the cancer cells, by suppressing anti-tumor responses, supporting angiogenesis, cancer cell migration, and invasion [3, 20]. Interestingly, due to the great plasticity of TAMs, it is possible to detect different subpopulations of these cells within distinct regions of the tumor tissue [20].

3.2.2 Role in Cancer and Dissemination

One of the first inflammatory cells to infiltrate solid tumors are Mφ. Contrarily to what was previously described, TAMs are composed of a mixed population of blood monocyte-derived Mφ and embryonic-derived tissue-resident Mφ [22, 23].

In the TME, through the production of cytokines, chemokines, and other factors, tumor cells are able to modulate Mφ function and characteristics in their profit, taking advantage of Mφ normal function in wound healing and tissue regeneration [3]. Production of CCL2 and IL-13 by tumor cells was shown to modulate TAM polarization into a M2-like activation state [24, 25], but many other factors present in the TME, as TGF-β or prostanglandin-E2, could possibly alter Mφ polarization [26, 27].

In the breast, CCL2 secretion by tumor cells was shown to be involved in the recruitment of inflammatory monocytes into the metastatic site and drive their differentiation into metastasis-associated Mφ (MAMs) [28]. CCL2 increases CCL3 expression by MAMs, which autologous signaling via CCR1 induces their retention in the lung, promoting metastasis [29]. In another report, CCL2 expression by $HER2^+$ tumor cells resulted in the production of Wnt-1 by intra-epithelial Mφ in the mouse, inducing the disruption of E-cadherin junctions between early cancer cells [24]. This process leads to an early dissemination and lung intravasation of the breast tumor cells, even before the primary tumor becomes palpable [24]. A similar process was observed in a model of hepatocellular carcinoma, where tumor cells drive the polarization of TAMs into

a M2-activation state via the canonical Wnt/β--catenin pathway, culminating with increased tumor growth, metastization, and immunosuppression [30].

In a mouse model of hepatocellular carcinoma, a role for CCL2-CCR2 axis in tumor growth was also described [31], supported by the inverse association of high CCL2 expression in human hepatic tumor tissues and post-surgical survival [31]. In human primary colon tumors, CCL2 is expressed in all tumor stages, being particularly highly expressed in tumor stage IV that developed metastasis in distant organs [32].

Contrarily, in a preclinical melanoma model, CCL2 was shown to direct the migration of γδ T-cells into the tumor, where they play a protective role by exerting an effective cytotoxicity against the tumor cells [33]. This example clearly shows that the same immune pathways might have distinct functions and effects in different tumor contexts.

While inflammatory monocytes have been shown to support metastization [28, 29], non-conventional patrolling monocytes, characterized as $CX3CR1^{+}Ly6C^{-}$, were reported to prevent melanoma lung metastasis through the recruitment of NK cells into the lung [34]. In an independent study, patrolling monocytes responded to primary melanoma tumors through the production of IL15, activating NK cells and the production of IFNγ, which inhibited a subsequent experimental lung metastization [35].

Interestingly, both in colon adenocarcinoma [22] and pancreatic ductal adenocarcinoma (PDAC) [23] mouse models, it was reported that the TME drives the in situ proliferation of embryonic-derived tissue-resident Mφ. In the colon, increased levels of CSF-1 during tumor progression where driving the mechanism, and antibody blocking of CSF-1 receptor (CSF-1R) was able to reduce the number of these cells in the mice colon, resulting in reduced tumor incidence and tumor size [22]. Transcriptional profiling of TAMs derived from tissue-resident Mφ revealed that these cells expressed higher levels of M2 genes and genes involved in extracellular matrix (ECM) remodeling [e.g., arginase-1 (arg-1), metalloproteases (MMPs)] than monocyte-derived TAMs [22, 23]. In fact, experiments with $CCR2^{-/-}$ mice in both PDAC and colon adenocarcinoma models demonstrated that inhibition of monocyte recruitment into the tumor site is not enough to inhibit tumor growth [22, 23]. A $CXCR4^{+}$ TAM population expressing high levels of ECM-remodeling genes, which resembles the embryonic-derived TAM in the mouse, was also detected in human PDAC samples, suggesting its involvement in the modulation of the pathology-associated fibrosis [23]. Modulation of the ECM by TAMs opens the way for tumor cells dissemination in the tissue, therefore prompting metastasis formation [2].

Both IL-4 and IL-13 signal through IL4Rα chain, activating STAT6 pathway, which is known to be associated with tissue remodeling and vascularization [3]. $STAT6^{-/-}$ mice were reported to be protected from tumor development in a colitis-associated cancer model [36], presenting reduced proliferation and increased apoptosis of epithelial cells, reduced pro-inflammatory cytokines and chemokines, and reduced accumulation of $CD11b^{+}Ly6C^{hi}CCR2^{+}$ cells. In a pancreatic tumor model, IL-13 was reported to polarize TAMs into a M2-phenotype, which in turn release CCL2 and IL1ra, driving pancreatic tumorigenesis [25]. Another study described an intricate communication between TAMs and tumor cells in the pancreas. The production of CCL18 by TAMs enhanced the aerobic glycolytic pathway in tumor cells, increasing lactate levels which modulate TAM phenotype into M2-like [37].

The PD-1/PD-L1 is one of the most studied immune checkpoints, with an important role in the regulation of T-cell function, being currently used as a drug target in the clinic. Both tumor cells and TAMs can express PD-1 ligands, which block T-cells via PD-1 [3, 21]. Remarkably, it was recently reported that TAMs also express PD-1, both in the murine and human setting, and that $PD\text{-}1^{+}$ TAMs presented a M2-phenotype, expressing higher levels of CD206 and lower levels of MHCII in comparison with their negative counterparts [38, 39]. Supporting the pre-clinical data, in human colorectal cancer the frequency of PD-1 expressing TAMs was positively correlated with disease stage [38].

The TME is usually characterized by a deprivation of nutrients and oxygen, due to the uncontrolled tumor cells growth. The aerobic glycolysis metabolism of tumor cells induces an increase in the local lactate levels, and it was recently reported that Mφ can sense hypoxia and lactate gradients, resulting in patterns of Mφ expressing arginase-1 and VEGF, therefore promoting angiogenesis and tumor growth [40].

The mechanisms described above represent only a fraction of the pathways by which Mφ influence tumor growth and dissemination, and a lot remains poorly understood.

3.2.3 Targeting Strategies

The pro-tumoral role of Mφ and other myeloid cells infiltrating tumors make these cells good therapy targets. In agreement, during the past years, a huge effort has been made to develop drugs able to target TAMs, eliminating them from the tumor tissue or even reprogramming their activation state and function. The results of several pre-clinical studies indicate that targeting of TAMs might be an efficient strategy to limit tumor growth and metastization, and enhance the response to other therapies.

Tumor cells have been reported to express on their surface molecules that allow them to avoid innate immune surveillance [3]. One example is CD47, which was reported to be expressed by several tumor cells [41, 42], and serves as an anti-phagocytic signal by binding SIRPα on the membrane of Mφ [3, 41, 42]. In a small-cell lung cancer model, blocking of CD47-SIRPα using an anti-CD47 therapy induced Mφ-mediated phagocytosis of the tumor cells and reduced tumor growth [41]. *Gholamin* et al. have demonstrated that CD47 is highly expressed in several malignant pediatric brain tumors, and that a humanized anti-CD47 antibody (Hu5F9-G4) presents therapeutic activity both in vitro and in vivo against clinical-derived human xenograft mouse models, without affecting the normal cells of the central nervous system [42].

Since Mφ function is highly dependent on CSF-1, several CSF-1R blocking therapies are currently under investigation [43–45]. In a mouse model of glioblastoma multiforme, although the treatment with a CSF-1R-inhibitor under clinical trial (BLZ945, Novartis) effectively reduced tumor size, more than 50% of the animals suffer rebound with high grade tumors [43]. Interestingly, this resistance was TME-driven, since TAMs present in rebound tumors upregulated insulin-like growth factor 1 (IGF-1), which results in activation of IGF-1R and PI3K signaling in glioma cells, supporting tumor growth and malignancy [43]. A therapeutic approach combining both CSF-1R blocking with PI3K or IGF-1 blocking increased the survival rate of the animals with recurrent tumors [43].

Inhibition of CSF-1R with PLX3397 (Plexxikon) in a pre-clinical model of hepatocellular carcinoma altered the polarization of TAMs into a M1-profile, resulting in increased numbers of CD8 T-cells, delayed tumor growth, increased survival of the mice [45]. Interestingly, an independent study demonstrated that a combinatory therapy of anti-CSF-1R and CD40-agonist also induced a reprogramming of TAM before their depletion, consequently boosting an anti-tumoral T-cell response against several transplantable tumor models [44].

These are just few examples on how targeting of TAMs can be helpful in the treatment of cancer, but it also points out the obstacles that can arise in the clinics from the use of mono-therapy. Pre-clinical data show that a more efficacious approach could be the targeting of more than one pathway by which TAMs promote tumor progression.

3.3 Tumor-associated Neutrophils

3.3.1 General Characteristics

Neutrophils, also known as polymorphonuclear leukocytes (PMN), are the most abundant white blood cells in the human circulatory system, typically representing more than 70% of all leukocytes [46, 47]. They are the first line of defense of the innate immune system, being able to detect and destroy invading pathogens through phagocytosis and intracellular degradation, degranulation of antimicrobial products [i.e., reactive oxygen species (ROS), antibacterial peptides, and enzymes]

and production of neutrophils extracellular traps (NETs) entrapping microorganisms [46]. During the differentiation and maturation process from hematopoietic stem cells, neutrophils undergo extensive changes and acquire distinct mature phenotypic and functional properties [47].

Neutrophils are short-lived cells under steady state condition, but, once migrated into tissues in response to inflammatory stimuli, their longevity is increased several fold [48]. During their persistence in tissues, neutrophils can exert complex activities, including orchestration of the immune response, but during the late final phases of acute inflammatory responses, they are involved in the resolution of inflammation through the production of pro-resolving lipid mediators [46, 47, 49–51]. Intra-vital imaging and animal models showed that neutrophils recruited at sites of damage do not undergo apoptosis once their life span ended, but they leave the site of tissue damage in a process termed "reverse transmigration" [49, 52]. Recent data indicate that leukotriene B4 induces cleavage of JAM-C by neutrophil elastase, driving neutrophil reverse transmigration in vivo [53].

Neutrophils are heterogeneous and versatile cells able to modulate inflammatory and immune responses. Plasticity of neutrophils is particularly evident among those infiltrating tumors. Tumor-associated neutrophils (TANs) undergo the most impressive phenotype changes. In fact, mirroring macrophages, TANs can be divided in two distinct functional subpopulations, N1 and N2 neutrophil. N1 neutrophils are mature cells characterized by anti-tumorigenic properties [52, 54]. On the contrary, N2 neutrophils are more immature cells and exert pro-tumorigenic properties, expressing high levels of MMP-9, Arg-1, and VEGF, factors promoting angiogenesis, facilitating an immune suppressive TME, and increasing cancer dissemination [55–58].

3.3.2 Role in Cancer and Dissemination

Neutrophils have traditionally received little attention in the cancer field, partly because their limited life span and fully differentiated phenotype seemed at odds with the chronic nature of cancer and has long been considered meaningless [57, 59]. Interest in neutrophil cell biology in the context of cancer has increased in the last years, also thanks to the yin/yang role of these cells on tumor development.

Neutrophils are recruited into growing tumors in response to CXC chemokines released by cancer and stromal cells [57]. The axis CXCL8-CXCR2 is among the most relevant for neutrophils recruitment, as shown, for instance, in human head and neck squamous cell carcinoma (HNSCC) or hepatocellular carcinoma [60–63]. Neutrophils play a role in the carcinogenesis process by releasing nitric oxide derivatives and ROS, promoting genetic instability and DNA point mutations [18, 55, 64]. Release of neutrophil elastase (NE) can also favor tumor cell proliferation and is involved in neutrophil mediated epithelial-to-mesenchymal transition [65]. CXCR2 deletion suppresses inflammation-induced carcinogenesis in mice, underlining the crucial role exerted by neutrophils in this process. In agreement, aberrant accumulation of neutrophils, documented in a wide variety of tumors, is often associated with poor clinical outcomes [63, 66, 67].

In general, N1 neutrophils were found in early stage tumors, whereas N2 TANs are predominantly found in established tumors [68]. It is known that type I Interferons (IFNs) are main inducers of N1 TANs, while TGF-β promote the acquisition of a N2 pro-tumoral phenotype [59, 69]. In HNSCC, expression of MMP-9 was increased in TANs in comparison to any other cell type in the tumor [70], while in hepatocellular carcinoma, increased number of TANs correlated with a higher angiogenic response [62]. Direct proof for neutrophils being the major tumor-associated leukocyte type expressing MMP-9 was recently provided in a study employing human xenografts and syngeneic murine tumors [71]. Also in melanoma or fibrosarcoma it was found that TANs are major source of MMP-9 and VEGF, and elimination of TANs resulted in reduced tumor growth [72].

A plethora of cytokines and proteins are stored within neutrophil granules and can be released in the TME [73]. Neutrophil-derived chemokines can influence tumor fate either indirectly, through the recruitment and activation of innate and adap-

tive immune cells [73], or directly, thanks to their capacity to modulate angiogenesis and cell proliferation [74, 75].

Recently, it was proposed that NETs could enhance adhesion of escaped circulating tumor cells and formation of distant metastases [76]. Activated neutrophils can promote metastasis by stimulation of tumor invasion at the primary site, wherein a "premetastatic phase" tumor-derived factor stimulates hematopoietic mobilization and tissue-specific responses, preparing a distant site for metastatic seeding. Wculek et al. showed in orthotopic mammary tumor-bearing mice that neutrophils accumulated in the lungs before cancer cells and their number increased during metastatic progression [77]. Neutrophils in the premetastatic lung augmented the tumorigenic potential of cancer cells in vivo and in vitro. In addition, reduction of TANs in prostate carcinomas seems to reduce angiogenesis and tumor cell intravasation [78].

Although a growing body of literature points to activated neutrophils driving tumor progression, this is not a universal finding. Granot et al. showed that neutrophil accumulation in the lung protected mice from mammary tumor metastasis, an effect that was mediated by reactive oxidant generation and tumor-secreted CCL2 [79]. Blaisdell et al. identified a protective role for neutrophils in a mouse model of PTEN-deficient uterine cancer [80]. In this model, neutrophils were recruited by the hypoxic tumor microenvironment, and their infiltration led to detachment of tumor cells from the basement membrane, reduction in tumor growth and metastasis. In addition, TANs were associated with good prognosis in patients with gastric and colorectal cancer [81, 82]. Galdiero and coworkers also showed for the first time that higher TANs density was associated with better response to 5-FU-based chemotherapy, while in patients only treated with surgery, TANs levels correlate with poor prognosis [82]. This suggests that the predictive role of TANs needs to be re-addressed in consideration of the therapeutic history of the patients.

The contradictory roles of neutrophils may reflect differences in tumor phenotypes and underlines the high plasticity and heterogeneity of these cells. The potential for TANs to be friends or foes in cancer points to the role of tumor microenvironment in conditioning neutrophils polarization. However, whether neutrophil heterogeneity and polarization in cancer is dependent on the tumor type is yet to be defined.

3.3.3 Targeting Strategies

As outlined above, growing evidences suggest an important role of neutrophils during tumor initiation, growth, and dissemination. As a consequence, interest is increasing on the possible prognostic role of TANs and on the therapeutic options to target neutrophils. In several human tumors, neutrophil infiltration was correlated with poor prognosis [66, 83–85] and with high tumor grade or more aggressive tumors [86, 87]. TANs were able to predict mortality in NSCLC [88, 89], but were associated with good prognosis in gastric and colorectal cancer [82, 90, 91].

At the moment, one of the most used predictive biomarker is the so-called neutrophil-to-lymphocyte ratio (NLR) measured in the peripheral blood. An elevated NLR is considered an indicator of inflammation and is associated with worse outcomes in many solid tumors, both in early and advanced stages of cancer [92–97]. Despite the number of studies supporting the validity of NLR as predictive biomarker in cancer, its prognostic value is controversial. In fact, levels of circulating neutrophils can be affected by several factors and do not necessarily mirror TANs, putting in place the need for further studies.

Detailed studies on neutrophils in the tumor microenvironment, including modern approaches evaluating genome-wide expression profiles of the population, potentially at single cell level, will add insight into neutrophil heterogeneity and may form the basis for targeted approaches against populations that drive tumor progression.

A first strategy to target neutrophils is inhibiting their trafficking and/or activation. Agents developed for the treatment of inflammatory and autoimmune diseases can be used also to target TANs. CXCR2 antagonists, developed to treat chronic obstructive pulmonary disease, decrease absolute neutrophil counts, reducing inflammation

[98]. Anti-CXCL8 antibodies that inhibit CXCL8–CXCR1/2 signaling pathway or small molecules targeting CXCR1 and/or CXCR2 decreased tumor growth and progression in mouse models [78, 99–101]. CXCR2 inhibition has also been associated with enhanced response of both tumor and micrometastases to chemotherapy treatment [102]. In addition, inhibitors of neutrophil-elastase displayed promise in lung cancer mouse models [103]. Another pathway under intense investigation is the IL-23–IL-17 axis [101]. As we acquire more knowledge about the plasticity of neutrophils, new approaches will emerge based on N1/N2 axis. Andzinski et al. showed that type I interferons skew TANs versus an anti-tumor N1 phenotype in tumor-bearing mice, and similar changes in neutrophil activation were observed in melanoma patients receiving type I interferon [69].

As previously mentioned, there is also growing evidence for NETs to drive tumor dissemination. There are currently several experimental approaches that can deplete NETs, including DNase treatment, antibodies against NET constituents, and small molecule inhibitors of signaling pathways required for NETosis [104]. Since neutrophils turnover in the tissue is very high, they could be potentially mediators for delivery of antineoplastic agents as "Trojan horse." As a proof of principle, Chu et al. observed in murine melanoma that neutrophils enhanced the delivery of nanoparticles to the tumor and augmented the effect of antibody-mediated immunotherapy [105]. Moreover, another promising approach is the use of anti-tumor monoclonal antibodies (mAbs) to activate the ADCC reaction in neutrophils. Upon Fc receptor activation, neutrophils produce ROS and release mediators with direct anti-tumor potential [106].

Many questions remain open and we need to study deeper the molecular mechanisms regulating the link between neutrophils and cancer to identify new prognostic and predictive biomarkers. Given the dual role of neutrophils in cancer, the consequences of depleting anti-tumor neutrophils together with tumor promoting cells are still unclear, reinforcing the importance of novel biomarker discovery. Cancer immunotherapy should drive to more personalized therapeutic approach. As we acquire knowledge about the cues which regulate neutrophil diversity, new approaches for their therapeutic modulation are expected to emerge.

3.4 Myeloid-derived Suppressor Cells

3.4.1 General Characteristics

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells first described in 2007 [107]. They arise from myeloid progenitors which fail to differentiate into mature dendritic cells (DCs), granulocytes, or Mφ [108, 109].

Three subsets of human MDSCs were defined: monocytic (M-MDSCs), polymorphonuclear/granulocytic (PMN-MDSCs), and early stage (e-MDSCs). M-MDSCs are identified as $CD11b^{+}CD14^{+}HLA\text{-}DR^{-/lo}CD15^{-}$ cells, PMN-MDSCs are defined as $CD11b^{+}CD14^{-}CD15^{+}$ or $CD11b^{+}CD14^{-}CD66b^{+}$ cells, while e-MDSCs, that lacks myeloid lineage markers characteristic of monocytic and granulocytic subsets, express CD33 and are $HLA\text{-}DR^{-}$ [110]. M-MDSCs and PMN-MDSCs include Mφ or neutrophils in different maturation stages, respectively, while e-MDSCs are immature cells [111, 112]. Early studies in the mice showed the existence of murine M-MDSCs and PMN-MDSCs in inflammatory conditions, while the mouse equivalent of e-MDSCs is yet to be identified [110]. Murine PMN-MDSCs are defined as $CD11b^{+}Ly6G^{+}Ly6C^{lo}$ cells, while M-MDSCs are $CD11b^{+}Ly6G^{-}Ly6C^{hi}$ cells.

Generally, human MDSCs are not present in healthy individuals but appear in cancer and other pathological conditions characterized by chronic inflammation that represents the driving force leading to the development of these cells [113, 114]. Accumulating evidences have shown that MDSCs also regulate immune responses during infections, acute and chronic inflammation, traumatic stress, sepsis and transplantation (reviewed in [108]).

MDSCs exert a general role in the suppression of T-cell, NK cell, and DC activity, support-

ing immune escape and tumor progression, and are the major obstacle to anti-tumor immunity [111, 115–118]. The strong suppressive activity exerted by MDSCs is associated to peculiar gene expression profiles resulting in the maintenance of a high oxidative stress environment. The importance of MDSCs in cancer is demonstrated by preclinical data indicating that their elimination in tumor-bearing mice restores the immune response and enhances the anti-tumor effects of immunotherapy.

3.4.2 Role in Cancer and Dissemination

MDSCs are recruited in the TME by different chemokines and sustain human cancer stemness [119–122]. CCL2, CCL5, and CSF-1 are involved in the recruitment of M-MDSCs, while CXCL1, CXCL5, CXCL6, CXCL8, and CXCL12 mediate PMN-MDSCs migration and degranulation through CXCR1 and CXCR2 signaling. Tumor cells, myeloid cells, and certain subset of regulatory T-cells (Treg) express CXCL8 and recruit neutrophils and PMN-MDSCs to TME. CXCL17 is also involved in recruitment of PMN-MDSCs and promotes angiogenesis partially by inducing VEGF expression in monocytes and endothelial cells [23, 24, 123].

Tumor cells can promote expansion of MDSCs trough the production of factors stimulating myelopoiesis and inhibiting maturation of differentiated myeloid cells. Prostaglandins, cyclooxygenase 2, IL6, IL-10, CSF-1, GM-CSF, and VEGF are some of the molecules that can promote expansion of myeloid suppressor cells [124–126]. Most of these factors trigger MDSCs expansion through Janus kinase (JAK) protein family members and signal transducer and activator of transcription 3 (STAT3).

In acute myeloid leukemia, extracellular vesicles (EVs) secreted by tumor cells play a key role in the formation of MDSCs via the conversion of normal myeloid cells and changing the normal myelopoiesis. The Mucin 1 (MUC1) oncoprotein was identified as the critical driver of MDSCs expansion mediated by EV: it has been shown that MUC1 induces increased expression of c-myc in EVs, affecting cell cycle proteins and inducing MDSCs proliferation [119, 127].

The generation of MDSCs is also promoted by persistent endoplasmic reticulum (ER) stress [128]. In addition, ER stress modulates the immunosuppressive capacity of tumor-infiltrating MDSCs by increasing expression of Arg-1, iNOS, and NOX2. Therefore, agents reducing ER stress could restore anti-tumor immunity by inhibiting suppressive MDSCs exacerbated by ER stress [128].

Once in the TME, MDSCs promote angiogenesis and contribute to establish pre-metastatic niches, enhancing metastasis [129–131]. In addition, MDSCs can contribute to recruit other immunosuppressive cells, such as regulatory T-cells (Treg). Also, MDSCs can reduce effectiveness of Chimeric Antigen Receptor (CAR) T-cell therapy by providing immunosuppressive profile within solid tumors [111, 132]. MDSCs are activated by tumor-derived factors. Several proinflammatory cytokines are involved in the activation of MDSCs, including IL-6, IL-1β, TNF-α, IFN-γ [126]. However contradictory data were obtained when one or more of these mediators were knocked down, suggesting that further studies are mandatory.

MDSCs and their secreted cytokines, such as IL-6, TNF-α and IL-1β, would be the key mediators in promoting tumor progression [122]. As already mentioned, MDSCs have a fundamental ability to suppress immune cell functions through a variety of mechanisms, the most relevant being the ability to suppress T-cell proliferation and cytotoxicity via release of soluble mediators such as IL-10, Arg-1, and nitric oxide (NO) and exhaustion of some amino acids [122, 133]. NO and ROS produced by MDSCs can promote apoptosis of T-cells [111, 134, 135]. Anergy and apoptosis of T-cells is also a consequence of the interaction of membrane molecules on MDSCs and T-cells, such as PD-1 and PD-L1 or Galectin 9 and T-cell immunoglobulin and mucin domain-3 [122]. In addition, it has been described that MDSCs are involved in the inhibition of NK cell activation and can promote expansion of immune-suppressive cell populations, such as Treg cells, through IL-10 and TGF-β release [136–138]. IL-10 secreted by MDSCs also promote M2-Mφ polarization

while down-regulating IL-12 expression by M1-Mφ [139]. Once in the tumor, MDSCs can also promote novel vessel formation and release matrix-bound VEGF by MMPs, in particular MMP-9.

Recent studies focused on energy metabolic pathway of MDSCs and its impact on immunosuppressive function showed that tumor-infiltrating MDCSs utilize fatty-acid β oxidation (FAO) as primary source of energy along with increased mitochondrial mass and high rate of oxygen consumption [140]. Blockage of FAO could significantly inhibit the immunosuppressive effect of MDSCs in humans and could be used as therapeutic strategy.

3.4.3 Targeting Strategies

Nowadays novel trends in anticancer therapies are focused on targeting tumor-related immune-suppression responses [141, 142]. Given their multiple functions, MDSCs can be considered as one of the major orchestrators of the immunosuppressive network contributing significantly to tumor progression and metastatization, correlating negatively with prognosis and overall survival [111, 143–145]. Therefore, MDSCs have captured considerable interest in the last few years as prime target for cancer immunotherapy [2, 7, 35, 146].

Strategies to target MDSCs are gradually emerging with promising results. Increasing numbers of preclinical and clinical studies were performed in the last years targeting MDSCs in cancer patients [see for a review [147]]. The list of ongoing clinical trials includes patients with chronic myeloid leukemia, NSCLC, hepatocellular carcinoma, metastatic and recurrent renal cell cancer, glioblastoma, sarcoma, and many others [147]. Inhibition of tumor growth and survival prolongation is obtained modulating MDSCs by three main ways: (1) inhibiting immunosuppressive activity of MDSCs, using STAT3 inhibitors [148, 149], phosphodiesterase type 5 (PDE5) inhibitors [150], and class I histone deacetylase [151, 152]; (2) blocking MDSCs recruitment to the tumor site, by, for example, CCR5 antagonists and IL-18 or CCL2 inhibitors [153, 154]; and (3) regulating myelopoiesis and depletion of MDSCs in the tumor-bearing hosts, thanks for instance to the treatment with all-trans-retinoic acid (ATRA), tyrosine-kinase inhibitors, and chemotherapeutic agents [147, 155–157].

Blockade of the activation of STAT3 by several STAT3 oligonucleotide inhibitors, in particular AZD9150, reduced MDSCs and Treg. AZD9150 is used to improve therapeutic efficacy in combination with ICI in different phase I/II clinical trial [149].

Another possibility to target MDSCs is through the modulation of their metabolic pathways. Recent studies showed that tumor-infiltrating MDCSs utilize FAO as primary source of energy along with increased mitochondrial mass and high rate of oxygen consumption [140]. Pharmacological inhibition of FAO, in combination with low-dose chemotherapy and adoptive cellular therapy, decreased production of inhibitory cytokines by MDSCs, thus reducing their immunosuppressive effects and inducing a significant anti-tumor effect [140, 158]. Finally, the development of multifunctional nanoparticle systems for effective targeting of MDSCs is a novel strategy for the manipulation of these cells [159].

Since MDSCs, opposite of Treg, are not present in steady-state conditions, their targeting has possibly no side-effects. In addition, targeting MDSCs in combination with ICI, antagonists, or chemotherapeutic reagents is more effective in tumor growth inhibition [142, 146, 155]. Several trials are ongoing where ICI are associated with strategies to target MDSCs. For instance, immune checkpoint blockade (ICB) with anti-CTLA-4 or anti-PD1/PD-L1 showed de novo resistance in metastatic castration-resistant prostate cancer (mCRPC) therapy, while combination of ICB agents and neutralizing agents that targets MDSCs could preserve T-cell function and showed robust synergetic response in mCRPC treatment [160].

Entinostat, a class I histone deacetylase inhibitor, inhibited the immunosuppressive function of both PMN-MDSCs and M-MDSCs and, in combination with anti-PD-1 antibodies, significantly increased survival and delayed tumor growth in mice with lung and renal cancer [151].

Given that the number of MDSCs correlates with tumor progression and low success of immu-

notherapy, an efficient personalized medicine is important to define the presence of all the different MDSCs subsets and the mechanisms through which they can suppress the anti-tumor response [122, 155]. Along this line, the development of strategies to identify the different subsets of MDSCs and distinguish these cells from neutrophils and macrophages is a challenge for developing and expanding the existing panels of markers.

3.5 Concluding Remarks

The TME exerts essential roles in the development and regulation of tumor growth. Innate immune cells, in particular TAMs, TANs and MDSCs, are integral components of TME. It is expected that innate immune cells exert a role in promoting the immune response against tumor growth; however, data collected over the years demonstrate that cancer cells can subvert the anti-tumoral properties of innate immune cells. TAMs exposed to the TME acquire an M2 phenotype and became the major orchestrator of CRI. TAMs promote survival and proliferation of cancer cells, angiogenesis, cancer cell migration, and invasion [2, 3, 7, 11, 20]. Neutrophils-infiltrating tumors are more immature cells exerting pro-tumorigenic properties and expressing factors promoting angiogenesis, facilitating an immunosuppressive TME, and increasing cancer dissemination [55–58]. Finally, accumulating evidences demonstrated that MDSCs play a pivotal role in the immunosuppressive TME and correlate with tumor progression [122, 155].

All together, these observations strongly suggest that targeting tumor-infiltrating myeloid cells represents a promising therapeutic tool against cancer, and recent or ongoing efforts further support this hypothesis.

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4 B-Cells in Cancer Immunology: For or Against Cancer Growth?

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4.1 Introduction

In the 1960s, B-cells were first defined in birds when researchers found that removal of the bursa in newly hatched chicks severely impaired the ability of the adult birds to produce Abs [1, 2]. A decade later, it was found that mammalian B-cells are derived from bone marrow and develop into plasma cells that are the source of antibodies (Abs). Over the years, most studies on B-cell function in immune response have focused on antigen presentation and antibody production. However, recent advances in B-cell biology have capitalized on old findings and demonstrated that B-cells can also act as effector cells or as regulatory cells [3, 4].

B-cells are often overlooked in tumor immunology, likely because of the common notion that humoral and cytolytic responses work in opposition. The field of tumor immunology has focused on $CD8^+$ T-cells due to their ability to directly kill tumor cells, as well as the close association between tumor-infiltrating $CD8^+$ T-cells and cancer patients' survival [5]. To date, the role of B-cells in tumor immunity has remained largely elusive. Results from different research groups are somewhat controversial. In this chapter, we review the roles of B-cells in tumor immunology, which may either positively or negatively affect tumor growth and patient outcomes.

4.2 CD40-Activated B (CD40-B) Cells

CD40-activated B (CD40-B) cells are thought to be an excellent source of professional antigen-presenting cells (APCs) for antigen-specific tumor immunotherapy. They have demonstrated potent effects on cellular immunotherapy of cancers [6–17]. CD40-B-cells induce potent expansion of antigen-specific $CD4^+$ and $CD8^+$ T-cells, including naïve $CD8^+$ T-cells [6–9, 12, 16]. One reason that dendritic cells (DCs) are considered as excellent APCs in tumor immunotherapy is that they can powerfully prime naïve T-cells, while resting B-cells cannot. Resting B-cells poorly express costimulatory molecules, resulting in immune tolerance regarding the induction of naïve T-cells. Recent studies have shown that activation of mouse and human B-cells using CD40L in vitro upregulates the expression of major histocompatibility complex (MHC) I, MHC II, and costimulatory molecules on B-cells [6–9, 13, 14, 16]. These B-cells present exogenous antigens by MHC class I or II molecules and stimulate antigen-specific T-cells [7, 8]. CD40-B-cells induce T-cell proliferation, interferon-γ (IFN-γ) production, and specific cytotoxic T lymphocyte (CTL) responses [6–9, 11–15]. In mouse models, it has been shown that CD40-B-cells directly present antigen to naïve $CD8^+$ T-cells, in order to induce the generation of potent T effectors which are able to secrete cytokines and kill target cells [16]. Moreover, CD40-B-cells express the full lymph node homing triad CD62L, CCR7/CXCR4, and leukocyte function antigen-1 (LFA1), suggesting that they could co-localize with T-cells in the T-cell-rich areas of secondary lymphoid organs [11, 15]. This will facilitate CD40-B-cell and T-cell contact for antigen presentation.

Using a metastatic mouse model, Li et al. provided direct experimental evidence that the augmented antitumor activity by anti-CD40 monoclonal antibody (mAb)-stimulated tumor-draining lymph node (TDLN) cells requires the presence of APCs, e.g., B-cells as well as DCs. They found that anti-CD40 mAb augments antitumor responses of TDLN cells via ligation to CD40 on both B-cells and DCs [17].

Typically, TDLN cells are composed of approximately 60% $CD3^+$ T-cells, 30% $CD40^+$ B-cells, and 5% DCs. In a murine sarcoma model, anti-CD3−/anti-CD40-activated MCA205 TDLN T-cells secreted significantly higher amount of IFN-γ in an antigen-specific manner (in response to MCA205 tumor, but not to MCA 207 tumor), in comparison with solely anti-CD3-activated TDLN T-cells (Fig. 4.1a). However, when B-cells were depleted from MCA205 TDLN cells, anti-CD3/anti-CD40 activation could not increase the IFN-γ anymore. This effect is very similar to DC depletion (Fig. 4.1a). In vivo, adoptive transfer of anti-CD3−/anti-CD40-activated MCA205 TDLN T-cells mediated significantly higher MCA205 tumor regression in a pulmonary metastasis setting, compared to anti-CD3-alone-activated TDLN T-cells (Fig. 4.1b). However, B-cell removal significantly reduced the therapeutic efficacy con-

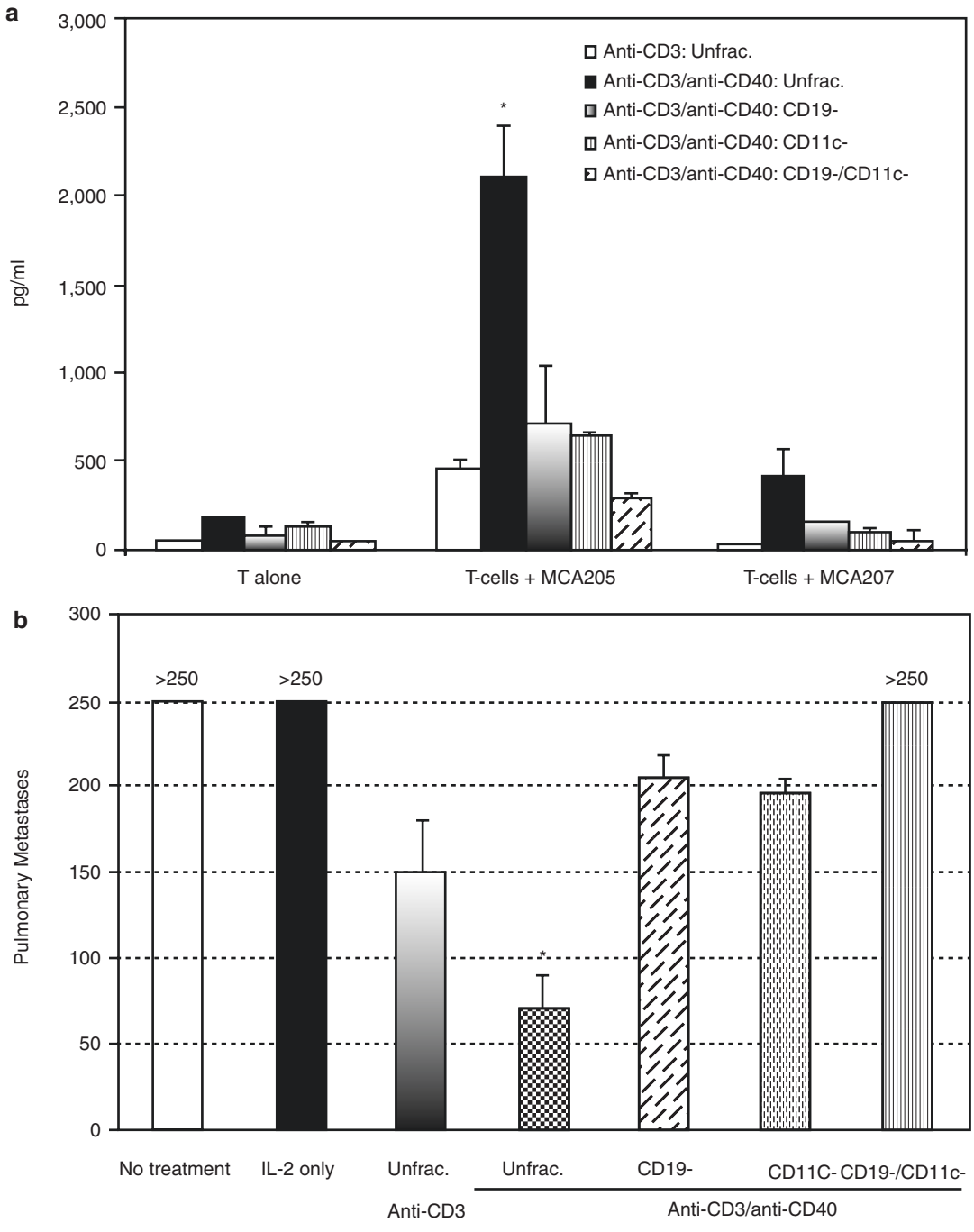

Fig. 4.1 Anti-CD40 mAb augmented antitumor responses of anti-CD3-activated TDLN cells via ligation to CD40 on both B-cells and DCs. (**a**) Activated total unfractionated (Unfrac) TDLN cells were co-cultured with MCA 205 vs. MCA 207 tumor cells to determine IFN-γ production. B-cells were removed by CD19 depletion ($CD19^-$), and DCs were removed by CD11c depletion ($CD11c^-$). (**b**) Activated total TDLN (Unfrac) cells or B-cell, DC-depleted TDLN cells ($CD19^-$ and/or $CD11c^-$) TDLN cells adoptively transferred into tumor-bearing mice for therapy. $*p < 0.05$ compare with any other group in (**a**, **b**), respectively (adapted by permission from the American Association of Immunologists, Inc. Copyright 2005: Li et al. [17])

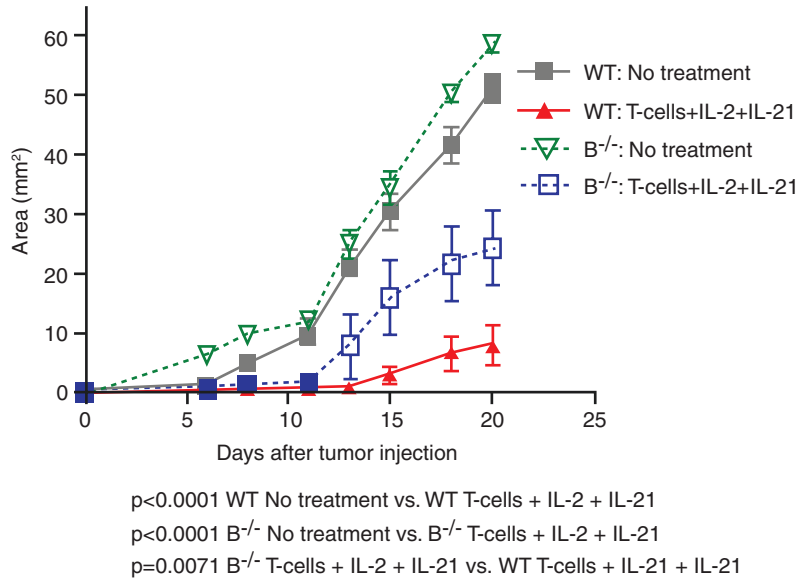

Fig. 4.2 Requirement for host B-cells in T-cell transfer + IL-2 and IL-21 administration-elicited antitumor immunity (adapted by permission from the American Association for Cancer Research: Iuchi et al. [18])

ferred by CD40 engagement, and so did DC removal. Together, these studies indicate that B-cells, as well as DCs, are required in the generation of potent antitumor T effector cells from TDLN cells via simultaneous targeting of CD3 on T-cells and CD40 on B and dendritic cells.

In a separate study, Iuchi et al. reported that host B-cells were required for adoptive transferred T-cells to mediate optimal antitumor immunity [18]. Tumor-bearing mice were treated with adoptive transfer of T-cells accompanied with IL-2 and IL-21 administration in wild-type and B-cell knockout ($B^{-/-}$) animals, respectively. They found that tumor growth inhibition was significantly diminished in the B-cell-deficient mice after T-cell + IL-2 + IL-21 combined therapy (Fig. 4.2).

In contrast to DCs, large numbers of B-cells can be obtained from the blood of patients after ex vivo expansion (up to 1000-fold) in the presence of CD40L [6]. For example, only about 10^6 DCs can be generated from 10 ml of blood, while 10^9–10^{10} B-cells can be produced from the same volume of the blood sample. Additionally, CD40-B-cells can be continuously expanded in long-term culture (>65 days) without the loss of APC functionality [6]. Therefore, CD40-B-cells have the advantage over DCs in terms of isolation, generation, and long-term expansion. These characteristics make CD40-B-cells a promising alternative as cell-based vaccines.

In current B-cell vaccine preparations, activated B-cells can be loaded with antigens by pulsing with peptides, proteins, tumor lysates, or by transfection with DNA or RNA, or transduction with viral vectors [9, 10, 19]. Coughlin et al. [9] loaded RNA on CD40-B-cells from pediatric patients. Vaccination using these B-cells resulted in simultaneous targeting of multiple antigenic epitopes and induced CTLs. Chung et al. [10] reported that B-cells stimulated with iNKT (CD1d-restricted invariant T-cells) ligand alpha-galactosylceramide (alphaGalCer) could directly prime CTLs and generate long-lasting cytotoxic antitumor immunity in vivo. Furthermore, Garbe et al. [19] reported that semi-allogeneic fusions of microsatellite instability (MSI) tumor cells with B-cells primed B-cells to induce MSI-specific T-cell responses.

4.3 Tumor Killer B-Cells

B-cells can directly kill tumor cells through antibody (Ab)-independent mechanisms [20]. Recent studies have shown that B-cells express death-

inducing ligands and can therefore mediate cell death under many circumstances. Evidence has emerged that B-cells express Fas ligand (FasL), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), programmed death ligands 1 and 2 (PD-L1 and PD-L2), and granzyme B (GrB), which are potentially involved in B-cell-mediated direct cytotoxicity against tumor cells [21–29].

Due to the well-known fact that B-cells can produce Abs which lead to CDC and ADCC, as well as the recent findings that B-cells may kill tumor cells directly through antibody-independent mechanisms, it is hypothesized that appropriately sensitized and activated B-cells can function as effector cells to mediate antitumor immunity. Indeed, Li et al. [30] proved that in vivo sensitized and in vitro activated B-cells could mediate tumor regression in cancer adoptive immunotherapy. In vivo sensitized TDLN cells were activated and expanded in vitro with LPS/anti-CD40, resulting in B-cell proliferation and differentiation. These activated B-cells were then adoptively transferred into tumor-bearing recipients for therapy. These tumor-primed and tumor-activated B-cells significantly reduced lung metastases in an adoptive immunotherapy model (Fig. 4.3). Furthermore, total body irradiation (TBI) could enhance the antitumor activity of the adoptively transferred B-cells. This study represents one of the early studies demonstrating that effector B-cells could confer antitumor immunity after adoptive transfer into tumor-bearing mice [30].

Using a murine 4T1 pulmonary metastatic model, it was found that adoptive transfer of 4T1-primed and LPS−/anti-CD40-activated TDLN B-cells significantly inhibited 4T1 pulmonary metastasis in tumor-bearing mice [31] (Fig. 4.4). The efficacy mediated by B-cells was comparable to that mediated by an equal number of T-cells, which served as a positive control in the experiment (Fig. 4.4a). Of note, adoptively transferred 4T1 TDLN T + B-cells mediated inhibition of the spontaneous pulmonary metastasis of 4T1 in a dose-dependent manner (Fig. 4.4b).

This study also showed that activated 4T1 TDLN B-cells caused tumor cell lysis directly in vitro in the absence of Ab and other effector cells and this direct cytotoxicity was tumor specific (Fig. 4.5). In these experiments, 4T1 mammary carcinoma murine tumor-primed TDLN B-cells were activated with LPS and anti-CD40 mAb, washed thoroughly, and then co-cultured with 4T1 tumor cells or irrelevant tumor controls, Renca (renal cell carcinoma) and TSA (sarcoma).

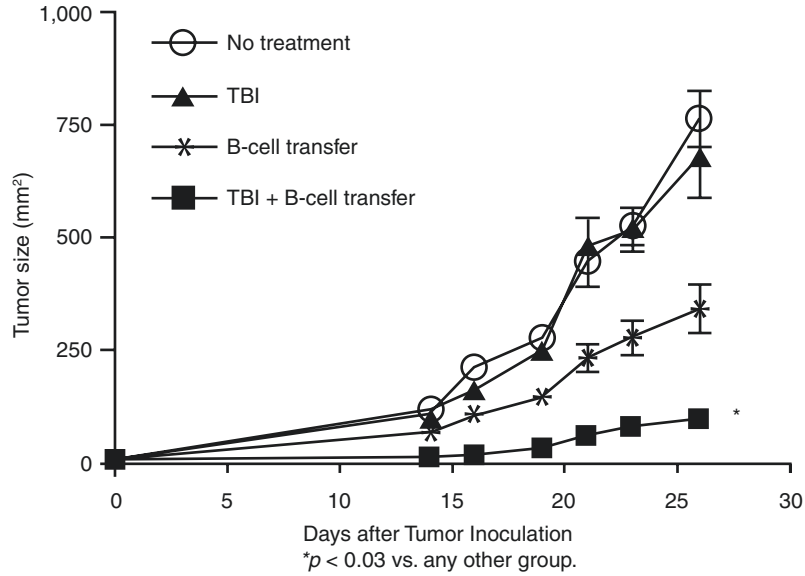

Fig. 4.3 TBI (total body irradiation) significantly augmented the therapeutic efficacy of adoptively transferred B-cells in the s.c. D5 tumor model (adapted by permission from the American Association of Immunologists, Inc. Copyright 2009: Li et al. [31])

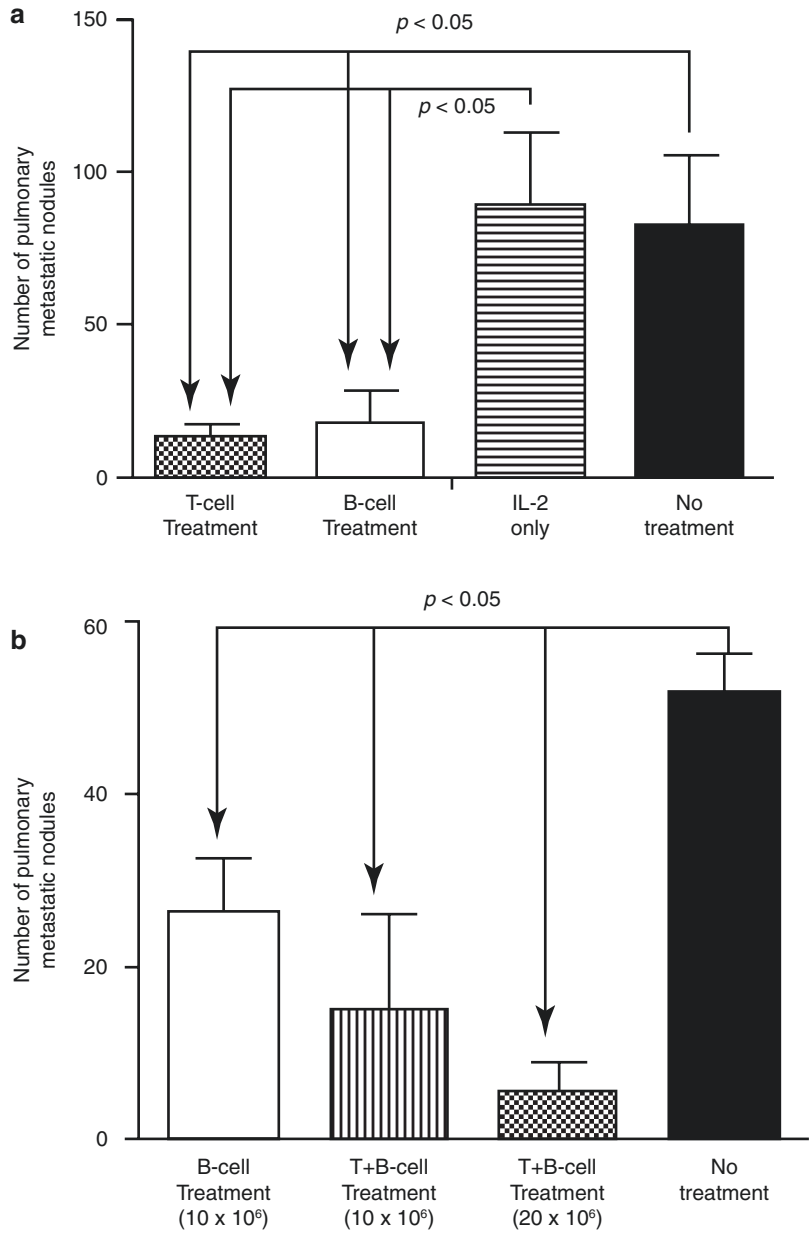

Fig. 4.4 (**a**) Adoptively transferred 4T1 TDLN B-cells mediated effective inhibition of the spontaneous pulmonary metastasis of 4T1 breast cancer cells similarly to equal numbers of T-cells. (**b**) adoptively transferred 4T1 TDLN T + B-cells mediated inhibition of the spontaneous pulmonary metastasis of 4T1 better then B-cells alone, and the efficacy was dose dependent (adapted by permission from the American Association for Cancer Research: Li et al. [31])

The effector B-cells killed 4T1 cells directly in a dose-dependent way and were significantly more effective than their killing of the control tumors. These data support the conclusion that tumor antigen-primed and in vitro activated B-cells are able to kill tumor cells independent of Ab or complement. However, the mechanism(s) by which the killer B-cells lyse tumor cells directly in such a setting remains to be identified.

In line with these findings, Kemp et al. demonstrated that CpG-A oligodeoxynucleotide (CpG-A ODN) stimulation of human PBMCs leads to high

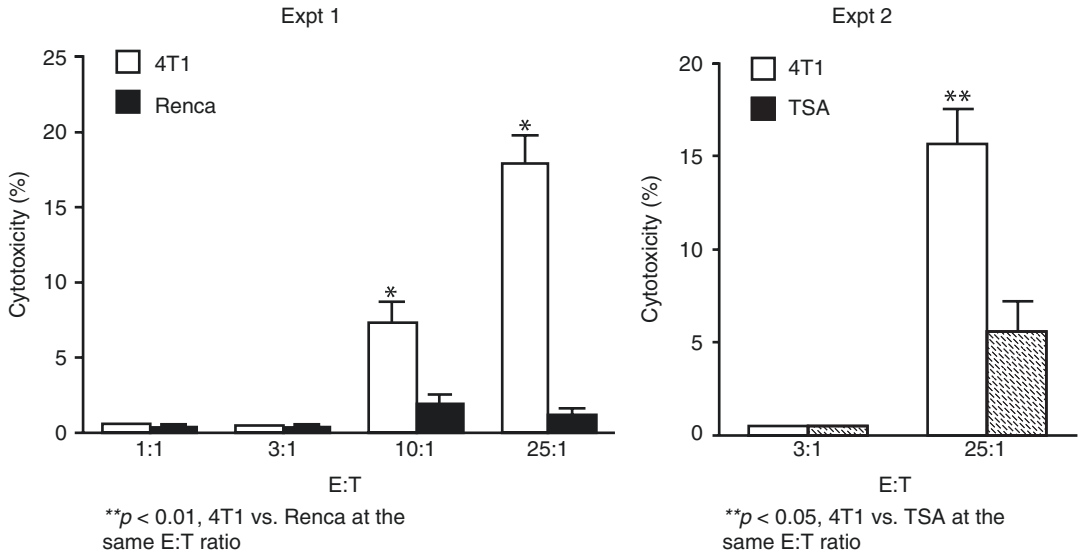

Fig. 4.5 Activated 4T1 TDLN B-cells mediate direct and tumor-specific cytotoxicity of 4T1 cells (adapted by permission from the American Association for Cancer Research: Li et al. [31])

levels of functional TRAIL/Apo-2L expression on B-cells, and these B-cells mediate TRAIL−/Apo-2L-dependent tumor cell lysis [25].

Additional studies support the observation that B-cell can function as effector cells in antitumor responses. For example, Penafuerte et al. reported that B effector cells activated with a chimeric protein consisting of IL-2 and the ectodomain of TGF-β receptor II (also known as FIST) induce potent antitumor immunity [32]. In this study, the B effector cells were characterized by the production of TNFα and IFN-γ and potent antigen presentation properties [32]. In addition, Forte et al. found that administration of a specific CD73 inhibitor, adenosine 5′-(α, β-methylene) diphosphate (APCP), to melanoma-bearing mice induced significant tumor regression [33]. They observed that after APCP administration, the presence of B-cells in the melanoma tissue was more than that observed in control mice. This was associated with the production of IgG2b within the melanoma, implying a critical role for B-cells in the antitumor activity of APCP [33]. Together, these studies suggest that the mechanisms underlining B-cell-mediated antitumor immunity may involve multiple cellular and molecular events, as well as direct killing of the tumor cells.

4.4 Tumor-Infiltrating B-Cells (TIL-Bs) in Cancer

Tumor-infiltrating B-cells (TIL-Bs) have revealed controversial roles in antitumor immunity. They have been found in breast cancer [34–36], ovarian cancer [37], lung cancer [38], colorectal cancer [39, 40], cervical cancer [41], cutaneous melanoma [42], and prostate cancer (CaP) [43]. A few studies have indicated that TIL-Bs are correlated with favorable survival of patients [36, 37, 42, 44, 45], lower relapse rate [41], or low metastasis [42]. In a study on immune infiltrates in high-grade serous ovarian cancer, it was revealed that intraepithelial $CD20^+$ TIL-Bs are associated with increased disease-specific survival [37]. Importantly, the association between the immune infiltrates and survival

was dependent on histological subtype, because immune infiltrates were less prevalent in the other histological subtypes compared to the high-grade serous cases [37]. In breast cancer, TIL-Bs are present in about 24% of tumors and comprise up to 40% of the lymphocytic infiltrates [34]. TIL-Bs have been shown to undergo antigen-driven clonal proliferation and affinity maturation in situ [35]. Very recently, in a large patient cohort of different histological and biological subtypes, Mahmoud and colleagues provided evidence for a favorable outcome when high numbers of $CD20^+$ TIL-Bs were present [36]. Additionally, TIL-Bs may be involved in humoral immune response in situ. Using recombinant Ab cloning techniques, Hansen et al. reported an antigen-driven humoral immune response directed against β-actin exposed on apoptotic mammary carcinoma cells [46]. Yasuda and coworkers [47] identified TIL-Bs which produce tumor-specific Abs against mutated p53. Maletzki et al. [40] also reported that TIL-Bs from colorectal carcinoma show an activated immunophenotype ($CD23^+$, $CD80^+$) and produce IgGs that specifically bind to allogeneic target tumor cells.

On the other hand, TIL-Bs may produce cytokines contributing to tumor development. It has been reported that TIL-Bs in castration-resistant CaP produce lymphotoxin by an inflammation-responsive IκB kinase (IKK)-β-dependent pathway, which then in turn activates IKK-α and STAT3 in tumor cells to enhance hormone-free tumor survival [43]. In this study, B-cell infiltration was detected in 100% of human CaP samples, while B-cells were undetectable in normal prostate or benign prostatic hyperplasia [43]. Castration-resistant CaP growth was delayed in mice reconstituted with bone marrow from $JH^{-/-}$ mice, which lack mature B-cells [43]. It was further found that these CaP allografts exhibited IKK-α nuclear translocation, which was dependent on IKK-β in B-cells. IKK-β deletion abolished lymphotoxin expression by B-cells. When lymphotoxin-β was ablated in B-cells, growth of castration-resistant CaP was delayed. Similarly, another study showed that tumor-infiltrating T and B-cells were not associated with long-term survival of patients with non-small-cell lung cancer [38].

The roles of TIL-Bs may be complicated, since the tumor environment is dynamic and changes during tumor onset and progression. TIL-Bs need to contact other immune cells or tumor cells to be activated or regulated, so their contributions to immune responses are likely to vary in different cancers and during the course of cancer.

4.5 Resting B-Cells and Regulatory B-Cells in Cancer

In contrast to activated B-cells, there is abundant evidence indicating that resting B-cells can promote the development or progression of cancer. Resting B-cells are small B-cells in the G0 stage of cell cycle, prior to activation. Studies have shown that B-cell-deficient mice exhibit enhanced T-cell antitumor activity and significant improvement in survival rate [48–52]. It has been reported that there are increased effector T-cells [48], increased T-cell infiltration of tumors [52], higher Th1 cytokine and antitumor CTL response [49, 51, 52], and even reduced T regulatory cell (Treg) frequencies [53] in these B-cell-deficient mice. Some studies explored the possible mechanisms involved. B-cells present in the priming phase result in disabled $CD4^+$ T-cell help for CTL-mediated tumor immunity [51]. B-cells produce IL-10 which can repress antitumor immunity [49, 54]. Similarly, Abs were shown to promote primary tumor formation in a transgenic mouse model of inflammation-associated carcinogenesis [55]. Autoantibody responses to self-proteins triggered by cancer vaccines may influence the efficacy of vaccination [56]. Additionally, B-cells have been shown to have other pro-tumorigenic roles. For example, enhanced NK cell antitumor activity has been reported in B-cell-deficient mice [48, 50, 52]; however, the mechanisms are poorly understood.

We hypothesize that the effects of B-cells on antitumor immunity depend on the presence of B-cell subsets mainly involved under certain tumor conditions. In the past two decades, investigators have identified B-cell subsets which are capable of suppressing the immune response. Suppression of an immune response was first reported in 1974 where spleen B-cells were found to impair delayed-type hypersensitivity (DTH) responses in guinea pigs [57, 58]. This finding led to the conclusion that DTH responses and T-cell function can be regulated by suppressor B-cells. Subsequently, convincing data have demonstrated that IL-10-producing B-cells, termed regulatory B-cells (Bregs) by Mizoguchi et al. [59], can suppress inflammatory responses in experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), and colitis [59–61]. Recently, Bregs and their potential immunomodulatory activities have been examined in several immune-related diseases. In the majority of these studies, the function of Bregs is dependent on IL-10 production, whereas the mechanisms are still undefined partly because of conflicting results regarding the phenotypic characterization of IL-10-producing cells. For example, the following B-cells have been reported as putative mouse Bregs: $CD1d^{high}$ subset of B-cells in chronic colitis in TCRα-deficient mice [59], $CD21^{high}CD23^{low}$ B-cells in contact hypersensitivity (CHS) mouse model [62], $CD21^{high}CD23^{high}$ T2-MZ precursor B-cells in CIA model [63], $CD1d^{high}CD5^{+}$ B-cells (termed B10 cells by Yanaba et al.) in CHS [64] and EAE models [65], $CD138^{+}CD19^{+}$ plasmablasts in *Salmonella typhimurium* infection [66], and T-cell Ig domain and mucin domain protein (TIM)-1^{+} B-cells [67]. For human, $CD19^{+}CD24^{hi}CD38^{hi}$ B-cells have been found as putative Bregs [68, 69].

Triggering Toll-like receptors (TLRs) [70–72], the BCR [64], CD40 [73], or combinations thereof have been shown to promote IL-10 production by B-cells. BCR-mediated Ca^{2+} flux appears to be required for IL-10 production, since B-cells deficient in the calcium sensors stromal interaction molecule (STIM) 1 and STIM2 have a profound defect in IL-10 secretion and abrogated suppression abilities in vivo [74]. Nuclear factor of activated T-cells (NFAT) 1, a transcription factor, is involved in Ca^{2+}-dependent IL-10 production [74]. Therefore, their proposed model for IL-10 production by B-cells is that, after BCR stimulation, STIM and Orai-dependent Ca^{2+} increase by store-operated Ca^{2+} entry (SOCE) activates calmodulin/calcineurin and then NFAT1, leading to IL-10 expression. In addition, the TLR signaling pathway is also required for IL-10 secretion [70–72]. Given that TLR stimulation does not induce Ca^{2+} mobilization in B-cells, crosstalk between Ca^{2+} and Ca^{2+}-independent TLR cascades may be involved in IL-10 production.

IL-10 is an immunomodulatory cytokine and inhibits Th1 polarization, prevents Th2 responses, and suppresses pro-inflammatory cytokine production by monocytes and macrophages [75]. So far, the potential role of Bregs in tumor immunology is not clear, but several studies suggest that Bregs can negatively regulate antitumor immunity. Using a mouse chemical carcinogenesis model, Schioppa et al. found that resistance to papilloma development in $Tnf^{-/-}$ mice was associated with a significant reduction in IL-10-producing B regulatory cells alongside an increase in IFN-γ-producing $CD8^{+}$ T-cells in the spleen [54]. In this study, $Tnf^{-/-}$ mice were resistant to chemical carcinogenesis of the skin. LPS-stimulated $CD19^{+}$ B-cells isolated from $Tnf^{-/-}$ mice produced less IL-10. These mice had a reduced absolute number of $IL\text{-}10^{+}CD19^{+}$ B-cells in their spleens, and $Tnf^{-/-}$ mice were deficient for $CD19^{+}CD21^{high}$ B-cells. The authors speculated that resistance to carcinogenesis in $Tnf^{-/-}$ mice may result from increased $CD8^{+}$ IFN-γ-producing T-cells and decreased IL-10-producing B-cells. In another study, Horikawa et al. reported that production of IL-10 by Breg inhibits lymphoma depletion during CD20 immunotherapy in mice [76]. They found that

adoptive transfer of CD1d^{high}CD5^{+} B-cells (that are enriched for B10 cells) eliminates the therapeutic benefit of CD20 mAbs in mouse lymphoma model. The transferred B10 cells in this model downregulated the expression of MHC II molecules and CD86 on macrophages and reduced LPS-induced nitric oxide and TNF-α production by macrophages, indicating that B10 cells suppress the antitumor response at least partly by downregulation of macrophage activity. Our unpublished data support that Bregs play a negative role in antitumor immunity. In melanoma and breast carcinoma models, depletion of IL-10-producing B-cells from TDLN cells resulted in the generation of potent effector B-cells which dramatically inhibit tumor metastasis after adoptive transfer in two genetically distinct immune competent hosts, B6 and Balb/c mice, respectively.

Although little is known about the mechanisms by which Bregs undermine effective antitumor immunity, several possibilities are suggested by studies on inflammation and autoimmunity. Bregs impair Th1 immune responses. The initial finding about Th1 response regulated by Bregs was reported by Skok et al. [77]; they found that IL-10 produced by B-cells is involved in the feedback regulation of Th1 development. It has been reported that Bregs suppress the Th1 cell-mediated immune reactions in a number of mouse models, including EAE, CIA, CHS, and diabetes mellitus [60, 61, 64, 65, 72, 78, 79]. Fillatreau et al. [60] reported that B-cell IL-10 deficiency correlates with enhanced type I autoreactivity; in addition, transfer of IL-10^{+} B-cells was found to result in resolution of EAE, characterized by enhanced encephalitogenic Th1 response. Later, Lampropoulou et al. [72] showed that TLR signaling in B-cells suppresses inflammatory T-cell responses (both Th1 and Th17) and stimulates recovery from EAE. Similarly, using mouse model of CIA, Mauri et al. showed that transfer of IL-10-producing B-cells inhibits T helper type 1 differentiation and prevents arthritis development [61]. Yanaba et al. [64] also revealed that CD1d^{high}CD5^{+} B-cell transfer normalized inflammation in CHS model. Using NOD mouse model of type 1 diabetes (T1D), Hussain et al. found that BCR-stimulated B-cells produce IL-10 and attenuate islet inflammation by polarizing CD4^{+} T-cell response toward a Th2 phenotype [79].

Bregs induce the differentiation of Tregs. Given that μMT$^{-/-}$ B-cell-deficient mice display reduced Treg frequencies in comparison with wild-type mice [53] and that these mice develop exacerbated EAE and Ag-induced arthritis (AIA) [60, 80], a role for Bregs in modulating Tregs was proposed. Several disease models have demonstrated that IL-10 produced by Bregs is important for the generation and/or maintenance of Tregs. Sun et al. reported that after oral tolerance induction, Treg cells increase much more in WT than in μMT$^{-/-}$ mice. However, adoptive transfer of B-cells before treatment normalized Treg cell development in μMT$^{-/-}$ mice [81]. In this study, they found that sublingual tolerization with OVA/CTB (Ag conjugated to cholera toxin B subunit) enhances the tolerogenic activity of B-cells and their production of IL-10, which was associated with the generation of Ag-specific Foxp3^{+}CD25^{+}CD4^{+} Tregs [81]. This relationship between Bregs and Tregs is further supported by the results from mouse models of airway sensitization. These results showed that Bregs prevent and reverse allergic airway inflammation via FoxP3^{+} T regulatory cells [82, 83]. Additionally, Bregs can induce the differentiation of T regulatory 1 (Tr1) cells [84–86]. Gray et al. [84] reported that autoimmune inflammation could be protected by the induction of Bregs which induce T-cell-derived IL-10. Blair et al. [86] used the transitional 2 immature (T2) B-cells stimulated with agonistic anti-CD40 (T2-like Bregs) to convert autologous effector T-cells into Tr1 cells. Sayi et al. [85] also showed that B-cells activated by *Helicobacter* TLR-2 ligands produce IL-10 and induce IL-10-producing CD4^{+}CD25^{+} Tr1 cells

Table 4.1 Phenotypic characterization of B-cell subpopulations

	Marker		Source	References
Resting B-cells	Human	$CD19^+CD38^-IgD^+CD27^-$	Tonsils	[87, 88]
		$CD38^-IgM^+IgD^+CD27^-$	Blood	[88]
	Mouse	$IgM^{low}IgD^{high}HSA^{low}CD21^{int}CD23^{bright}Mel14$ $(CD62L)^{bright}CD44^{int}CD69^-$	Lymph node	[89]
		$IgM^{high}IgD^{high}CD23^{bright}$	Spleen	[90]
CD40 B-cell	Human	$CD19^+CD38^+CD80^+CD86^+CD71^+$ $CD95^+CPM(carboxypeptidase\text{-}M)^+$	Tonsils	[87]
		$CD19^+CD23^+CD54^+CD58^+CD80^+$ $CD86^+MHCI^{high}MHCII^{bright}$	Blood	[6]
	Mouse	$B7.1^{high}B7.2^{high}ICAM^+MHCI^{high}$ $MHCII^{bright}$	Spleen	[90, 91]
Putative Breg	Human	$CD19^+CD24^{high}CD38^{high}$	Blood	[68, 69]
	Mouse	$B220^+CD1d^{high}CD21^{intermediate(int)}$ $CD62^{low}IgM^{int}CD23^{high}$	Lymph nodes[a]	[59]
		$B220^+CD21^{high}CD23^{low}$	Spleen in CHS model	[62]
		$B220^+CD21^{high}CD23^{high}$ $IgM^{bright}CD1d^{high}$	Spleen in CIA model	[63]
		$CD1d^{high}CD5^+$ $CD19^+$ $B220^+$	Spleen in CHS model	[64]
		$CD1d^{high}CD5^+$ $CD19^+$	Spleen in EAE model	[65]
		$CD138^+CD19^+$	Spleen of mice infected with *Salmonella*	[66]
		TIM-1(T-cell Ig domain and mucin domain protein)$^+CD19^+$	Spleen	[67]
TIL-Bs	Mostly unknown. Related to cancer types and progression			
	Human	$CD19^+CD20^+$ $CD23^+CD80^+$	From colorectal carcinomas	[40]
Killer B	Unknown			

[a]From TCRα-deficient mice

depending on TCR signaling and a direct T-B-cell interaction through CD40/CD40L and CD80/CD28 pathways.

4.6 Concluding Remarks

B-cells are phenotypically and functionally heterogeneous. Characterization of B-cell subpopulations is shown in Table 4.1. B-cells play multiple roles in tumor immunity (Fig. 4.6). On the one hand, accumulating literature indicates that B-cells are significantly involved in antitumor responses. In this regard, B-cells present tumor antigens to T-cells to generate antitumor CTLs. Upon tumor antigen stimulation, B-cells can differentiate into plasma cells to produce antibodies to target tumor cells via ADCC and/or CDC. In addition, B-cells may act as killer cells to directly cause tumor cell lysis in the absence of antibodies. B-cells migrate to tumor tissue and become TIL-Bs which may induce humoral immune response or act as killer cells in situ. On the other hand, regulatory B-cells have been described which downregulate antitumor responses by producing immunomodulatory cytokine IL-10, suppressing Th1 immune responses, and enhancing Treg and Tr1 responses. Further characterization of B-cell subsets responsible for these conflicting functions demonstrated in tumor immunity and understanding of the molecular mechanisms involved would help

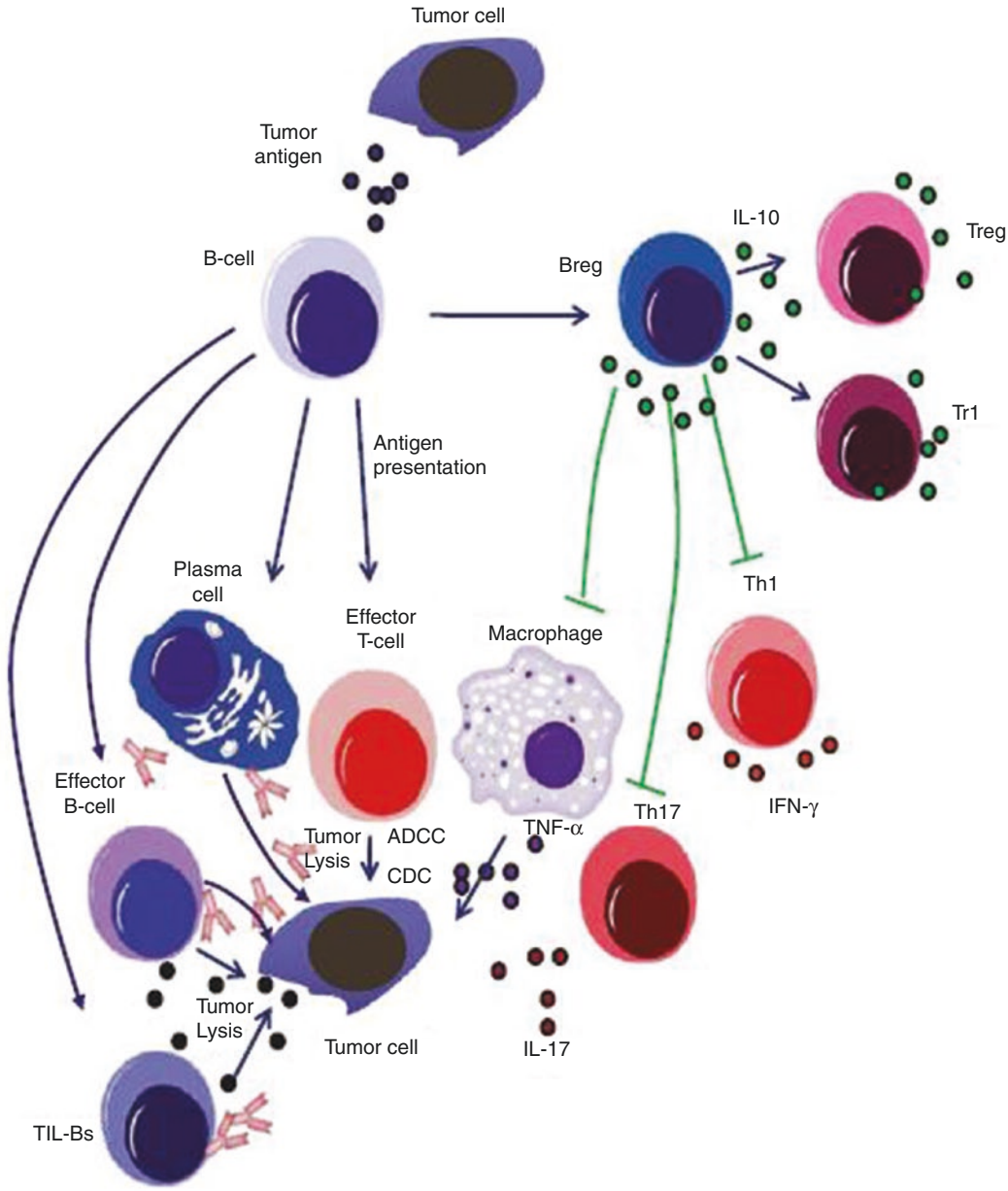

Fig. 4.6 Potential roles played by B-cells in tumor immunity. *ADCC* antibody-dependent cellular cytotoxicity, *CDC* complement-dependent cytotoxicity, *TIL-Bs* tumor-infiltrating B-cells

develop novel clinical strategies for cancer immunotherapy.

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Competing Interests The authors have declared that no competing interest exists.

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The Roles of CD4+ T-Cells in Tumor Immunity

5

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5.1 Introduction

The immune system is not only responsible for the specific defense against pathogens like viruses, bacteria, and parasitic worms but is also involved in cancer prevention and suppression. In the past few decades, it has been accepted that both innate and adaptive immune systems contribute to the early detection and regression of tumors. Crosstalk between these two arms may be a requisite factor for the initiation of efficient and optimal immune responses against the tumors. Till date, the majority of studies has focused on the responses of CD8+ cytotoxic T lymphocyte (CTL) or dendritic cells (DCs) against tumor-associated antigens presented by major histocompatibility complex (MHC) Class I. However, a significant number of studies have demonstrated that cancer cells might evade these cells through down-regulation/loss of MHC class I. Interestingly, CD4+ T-cells could well respond to MHC Class I negative tumors—those resistant to CTL lysis. Additionally, evidence has emerged that successful elimination of tumors required the cooperation of CD4+ and CD8+ T-cells, and CD8+ fail to adequately function in the dearth of adequate help from CD4+ T-cells [1]. This has highlighted the critical roles these cells [2]. In other words, CD4+ T-cells contribute in facilitating the initial activation and expansion of CD8+ T-cells directly (CD40–CD154 interaction) and indirectly (IL-2 production). Apart from the recruitment of CD8+ T-cell responses, some unconventional effects of CD4+ T-cells in tumor rejection have been suggested, including the cytotoxic effect on tumor cells, upregulation of the expression of MHC molecules, inhibition of angiogenesis, and induction of tumor dormancy (reviewed in [3]).

Effector CD4+ T-cells can be divided into multiple types. In addition to the classical T-helper (Th)1 and Th2—which were considered the only two Th cells for a long time—other subsets have been discovered in recent decades. The newly identified Th cells include Th17, follicular helper T (Tfh) cell, Th9, and Th22, each with a characteristic cytokine profile and individual transcription factors. Apart from the aforementioned effector cells, various subsets of CD4+ regulatory T-cells (Tregs) with suppressive function had been identified, including natural Tregs (nTregs), interleukin (IL)-10-producing type 1 Tregs (Tr1), and transforming growth factor (TGF)-β-producing Th3 cells.

Our understanding of the critical role of CD4+ T-cells in orchestrating immune responses during cancer has grown dramatically in recent decades. In the past decades, several studies have been conducted, implying the presence of different subtypes of T-cells in the peripheral blood of cancer patients as well as their tumor biopsies. These studies conducted have shown that the immune system and developing tumors are intimately intertwined. Hence, awareness of the exact roles of critical cells that contribute to cancer is required for the emergence of a novel therapeutic strategy for cancer treatment. Although all the CD4+ T-cells subsets are present in the tumor site, they do not contribute equally. Moreover, some of these cells may have both positive and negative roles during the process of tumor eradication. It is accepted that some particular types of CD4+ T-cells—specifically Th1 cells—can effectively eradicate or arrest the growth of large, established tumors. There is some evidence of the direct negative role of some effector subsets of Th cells, such as Th17, in certain types of cancer. Moreover, some other subgroups, such as Th2 cells, could inhibit the differentiation of Th1 cells, one of the

most potent players in anti-tumor immunity. Aside from effector T-cells that contribute to the initiation of tumor-specific immunity, Tregs have been proven to play a role an active and significant role in the progression of cancer, probably through the suppression of anti-tumor mediated responses. These cells have central roles in the maintenance of self-tolerance in healthy individuals, which protect them from developing autoimmunity. However, a growing body of evidence suggests that the regulation of effector T-cell by the Tregs causes impairment of immune response against tumor cells. In other words, CD4+ Tregs hamper the function of antigen-specific T-cells that recognize tumor antigens and maintain T-cell tolerance to self-antigens. Hence, the elimination of Tregs in cancer patients—and within the tumor microenvironment, in particular—seems essential for a successful cancer therapy [4]. Despite the initiation of anti-tumor immunity by effector T-cells, tumor cells have evolved different tumor-immune escape mechanisms. This might explain the progression of tumor cells in the presence of T-cell-mediated immunity and defeat conventional cancer immunotherapy. Down-regulation of target antigen expression and antigen-presenting machinery and the promotion of regulatory responses in tumors are examples of primary tumor immune escape mechanisms. Considering the suppressive function of Tregs, and also a correlation of their accumulation in tumor sites with intratumoral angiogenesis [5], it can be expected that these cells contribute to promoting cancer through tumor immune escape and angiogenesis [4].

An understanding of the role of different subsets of CD4+ T-cells in the solid tumor and hematological malignancies has evolved rapidly over the past few years. Although the vast majority of those studies have provided several valuable findings related to roles of CD4+ T-cells in cancer, controversy remains in other studies. In this context, the exact role of each subset of effector CD4+ T-cells, as well as Tregs, may change current cancer therapeutic options when combined with our previous understanding of the functions of other cells (e.g., CD8+ T-cells) during cancer. Although the innate immune cells, humoral immune responses, and CD8+ T-cells contribute significantly to the initiation of anti-tumor immunity, in this chapter, the role of only CD4+ T-cells subsets will be discussed in detail.

5.2 Adaptive Immune Responses During the Cancer

For decades, recognizing and destroying tumor cells were conceived as one of the duties of the immune system. Generally, the immune system could be divided into two major arms, including the primitive innate immune system and the acquired or adaptive immune system. In the former arm, different critical immune cells provide protection against various pathogens and foreign agents. These cells include DCs, macrophages, granulocytes, mast cells, natural killer (NK) cells, gamma delta T-cells (γδ T-cells) cells, as well as natural killer T (NKT) cells. On the other side, the latter arm primarily consists of T lymphocytes and B lymphocytes, and also their associated mediators (e.g., cytokines and antibodies). Every day, a large number of cancer cells are detected with the cooperation of both innate and adaptive immunities. Regarding developed tumors, these two arms start working together against the cancer cells long before it becomes clinically apparent, but because of several possible reasons, failed to detect them well or destroy them. Considering several studies of increased susceptibility to tumor development in immunodeficient mice, the critical role of different components of both the innate and adaptive immune systems in preventing cancer can be better understood. The increased incidence of cancer in patients with some types of primary immunodeficiencies—as the results of dysregulation of the immune response (regardless of exposure to an infectious organism)—can confirm this context in humans [6]. Moreover, the higher prevalence of cancer among patients who use a variety of immunosuppressants, such as kidney transplant recipients, also imply the critical role of the immune system function in destroying cancer cells [7, 8]. The fact that the HIV/AIDS population appears to be more susceptible to almost all cancers also supports the role of the immune system, specifically

CD4+ T-cells, in prevention of cancer development [9]. There are some reported associations between the tumor infiltration by T-cells with an improved prognosis for different types of tumors, which highlight the pivotal role of the adaptive immune system during cancer [10, 11].

Thanks to the ability of a generation of a diverse repertoire of antigen receptor specificities via DNA recombination, adaptive immunity is able to recognize a great variety of different antigens, including exogenous (entering from outside) and endogenous (generated within the cells). Hence, it is expected that both the arms of the immune system rise against the newly developed cancer cells. Typical tumors usually contain all immune cell types related to both innate and adaptive immune systems, including macrophages, DCs, NK cells, mast cells, B-cells as well as several subsets of T-cells. Although the adaptive immune system elicits other cell responses as well as directly acting against the tumor cells, the dual nature of the adaptive immune system has made it a double-edged sword in cancer. Indeed, some individual subsets of lymphocytes try to impair immune responses. In a healthy condition, it is beneficial and could significantly prevent organ damage and also the development of autoimmunity. However, in the patients with cancer, impairment of immune system means allowing tumor cells to grow. In theory, it was expected that reversing the impaired immune responses might promote anti-tumor immunity. Interestingly, this theory was converted to reality after the first successful immunotherapy in cancer patients. Today, there are some limited checkpoint inhibitors, including PD-L1 inhibitors (atezolizumab, avelumab, and durvalumab), two PD-1 inhibitors (nivolumab and pembrolizumab), and a CTLA-4 inhibitor (ipilimumab), which have shown to be helpful in treating different types of cancer. Although cellular immunity is thought to be more critical than humoral immunity in cancer, there is mounting evidence to support the fundamental role of B-cells in tumor immunology. In fact, these types of cells could play different roles, which aid the shrinkage of the tumor. In addition to the classical functions of antibody production, these cells also could act as antigen presenting cells (APCs), provide co-stimulation, and also secrete cytokines that promote both innate and adaptive immune systems [12]. The role of B-cells in tumor immunity is beyond the scope of this chapter and will not be discussed.

5.3 T-Helper Cells Differentiation and Function

5.3.1 Overview of CD4+ T-Cell Subsets

T-cells are classically divided into two major groups, including CD8+ T-cells and CD4+ T-cells, which become activated when they are presented with MHC-I and MHC-II, respectively. Each of these groups contains effector and regulatory subsets, which act in opposite directions. CD4+ T-cells (also called Th cells) play critical roles in instigating and shaping adaptive immune responses. Th lymphocytes are prototypic members of T-cells, which augment both humoral and cellular immune responses. In addition to their roles in modulating immune responses to different types of pathogens and chronic infectious agents, they are capable of enhancing immunity against the tumors. These cells can act through activation of other tumor-specific cells or even direct recognition of antigen on MHC-II-expressing tumor cells, followed by hindering tumor growth or inducing tumor cell death. During an immune response, both the activation of naïve T-cells stimulus through the T-cell receptor (signal 1) and the interaction between appropriate costimulatory molecules (signal 2) are required. Receiving enough signals results in the robust clonal expansion of naïve T-cells and their differentiation into either effector or memory cells.

5.3.2 Differentiation

The identification and characterization of CD4+ T-cell lineage subsets began with a description of Th1 and Th2 cells [13]. Subsequently, different other subsets of effector CD4+ T-cells in the past 10 years—including Th17, Tfh, Th9, and Th22—have filled the gaps and deficiencies existing in

the previous simplistic Th1/Th2 paradigm. Moreover, another different subset, with a suppressor function, could be differentiated from the naïve T-cells, called iTregs. These large subsets are defined by their pattern of cytokine production and function.

As regards the traditional Th1/Th2 dichotomy, Th1 cells make interferon-gamma (IFN-γ) their signature cytokine and tend secret IL-2 and tumor necrosis factor-alpha (TNF-α). However, Th2 cells produce IL-4 in a significant amount of their signature cytokine. The production of IL-5, IL-13, and sometimes IL-9 is another characteristic of these cells [14]. The presence of both IL-2 and IL-4 in the microenvironment of activated naïve T-cells is essential for the differentiation of Th2 cells. Interestingly, IL-4 secreted from Th2 cells acts in an autocrine manner to induce Th2 differentiation. Subsequently, it was found that IL-4 neutralization as well as the addition of IL-12 to the culture cause Th1 differentiation. The involvement of T-bet as a master regulator, STAT1 (triggered by IFN-γ), and activated STAT4 (by IL-12) contribute to the development of Th1 cells. On the other hand, GATA3 as a primary master regulator and STAT6 activation (by IL-4) are required for Th2 differentiation from the naïve T-cells [15].

Th17—the next discovered subset of Th cells—was characterized as the major source of IL-17A, IL-17F, and IL-22, which could not be secreted by previously defined Th cells. First, it was reported that the presence of both TGF-β1 and IL-6 is essential for the generation of T17 cells. Subsequently, it was reduced to only IL-6 [15]. Aside from the previously described Th cell subsets, Th9 could also be considered a critical one, with emerging evidence of involvement in a different type of disease. These IL-9-producing cells are differentiated from activated naïve T-cells in the presence of TGF-β and IL-4. In addition to PU.1, the critical transcription factor for Th9 differentiation, other players—such as STAT6, IRF4, and GATA3—were suggested to be involved [16–19]. Aside from the IL-9, IL-4, and TGF-β, multiple cytokines, including IL-1, IL-2, IL-23, IL-25, and IL-33, affect Th9 priming and IL-9 production (reviewed in [20]).

Before the identification of Th22, IL-22 production was thought to be mainly associated with Th17 cells. However, IL-22 is now recognized to be not only produced by Th17 but also the Th22 subset. In fact, the highest IL-22 levels are present in Th22 cells, while Th22 subset completely lacks expression of IL-17A [21, 22]. In this subset, with the CCR6+CCR4+CCR10+ phenotype, AHR plays a vital role in IL22 expression as the key transcription factor [23]. Moreover, two cytokines of IL-6 and TNF-α have also been found to be essential for Th22 differentiation. Tfh is another subset of CD4+ T-cells, which provides a helper function to B-cells; this is characterized by the expression of CXC chemokine receptor 5 (CXCR5) along with other markers, including expression of inducible co-stimulator (ICOS), PD-1, cytokine IL-21, and transcription factor B-cell lymphoma 6 (BCL-6). Among the cytokines, IL-6 and IL-21 are indispensable for Tfh differentiation, and BCL-6 is a master regulator of this effector T-cell subset. In addition to the generation of Tfh cells from naïve T-cells, there is some evidence that this type of cell could be generated from other effector T-cells. Additionally, several signaling pathways are involved in the differentiation or inhibition of Tfh cells (reviewed in [24]).

Aside from effector CD4+ T-cells, iTregs is a subset, with suppressor function. This subset is different from CD4+ nTregs that develop in the thymus during positive and negative selection. iTregs are formed in the periphery from activated naïve CD4+ T-cells. However, both iTregs and nTregs maintain peripheral tolerance through the suppression of aberrant immune responses and protect from potential autoimmune responses [25]. Tregs—regardless of their origin of development—are vital in the prevention of autoimmunity. Three main subsets of iTregs have been described, including Th3 [26], Tr1 cells [27], and the recently described iTr35 Tregs [28]. These three subsets of iTregs are induced via TGFβ, IL-10, and IL-35, respectively. In contrast to Th3 cells, neither of the Tr1 and iTr35 subsets expresses or requires the transcription factor Foxp3.

5.3.3 Functions

Th1 cells—which involve cell-mediated immunity—deal with viral infections and some bacteria, certain autoimmune diseases, and, most importantly, anti-tumor immunity. This arm of cellular immunity is triggered by replicating intracellular pathogens, which is followed by the inhibition of Th2 differentiation. In contrast, Th2 cells are involved in the promotion of specific humoral immunity, and usually deal with some bacteria, toxins, and allergens. These cells also cause certain autoimmunity conditions and major types of allergies. Moreover, it is believed that Th2 immunity interferes with anti-tumor immunity through the inhibition of Th1-related responses. In contrast to Th1, Th2 promotes during helminth infection and skews responses toward a Th2 phenotype in an autocrine manner. Because of the significant role of Th1 and Th2 cells in the adaptive immune system, any long-lasting alteration in the Th1/Th2 balance without normalization may be associated with different types of diseases. For example, some autoimmune diseases are believed to be the results of higher Th1 or Th2-related responses [29]. Interestingly, it was suggested that the outcomes of some autoimmune diseases could be predicted during pregnancy, based on an alteration in the Th1/Th2 balance [30]. Additionally, the regulation of the Th1/Th2 ratio was effective in treating some of those conditions.

The emergence of Th17 shed light on different other functions of effector CD4+ T-cells. These cells participate in antimicrobial immunity and fight against extracellular bacteria/fungi. Th17 cells have inhibitory effects on the regulatory function of Tregs. According to these roles and distortion of the Th17/Treg balance favoring the pro-inflammatory Th17 in the majority, this critical subset was identified as a critical player in the majority of inflammatory and autoimmune diseases [31]. With regard to the role of Th17 in tumor-immunity, there are controversial findings. However, the accumulation of Th17 cells in many different types of tumors in comparison with healthy tissues demonstrated their significant contribution to cancer, which is discussed below.

The exact roles of Th9 and Th22 have not been described as well as other three discussed Th cells. There is some evidence of the contribution of IL-9 in inflammation, allergic diseases, and autoimmune conditions. Despite the elusive functions of Th9 cells in cancer, these cells activate not only the determining signaling pathways for tumor regression but also exert anti-tumor immunity [32]. Because of the high secretion of proinflammatory cytokines, such as IL-22 and TNF-α by Th22 cells, it is not surprising that these cells contribute to the pathogenesis of inflammatory and autoimmune diseases [33]. The significant alteration in the Th22-related responses in different cancers also suggests the pivotal role of this subset of CD4+ T-cells in cancer [34–36]. Tfh cells, which their primary function is to provide help to B-cells are other involved cells in regulation of immune system [37]. Moreover, considering the increase in circulating Tfh cells as well as its correlation with disease activity and autoantibody production in multiple autoimmune diseases, these cells could be referred to as the central players during autoimmunity [38]. Regarding the role of Tfh cells in different types of cancer, there is little available data on their correlation with cancer progression and survival rates, which will be discussed in this chapter.

The regulatory function of Tregs is essential to prevent autoimmunity. These cells are involved in a wide range of diseases, including infection, autoimmunity, and cancer. Perturbations in the development and/or function of Tregs can manifest themselves as severe autoimmune conditions. For example, the lack of the function/number of Tregs during a large number of autoimmune diseases had been proposed earlier. Conversely, some persistent infections, such as chronic hepatitis B virus (HBV) infection, were found to be associated with the regulatory function of Tregs or increase in the level of their related cytokines. This led to some approaches being proposed to cure HBV, based on the impairment of regulatory responses. These suppressive cells act through by not only depending on a single mechanism of suppression but also have an arsenal of regulatory mechanisms at their disposal [39]. The promotion

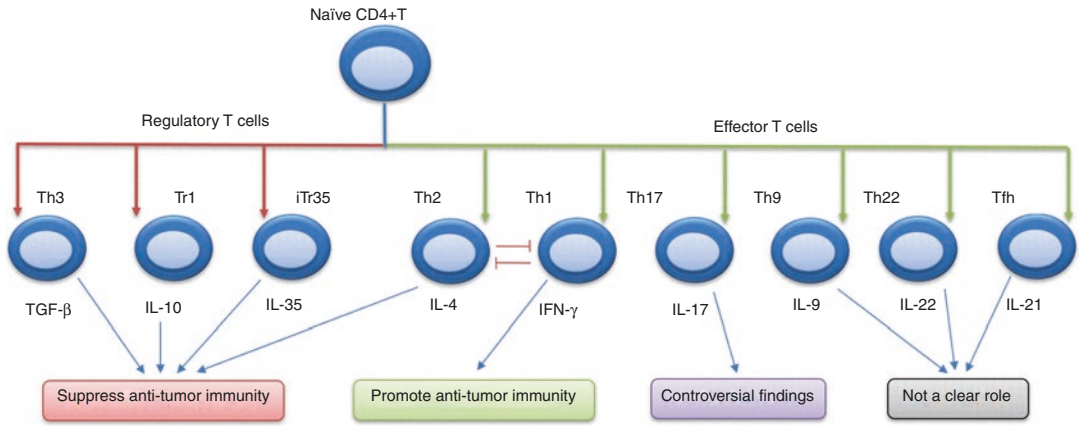

Fig. 5.1 Differentiated CD4+ T cells can be classified into regulatory and effector arms. Both Tregs and effector T cells inhibit each other. In the effector arm, Th1 and Th2 responses mutually inhibit each other. Any of the differentiated CD4+ T cells produce several cytokines, while the related master cytokine has been written. Generally, it has been accepted that Tregs and Th2 cells seem to inhibit anti-tumor immunity. Conversely, Th1 cells lead to enhancement of anti-tumor immunity. Th17 cells act as a double-edged sword in anti-tumor immunity. Although recent studies have shed light on the probable role of recently described CD4+ T cells, including Th9, Th22, and Tfh cells, there is no consensus about their roles against tumors

of tumors is another negatively described outcome of high numbers of Tregs. Indeed, it seems that large numbers of these cells in tumors cause impairment of anti-tumor immunity. The differentiation of CD4+ T-cells and their suggested roles in relation to anti-tumor immunity have been presented in Fig. 5.1.

5.4 T-Helper Cells in Tumor Microenvironment and Their Roles in Inducing Anti-tumor Immune or Immune System Exhaustion

The tumor microenvironment is complex and dynamic, consisting of several components, including tumor parenchyma cells, fibroblasts, mesenchymal cells, blood, lymph vessels, as well as tumor-infiltrating immune cells and their products (e.g., cytokines and chemokines). The intratumor cell types seem to play different roles in the natural life of the tumor, which cause tumor suppression or the opposite. T-cells are critical players in tumor immunity, which migrate into the tumor microenvironment randomly or undergo fully ballistic migration [40]. As it was briefly described, T-cells consist of various anti-tumor effector and regulatory subsets. Though they are a vital part of the immune system that actively plays a role in the tumor microenvironment, their dysfunction in solid tumors results from multiple mechanisms. The tumor-infiltrating lymphocytes (TILs) are increasingly significant predictors of tumor biology and outcome. More precisely, tumor-infiltrating T-cells were found to be associated with the improved clinical outcome as well as survival in patients with different types of cancer, including colorectal cancer [41], breast cancer [42, 43], and lung cancer [44, 45]. Although a significant anti-tumor role for CD8+ TILs was described in several studies, increasing evidence implicates the undeniable roles of CD4+ T-cells during cancer.

The exact role of CD4+ T-cells in induction or inhibition of tumorigenesis and metastasis is very complex and not yet fully clarified. However, accumulating evidence is pointing to the pivotal role of these cells in the modulation of the anti-tumor immune response. Different subsets of CD4+ T-cells do not act in the tumor microenvironment in the same ways. Some of these suppress tumors while some others promote tumors. The balance of these two major groups may be

essential in determining the outcome of immune responses within tumors. Accordingly, some TILs may be friends, while others may be foes [46]. The majority of the studies have shown that Th1 cells could be considered a positive prognostic factor in cancer [47]. Thereby, some strategies with the goal of promoting effector cells responses, such as an adoptive transfer of TILs in combination with IL-2 was suggested as an effective treatment approach for patients with metastatic melanoma [48]. In contrast, Tregs may accumulate in the tumor environment, which results in hindering tumor rejection via the suppression of tumor-specific T-cell responses [46]. Indeed, these cells are accepted as limiting anti-tumor immune responses, because of the prevention of induction of tumor-associated antigen-specific immunity as well as the inhibition of the effector function of cytotoxic T-cells and NK cells [49, 50].

5.5 Elimination of Tumor Cells by Effector T-Helper Cells and Tumor Evasion Strategies

The principal purpose of T-cell motility is to search for cognate antigen on APCs as well as target cells with the majority of tumors that are MHC-I positive in the early stages. This makes them recognizable by the specific anti-tumor CD8+ T-cells, which target them and progressively kill MHC-I positive cells. Aside from this subset, CD4+ T-cells also employ different strategies to target tumor cells or even stimulate other anti-tumor immunity arms [51]. The direct cytotoxicity of CD4+ T-cells was reported both in vitro and in vivo toward MHC-II positive tumor cells. Moreover, some indirect strategies to target MHC-II negative tumor cells have also been suggested [51].

Although T-cells function to eliminate tumors, there are a number of different strategies implemented by tumor cells to escape immune surveillance, including the loss/down-regulation of tumor antigen/MHC expression on tumor cells, resistance to apoptosis, accelerate apoptosis in activated T-cells, anergy induction, attraction of Tregs to the tumor, and stimulation of regulatory cytokines production (e.g., IL-10 or TGF-β) [52]. Increasing numbers of researchers believe that Tregs, as well as co-inhibitory molecules on effector T-cells, are exploited by several types of tumors to evade anti-tumor immunity [4, 53, 54]. Tumor antigens have the potential to elicit tumor-specific immune responses. However, cancer cells may lose their antigenicity, which could be considered one of the strategies of most studies for tumor-immune evasion [55]. If tumor cells are detected by T-cells, apoptotic evasion through the alteration or defection in apoptotic pathways is another barrier. The blockage of Fas or TNF receptors that can lead to apoptosis signaling and expression of decoy receptors to inhibit death receptor signaling are two examples of evading apoptosis strategies by tumor cells [56]. There is some evidence of the acceleration of T-cells apoptosis (e.g., FasL-mediated or TRAIL-mediated apoptosis induction) [56]. The detected tumor cells could also induce anergy in activated T-cells. Indeed, tumor cells may cause a problem in the induction of second signals by triggering the T-cell receptor either without adequate concomitant co-stimulation or high co-inhibitory signaling [52]. The negative roles of some co-inhibitory molecules, such as CTLA-4 and PD-1, in cancer, could also be confirmed by improving the survival of patients with metastatic melanoma following the blocking of the CTLA-4 by ipilimumab or PD-1 by nivolumab and pembrolizumab [57–59]. Inducing the exhaustion of T-cells through Tregs recruitment (probably by attracting them with chemokine CCL22 produced by tumor cells [60]) could also significantly limit anti-tumor immunity.

5.6 The Role of Effector T-Helper Cells in Cancer Immunity

5.6.1 Overview

As was pointed out in previous sections, effector CD4+ T-cells contribute to priming anti-tumor immunity through different major strategies,

such as exertion of direct anti-tumor activity, activation, and expansion of CD8+ T-cells as well as orchestration of antibody production [51, 61]. These have made Th cells the attractive immunotherapeutic approach for the treatment of cancer. There is much data on the roles of CD4+ T-cells in solid tumors as compared to the hematological malignancies. Traditionally, research in cancer immunity has focused almost exclusively on Th1/Th2 cell balance, which has been replaced by the Th17 and Tregs paradigm during the last decade. Recently, Th9, Th22, and Tfh cells were also found be involved in tumorigenesis, and this has been a hot topic among researchers in recent years. Moreover, the possible interaction between each of these subsets during human cancer remains to be elucidated. Two issues related to these cells had been discussed widely, including the proportion of Th1 cells/expression of Th1-associated genes in the baseline as well as the association of those with the outcomes, which have been discussed in this chapter.

5.6.2 T-helper 1

Among the different subsets of Th cells, Th1 is one of the most studied and critical ones in the mediation of anti-tumor immunity. In other words, it has been accepted that Th1-dominant immunity is essential for the induction of anti-tumor cellular immunity in vivo [62]. This role for Th1 immunity is likely due to the stimulation of both innate and adaptive immune response to tumors. IFN-γ, the principal Th1 effector cytokine, has been shown to be crucial for preventing and suppressing the development of cancers. Although most cell types in the body can produce IFN-γ, Th1 cells were identified as a primary source. This cytokine helps immune system to inhibit and kill tumor cells and impedes tumor growth through different mechanisms. For example, it seems that IFN-γ acts as a bridge between the Th1 cells and tumor-infiltrating antigen-presenting macrophages [63]. During this process, *macrophages could render tumor cells cytotoxic, either directly or indirectly*. This could lead to an acceleration of tumor eradication through the initiation of collaboration between innate and adaptive immunity. Boosting the MHC-I antigen-processing machinery that facilitates cytotoxic T-cells to recognize cancer cells [64], enhancing NK cells activity [65], and regulating their proliferation in vivo [66], inducing DC cytotoxic function [67], activation of effector cells (e.g., macrophages and neutrophils), inducing the infiltration of macrophages as well as CTL into the tumor, regulating CD4+ Th cell differentiation, and modifying anti-tumor cytokine responses [68–71] are some of the proposed mechanisms of the anti-tumor functions of IFN-γ. Moreover, IFNs could regulate the expression of a vast array of genes involved in tumor cell growth, proliferation, differentiation, survival, migration, and also other specialized functions favoring tumor shrinkage [72]. In this respect, it was shown that Th1 cytokine-enriched microenvironment hampered tumor growth [73]. In addition to the IFN-γ, related chemokines are also capable of recruiting effector immune cells into the tumor microenvironment, which can act in concert with tumor elimination [74].

In recent decades, a large number of studies have assessed the role of Th1 cells in patients with different types of cancer. There is a consensus among researchers with regard to the crucial role of Th1 cells in regressing tumors and impeding tumor growth. Generally, the enhancement of Th1 responses in several types of cancer, such as lung cancer [75], cervical cancer [76], and breast cancer [77], was demonstrated to be involved in augmenting anti-tumor responses.

Moreover, an elevated level of T-bet, the master transcription factor of Th1 cells, was reported in patients with high-grade bladder cancer in comparison to those with low/medium-grade bladder cancer [78]. In contrast to such evidence, there is some evidence implying a decrease in Th1-related responses. For example, suppressed levels of Th1-related cytokines in hypopharyngeal carcinoma [79] and oral squamous cell carcinoma [80] have been noted.

There are different pieces of evidence suggesting the correlation between the Th1-related responses and a favorable outcome in several tumor types. A high proportion of Th1 was asso-

ciated with a desirable outcome in colorectal cancer [81]. Indeed, those with high expression of the Th1 cluster had a prolonged disease-free survival. A better outcome as the result of increased expression of genes for Th1 adaptive immunity during colorectal cancer and, subsequently, presumably facilitation of the effector memory T-cell infiltration was also reported in another study [41]. Moreover, high Th1 infiltrated lymphocyte was found to be strongly associated with a better prognosis in patients with colorectal cancer [82]. A higher T-bet expression was reported to be a predictor of outcome in HER2-overexpressing breast cancer patients treated with trastuzumab plus docetaxel [83] and a predictor of patients' survival in bladder carcinoma [78]. In contrast, a weak Th1 response was found to be associated with the poor treatment response and prognosis in breast cancer patients [84]. The predominance of Th1 response after the cancer treatment was reported in both animal and human models also suggests the critical role of Th1 cells in establishing anti-tumor immunity [85].

In addition to solid tumors, different studies have suggested the importance of Th1 cells in the hemolytic cancers. Regarding acute lymphoblastic leukemia (ALL), it was found that the circulating levels of two of the most important Th1-related cytokines, IFN-γ and IL-12, were significantly higher than they were in the control group [86]. In contrast, Chen et al. [87] have demonstrated an observable decrease in both bone marrow and peripheral blood Th1 cells frequencies of newly diagnosed chronic myeloid leukemia (CML) patients. Similar to solid tumors, there is some evidence of better outcome in patients with more Th1-related responses. For instance, acute myeloid leukemia (AML) patients with high Th1 frequencies have shown a prolonged survival [88].

5.6.3 T-helper 2

Generally, among the two classical subsets of Th cells (Th1 and Th2), the majority of studies had focused on the role of Th1 rather Th2 cells. Although Th2 cells are capable of destroying the tumor by inducing tumor necrosis [70], the majority of studies on Th2 have suggested no beneficial role for them against the tumors. In fact, Th2 cells seem to promote tumor progression via counteraction of Th1 cells—key players in the initiation of anti-tumor immunity—as well as interfere with anti-tumor CTL activity. Moreover, IL-4 and IL-10 secreted by Th2 cells contribute to inhibit cell apoptosis [89] and promote regulatory responses through the inducement of the differentiation of Tr1 cells [90], respectively. However, Th2 master cytokine (e.g., IL-4) were also demonstrated to induce infiltrating eosinophils and macrophages, followed by the initiation of anti-tumor immunity [91–93]. IL-13 is another Th2 production, with both positive and adverse impacts on anti-tumor immunity. It causes tumor regression by inducing the activities of neutrophils and macrophages [94, 95] while protecting tumors through the suppression of IFN-γ and CTL activity [96]. Th2 cells-associated cytokines provide help for B-cells and facilitate IgG and IgE antibody responses. Although tumor-infiltrating B-cells may lead to favorable clinical outcome in some types of cancers [12], there is some speculation, related to the inhibition of anti-tumor immunity via B-cells, including the production of anti-tumor immunity cytokines (e.g., IL-10), the inhibition of production/activity of critical players against the tumor (IFN-γ, CTL, NK cells), and the masking of T-cell epitopes by secreted antibodies [97]. Considering the probable adverse effect of B-cells in anti-tumor immunity, the tumor promotion role of Th2 cells could be confirmed from another aspect.

Some studies have examined Th2 responses in cancer patients. A significant decrease in Th1, but not Th2 in renal cell carcinoma (RCC), has caused the skewing of Th1:Th2 balance toward more Th2 correlated with tumor stage and grade progression [98]. A distinct polarization of Th2-related cytokines, including IL-4 and IL-10, as well as suppressed Th1-related cytokines in those with oral squamous cell carcinoma [80] and cervical cancer [99] are other pieces of evidence for the skewing of Th1:Th2 toward Th2 phenotype. Moreover, because of increased Th2-associated

cytokines in hypopharyngeal cancer tissues and pericarcinoma tissues, it was suggested that this type of cell might promote cancer development as well as metastasis during hypopharyngeal carcinoma [79]. Th2 cells were also reported to associate with reduced patient survival pancreatic cancer [100]. However, it seems that Th2 cells do not play a significant role in patient's prognosis during colorectal cancer [81].

Regarding the hematological cancers, there is some evidence implementing the correlation of Th2 cell population and advanced disease. For example, shifting from the dominance of Th1-mediated immunity in chronic lymphocytic leukemia (CLL) patients toward Th2 phenotype by disease progression was reported [101]. In childhood ALL, a decreased Th2-related cytokines was observed [86], and also Th2 cytokine expression was suggested as the predictor of relapse [102]. In contrast to the CLL and ALL, Kiani et al. [103] reported no difference between the Th2 cytokines (IL-4, IL-10, IL-13) in CML and controls.

5.6.4 T-helper 17

Before the appearance of the Th17 subset, it was generally agreed that Th1 and Th2 are the only Th subsets that play significant roles in, respectively, the regression or promotion of tumors. Following the discovery of the third Th subset, Th17 cells, researchers have more focused on Th17/Treg instead of the classical Th1/Th2 paradigm. As mentioned, Th17 cells have recently been identified as important immune modulators in a variety of diseases, including autoimmune diseases as well as several types of cancer. In contrast to the role of Th17 cells in inflammation and autoimmunity [104], results from Th17 studies in different types of cancer have yielded conflicting results [105]. On the other word, there is enough evidence for considering it as a double-edged sword in cancer [106]. From the anti-tumor point of view, Th17 cells have shown both a direct eradication of tumors and the exertion of indirect anti-tumor effects through several signaling pathways. The capability of production of different effector cytokines by tumor-infiltrating Th17 cells as well as negatively correlated with the proportion of Th17 and Tregs, favoring the promotion of anti-tumor immunity and the prevention of immune system exhaustion. Moreover, the recruitment or activation of other tumor-specific immune cells, such as tumor-specific CD8+ T-cell and DCs, inducing the Th1-type chemokines CXCL9 and CXCL10, and promotion of IFN-γ secretion also contribute to the enhancement of Th17-associated anti-tumor responses [107]. On the other hand, the promotion of tumor angiogenesis as well as up-regulated prosurvival and proangiogenic genes as the result of Th17 activity could be considered the pro-tumor effects of these cells [107]. It was demonstrated that IL-17A triggers STAT3 activation in tumor cells, which leads to tumor growth [108]. Moreover, a vast majority of studies has shown that Th17 cell infiltration is detrimental in several types of cancer, which may be explained by the function of these cells as regulatory cells with the capacity to suppress anti-tumor immunity [106].

The majority of the murine and human studies implicate the increased proportion of Th17 cells as well as their associated cytokines in peripheral blood or in different types of tumor samples, such as RCC, gastric cancer, breast cancer, squamous cell carcinoma, lung cancer, hepatocellular carcinoma (HCC), cervical cancer [35, 76, 98, 109–118]. The enhancement of Th17 immune response in the adjacent tissues of sporadic colorectal cancer [119] or serum [120], making Th17 cells a valuable tumor marker in patients with colorectal cancer. Although the exact mechanism of Th17 cells up-regulation in tumor has remained unknown, some suggested mechanisms are responsible for the Th17 cell expansion and their migration into tumor microenvironments, such as chemokine-mediated recruitment of these cells into tumor sites as well as the function of tumor microenvironment factors (e.g., IL-1b, TNF-α, and IL-17) [121]. In contrast to different studies that had suggested the detrimental effects of Th17 cells in establishing anti-tumor immunity, there is paucity of evidence of the beneficial roles of Th17 cells in human cancer, such as ovarian cancer [122]. For example, impaired Th17 responses

were observed with the tumor progression in the tissue [81], which is inconsistent with some other cancers. The role of Th17 in the destruction of advanced melanoma was also previously deduced in a transgenic mouse model [123].

The association between the Th17 cells and poor outcome in different types of cancer, including HCC and colorectal cancer, has previously been reported [81, 124]. Interestingly, it was reported that Th17 cells' population dramatically decreased 2 weeks of radiofrequency ablation in lung cancer patients [109]. Moreover, there is some evidence of the Th17 response increasing as gastric cancer advanced [125]. However, in advanced colorectal cancer, it was reported that Th17 cells ratio in circulation to tumor tissues were decreased [126], which is consistent with the previous study that suggested Th17 cells can be recruited into the tumor microenvironment from the circulation [127].

In addition to the solid tumors, there are several findings with regard to the roles of Th17 in hematological malignancies, including multiple myeloma (MM), CLL, AML, non-Hodgkin lymphoma, as well as CML in bone marrow and/or peripheral blood. A significant elevation in the baseline and an induced frequency of Th17 cells were reported in both peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells in MM patients as compared to healthy controls [128]. Moreover, the suppression of Th1-mediated cytokines (e.g., IFN-γ) was observed as the result of a function of IL-17 and IL-22 combination, which suggests Th17 cells as the inhibitors of immune function in MM patients [128]. The elevated number of Th17 cells was seen in a different stage of AML in comparison with controls [129, 130], while a significant decrease in plasma IL-17 was found [129]. This unexpected finding may be explained by promoting the effect of Th17 independent of direct secretion of IL-17. In another study, in various stages of AML, a marked decrease in Th17 cells in the bone marrow microenvironment of newly diagnosed patients compared patients with complete remission, relapsed-refractory patients, or controls was observed [131]. Considering the role of IL-17 in MM pathobiology, it is conceivable that Th17 cells exert a pathogenetic role and are an important therapeutic target in MM and probably AML. Conversely, a significantly low percentage of Th17 cells present in lymphoma specimens from patients with B-cell non-Hodgkin's lymphoma was observed [132]. Because the higher number of Th17 and IL-17A levels was found to be associated with a less advanced clinical stage of CLL in a study containing untreated CLL patients in different clinical stages, the beneficial role of Th17 in CLL immunity was suggested [133]. Furthermore, a higher proportion of Th17 cells seems to be associated with a more favorable clinical course in CLL patients [134]. Hus et al. [133] found a positive correlation and a negative one between the Th17 percentages and iNKT and Tregs, respectively, in CLL patients. These results demonstrate the probable beneficial role of Th17 in CLL immunity. Regarding CML, considering the decrease in the frequency of Th17 in newly diagnosed CML patients in comparison with healthy controls, the protective role of Th17 cells in CML pathogenesis was suggested [135]. The association of high Th17 cell frequency in the AML patients with poor prognosis was also reported [88].

In summary, answering the question as to whether Th17 cells in tumor tissues are beneficial for patients through enhanced anti-tumor immunity, or cause promotion of tumor through an increase in inflammatory angiogenesis, is not easy. Indeed, it seems that Th17 cells play a role both in tumorigenesis and the eradication of an established tumor, which makes it a double-edged sword.

5.6.5 T-helper 9

Less than a decade ago, another CD4+ T-cell subset—characterized by the secretion of high levels of IL-9—was called the Th9 cell has identified [16]. Before the discovery of Th9, IL-9 was suggested as a promoter of some human hematological tumors, such as Hodgkin's lymphoma [136]. Indeed, high levels of IL-9 in the sera from Hodgkin's lymphoma patients, but not in non-Hodgkin's lymphoma patients or healthy con-

trols, implied the contribution of this cytokine to the development of Hodgkin's lymphoma. These effects may be explained by inducing regulatory responses via IL-9. The role of IL-9 in enhancing the suppressive functions of Tregs in vitro was another novel function against tumor regression via anti-tumor immunity [137]. This finding was subsequently confirmed by Hoelzinger et al. [138], who showed that IL-9 ablation enables CD8+ and CD4+ and activates the adaptive anti-tumor immunity. In contrast, there is enough evidence that Th9 cells activate both innate and adaptive immune responses, thereby harboring anti-cancer properties in solid tumors [139].

From the view of the beneficial role of Th9 cells, different studies were published during the last decade. The first report regarding the anti-tumor effect of Th9 cells was conducted using the murine melanoma model. As a result, it was concluded that the adoptive transfer of tumor antigen-specific Th9 cells could block tumor growth, which was reversed by anti-IL-9 [140]. This is consistent with the anti-tumor function of Th9 in an IL-9 dependent fashion. Interestingly, the efficacy of recombinant IL-9 administration was found to depend on the presence of mast cells, but not on the presence of T-cells or B-cells [140]. Because of the triggering mast cell activation by IL-9 [141], it was proposed that anti-tumor capacity of IL-9 and Th9 cells might be through the activation of mast cell [32]. In another study on metastatic melanoma patients, because of the association between the early increase in Th9 cell counts and improved clinical response during the nivolumab therapy, Th9 cells were suggested as cells with anti-cancer properties as well as a valid biomarker for anti-PD-1 response [142]. Lu et al. [143] have examined the role of IL-9/Th9 in a murine model of pulmonary melanoma; the anti-tumor effects of Th9 cells through provoking CD8+ CTL-mediated anti-tumor immunity were reported. Another interesting finding related to the function of Th9 cells was the recruitment of effector cells against tumor growth into the tumor sites [143]. Th9 cells also enhance anti-tumor immunity by triggering IL-21 in addition to IL-9 [144], and probably the involvement of IL-3, which could favor the survival of DCs [145]. Employing CD8+ T-cells' anti-tumor responses is another suggested mechanism underlying Th9 anti-tumor activities.

In studies of human cancer, some contradictory results have been reported. Ye et al. [146] found Th9 cells as a promoter of proliferation and migratory activity of lung cancer cells, which act through the regulation of immune responses in lung cancer cells in the tumor environment. During this process, the activation of STAT3 signaling pathway was responsible, which works in a manner opposite to IFN-γ activated STAT1 signaling that suppresses lung cancer cell proliferation and migration. A recent study on HCC patients had shown a significantly higher frequency of circulating IL-9-producing Th9 cells (specifically in peritumoral and tumor tissues) in comparison with healthy controls [147]. Additionally, because the association of higher tumor-infiltrating Th9 frequency had shorter disease-free survival period in the patients studied, the tumor-promoting role of Th9 cells in HCC, probably through CCL20 and STAT3 pathways, were suggested [147]. Taken together, Th9 cells seem to be new players in exerting anti-tumor activities, perhaps through the promotion of the activation of innate and adaptive immune responses as well as by triggering cancer cell death [32]. However, further studies are required to explain the controversial results regarding the role of Th9 cells in human cancer.

5.6.6 T-helper 22

Recently, the Th22 subset was identified as a new human Th subset, characterized by abundant secretion of IL-22 and TNF-α, but not IL-17 or IFN-γ, which makes it clearly separate from the Th17 and Th1 subsets [21, 148, 149]. There is some growing evidence regarding the role of Th22 and in the pathogenesis of inflammatory and autoimmune diseases, including psoriasis and rheumatoid arthritis [150, 151]. Mounting evidence suggests that Th22 cells are other critical players during the human cancers [34–36].

Before focusing on the Th22 cells in cancers, some evidence was found that is related to high

levels of IL-22 in the tumor tissues or peripheral blood of cancer patients [152–155]. Some of them interpreted it as the involvement of Th17 cells, but not Th22 cells. The higher expression of the cytokine and also its receptor was found to positively relate to invasion and metastasis in pancreatic ductal adenocarcinoma [156]. In contrast, some studies reported that IL-22 levels were lower in patients with HCC than in healthy controls [157]. Subsequently, different authors have evaluated the possible role of another subset of IL-22-producing cells (L-22(+) IL-17(−) IFN-γ(−)CD4(+) T-cells), which could be distinguished from Th17 cells. These cells seem to contribute to tumorigenesis through STAT3 and probably epigenetic alterations, which was recently concluded in the study of Th22 cells' interaction and colon cancer cells [158]. As one of the first studies on the role of Th22 in tumor immunity, it was demonstrated that Th22 cells are increased in tumor tissues in gastric cancer [159]. It was thereby suggested that these newly identified cells contribute to tumor progression and predict poorer patient survival. Moreover, targeting of these cells in patients with gastric cancer was also proposed as a therapeutic option. The same year, these results were confirmed by another study, and positive correlation between Th22 cells and Th17 cells was also reported [34]. Soon, new evidence for the involvement of Th22 cells in other solid tumors, such as HCC, gastric cancer, colorectal cancer, cervical cancer, ovarian cancer, and pancreatic cancer, was revealed. A few years ago, the elevation of both serum IL-22 and IL-22 in HCC tissues was observed, as compared to the normal tissues or healthy controls [35]. Interestingly, an increase in Th22 cells was correlated with the advancement of the tumor stage. Another consequence was finding a correlation between the frequency of Th22 cells and serum IL-22 in HCC patients, which was not true in healthy controls [35]. Considering the role of IL-22 in leading to tumor growth, the inhibition of apoptosis, and the promotion of metastasis in HCC patients [153], it was suggested that Th22 cells contribute to the pathogenesis of HCC through the production of IL-22. In a study of gastric cancer patients, increases in circulating Th22 cells in the peripheral blood of gastric cancer patients with tumor progression or tumor tissues in another, which were associated with tumor progression and predicted poorer patient survival, were revealed [34, 159]. Huang et al. [160] have found a higher prevalence of Th22 cells in tumor tissues as compared to paratumoral tissues in colorectal cancer. However, those results are probably inconsistent with the lower in percentages of Th22 cells (IL-22 mRNA in tumors were determined by real-time PCR) in colorectal cancer compared to healthy individuals and negatively correlated with the pathological stages of cancer [161]. Regarding cervical cancer, the possible adverse role of Th22 cells was suggested when a higher proportion of these cells was recorded in the peripheral blood of the patients compared to healthy controls [76]. Another significant finding was a correlation of aggregation of Th22 cells with lymph node metastases in cervical cancer patients [76]. Additionally, a positive relationship between Th22 and Th17 cells, but a negative correlation between Th22 and Th1 cells were reported [76]. In a study of patients with epithelial ovarian cancer, in addition to an elevation of Th22 responses, associations of both Th22 and IL-22 with the stage of disease were observed [162]. Stimulation of colon cancer proliferation via Th22 cells was also suggested in colon cancer [158]. Increasing IL-22-producing T-cells in tumor tissue of patients with pancreatic cancer, and its positive correlation with increased tumor, node, and metastasis (TNM) as well as poorer patient survival also caused the suggestion of blockade of IL-22 signaling as a viable method to treat these patients [163].

Th22 cells conjointly contribute to the pathogenesis of AML, which led to the introduction of Th22 as a novel biomarker to assess patients at risk [129]. Moreover, Th22 cells (and also IL-22) were negatively correlated with Th1 cells in newly diagnosed AML patients [129].

Considering the increase of Th22 population, newly diagnosed AML patients—in comparison with controls as well as no significant difference between the circulating Th22 cells in those who achieved complete remission and controls—it was suggested that Th22 cells participate in the development and progress of AML [129].

However, a lack of correlation between the Th22 cells and plasma level of IL-22 in newly diagnosed AML patients suggest the involvement of another source(s) of IL-22 in those patients [129]. A bright increase in Th22 (examined by flow cytometry) plasma IL-22 concentration (measured by ELISA), and AHR expression (analyzed by RT-PCR) in both groups of newly diagnosed with T-cell ALL and those who achieved complete remission was observed, when compared to the healthy controls [164]. It is interesting to note that in that study, a positive correlation of Th22 cells with Th17 or Th1 cells was also reported. In the evaluation of Th22 responses (frequency of Th22 cells, AHR expression, but not IL-22 concentration) in the peripheral blood and bone marrow of CML patients, it was revealed that the frequencies of these cells were profoundly lower in newly diagnosed CML patients than healthy controls [87]. Conversely, Lu et al. [165] have reported an elevation in the frequency of Th22 cells in the peripheral blood of newly diagnosed B-cell non-Hodgkin's lymphoma patients. Interestingly, the frequency of Th22 cells was correlated with poorer response to chemotherapy and also decreased following chemotherapy. Similar to several other reported blood cancers, increased levels of Th22 cells in MM was reported in different studies, which was associated with therapeutic outcome (decline in complete remission patients following chemotherapy), clinical stage (higher frequency in higher stages), and a poor prognosis [166, 167].

Altogether, these results suggest that Th22 cells may be involved in the development of both solid tumors and hematological malignancies, and tumor-infiltrating Th22 cells may be suitable therapeutic targets in those patients. According to the results of some studies, it is conceivable that other unknown sources of IL-22 can be involved in cancers.

5.6.7 T-Follicular Helper

Tfh cells provide help to B-cells in the lymph node and express essential cytokines and chemokines, such as IL-4, IL-21, and CXCL13, to promote B-cell immunity. IL-21, a key Tfh-related cytokine, was found to have a high ability to establish anti-tumor immunity through induction of tumor-reactive CD8+ T-cells with cytotoxic effects [168]. In the past decade, several attempts have been made toward clearing the role of Tfh cells in different types of cancer. In the evaluation of HBV-associated HCC patients, it was observed that Tfh cells have significantly declined in HCC patients as compared to healthy controls [169]. Interestingly, the higher Tfh population was also found at the tumor site when compared to the non-tumor regions. Correlation of Tfh cell numbers with the disease progression and reduced disease-free survival were other important findings, which have highlighted the critical role of these cells in HCC development among the HBV-infected individuals [169]. The impairment of HCC-specific Tfh cells, probably due to an elevation in the expression of PD-1 and PD-L1—which was associated with advanced tumor stages—was also reported [170]. The beneficial role of Tfh cells was also previously suggested in patients with breast cancer. Gu-Trantien et al. [171] have found that not only higher Th1 but also Tfh cells in breast cancer patients are significantly associated with better outcome. In another study, the impairment of Tfh cells was the result of elevation in the expression of TIM-3 and PD-1, but no alteration in the frequencies of circulating Tfh cells in breast cancer patients was reported when compared to healthy controls [170]. The significantly lower frequency of Tfh cells in the peripheral blood of non-small-cell lung carcinoma patients than in healthy subjects and the positive correlation of frequency of tumor-infiltrating Tfh cells with survival time from the date of surgery was another evidence of the involvement of Tfh cells in anti-tumor immunity [172]. These cells may also have a role in antibody class switching of B-cells toward the promotion of anti-tumor immunity, which was previously shown in prostate cancer [173]. In contrast to the majority of solid cancer, hematological cancer patients, including non-Hodgkin lymphoma [174] and CLL [175], showed an increase in the circulating Tfh in CD4+ T-cells as compared to the normal subjects.

5.7 Regulatory T-Cells in Cancer Immunity

Tregs have been found to be the critical players in almost all types of immune-related conditions. In the majority of autoimmune diseases, the impaired activities of these cells seem to be responsible. Conversely, the same cells have an entirely opposite role in cancers. In fact, exerting the suppression of both innate and adaptive immunity by Tregs could cause the promotion of the progression of the disease in cancer [53]. In addition to the impairment of anti-tumor immunity, it was suggested that these cells represent significant hurdles toward successful immunotherapy [176]. In contrast to autoimmune diseases, which are usually associated with a decreased number of Tregs, in cancer individuals, these cells were found to downregulate the activity of effector T-cells against tumors, resulting in T-cell impairment in cancer-bearing hosts and favoring tumor escape from immune response [53]. Generally, Tregs could be divided into two major groups: Those derived from the thymus (nTregs) or those that arise extrathymically in the periphery (iTregs). Till date, different subsets of T-cells with regulatory function have been identified, including Foxp3+ T-cells, Th3, Tr1, iTr35+ T-cells, etc., that seem to contribute in the exhaustion of anti-tumor activities [177]. Different mechanisms of action related to Tregs have been suggested, including the production of inhibitory cytokines (e.g., IL-10, TGF-β, and IL-35), using direct cytolysis to mediate suppression, metabolic disruption, DC modulation, etc. [178].

The involvement of Tregs in peripheral blood and tumor tissues in the patients with different types of human cancer has been observed [179–182]. In fact, these cells seem to contribute to attenuate host anti-tumor immunity. This has led to the emergence of the idea of blocking Tregs migration or function to defeat human cancer [60]. Indeed, these cells contribute to suppression of anti-tumor responses and mediate immune tolerance favoring tumor growth. The fact that the suppression of Tregs could lead to an enhancement of anti-tumor immunity was suggested, which may be related to the function of proinflammatory cytokines [183]. A significant decrease in the activated Tregs and naïve Tregs in the peripheral blood of patients with different stages of RCC was reported [98]. Indeed, the balance of Th17 and Tregs was skewed toward the Th17 profile, as the tumor stage and grade progressed [98]. In contrast, Liotta et al. [184] have shown a significant elevation in Tregs frequency in TILs, with no important differences between the peripheral blood of controls and patients with RCC, which were associated with worse prognosis.

The disappearance of tumor-infiltrating Tregs following neoadjuvant chemotherapy in breast cancer patients could confirm the harmful role of these cells during cancer [185]. Contradictory results are associated with the role of regulatory responses in different types of cancer. Although expression of FoxP3, a critical transcription for Tregs, was associated with the worst overall survival in breast cancer [186, 187], the positive impact of Tregs was suggested in colorectal cancer [81]. Moreover, there is some growing evidence of the protective role of Tregs during cancer [188].

As one of the first studies on the role of Tregs in hematologic cancers, Beyer et al. [189] showed the increased frequency of Tregs in CLL patients, specifically in untreated or progressing patients. Since that time, different studies have been published, which confirmed the previously reported data [190, 191]. Moreover, increasing the proportion of Tregs in CLL patients, particularly those with progressive disease, seems to be associated with a higher suppressive function on the anti-tumor immunity, which is followed by the expansion of leukemic cells as well as disease progression [192]. The critical role of Tregs in other types of leukemia was also reported. It was found that the percentages of these suppressor cells were significantly higher in AML patients in comparison with healthy controls [193]. More interestingly, the Tregs frequency negatively correlated with clinical improvement after six cycles of chemotherapy. The same story of the promotion of Tregs-related responses has been reported in ALL [194] and CML [195].

5.8 Clinical Therapeutic Implications: Focused on T-Cell-based Therapies

Despite the advancements in the understanding of cancer over the last few decades, the therapeutic efficacy of cancer treatments is currently still poor. Traditional cancer treatments mainly consist of surgery, chemotherapy, and radiation therapy. However, these approaches have demonstrated insufficient efficacy for a large number of patients with late-stage disease. Additionally, because of several side effects of chemotherapy and radiation therapy, more effective methods with fewer side effects are required to be employed for cancer patients. The employment of body's own immune system to shrinkage tumor is associated with significantly less severe adverse events, as compared to chemotherapy. In recent decades, cancer immunotherapy has emerged as a promising therapeutic option for cancer individuals.

As discussed, during the majority of the cancers, the dysfunction of the immune system has caused the development and promotion of a tumor. This could be confirmed by several reports of T-cells exhaustion in those with cancer. Hence, it could be expected that reversing the functional impairment of the tumor-specific T-cells is a potent strategy to induce tumor regression. Manipulating the immune system to promote anti-tumor immunity is the primary aim of cancer immunotherapy. To reach this goal, multiple immunotherapeutic approaches could be employed. For instance, different strategies to deplete Tregs have been introduced to restore the impaired anti-tumor immunity, which are relying on monoclonal antibodies. Cancer vaccines and adoptive cell transfer are other approaches to improve the chance of tumor shrinkage and eradication. Additionally, using engineered T-cell receptor TCR- and chimeric antigen receptor (CAR)-transduced T-cells were found to be the promising approaches to treat cancer, which is not addressed here effectively. Side effects of immunotherapy could vary significantly from one approach to another (e.g., cancer vaccines, cytokines therapy, adoptive cell therapy, immune checkpoint inhibitors, and monoclonal antibodies). However, the main possible side effects of immunotherapy remained immunotoxicity and autoimmunity [196].

In theory, and based on animal models, we could be optimistic about these strategies to eradicate tumors; however, sometimes the results may be a little frustrating in clinics. Interestingly, these procedures could also be opportunities to reduce viral-related cancers (human papillomavirus, cervical cancer, HBV, HCC). Adoptive cell transfer (ACT) [197], T-cell targeting antibodies [197], and cytokine-based immunotherapy [198] are three approaches that are directly related to T-cells and have been reviewed below. Although all these strategies have been designed to promote anti-tumor immunity, each of them has its own characteristics. There is some evidence to support beneficial roles of combining immunotherapy approaches to enhance and broaden the anti-tumor activity [199].

5.8.1 Adoptive Cell Transfer

Adoptive T-cell transfer aims to expand anti-tumor T-cells via autologous TILs. During this procedure, patients could benefit from expanding their own T-cells in vitro, which is followed by the promotion of tumor-specific T-cells by reinfusion of ex vivo-expanded TILs. It can be considered an emerging field for cancer treatment, which has shown promise in recent trials. More precisely, this approach consists of the infusion of unmodified or engineered T-cells, capable of recognizing and eliminating cancer cells expressing TAAs by their surface receptors (T-cell receptors, or chimeric antigen receptors). ACT is efficient immunotherapies against metastatic *melanoma*, which have yielded durable and complete responses in those with refractory metastatic melanoma. CAR T-cell therapy is one of the forms of the ACT, which recently have got its FDA approval for the treatment of children and young adults with B-cell ALL. In contrast to the hematological malignancies, the results of trials on targeting solid tumors

by CAR T-cells are less spectacular; however, it could still be a treatment for solid tumors [200].

5.8.2 Inhibiting Regulatory Responses

As discussed, Tregs in the tumor microenvironment interferes with successful cancer immunotherapy. Thereby, the modulation of Tregs number/function in cancer is expected to hold promise for unleashing potent anti-tumor immunity. Accordingly, Tregs depletion and blockade could potentially override the development of effective anti-tumor immunity against the cancer cells. Recently, Taylor et al. have shown that a combination of these approaches could lead to more potent immunotherapy in claudin-low breast cancer patients [201]. A large number of murine studies has demonstrated that the depletion of Tregs from tumor microenvironment could potentially restore impaired anti-tumor immune responses [202–204]. As an example, Mattarollo et al. [203] selectively depleted of Foxp3+ Tregs in animal models of melanoma, which resulted in the inhibition of tumor growth as well as the enhancement of survival after receiving cancer vaccination with NKT cell adjuvants. As could be expected, via non-selective depletion of tumor-infiltrating Tregs, severe autoimmunity can occur in genetically susceptible individuals [205]. Moreover, the specificity of targeting of tumor-infiltrating Tregs and not confusing with effector lymphocytes because of some common molecules targeted for therapy is another concern related to the depletion of Tregs in cancer.

It is generally accepted that co-inhibitory pathways are the primary reason for failing the spontaneous immune-mediated tumor elimination during cancer. With regard to two different types of tumors (non-small-cell lung cancer and colorectal cancer), it was found those infiltrated Tregs upregulate immune checkpoints and are more suppressive than those isolated from non-tumor Tregs in vitro [206]. Among the exclusively expressed co-inhibitory molecules on T-cells, CTLA-4, and PD-1 have attracted many attentions during the recent decade. CTLA-4 (also known as CD152) was the first identified co-inhibitory molecule on the T-cells, responsible for blocking autoreactive T-cells in the early stages of T-cell activation [207]. It was also the first approved targeted immune checkpoint receptor in cancer (FDA approval for metastatic melanoma [208]). Another well-studied immune-checkpoint receptor related to cancer is PD-1, which could exhaust anti-tumor immune responses and then cause cancer immune resistance, once engaged by PD-L1 and PD-L2 [209]. Following the very promising results of the inhibition of the immune checkpoint, the FDA has approved some new drugs to target CTLA-4 (ipilimumab), PD-1 (pembrolizumab and nivolumab), and PDL-1 (atezolizumab) for the treatment of increasing numbers of cancers. Following proving of the efficacy of checkpoint blockade in metastatic melanoma [208], it was extended for the treatment of other cancers, such as prostate cancer [210], RCC [211], non-small-cell lung cancer [212], urothelial carcinoma [213], and ovarian cancer [214]. Recent findings also suggest that combined therapy may be more beneficial than monotherapy [215–218].

5.8.3 Cytokine-based Immunotherapy

The employment of two cytokines of IL-2 and IFN-α could be considered the first attempts for cytokine-based immunotherapy in patients with cancers, such as advanced melanoma [219, 220]. In

addition to IFNs and IL-2, the employment of several other cytokines with a potential of anti-tumor function, including granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-7, IL-12, IL-15, IL-18, and IL-21 seem to be useful to achieve better immunity [221]. Animal models have shown that cytokine-based therapy could enhance NK cell functions in cancer patients with the relatively non-toxic effects [222]. The ability of some cytokines to induce the differentiation and the stimulation of anti-tumor T-cells, as well as the inhibition of Tregs, makes them even more attractive. Because of the ability of T-cells to recognize neoantigens, promotion of effector T-cells response may be used as an effective immunotherapy approach to cancer [223]. The increasing knowledge regarding the roles of different subsets of Th cells in cancer has opened up several new avenues for research into its treatment. For example, because the Th1 response is essential for initiation of anti-tumor immunity, boosting Th1 response by cytokine-based therapies has attracted a lot of attention in recent times [198]. Additionally, the promotion of Th9 cell may lead to the establishment of novel Th9-dependent treatments of cancer [224]. In contrast, considering the impact of some suppressive cytokines, such as IL-10 and IL-35, on tumor progression, neutralization of these could be used in cancer immunotherapy [221, 225]. Targeted therapies were not only discussed for those involved in regulatory responses but also some pro-inflammatory cytokines. For example, the use of anti-IL-6 agents in human cancer was found capable of targeting malignant tumor cells as well as limit the interactions of cancer cells with their microenvironment [226]. There are also some cytokines, with the dual function, such as IL-17, IL-23, and TGF-β, which could act as both tumor promoters and tumor suppressors [221].

5.9 Concluding Remarks

Accumulating evidence indicates that CD4+ T-cells undeniably play a vital function in constraining tumor development. CD4+ T-cells could be categorized into various subsets, with each of them having a distinct role in tumor immunosurveillance. In general, these cells contribute to enhancing anti-tumor immunity through multiple mechanisms. The most well-studied Th cells with anti-tumor properties are Th1 cells. New evidence also implies the likely role of the newly identified subset, Th9, in exerting anti-tumor activities. However, some subsets of CD4+ T-cells, such as Tregs, inhibit anti-tumor immunity and play a direct role in promoting immune evasion. Some other subsets, such as Th2, also play an indirect role in inhibition of anti-tumor immunity. Moreover, some of the effector T-cells—such as Th17 and Th22—seem to contribute to tumorigenesis. These findings have made CD4+ T-cells the double-edged sword in cancer. The evaluation of frequencies of CD4+ cells subsets in patients with various types of cancers has suggested the probably different roles of specific subsets in various types of cancers. Table 5.1 summarizes the last findings on the involvement of different subsets of CD4+ T-cells in cancers. The recognition of exact roles of each subset of Th may be followed by more effective therapeutic options for cancer patients. Although there is a large number of studies regarding the role of various cancers, the exact role of some newly identified subsets, such as Th9, Th22, and Tfh, have remained uncertain. Contradictory results regarding the particular types of cancer is another challenge in the clarification of the CD4+ T-cells roles.

Table 5.1 The last findings about the roles of CD4+ T cells in cancers

CD4+ T cells	Role	Frequency/expression of related genes responses in cancers	Outcome
Th1	– Essential for the induction of anti-tumor cellular immunity	– Enhanced Th1 responses in several types of cancer, but not all	– A positive correlation between the Th1-related responses and a favorable outcome in several solid tumors and hematologic malignancies
Th2	– Although Th2 cells are capable of destroying the tumor by inducing tumor necrosis, the majority of studies on Th2 suggests no beneficial role for them against the tumors – It seems that Th2 cells tend to promote tumor progression via counteraction of Th1 cells	– Skewing Th1:Th2 balance toward more Th2 in the majority of cancers	– Th2 responses are probably associated with cancer development, metastasis, and reduced patient survival
Th17	– There is enough evidence to consider Th17 cells the double-edged sword in cancer	– Increased proportion of Th17 cells as well as their associated cytokines during different types of cancer was reported	– Association between the Th17 cells and poor outcome in various types of cancer. However, a few studies on the association of Th17 cells with a more favorable clinical course during some hematologic malignancies are available
Th9	– The majority of studies has reported anti-tumor activity of Th9 cells, probably through promotion, the activation of innate and adaptive immune responses as well as triggering cancer cell death	– More studies required to clarify	– Require more studies to clarify
Th22	– The possible adverse role of Th22 cells in priming anti-tumor immunity was suggested in different studies	– The majority of studies reported increased frequency of Th22 cells in solid and hematological cancers	– Correlation of Th22 cells frequency and poorer outcome
Tfh	– The high ability to establish anti-tumor immunity through induction of tumor-reactive CD8+ T cells with cytotoxic effects	– Contradictory results	– Probably correlation of Tfh cells' frequency and better outcome, but require more studies to clarify
Tregs	– Suppression of both innate and adaptive immunity favoring tumor promotion	– Increased in almost all the reported studies	– Associated with the poor outcomes

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Regulatory T-Cells and Th17 Cells in Tumor Microenvironment

6

Chang H. Kim

Contents

6.1 Introduction

Organs and tissues in the body are highly heterogeneous in producing tissue factors that affect the development and maintenance of immune cells. In general, the tissues in the body maintain highly tolerogenic conditions. This is important to prevent unwanted autoimmune or inflammatory responses to harmless antigens and immune stimulants. Tumors, formed in tolerogenic tissue environments, are naturally hypo-immunogenic and utilize a number of mechanisms to actively suppress the generation of effector T-cells [1, 2]. Tumors maintain tolerogenic environments to avoid anti-tumor immune responses. Tolerogenic tumors harbor high numbers of FoxP3$^+$ T-cells (commonly called Tregs). Despite the tolerogenic nature of the tumor microenvironment, tumors variably produce many factors that affect T-cell differentiation and maintenance. The numbers of effector T-cell populations in tumors are highly variable. Certain cancers are associated with chronic inflammatory conditions [3]. For example, cancers formed in certain tissues, such as the intestine and in patients with chronic infection, are exposed to microbes, which can form inflammatory conditions in tumors. Cancers formed under these conditions would be heavily influenced by inflammatory conditions. Necrotic tumor cells also induce inflammation through damage-associated molecular pattern (DAMP) receptors such as TLR2, TLR4, and the receptor for advanced glycation end products (RAGE) [4]. In addition, necrtotic cells in tumor microenvironments can generate Adenosine Triphosphate (ATP) and Nicotinamide Adenine Dinucleotide (NAD), which not only trigger the activation of purinergic receptors expressed

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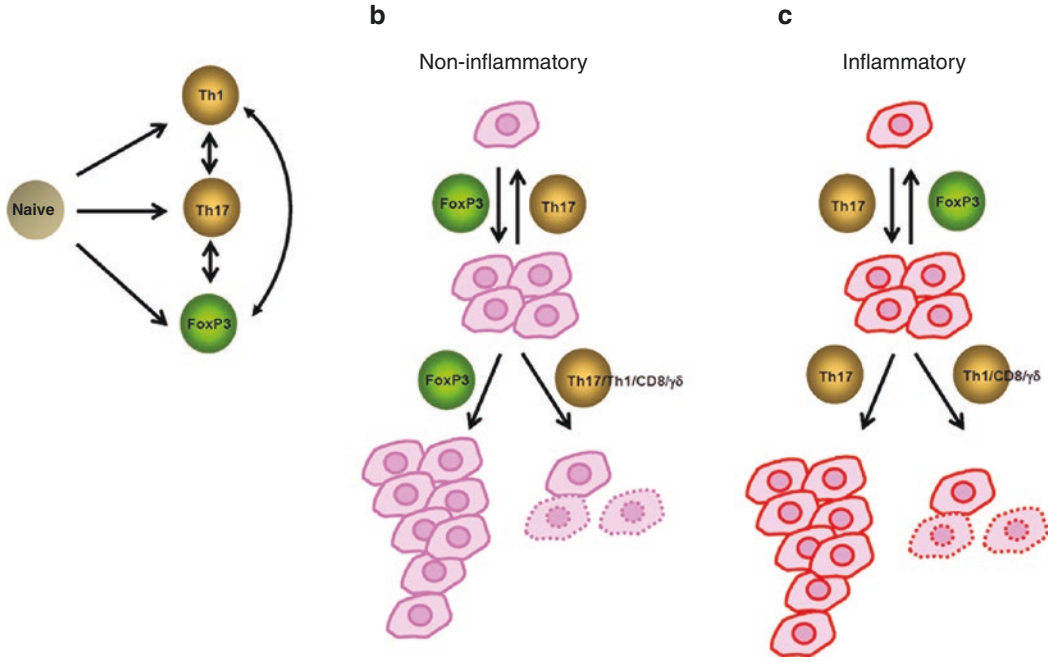

Fig. 6.1 Potential roles of FoxP3⁺ T-cells and Th17 cells in tumors. (**a**) FoxP3⁺ T-cells are made in the thymus as naïve-type FoxP3⁺ T-cells, which migrate to lymphoid tissues. These FoxP3⁺ T-cells can become the memory type after activation in secondary lymphoid tissues. Induced FoxP3⁺ T-cells with memory-type FoxP3⁺ T-cells and Th17 cells are made from naïve CD4⁺ T-cells. FoxP3⁺ T-cells suppress effector T-cells and other immune cells and decrease tissue inflammation. Th17 cells produce IL-17 cytokines to induce inflammatory responses. FoxP3⁺ T-cells, Th17 cells, and Th1 cells can also trans-differentiate into each other in appropriate cytokine and antigen priming conditions. (**b**) FoxP3⁺ T-cells can promote tumor growth by suppressing anti-tumor immune responses at early and late stages. On the other hand, Th17 cells can induce immune responses that lead to eradication of tumor cells in a manner similar to other effector, CD8⁺ and γδ T-cells. (**c**) In inflammatory conditions, FoxP3⁺ T-cells and Th17 cells have the potential to play different roles. Th17 cells cause inflammation in tissues; hence inflammatory tumors are formed and stimulated to grow. FoxP3⁺ T-cells suppress the function of Th17 cells and other inflammatory T-cells, leading to suppression of the tumorigenic process in inflamed tissues. It is thought that complex interactions and balances among these T-cells and other cell types determine the overall immune responses in tumors

by many cell types including immune cells but also are metabolized by ectoenzymes [5]. Inflammatory tumors harbor FoxP3⁺ T-cells and effector T-cells, including Th17 cells and Th1 cells [6, 7]. FoxP3⁺ T-cells can suppress the function of anti-tumor effector T-cells and other immune cells to promote tumorigenesis (Fig. 6.1). On the other hand, FoxP3⁺ T-cells can suppress tissue inflammation to prevent the emergence of tumor cells following chronic tissue inflammation. Effector T-cells produce inflammatory cytokines that promote tumorigenesis by increasing tissue inflammation and angiogenesis, but they can also promote anti-tumor immunity. An inverse correlation was observed between frequencies of FoxP3⁺ T-cells and effector T-cells such as Th17 cells and Th1 cells [8–10]. In certain cancers, the frequency of FoxP3⁺ T-cells increases, whereas that of Th17 cells decreases as cancers advance to more aggressive stages [10]. The presence of FoxP3⁺ T-cells and Th17 cells in tumors and associated tissues not only reflects the nature of tumor microenvironments but also indicates the types of active T-cell-mediated immune responses in tumors. In this chapter, we will discuss tumor factors that regulate T-cell differentiation into Tregs and Th17 cells, migration of the T-cell subsets into tumors and associated lymphoid tissues, and the functions of Tregs and Th17 cells in regulating anti-tumor immune responses.

6.2 Diversity of Tumor Microenvironments and Tumor Tissue Factors

The tumor microenvironment is highly heterogeneous, depending on tumor types and tissue sites. Together with tumor cells, fibroblasts, myofibroblasts, adipocytes, neuronal cells, endothelial cells, mast cells, and other tissue cells make up tumors. Moreover, immune cells are an important component of tumors and are mainly composed of T-cells, B-cells, innate lymphoid cells, and myeloid cells. Tumor-associated myeloid cells are heterogeneous as well and contain immature and mature myeloid cells. Myeloid-derived suppressor cells (MDSC) are highly heterogeneous and enriched in tumors [11]. MDSC are composed of multiple myeloid cell lineages at various different stages. Compared to mature myelocytes such as dendritic cells (DCs) and macrophages, MDSC do not highly express cytokines, co-stimulatory molecules, and MHC class molecules. Therefore, they poorly support anti-tumor effector T-cell responses. Moreover, MDSC express various molecules that dampen immune responses. MDSC produce peroxynitrite for nitration and nitrosylation of many proteins in the tumor environment [12, 13]. A major target protein for nitration and nitrosylation is TCR, which becomes ineffective at activating T-cells after the modifications [14]. They also express Arg1, inducible nitric oxide synthase (iNOS), and TGF-β1, among others [15]. Tumors also harbor many macrophages, which can be made from MDSC or myeloid progenitor cells [16]. Dendritic cells express indoleamine 2,3-dioxygenase (IDO) to regulate available tryptophan in tissue environments [17]. Other immune cells such as mast cells, NK cells, $CD8^+$ T-cells, and B-cells are frequently found in many tumor types.

The tumor environment is low in both oxygen and pH. Tumor cells rapidly divide and therefore vigorously consume oxygen supplied via blood vessels. Tumor cells mainly utilize the aerobic glycolysis pathway to generate energy [18]. This can accumulate lactic acid and protons, leading to low extracellular pH [19]. The most common pH range in tumors is 6–6.5. The low acidic tumor environment leads to immune cell anergy. For example, cytotoxicity and cytokine secretion by $CD8^+$ T-cells are impaired at the low pH range [20]. In addition to lowering pH, lactate plays an important role in fueling cancer cells and modulating immune cell phenotype by inducing M2 tumor-associated macrophages and inhibiting effector T-cell activity. This may be mediated in part through direct binding to N-myc downstream-regulated gene 3 (NDRG3) and activation of a hypoxia-inducible factor 1a (HIF-1a). In addition, many tumor-associated metabolites can control the metabolic milieus of tumors.

Cells in the tumor microenvironment can produce various cytokines and growth factors [21]. Some of these factors are drained into lymphatic vessels and form tumor-associated microenvironmental milieus in lymph nodes. Tumor-specific or tumor-associated antigens and dendritic cells harboring these antigens are drained or transported into lymph nodes for presentation to T-cells. Effector and regulatory T-cells can be made following this antigen priming process. The cytokine milieu is critical in determining the fate of differentiating T-cells in tumor-draining lymph nodes. Again, the type and amount of cytokines and other factors produced in tumors are thought to be highly variable among tumor types. Expression of IL-1α, IL-1β, IL-6, IL-11, and TNF-α was observed in colon carcinoma, colon adenoma, ovarian cancer, and gastric cancer [22–28]. IL-2 and IL-15 are expressed in melanoma. IL-10 and TGF-β are expressed in myeloma, colon cancer, lung cancer, and mammary carcinoma [29, 30]. Expression of IL-17, IFN-γ and IL-4 has been observed in certain tumor types [31–33]. Expression of M-CSF, GM-CSF, and IL-3 has been observed as well [34–36]. These tumor-derived hematopoietic cytokines regulate myeloid cell-mediated inflammation and affect T-cell activity in tumors. Chemokines such as CXCL chemokines (CXCL1, 3, 6, 8, 10, and 12) and CCL chemokines (CCL1, 2, 5, 17, 25, and 28) are expressed in various tumor types [37–40]. Growth and angiogenic factors such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and hepatocyte growth factor (HGF) are broadly expressed in a number of cancer types [41, 42]. The cell types producing these factors are not limited to tumor cells but can be from vari-

ous cell types in tumors. For example, tumor-associated macrophages produce both inflammatory and immunosuppressive cytokines such as IL-1, IL-6, IL-10, and TGF-β [43].

T-cell receptor (TCR) activation signals are modified by the signals from co-stimulatory and co-inhibitory molecules, which are expressed by tumor cells and tumor-associated antigen-presenting cells (APC) [44]. These molecules include B7-1, B7-2, programmed cell death-1 ligand (PD-L1), PD-L2, ICOS-L, B7-H2, B7-H3, B7-H4, and B7-H6. Among these, PD-L1-PD and B7-1/2-CTLA-4 pairs play important roles in the formation of Tregs in tumor microenvironments [45–47]. Moreover, TNF receptor family members such as OX40, GITR, 4-1BB, and CD40 are expressed in tumors and regulate anti-tumor immune responses [48, 49].

Inflammatory mediators are produced in tumors. Cyclooxygenase-2 (COX-2) is highly expressed in malignant tumors [50, 51]. COX-2 expression is induced in hypoxic conditions or by cytokines and growth factors [52]. COX-2 generates prostaglandin H2 from arachidonic acid, which is processed to generate major inflammatory mediators such as prostaglandin D2 (PGD2), prostaglandin E2 (PGE2), prostaglandin I2 (PGI2), and thromboxane A2 (TXA2). These mediators regulate angiogenesis and various aspects of inflammatory responses in tumors [50].

Some tumor types are under the influence of microbe-associated molecular pattern (MAMP) receptor ligands if tumors are formed in barrier tissues such as the intestine or in patients infected with pathogens. In mucosal tissues, decreased barrier functions due to tumorigenesis or pre-existing inflammation can lead to bacterial invasion and induction of inflammatory responses. Furthermore, tumors that are associated with infection by papillomavirus (uterine cervical carcinoma), hepatitis B virus (hepatocellular carcinoma), Epstein-Barr virus (Burkitt's lymphoma), human T-cell leukemia virus (adult T-cell leukemia), or herpes virus (Kaposi's sarcoma) would be influenced by viral MAMPs. MAMPs and DAMPs activate toll-like receptors (TLRs) [53]. TLR activation can induce tissue inflammation that promotes cancer [54]. MYD88 signaling is also required for activation of dendritic cells for proper formation of effector T-cells. Without proper MYD88 signaling, Th2 cells that are ineffective in anti-tumor immunity can be made [55]. TLR signaling can work together with STAT3 and notch pathways to activate MAPK and NF-kB, which promote the survival and proliferation of tumor cells [56].

Retinoic acid is an anticancer agent. Retinoic acids such as all-trans retinoic acid (ATRA) and 9-cis RA are produced from retinol (vitamin A) by retinol-metabolizing enzymes such as ADH and RALDH [57]. Epithelial cells and APCs in the intestine highly express these enzymes [58]. In addition, tissue cells in many other organs express RALDHs and produce RAs. RALDH2 expression is induced during immune responses to increase the concentration of RA available in local tissue environments. Inflamed tissues or tumors express RA-producing RALDHs at low levels but highly express RA-catabolizing CYP26 enzymes [59, 60]. In sum, the tumor microenvironment is made of highly diverse factors. Some are from tumor cells, while others are from tissue cells and immune cells. These factors have profound effects on immune cells in tumors and associated lymphoid tissues as discussed in detail later in this chapter. Another important characteristic of tumor microenvironments is high levels of ATP metabolites such as AMP and adenosine. Tumor-infiltrating T-cells such as Tregs express CD39 and CD73. CD39 is an enzyme that degrades ATP into AMP, and CD73 is an ecto-5′-nuclease that degrades AMP into adenosine [61]. Through the action of these enzymes, extracellular ATP is converted to AMP and then to adenosine, which activates adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3) on endothelial cells to produce angiogenic factors [62]. This can result in immunosuppression to dampen anti-tumor immune responses.

6.3 Generation of Tregs and Th17 Cells

FoxP3$^+$ Tregs are made in the thymus as natural FoxP3$^+$ T-cells. They are also induced in the periphery from naïve CD4$^+$ T-cells. In addition, IL-10-producing Tregs (Tr1 cells) are made from naïve

$CD4^+$ T-cells. Tregs produce suppressive cytokines such as IL-10, IL-35, and TGF-β [63–65]. These Tregs play critical roles in preventing autoimmune diseases. Tregs are generally made whenever effector T-cells are formed during immune responses. This is important to limit the potentially inflammatory activities of effector T-cells.

Induction of effector T-cells and Tregs occurs mainly in secondary lymphoid tissues. One reason for this is that naive $CD4^+$ T-cells that become effector T-cells and Tregs migrate mainly to secondary lymphoid tissues. However, memory/effector T-cells can trans-differentiate into each other at any tissue sites upon antigen priming (Fig. 6.1a). Th1 cells are the most readily made effector T-cells from naïve $CD4^+$ T-cells. IL-12, a cytokine produced from DCs, promotes the generation of Th1 cells. Th2 cells are made when IL-4 is abundant. Th17 cells are generated when IL-6, TGF-β, and other inflammatory cytokines are present during T-cell priming. MAMPs and TLR activation in tissues promote the generation of Th17 cells. Th1 cells are efficient in promoting cell-mediated immunity through production of IFN-γ. Th17 cells are effective at inducing inflammatory conditions through producing IL-17. A number of inflammatory cytokines, neutrophil-attracting chemokines, and inflammatory mediators are induced by IL-17 [66]. IL-2 is required for the induction of T-cell proliferation. IL-7 and IL-15 drive T-cell proliferation in an antigen-independent manner in lymphopenic conditions [67, 68]. IL-2 suppresses the formation of Th17 cells [69]. IL-4, while inducing Th2 cells, suppresses the formation of induced $FoxP3^+$ T-cells and Th1 cells [70, 71]. IL-27 promotes the generation of Tr1 cells [72, 73]. Expression or activation of specific transcription factors is required for the generation of specialized effector T-cells and Tregs. For example, RORγt, STAT3, and aryl hydrocarbon receptor (AHR) are important for Th17 cells. FoxP3 and STAT5 are important for the formation of induced Tregs. c-Maf and aryl hydrocarbon receptor (AHR) are important for formation of Tr1 cells [63, 64, 74]. Beyond cytokines, many other factors can modulate the generation of Tregs and Th17 cells. This subject has been exhaustively discussed elsewhere and therefore will not be covered in detail.

6.4 Impact of Tumor-Derived Factors on T-Cell Differentiation

Most T-cells in tumors are memory T-cells [75]. Both antigen-specific and nonspecific bystander T-cells would be present in tumors. In general, the presence of memory T-cells and $CD8^+$ T-cells is linked to positive prognosis in cancer patients. This indicates that it is beneficial to have these T-cells in tumors. About 30–50% of $CD4^+$ T-cells in various tumors formed in animals are $FoxP3^+$ T-cells [75]. Th17 cells are also found in tumors, particularly tumors formed in mucosal tissues [8, 76, 77]. In contrast, Th17 cells are hard to find in transplanted tumors in animal models at ectopic sites [75]. Many factors of the tumor microenvironment can promote the generation of $FoxP3^+$ T-cells. First, APCs in tumor environments are prone to generate $FoxP3^+$ T-cells. During infection, DCs uptake antigens and undergo maturation in response to TLR and cytokine receptor activation. Activated DCs emigrate tissue sites of infection and migrate into secondary lymphoid tissues through lymphatic vessels. Only mature DCs express MHC molecules and co-stimulatory molecules such as B7-1 and B7-2 at high levels. In tumors, the signals to maturate DCs are diverse and not as apparent as those in infection. Thus, APCs maturated in tumor microenvironment do not highly express the co-stimulatory molecules [78]. Moreover, tumor-associated APCs express co-inhibitory receptor ligands such as PD-L1 and PD-L2 [79, 80]. This negatively affects T-cell activation and differentiation. Therefore, DCs in or from tumors have low activation potentials for T-cells. This condition typically generates induced $FoxP3^+$ T-cells but not effector T-cells. Other APCs in tumors, such as macrophages and MDSC, are also ineffective in generating effector T-cells but are prone to induce Tregs [81].

As mentioned, the hypoxic condition in the tumors is another regulatory factor for T-cells [82]. It is expected that draining lymph nodes or tertiary lymphoid tissues within tumors have low oxygen levels. T-cells become $FoxP3^+$ T-cells when they are activated in hypoxia [83]. This is in part mediated by a transcription factor called HIF-1α The high glycolytic activity in tumors leads to accumulation of lactic acid [84–86]. This

promotes the generation of FoxP3+ T-cells. TGF-β1 is a characteristic cytokine produced in the tumor environment [87–89]. TGF-β1 is the most efficient cytokine which induces FoxP3+ T-cells in the periphery. Along with TGF-β1, IL-10 acts to suppress anti-tumor immune responses and the promotion of Tregs [90, 91]. IL-10 is produced by various cell types, including T-cells, myeloid cells, B-cells, and tumor cells.

The prostaglandin inflammatory mediator PGE2 is highly produced in the tumor environment. PGE2 induces FoxP3+ T-cells. This induction is mediated by EP4 and EP2 receptors [92, 93]. In this regard, inhibition of cyclooxygenase-2 (COX-2) decreased FoxP3 expression in tumors and reduced tumor burden [94]. Interestingly, FoxP3+ Tregs express COX-2 and produce PGE2 [95]. The PGE2 produced by Tregs suppresses effector T-cells. In addition, prostaglandin D2 (PGD2) acts on DCs to induce FoxP3+ T-cells [96]. This effect is mediated through the D prostanoid receptor and cyclic AMP-dependent protein kinase A. In this regard, enforced expression of COX-2 in head and neck squamous cell carcinoma led to expansion of IL-10+ FoxP3+ T-cells [97].

Commensal bacterial products that activate TLR2 are implicated in selectively promoting FoxP3+ T-cells and Th17 cells. Segmented filamentous bacteria (SFB) promote Th17 cells in the small intestine [98]. Certain bacterial groups such as *Clostridium* and *Bacteroides fragilis* promote the generation of FoxP3+ T-cells in the intestine [99, 100]. Tumors, formed in the intestine, female reproductive tract, and skin, are expected to be heavily influenced by commensal bacteria. In these tumors, bacterial MAMPs would activate APC and T-cells to regulate the generation of FoxP3+ T-cells and Th17 cells. Thus, depending on the bacterial group that is dominant in the tumor environment, FoxP3+ T-cells and Th17 cells can be differentially generated. In addition, short-chain fatty acids, such as acetate, propionate, and butyrate, are produced from dietary fibers by gut commensal bacteria [101]. These metabolites can regulate T-cells and epithelial cells and have anti-tumor activity in the colon [102–104].

As mentioned, retinoic acid is an important tumor factor. Retinoic acid affects the phenotype and numbers of T-cells and the growth and differentiation status of tumor cells. Retinoic acid promotes the generation of FoxP3+ T-cells but suppresses that of Th17 cells [105, 106]. Retinoic acid affects the development of DCs and generates tolerogenic DCs expressing Arg1 [107]. These DCs promote the generation of FoxP3+ T-cells but suppress the formation of Th17 cells. This function seems to be mediated through RARα. It is also reported that retinoic acid at low concentrations (i.e., 0.5–5 nM) is required for normal function of effector T-cells [108, 109]. Low concentrations of RA are found in the blood and bodily fluids in most tissues. In vitamin A deficiency, the migration and function of effector T-cells are severely impaired. As mentioned, tumor cells express CYP26 A1/B1/C1 enzymes and can decrease retinoic acid concentrations in tumors and associated tissues [59]. This hypo-retinoic acid condition would significantly affect the T-cell profile in tumors and associated lymphoid tissues. Moreover, retinoic acid can promote differentiation of tumor-associated MDSC into dendritic cells and macrophages [110]. In this regard, increased RA levels by inhibiting CYP26 enzymes had anti-tumor effects in experimental colon cancer formation [104].

6.5 Migration of Tregs and Th17 Cells into Tumors

Migration of T-cells, including Tregs and Th17 cells, is regulated by trafficking receptors such as chemokine receptors and adhesion molecules [111, 112]. Adhesion molecules such as selectins and integrins mediate rolling and firm adhesion of leukocytes on endothelial cell vessels [113, 114]. Chemokines induce integrin activation between rolling and firm adhesion of immune cells on endothelial cells. Chemokines also induce chemotaxis for migration of immune cells within tissues. Organs and tissues express distinct and overlapping chemokines and adhesion molecules for regulation of immune cell migration [115]. Since tumors are formed within specialized organs and tissues, there are similarities in expression of trafficking signals between normal tissues

and tumors formed within the tissues. Compared to normal tissues, however, tumors have altered expression of chemokines and adhesion molecules [116]. The trafficking signals and receptors required for T-cell migration into the intestine are well established. In the intestine, CCL20 and CCL25 are, respectively, expressed in the subepithelial cell dome (SED) of Peyer's patches and by small intestinal epithelial cells [117–120]. Endothelial cells in the intestine, Peyer's patches, and mesenteric lymph nodes express mucosal addressin cell adhesion molecule-1 (MAdCAM-1) [121]. T-cells migrating to the small intestine express CCR9 and α4β7 [122–124]. Memory T-cells migrating to the Peyer's patches express CCR6 [125, 126]. Naïve T-cells migrating to Peyer's patches, MLN, and PLN express CCR7, α4β7, and CD62L [127]. Memory T-cells migrating to other tissues or inflamed tissues variably express CCR1-6, CCR8, CCR9, CCR10, CXCR3, CXCR5, and CXCR6 [115]. Effector T-cells frequently express P-selectin glycoprotein ligand-1 (PSGL-1), E-selectin ligand-1 (ESL-1), CXCR3, CCR5, and CCR4 [112, 127].

The trafficking receptors of Tregs and Th17 cells have been determined. FoxP3$^+$ T-cells that are made in the thymus express CCR7, CXCR4, and CD62L [128, 129]. FoxP3$^+$ T-cells activated or induced in the periphery express memory-type trafficking receptors that are frequently expressed by Th1 or Th2 cells. Th17 cells express most memory-type chemokine receptors [130, 131]. CCR6 is a characteristic chemokine receptor expressed by most Th17 cells. In general, FoxP3$^+$ Tregs and Th17 cells follow the trafficking pattern of conventional naïve and memory/effector T-cells. Conventional naïve CD4$^+$ T-cells expressing CCR7 and CD62L lose these receptors upon T-cell activation in the secondary lymphoid tissues and migrate into non-lymphoid or inflamed tissues. Various tissue factors influence the expression of trafficking receptors on FoxP3$^+$ T-cells and Th17 cells [132, 133]. For example, retinoic acid acts on T-cells undergoing activation to induce gut homing receptors such as CCR9 and α4β7. FoxP3$^+$ T-cells and Th17 cells express these gut homing receptors and migrate to the intestine [105, 134]. In vitamin A deficiency, the number of FoxP3$^+$ T-cells and Th17 cells in the gut is significantly decreased in part because most T-cells do not migrate to the small intestine [135]. In addition, TGF-β1 is a major cytokine that induces the expression of CCR6 on FoxP3$^+$ T-cells and Th17 cells [130]. Moreover, IL-2 is a cytokine that effectively downregulates CCR6 expression induced by TGF- β1. Thus, cytokines and tissue factors can co-regulate the expression of trafficking receptors on T-cells.

Researchers have been searching for chemokines that regulate immune cell trafficking and anti-tumor immune responses [136–140]. Chemokines such as CCL3-5, CCL20, and CXCL10, often expressed in inflamed tissues, are also expressed in tumors [141–146]. Chemokines induce chemotaxis of immune cells and tumor cells. They can co-stimulate T-cells and promote angiogenesis [147, 148]. CCR2-10 and CXCR3-5 regulate T-cell trafficking in various tumors [139]. Most of these receptors are highly expressed by FoxP3$^+$ T-cells and Th17 cells in mice and humans [112, 128–131, 149]. CCL17 and CCL22 are highly expressed in gastric cancer with CCR4-expressing FoxP3$^+$ T-cells [138]. CCR7 is expressed by some T-cells in colorectal cancers and is predictive of positive prognosis [150]. CXCR4$^+$ T-cells are increased in lung adenocarcinoma [151]. Chemokines expressed in tumors also attract hematopoietic progenitors, myeloid cells, NK cells, and CD8$^+$ T-cells [11, 143, 152]. Chemokine signals are highly heterogeneous among different tumors. They are shaped by tissue-specific and inflammatory microenvironments in tumors. Therefore, it is difficult to find universal trafficking signals which govern T-cell trafficking in many tumors.

Our group investigated the trafficking receptors expressed by tumor-infiltrating FoxP3$^+$ T-cells [75]. FoxP3$^+$ T-cells account for 25–50% of CD4$^+$ T-cells infiltrating A20, CT26, 4T1, and B16 tumors. Most of these FoxP3$^+$ T-cells are memory CD44$^+$ CD62$^-$ T-cells, which are downregulated for CD62L and CCR7. Downregulation of CCR7 was critical for the migration of FoxP3$^+$ T-cells into tumors, as CCR7^{high} FoxP3$^+$ T-cells were not efficient at migrating into tumors [75]. Downregulation of CCR7 and CD62L occurs in tumor-draining lymph nodes during antigen priming. Therefore, migration of T-cells into secondary lymphoid tissues is required to acquire a

proper trafficking receptor phenotype for migration into tumors. While downregulated for CCR7 and CD62L, tumor-infiltrating FoxP3⁺ T-cells express CCR8 and CXCR4 at high levels [75]. This trafficking receptor phenotype reflects the differentiation status of the tumor-infiltrating T-cells and/or the trafficking receptor requirement for FoxP3⁺ T-cell migration into the tumors. Induction of FoxP3⁺ T-cells from FoxP3⁻ T-cells in tumors is not efficient [75]. Thus, the tumor-infiltrating FoxP3⁺ T-cells in these tumors are largely from the FoxP3⁺ T-cells made in the thymus or secondary lymphoid tissues rather than FoxP3⁺ T-cells induced directly in tumors. However, this can be quite different in other types of tumors where the tumor microenvironment is more conducive in priming T-cells for differentiation into Tregs. For example, TGF-β cytokines are expressed in many tumors and efficient in inducing Tregs [89]. However, this depends on migration of naïve T-cells into tumors which is not likely in most tumor types. In this regard,

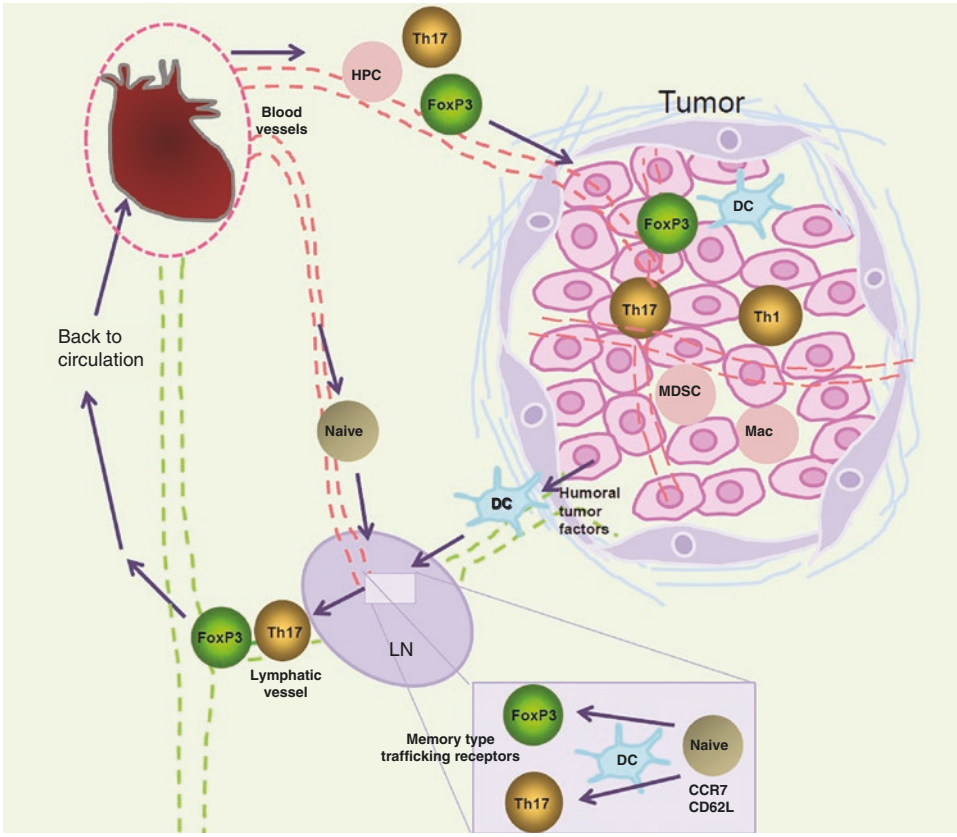

Fig. 6.2 Migration of FoxP3⁺ T-cells and Th17 cells into tumors. Natural FoxP3⁺ T-cells made in the thymus can migrate into lymph nodes, but cannot migrate directly into tumors unless tumors are formed in lymphoid tissues. FoxP3⁺ T-cells can migrate into tumors after they are antigen-primed in secondary lymphoid tissues and gain the memory-/effector-type trafficking receptors. Loss of CCR7 and CD62L occurs during antigen priming and is required for migration of antigen-primed FoxP3⁺ T-cells into tumors. Induced FoxP3⁺ T-cells in the tumor-draining lymph nodes can migrate into tumors, as they decrease the expression of CCR7 and CD62L but upregulate memory-/effector-type trafficking receptors such as CCR4, CCR5, CCR8, CCR10, and/or CXCR4. Dendritic cells (DCs) transport and present tumor antigens and play important roles in generating FoxP3⁺ T-cells and Th17 cells in lymph nodes. Soluble tumor-derived factors are collected in draining lymph nodes, and some of these factors affect T-cell priming and differentiation. In tumors, macrophages (Mac), DCs, and MDSC suboptimally activate T-cells in tumors. These APCs play potentially important roles in maintaining the phenotype of FoxP3⁺ T-cells and Th17 cells in tumors. There is no such thing as tumor-specific trafficking receptors. Instead, T-cells variably use conventional trafficking receptors to migrate into different tumors

generation of Tregs from non-Treg effector cells such as Th17 and Th1 cells is a potentially important pathway to generate Tregs in tumors [153, 154]. In tumors, FoxP3$^+$ T-cells appear highly stable in maintaining their FoxP3 expression. Th17 cells would probably utilize the same tissue- or inflammation-associated trafficking signals utilized by Th17 cells.Th17 cells are prevalent in the gastrointestinal (GI) tract and other mucosal tissues. High numbers of Th17 cells were observed in aggressive forms of GI cancers [8, 76, 77]. Thus, these tumors would have trafficking and cytokine signals appropriate for recruitment and maintenance of Th17 cells or their progenitors. For example, CCL20 is expressed by cervical cancer and recruits Th17 cells [155]. Migration of FoxP3$^+$ T-cells and Th17 cells into tumors and draining lymph nodes is summarized in Fig. 6.2.

6.6 Impact of Tregs and Th17 Cells on Anti-Tumor Immune Responses

The presence of T-cells in tumors is a highly reliable prognostic factor for survival of cancer patients [156, 157]. There is a strong positive correlation between patient survival and frequencies of memory CD4$^+$ T-cells and CD8$^+$ T-cells in many cancer types. Tumorigenesis is increased in pan-T cell- or γδT cell-deficient animals or humans [158]. Strikingly, αβ T-cells have a small negative effect on tumor numbers, but exert a greater positive effect on tumor size. This implies that αβ T-cells are composed of heterogeneous subsets with different functions, and some of these T-cells may even promote tumor growth. FoxP3$^+$ T-cells and other regulatory T-cells are likely the T-cells that suppress anti-tumor immune responses. FoxP3$^+$ T-cells can inhibit anti-tumor immune responses and promote tumor growth [159]. Many FoxP3$^+$ T-cells are self-reactive and effective in preventing autoimmune diseases. The same function can be used to promote tumor growth. This is because tumor cells basically express self-antigens and FoxP3$^+$ T-cells can effectively suppress immune responses to self-antigens [160]. In line with this, the frequencies of FoxP3$^+$ T-cells in many tumor types are inversely correlated with patient survival rates [157, 161]. However, lack of correlation or positive correlation has been noticed as well [162, 163]. A good example is colorectal carcinoma, in which high frequencies of FoxP3$^+$ T-cells are associated with a favorable prognosis [6]. It is expected that FoxP3$^+$ T-cells can even prevent the formation of some tumors by suppressing tissue inflammation at early stages of tumorigenesis. Therefore, FoxP3$^+$ T-cells have the potential to either promote or suppress tumorigenesis depending on tumor types, tissue sites, and immune responses. The potentially complex functions of Tregs in tumorigenesis are depicted in Fig. 6.1.

It has been observed that Th17 cells can promote CD8$^+$ T-cell-mediated anti-tumor immune responses in a mouse model [164]. Moreover, polarization of CD8$^+$ T-cells into Tc17 cells increased their anti-tumor immunity [165]. Th17 cells may become Th1 cells or activate CD8$^+$ T-cells to increase anti-tumor immunity. Paradoxically, Th17 cells can cause inflammation to initiate development of inflammatory tumors at early stages of tumorigenesis. In colorectal cancer, Th17 cells are linked to poor prognosis, whereas Th1 cells are positively linked to patient survival [166]. The major cytokine product of Th17 cells, IL-17, can induce tissue inflammation and the expression of certain angiogenic factors, including CXCL8, MMP-2, MMP-9, and VEGF [167]. For example, colon cancer-derived Th17 cells triggered the production of the aforementioned tumor-promoting factors by tumor-associated stroma. On the other hand, they recruit beneficial neutrophils through IL-8 secretion and drive cytotoxic T-cells into tumor tissues by producing chemokines [168]. The function of Th17 cells in cancer can be complex

and appears to be determined again by cancer type, stage, and site. The potentially complex functions of Th17 cells in tumorigenesis are depicted in Fig. 6.1.

Apart from their effector functions, the frequencies of FoxP3$^+$ T-cells and Th17 cells reflect the context of the tumor microenvironment. Non-

inflammatory tumors with low expression of IL-6 and other inflammatory cytokines would have high numbers of FoxP3+ T-cells, whereas inflammatory tumors with high expression of inflammatory cytokines would harbor high numbers of Th17 cells. Tumors are heterogeneous in the tumor microenvironment even within the same group of cancers, and not all tumors fit into the inflammatory vs. non-inflammatory tumor model. While there is an inverse correlation between FoxP3+ T-cells and Th17 cells, both T-cell subsets can be increased or decreased depending on the balance of cytokines and other tissue factors. An example for this situation is invasive ductal breast carcinoma [167].

As discussed throughout this article, FoxP3+ T-cells and Th17 cells play both positive and negative roles in regulating anti-tumor immune responses (Fig. 6.1). Despite the presence of these T-cells, some tumors still develop and grow. Thus, these T-cells by themselves may not effectively mount anti-tumor immune responses. More detailed studies on FoxP3+ T-cells and Th17 cells in various tumors can provide systematic information regarding the tumor microenvironment and therapeutic interventions. It is important to develop novel strategies to boost the beneficial effects of the T-cell subsets and to suppress their tumor-promoting effects. The key is to alter tumor microenvironment to regulate these T-cell subsets. This is expected to be achieved through control of antigen-presenting cells, metabolism, cytokines, chemokines, co-stimulatory/inhibitory receptors, inflammatory mediators, and nuclear hormone receptor ligands such as retinoic acid. Regulation of multiple factors at the same time would provide more effective strategies in tipping the T-cell balance toward tumor-eradicating immune responses. A

one-size-fits-all approach is not likely to be effective in changing the microenvironment and T-cell activity in all tumors. In this regard, another point is that anti-tumor therapy strategies should be tailor-made based on cancer type, tissue site, and tumor microenvironment. Chimeric antigen receptor (CAR)-based or PD-1-based therapies utilizing or targeting these T-cells would be also promising strategies [169]. It is expected that application of wrong immunotherapy strategies to regulate the T-cell subsets would even exacerbate malignant diseases in cancer patients.

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7 T-Cell Metabolism and Its Dysfunction Induced by Cancer

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Contents

7.1 Introduction

During its life, T-cell goes across several stages of development that takes place in different organs or tissues. These dissimilar microenvironments, where T-cell differentiation takes place, will require different metabolic processes and metabolic demands. Immunometabolism refers to the integrative study of the biochemical reactions that provide building blocks and energy to fulfill the varying demands in the life of immune cells and particularly T-cells.

7.2 T-Cell Life

The development of a T-cell response begins when circulating naive T-cells (T_N) encounter an antigen presented by an antigen-presenting cell such as dendritic cells, macrophages, or B-cells. In the proper context (co-stimulatory molecules and cytokines), the antigen-specific T-cell is activated. Then, after TCR interaction with the cognate antigen, a cascade of intracellular events

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occurs that leads to activation, proliferation, and differentiation. Effector T-cells (T_{EFF}) produce cytokines or, in the case of $CD8^+$ T-cells, granzymes and perforin to control the infection and to kill pathogen-infected cells or transformed cells. After the infection or inflammatory process is controlled, T-cells undergo a contraction process, where most effector T-cells die by apoptosis, but the others establish a pool of memory T-cells (T_M), including different subsets, such as effector memory T-cells (T_{EM}), central memory T-cells (T_{CM}), and stem cell memory T-cells (T_{SCM}), conferring long-term protection [1]. However, if the source persists (i.e., chronic infection), T-cell response will become progressively exhausted because of the continued TCR signaling with the persistent antigen. Exhausted T-cells (T_{exh}) have poor effector functions and overexpress multiple inhibitor receptors, such as PD-1, that attenuate signaling downstream of the TCR [2].

The subsets of antigen-experienced T-cells are heterogeneous and differ in their function, localization, and phenotype. T_N are T-cells that have not yet encountered their cognate antigen, have a high proliferative potential, and are located in the blood and lymph nodes. T_{EFF} are short-lived, and they produce cytokines, or molecules involved in cytolysis, and have a low proliferative potential. T_{EFF} are considered as terminally differentiated T-cells. Memory T-cells have been divided in at least two subsets, based on the expression of homing and chemokine receptors, their localization, proliferative capacity, and on their function. T_{CM} cells, similar to T_N cells, are also located in the blood and migrate to secondary lymph nodes. T_{CM} cells produce low levels of inflammatory cytokines, such as interferon-gamma (IFN-γ), and they can secrete high levels of IL-2. T_{EM} cells have the potential to home into non-lymphoid tissues, they produce high levels of inflammatory cytokines, and their cytotoxic potential is higher with respect to T_{CM}; thus, T_{EM} cells have similar effector functions compared to T_{EFF}; nevertheless, the proliferative potential of T_{EM} cells is lower compared to T_{CM} and T_N cells [3–6].

The characterization of the different subsets of T-cells is still a matter of debate; because, among other things, the differentiation of T-cells is a process exquisitely regulated, where metabolism has an essential role. Several metabolic pathways have been recently described that modulate and participate in the differentiation process of T-cells.

7.3 Catabolism and Anabolism of T-Cells Throughout the Different States of Maturation: Naive, Effector, and Memory Cell

7.3.1 Naive

Quiescent T-cells (T_N and T_M cells) have energetic demands relatively smaller than activated T-cells. Resting T-cells have a low rate of nutrient consumption; thus, the catabolic pathways for ATP production favor the highest ATP yield, using fatty acid oxidation (FAO) and the mitochondrial tricarboxylic acid cycle (TCA or Krebs cycle) [7–9]. Alternatively, glucose or amino acids may fuel TCA [7].

Quiescent T-cells have the ability to persist for long periods and to respond to the antigen-presenting cell. However, T_N cells exhibit a greater clonal expansion than T_M cells. Also T_M cells differ from T_N cells in that they have relatively fewer requirements of co-stimulating signals for their activation, proliferation, and function [10], possibly because, among other factors, T_N cells have less mitochondria than T_M cells [11].

7.3.2 Activation

Upon antigen presentation, naive $CD8^+$ T-cells are activated. The activation of T-cells requires the TCR receptor and other co-stimulatory molecules, such as CD28 and CD27; during this process activation markers are expressed on the surface of T-cell, for instance, CD137 and CD69 among others (Fig. 7.1). Co-stimulation is followed by a fast peak of glucose consumption and the enhancement of glycolytic metabolism [7]. Thus, activation is accompanied by increased

expression of the glucose transporter Glut1 and a significant immediate increase in glycolytic flux in both human $CD4^+$ and $CD8^+$ T-cells. Undergoing a "Warburg-like" switch allows to increase the glycolytic metabolism upon activation of $CD4^+$ and $CD8^+$ T-cells, though, $CD4^+$ T-cells exhibit higher basal levels of glycolysis, possibly because hexokinase II (HKII), pyruvate kinase (PKM2), and lactate dehydrogenase (LDH) levels are all increased in $CD4^+$ T-cells in comparison to $CD8^+$ subset [12]. Interestingly, cytokines production in $CD8^+$ T-cell is dependent on mitochondrial metabolism [12]. In another study, Yin et al. showed that in vitro activation of $CD4^+$ T-cell increases both glucose and glutamine metabolism, where pyruvate might be oxidized by the mitochondria or reduced into lactate. Interestingly, the production of pro-inflammatory cytokine IL-17A is dependent on both last processes, and the IFN-γ production is dependent only on pyruvate oxidation [13], suggesting that the metabolism of Th17 cells differs from Th1 cells.

When T-cells are activated, anabolic processes such as protein and lipid synthesis are increased, whereas catabolic processes like beta-oxidation are suppressed [7]. Also the synthesis of metabolites such as polyamines, cholesterol via fatty acid (FA) synthase, and the pentose phosphate intermediates enhances T-cell activation [12].

After T-cell activation via TCR and CD28, phosphatidylinositol-3,4,5-trisphosphate (PIP3) is produced via phosphoinositide 3-kinase (PI3K) activation. The oxidative phosphorylation (OXPHOS) rate might be increased when calcium is introduced into the mitochondria after being released from endoplasmic reticulum via PIP3 promotion [7]. PIP3 activates downstream signaling components, AKT being the most notable. mTOR and AKT are at the center of a conserved mechanism that permits eukaryotes to integrate nutrient and growth factor signals and, in turn, control proliferation and cell growth [14]. A continuous stimulation of TCR depletes calcium in the endoplasmic reticulum allowing the extracellular calcium entrance. The permanent aperture of the calcium release-activated channels (CRACs) allows the extracellular flux of calcium supporting an effective activation of T-cell [7]. Recent studies suggest that the entrance of

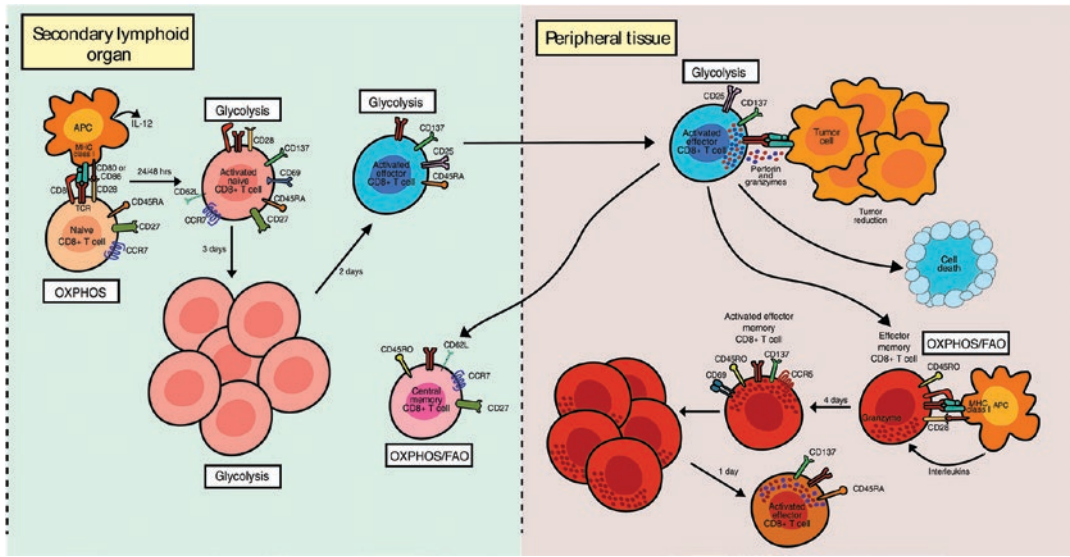

Fig. 7.1 Activation, differentiation, and metabolic profile of T-cells. After activation, naive T-cells proliferate and differentiate in the lymph nodes until they acquire effector functions; then effector T-cells migrate to peripheral tissues. When the infection or inflammatory process is resolved, most of the effector T-cells undergo cell death, and a small proportion differentiate into memory T-cells. During this different maturation stages, the metabolism must change in order to fulfill the nutritional and energetic requirements of T-cells

calcium through the CRACs induces mobilization of the mitochondria into the immune synapses, where the accumulation of CRACs has been found. High levels of cytosolic calcium activate transcription factors that regulate the T-cell response, such as NFAT1 and NF-$_{\kappa}$B, the last being involved in IL-2 production [15].

T-cells undergo metabolic conversion from OXPHOS to glycolysis when they are activated. Nevertheless, in the presence of rapamycin, antigen-stimulated CD8^{+} T-cells increase both OXPHOS and glycolysis. Rapamycin inhibits the mammalian target of rapamycin (mTOR) pathway. Rapamycin treatment also augments the formation of memory cell precursors, and their progenies live longer than memory cells. Interestingly, rapamycin-treated cells endure glucose and IL-2 withdrawal in vitro. Nevertheless, oligomycin inhibition of OXPHOS in rapamycin-treated T-cells causes mitochondrial hyperpolarization, decreased ATP, and increased reactive oxygen species (ROS) formation [16].

7.3.3 Proliferation

The frequency of specific T-cells for any given antigen is low before activation (1/100,000). Following antigen presentation in an appropriate context, antigen-specific T-cells undergo a process of massive proliferation, increasing up to 10,000- to 50,000-fold in their number [1, 17]. For sustaining this massive proliferation, it is necessary that several biosynthetic metabolic pathways become activated, and the mitochondria play an important role in these processes.

The mitochondria of proliferative T-cells take a biosynthetic role where pyruvate and glutamine are intermediary molecules of other biosynthetic pathways. Pyruvate is the last product of glycolysis and is imported into the mitochondria; there, pyruvate is transformed in acetyl-CoA, which will be converted into citrate. The latter can continue through Krebs cycle possibly supporting amino acid and fatty acid (FA) synthesis. Although T-cells could import extracellular lipids, they rather produce de novo lipids to synthesize new membranes during cell growth and proliferation. Amino acids such as aspartic acid, asparagine, arginine, glutamine, and proline are synthesized from Krebs cycle intermediaries. Additionally, glutamine is the amine donor for purine base synthesis during the proliferation of T-cells [18].

The upregulation of the calcium-dependent dehydrogenases supports higher levels of NADH that are used for maintaining mitochondrial respiration. The reduced cofactors (NADH and FADH) produced from this cycle will feed OXPHOS, which will maintain the mitochondrial membrane potential (ψ), thus suppressing apoptosis of proliferative lymphocytes [7]. The persistent saturation of the OXPHOS might increase the production of ROS, which might stimulate the biosynthesis of nucleotides and in consequence might promote T-cells that enter into the S-phase of the cell cycle. In this case, ROS would act as second messenger indicating suitable energetic conditions to support T-cell proliferation [7].

7.3.4 Effector

Although effector T-cells (T_{EFF}) change their metabolic program from OXPHOS to aerobic glycolysis allowing the macromolecular synthesis [2], both energetic metabolisms might fuel T-cell proliferation [19]. However, it has been reported that only aerobic glycolysis is required to support the effector function of CD4^{+} T-cells, like the ability to produce IFN-γ. In this regard, GAPDH binds to AU-rich elements within the 3'UTR of IFN-γ mRNA as a posttranscriptional control, reducing protein translation. This GAPDH-mediated inhibition may be controlled by its metabolic function or by the expression level of GAPDH within CD4^{+} T-cells [19]. On the other hand, Tripmacher et al. reported that the cytokine synthesis is not affected by the myxothiazol inhibition of OXPHOS or by the absence of glucose; instead CD4^{+} T-cell proliferation strongly depends on glucose availability [20]. T_{EFF} cells that fulfill this energetic change are rendered

dependent on glucose and possibly lose the ability to obtain energy from other substrates.

Alternatively, it has also been described that T_{EFF} cells can acquire external FA and store the excess in cytoplasmic lipid droplets [21]. The reduction in glycolytic flux in pro-inflammatory T-cells from rheumatoid arthritis (RA) leads to deficiencies in ATP and pyruvate, which triggers fatty acid biosynthesis and the formation of lipid droplets. This metabolic phenotype is associated with the tissue migration of RA T-cells in vivo [22]. Recently, it has been shown that regulatory T-cells (T_{reg}, $CD4^+$ $Foxp3^+$) uptake FA at a higher rate than T_{EFF} cell subsets, supporting the role of FA metabolism for T_{reg} function [23].

It has been reported that effector memory $CD8^+$ T-cells that re-express CD45RA exhibit multiple characteristics of senescence. Although $CD8^+$ T_{EM} cells have potent functional activity, these cells can accumulate nonfunctional giant mitochondria increasing the mitochondrial mass [24]. On the other hand, Van der Windt et al. found that T_{EFF} cells have less mitochondria than T_M cells possibly because after Ag presentation, the latter proliferates while infection is present and eventually outpaces its own mitochondrial biogenesis; other possibility might be that the mitochondria is unequally segregated during T-cell division. Interestingly, the secondary T_{EFF} expansion is more prolonged than primary T_{EFF} [11].

7.3.5 Memory

After antigen stimulation, T-cells might follow different fates where the levels of glycolysis can act as a metabolic rheostat determining the fate of the immune response. Whereas low levels of glycolysis favor the long-term response with the establishment of memory cells (T_{CM}, T_{EM}, T_{SCM}) and the upregulation of transcription factors (such as *Tcf7*, *Lef1*, and *Bcl6*) associated with this phenomenon, a high glycolytic metabolism supports a T_{EFF} cell profile. This profile is characterized by short-lived cells with strong effective responses, accompanied by a high expression of transcription factors such as *Blimp-1* [9].

A substantial spare respiratory capacity (SRC) is maintained in the mitochondria of $CD8^+$ T_M cells to produce energy under conditions of increased activation or stress; this extra mitochondrial capacity is thought to be important for long-term cellular survival and function [25]. It has been shown that the SRC of $CD8^+$ T_M cells is dependent on the ability of the mitochondria to promote fatty acid oxidation, because FAO, at least partly, provides substrates (long-chain fatty acids, medium- or short-chain fatty acids) for OXPHOS, showing the link between lipid metabolism (burning fat) and cellular longevity in the immune system [11]. In addition, glutamine might also contribute to OXPHOS in T_M cells [11].

As it has been already mentioned, in the differentiation to memory cells, a shift back to mitochondrial OXPHOS is fueled at least in part by FAO [2]. The overexpression of carnitine palmitoyltransferase 1a (Cpt1a), a rate-limiting in FAO, results in increased numbers of $CD8^+$ T_M cells [25]. The pharmacological modulation of FAO can also enhance $CD8^+$ T_M development after vaccination; nonetheless, deletion of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), which is a molecule that downregulates T-cell activation and is an important signal for promoting (inducing) FAO, impairs the development of T_M cells [8]. Additionally, O'Sullivan et al. reported that T_M cells use extracellular glucose to support OXPHOS and FAO, based on their finding that $CD8^+$ T_M cells acquire substantially fewer long-chain FA from the microenvironment than $CD8^+$ T_{EFF} cells. These same authors suggest that lipids must be first synthesized to generate substrates needed for FAO; accordingly, the lysosomal acid lipase (LAL) is expressed in T_M cells sustaining lysosomal lipolysis and boosting memory T-cell development after infection. LAL hydrolyzes triacylglycerol (TAG) and cholesterol esters (CE) to generate cholesterol and free FA in cell lysosomes and participates in mobilization of FA for FAO and T_M cell development, suggesting a futile cycle that allows T_M cells to preserve

glycolytic and lipogenic machinery and maintaining mitochondrial health over long periods of quiescence [21].

Additionally, IL-15, a cytokine that favors survival and self-renewal of T_M cells, promotes the mitochondrial biogenesis favoring the T_M phenotype and enhancing SRC [25]. One characteristic of $CD8^+$ T_M cells is the ability to mount a stronger and faster response to reinfection, supported by mitochondrial ATP production that also facilitates the activity of the glycolytic enzyme hexokinase (HK). It has been shown that this enzyme can be bound to the mitochondrial outer membrane of $CD8^+$ T-cells. HK is quickly recruited to the mitochondria in response to Akt response upon activation. This configuration allows the rapid function of this first enzyme of glycolysis, increasing its accessibility to mitochondrial ATP. This process facilitates the rapid activation of glycolysis that supports proliferation. Mitochondrial HK dissociation impairs proliferation and the rapid engagement of glycolysis, decreasing the secondary effector T-cell response [11]. In contrast, the overexpression of the glycolytic enzyme phosphoglycerate mutase-1 (Pgam1) does not allow the differentiation to long-term memory $CD8^+$ T-cells [9].

Also because AMPK has emerged as an important regulator of memory $CD8^+$ T-cells, a pharmacologic activation of AMPK with metformin might result in enhanced differentiation to $CD8^+$ T memory cells [8].

Recently, the key role of Opa1 in the T_M cell generation has been studied; this protein participates in joining the mitochondrial inner membranes allowing the mitochondrial fusion. Tight mitochondrial cristae organization in T_M cells maintains closely associated electron transport complexes (ETC), which makes the activity of ETC more efficient, favoring a redox balance and allowing continuous entrance of pyruvate into the mitochondria. Survival of Opa1-deficient T_M cells is severely impaired, possibly because FAO cannot be efficiently engaged [26]. T_M cells ensure that any generated pyruvate will efficiently feed into the TCA cycle; in consequence the mitochondrial cristae are tightly configured maximizing the performance of OXPHOS [26].

7.4 Metabolism of T-Cell Is Modified in the Tumor Microenvironment

Cancer cells inhibit anti-tumor immunity through both immunomodulatory receptors and the creation of an immunosuppressive microenvironment. Development and effective function of T-cells are also affected by both nutrient deficiencies, and the excess of waste products of tumor cell metabolism favors acidosis, such as lactate and other metabolites.

7.4.1 Glucose Limitation

Glucose limitation is one of the most important traits of tumor microenvironment. Competition for glucose occurs between tumor cells and tumor-infiltrating lymphocytes (TILs) in the tumor microenvironment, where the nutritional state is crucial in the functionality of TILs. Under nutrient restrictions T-cells can become hyporesponsive; when nutrient deprivation of T-cells is prolonged, even if tumors are highly antigenic, cytokine production is dampened and becomes relatively irreversible [27].

Schurich et al. compared the metabolic profile of $CD8^+$ T-cells originated from subjects affected by two chronic virus infections, CMV and HBV. While CMV-specific T-cells supply their energetic demands by the use of both glycolysis and OXPHOS to exert effector functions, the HBV-specific $CD8^+$ T-cells, which show an exhausted phenotype, characterized by expression of inhibitory molecules like Tim-3 and CTLA-4, do not use OXPHOS and show a dependence on glycolysis [28]. These exhausted cells also present a higher Glut1 expression. When these T_{exh} cells are forced to use OXPHOS, they present a decrease in cytokine production and an increase in the expression of co-inhibitory molecule PD-1. Interestingly, the pro-inflammatory

cytokine IL-12 can stimulate a recovery of HBV-specific effector function and a reduction of PD-1 expression on $CD8^+$ T-cells [28].

Glucose limitation in the early stages of differentiation of the $CD8^+$ T_{EFF} cells promotes T-cell exhaustion, but if there is a recovery of glucose levels after the antigen presentation, the development of T_M cells can be completed. However, if the glucose limitation persists, the exhausted phenotype will be promoted and supported by PD-1 expression [2]. PD-1 signaling reduces glycolysis and promotes FAO in total $CD4^+$ T-cells [29]. PD-1 signaling suppresses Akt/mTOR and aerobic glycolysis and also inhibits PGC-1α and mitochondrial depolarization [27, 30]. On the other hand, mTOR signaling has been shown to contribute to mitochondrial depolarization in early T_{exh} cells. A possible explanation for this apparent contradictory phenomenon is that, mTOR signaling might be activated by other pathways such as Ras, PI3K, PTEN, or p53, which has been reported to occur in cancer pathogenesis [14, 31]. The blockade of the PD-1 pathway reverts the metabolic depression in T_{exh} cells that has an intermediate expression of inhibitory receptor PD-1, whereas limiting mTOR activity by rapamycin improves the mitochondrial fitness of early T_{exh} cells [2]. In a study by Chang et al., the blockade of CTLA-4, PD-1, or PD-L1 with antagonist antibodies in transplanted mice with progressing tumors (D42m1-T3 cells) increases glucose levels in the extracellular tumor milieu, allowing TILs to restore their glycolytic capacity and their effector function. The authors also noted that this checkpoint blockade might be most effective against tumors with higher glycolytic rates [27].

Several $Ca2^+$ channels expressed on plasma membrane (PMCA), mitochondrial membrane (MCU), and ER membrane (SERCA) modulate cytosolic $Ca2^+$ levels. Glycolysis also suppresses SERCA-mediated $Ca2^+$ reuptake activity, allowing the activation of nuclear factor of activated T-cell (NFAT) signaling and anti-tumor responses. In contrast under glucose deprivation, levels of phosphoenolpyruvate (PEP, a glycolytic metabolite) diminish, allowing the $Ca2^+$ reuptake activity of SERCA. These low levels of $Ca2^+$ lead to defective NFAT signaling and effector functions [10]. Reprogramming TILs through phosphoenolpyruvate carboxykinase 1 (PCK-1) overexpression in T-cells increases PEP production, yielding stronger anti-tumor responses in glucose-deprived cells. Lactic acid and FAs might be replenishing Krebs cycle for PEP production [10].

Effector functions of T-cells are affected by low levels of glucose or high lactate conditions, loss of glycolysis impairs the ability to produce IFN-γ and maintain intracellular calcium in T_{EFF} cells. Nonetheless, Angelin et al. found that T_{reg} cells were not affected by low glucose and high lactate conditions. In these cells, Foxp3 negatively regulates Myc, and as a consequence, glycolysis diminishes. Foxp3 also promotes OXPHOS regenerating NAD^+ that reduced to NADH by LDH. GAPDH activity in T_{EFF} cells, which is a key role enzyme of glycolysis, is inhibited by high levels of NADH; in contrast, T_{reg} cells are less dependent on glycolysis. This T_{reg} metabolic phenotype might be deleterious when the immune response is needed to destroy cancer cells [32].

7.4.2 Hypoxia

Hypoxia is an important characteristic of the tumor microenvironment, and there are some reports that show the presence of infiltrating lymphocytes in solid tumors, such as lung, breast, esophageal, and colorectal carcinoma, among others [33–36]. However, the efficiency of their effector capacity has been questioned. Remarkably, during the development of the $CD8^+$ T-cells in the lymphoid organs, lymphocytes are exposed to low oxygen tensions, where pO_2 in the spleen and thymus is around 0.5–4.5 kPa (0.5–4.5%) and 0–2.3 kPa (0–2.3%), respectively. pO_2 in the skin epidermis and the hematopoietic stem cell niche is frequently <1 kPa (<1%) [37].

Caldwell et al. reported that in $CD8^+$ T-cells cultured under hypoxia (2.5% O_2), the expression of vascular endothelial growth factor (VEGF), which contains hypoxia-responding elements, is induced, and the expression of IL-2

and IFN-γ (hypoxia-independent gene products) is suppressed. Nevertheless, the effector capacity (Fas Ligand- and perforin-dependent lethal hit delivery) of differentiated $CD8^+$ T-cells was not affected by this condition. Also, $CD8^+$ T-cells express high cell surface density of TCR/CD3 complex and cell adhesion of LFA-1 under hypoxia [38]. In contrast, Nakawama et al. found that low oxygen concentration (3–4% O_2) enhances the induction of antigen-specific $CD8^+$ T-cells, but the combination of hypoxia and acidosis abrogates this enhancement. Remarkably, the mechanism of the hypoxia-associated induction of antigen-specific $CD8^+$ T-cells is not associated with HIF-1α expression [37].

On the other hand, glucose metabolism has particular patterns among the different subtypes of T-cells in hypoxia. The levels of expression of the glucose transporter Glut1 allow to distinguish among different subtypes of $CD4^+$ and $CD8^+$ T lymphocytes. The levels of Glut1 also correlate with proliferation under normoxic conditions; in contrast, under hypoxia, higher Glut1 levels are found but with diminished proliferation rates. Under both conditions, lymphocytes conserved the capacity to produce IL-2. T-cells with high expression of Glut1 show an increased ratio of $CD8^+/CD4^+$ lymphocytes [39].

7.4.3 Lactate and Acidosis

Immune signature database analyses have denoted a negative correlation between LDHA expression and T-cell activation markers in human melanoma patients [40]. High levels of lactic acid in the microenvironment contribute to T-cell and NK cell metabolic dysfunction preventing upregulation of NFAT and diminishing IFN-γ production [40]. Lactic acid also reduces proliferation and cytokine production by PMA-stimulated $CD8^+$ T-cells up to 95% and 50%, respectively. After replacement with new lactate-free medium for 24h, $CD8^+$ T-cells restore their function [41].

In human renal cell carcinoma, the levels of intra-tumoral interstitial lactate are elevated. Although glucose levels do not diminish, TILs from human renal cell carcinoma are unable to efficiently consume and metabolize glucose. TILs also present small and fragmented mitochondria that are hyperpolarized and produce large amounts of ROS [42].

Sodium lactate inhibits $CD4^+$ T-cell motility via glycolysis interference; it also induces a switch to Th17 cell differentiation, which produces the pro-inflammatory cytokine IL-17. On the other hand, lactic acidosis affects $CD8^+$ T-cell motility, and this phenomenon is independent of glycolysis control. Lactic acidosis also decreases cytolytic activity of $CD8^+$ T-cells in vitro [43].

Finally, acidosis has been shown to have protumoral effects per se, and low pH culture promotes inhibition of cytotoxic activity on $CD8^+$ T-cells against target tumor cells. Acidosis also inhibits cytolytic degranulation and cytokine production and blocks the induction of antigen-specific $CD8^+$ T-cells in vitro [37].

7.5 Concluding Remarks

The study of the immune response was traditionally focused on the characterization of effector function, cytokine regulation, and differentiation process of the immune cells, whereas metabolism was overlooked. Nowadays, the study of immunometabolism has become increasingly relevant, because it has emerged that the nutritional levels found in the microenvironment can affect the differentiation process and effector functions of immune cells. Some diseases, like cancer, can alter the microenvironment where immune cells will try to exert their function. Therefore, in order to reach success in the distinct forms of immunotherapy, it will be necessary to understand first how metabolism affects these immune cells. Using this approach, the study of immunometabolism has revealed some clues in regard to how diminishing the exhausted phenotype or reinforcing the immunologic memory boosts an anti-tumor T-cell response. Thus, the knowledge in this field is now opening novel therapeutic approaches.

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8 The Role of Exhaustion in Tumor-Induced T-Cell Dysfunction in Cancer

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Contents

8.1 Introduction

T lymphocytes are essential components of the immune system and are divided into two major functional types: helper and cytotoxic T-cells. Helper T-cells ($CD4^+$) release an array of cytokines and orchestrate diverse immune responses, which integrate both adaptive and innate effector mechanisms. Cytotoxic T-cells ($CD8^+$ effector T-cells) are primarily involved in the recognition and elimination of body cells compromised by intracellular pathogens or oncogenic transformation.

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A major focus of immunotherapeutic strategies to boost endogenous antitumor immunity has been the stimulation of T-cells. However, despite homing to tumor sites, infiltrating T-cells seldom control tumor growth, because the tumor microenvironment contains a wide array of suppressive mechanisms that allow tumors to escape T-cell effector functions.

Induction of tolerance by T-cell anergy has been regarded as a mechanism responsible for T-cell hyporesponsiveness in cancer patients. However, cancer is also regarded as a chronic disease, similar to chronic viral infections, where T-cells are continuously stimulated. Thus, with chronic stimulation, tumor-specific T-cells gradually become less functional, until they undergo cell death, a phenomenon known as

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T-cell exhaustion. This chapter will focus on the latter mechanism and its role in cancer-induced T-cell dysfunction.

8.2 T-Cell Activation

T-cell activation requires two signals delivered by antigen-presenting cells (APCs). The first signal involves the presentation of the antigen by APCs, in the form of peptides bound to MHC class I or class II molecules, to the T-cell receptor (TCR), expressed on the surface of the T-cell. The second signal, or costimulatory signal, stimulates T-cells in conjunction with the antigen. The molecules expressed on APCs engage their corresponding costimulatory receptors on the surface of T-cells. CD80 (B7-1) and CD86 (B7-2) are well-characterized costimulatory signal molecules, which interact with CD28 expressed on the T-cell membrane [1] (Fig. 8.1). CD28 is the primary costimulatory molecule for naïve T-cells; this molecule is essential for initiating T-cell responses. The interaction of CD80 and CD86 with CD28, together with TCR signaling, promotes the expansion, along with differentiation of antigen-stimulated T-cells into effector and memory cells. The interaction of CD28 with its ligands (1) enhances the production of interleukin (IL-) 2, as well as other cytokines, (2) promotes energetic metabolism, (3) induces the cell cycle progression, (4) promotes T-cell survival, and (5) maintains T-cell responsiveness upon subsequent restimulation [2].

Although costimulatory molecules were initially identified as stimulators of T-cell responses, some of these receptors inhibit T-cell function [1]. For example, cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) is a CD28 homolog that also binds to CD80 or CD86. CTLA-4 expression is inducible after T-cell activation and is involved in the induction, along with maintenance of tolerance, as its ligation inhibits IL-2 production, thus blocking cell cycle progression [1].

After the discovery of homologs of CD28/CTLA-4 and their ligands, many other coinhibitory molecules have been identified, some of which include the inducible T-cell costimulator (ICOS or CD278) with its ligand CD275 (ICOS-L, B7h, B7-RP), the inhibitory programmed death-1 (PD-1, CD279) with its ligands PD-L1 (B7-H1, CD274) as well as PD-L2 (B7-DC, CD273), and the B- and T-lymphocyte attenuator (BTLA, CD272) which binds the herpes virus entry mediator (HVEM). BTLA is an additional receptor of the immunoglobulin superfamily that negatively regulates T-cell activation. In addition, HVEM interacts with another negative regulator of T-cells, CD160. Recent studies of the lymphocyte activation gene-3 (LAG-3, CD223) suggest that this molecule also plays an important role in the regulation of T-cell responses. Moreover, the T-cell immunoglobulin domain and mucin domain-3 (TIM-3), with its ligand galectin-9, are involved in terminating Th1 cell responses and establishing tolerance [3, 4].

T-cells that recognize antigens in the absence of costimulation either fail to respond and undergo cell death or enter a state of unresponsiveness. Thus, costimulation is a key factor in the outcome of T-cell interactions with the antigen. Significant efforts have been undertaken to characterize costimulatory molecules in order to augment antitumor responses; recent evidence has demonstrated the importance of coinhibitory molecules in the inhibition of immune responses. Thus, interference with these regulatory pathways has gained interest as a potential strategy for cancer therapy [1].

8.3 T-Cell Anergy

Tolerance is a mechanism that renders antigen-specific T-cells (self-tolerance) hyporesponsive and prevents autoimmunity. Central and peripheral tolerance are two mechanisms involved in T-cells' unresponsiveness to self. Peripheral tolerance is a mechanism that promotes T-cell functional inactivation after antigen encounter [5]. Central tolerance is not part of the objective of this review and is comprehensively covered elsewhere [6]. Peripheral tolerance protects the host from autoimmune diseases and has been suggested to play an important role in the induction of T-cell dysfunction in cancer patients.

Anergy is one of the mechanisms for inducing peripheral tolerance in which, subsequent to anti-

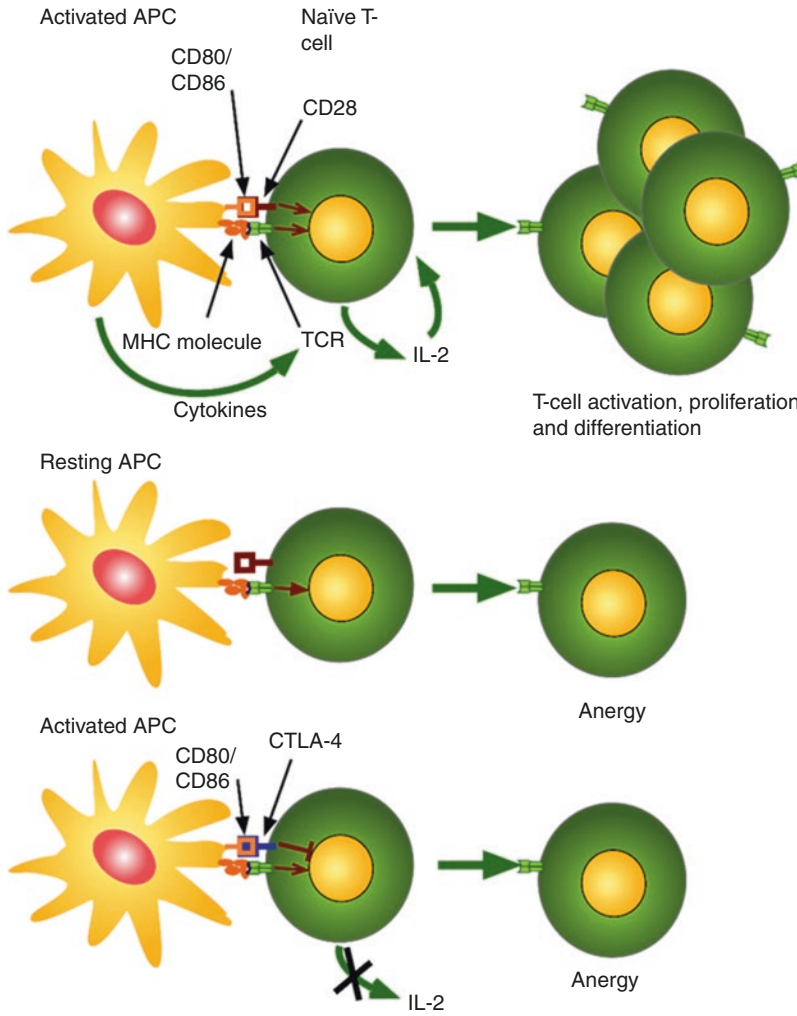

Fig. 8.1 T-cell activation requires recognition of the antigen and costimulatory signals. Inflammation generated by tissue damage or infections activates antigen-presenting cells (*APCs*) and stimulates the expression of costimulatory molecules, such as CD80/CD86. Presentation of the antigen to the T-cell receptor (*TCR*), in the context of major histocompatibility complex (*MHC*) molecules and CD80/CD86 that interact with CD28, stimulates the expansion and differentiation of naïve T-cells (*top panel*). Resting APCs express few or no costimulatory molecules and fail to activate T-cells, and this leads to anergy (*middle panel*). CTLA-4 is a coinhibitory molecule that binds CD80 and CD86 and is upregulated on activated T-cells. CD80/CD86-CTLA-4 interactions inhibit T-cell responses and also mediate anergy

gen encounter, the T-cell is intrinsically and functionally inactivated [5]. The cell remains alive in this hyporesponsive state for an extended period of time. Anergic T-cells neither produce nor respond to proliferative signals and are unable to exert effector functions, such as cytolysis or cytokine secretion. As there are many mechanisms that induce T-cell hyporesponsiveness, each one has its own characteristics, as well as mechanisms that maintain this state [7]; hence, the use of the term anergy has been controversial. In this chapter we will refer to anergy as a state of hyporesponsiveness resulting from stimulation by an antigen without costimulatory and inflammatory signals. A characteristic of anergy is that it must be cell autonomous, which distinguishes this process from immunoregulation mediated through other regulatory cells, such as regulatory T-cells (Tregs) [8, 9].

There are at least five distinct sets of circumstances that lead to T-cell anergy [5, 7]: (1) TCR ligation in the absence of full costimulation, (2) exposure to partial agonists and peptides with minor sequence differences from native agonist antigenic peptides that exhibit reduced avidity for TCR ligation, (3) full signaling without IL-2 receptor-driven cell division, (4) TCR ligation in the presence of IL-10 or transforming growth factor-β (TGF-β), and (5) anergy induced through CTLA-4 or other coinhibitory molecules (Fig. 8.1).

Thus, anergy is the consequence of factors that negatively regulate proximal TCR-coupled signal transduction, together with active transcriptional silencing, which is reinforced through epigenetic modifications [10]. This state of nonresponsiveness is molecularly distinct from T-cell exhaustion. T-cell anergy is induced upon the first encounter with the antigen and is quickly initiated, in contrast with T-cell exhaustion, which is progressive. Gene expression profiles show that anergy is partially distinct to exhaustion. Genes, such as Rnf128 (Grail), Egr2, Egr3, Dgka, and Cb1b, are upregulated in anergic (but not in exhausted) T-cells, whereas NFAT is upregulated under both conditions [11–13]. The detailed characterization of the differences between anergy and T-cell exhaustion will have important implications for therapeutic interventions in immune-mediated diseases and chronic infections.

8.3.1 T-Cell Anergy in Cancer

Anergy has been proposed to play a role in the impairment of T-cell function in human cancers. For starters, tumor cells are poor APCs, as these cells express antigens on MHC class I molecules but do not express costimulatory molecules to provide a second signal for full T-cell activation; thus, tumor-infiltrating lymphocytes (TILs) are rendered anergic [14]. In addition, immature myeloid-derived dendritic cells (iDCs), plasmacytoid dendritic cells (pDCs), and myeloid-derived suppressor cells (MDSCs), together with tumor-associated macrophages (TAMs) potentially induce anergy in TILs [10, 15, 16]. Several studies have shown that human tumor cells, iDCs, pDCs, MDSCs, and TAMs, express high levels of coinhibitory molecules, such as PD-L1, PD-L2, ICOS-L (B7-H2, CD275), and B7-H3 (CD276), indicating a poor costimulatory, as well as a high inhibitory anergy-promoting environment. Evidence that cancer induces T-cell anergy comes from studies where the transfection of CD80 in tumor cells or the blockage of the B7 family coinhibitory molecules results in reduced tumor growth or tumor rejection in mouse models [3, 15–17].

Analysis of the functional state of TILs has shown that these cells are characterized by impairment of cytolytic activity, decreased cytokine secretion, reduced expression of IL-2Rα (CD25), and diminished activation of extracellular signal-regulated kinase (ERK) after TCR activation. Thus, T-cell anergy occurs in the tumor microenvironment of some human cancers [18–20]. Nevertheless, direct evidence of anergic T-cells has been difficult to obtain due to the lack of surface markers to identify this state [10].

Based on mouse tumor models, the induction of antigen-specific T-cell anergy has been suggested to be an early event in the progression of tumors, which occurs in the equilibrium phase of immunoediting, before immunosuppression takes place in advanced tumors (escape phase) [14, 21]. However, Klein et al. showed that highly immunogenic tumors evade immunosurveillance due to antigen overload and an insufficient number of tumor-specific T-cells, resulting in the exhaustion of the immune cells [22]. Thus, from a temporal perspective, T-cell anergy may predominantly occur during the early stages of tumor progression, whereas T-cell exhaustion might play a crucial role in T-cell dysfunction during the late stages of cancer [14].

8.4 T-Cell Exhaustion

T-cell exhaustion has been defined as a stage of T-cell differentiation where T-cells have poor effector functions and sustained coinhibitory receptor expression, along with a transcriptional state distinct from that of functional effector or memory T-cells [23]. Originally, this phenomenon was identified in chronic viral infections in mice

and later in chronic viral infections in humans, e.g., human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) [23–26]. Chronic bacterial and parasitic infections have been demonstrated to promote T-cell exhaustion; also, cancer has been suggested to induce a similar phenomenon [24, 27, 28].

During chronic infections, antigen-specific $CD8^+$ T-cells initially acquire effector functions, but gradually become less functional as the infection progresses. The dysfunction of exhausted T-cells is hierarchical, showing the initial loss of properties, such as cytotoxic activity and proliferative potential, together with IL-2 synthesis; followed by diminished tumor necrosis factor-alpha (TNF-α) secretion; and subsequent loss of interferon-gamma (IFN-γ) production during the late stages of exhaustion. Finally, during the most extreme stages of exhaustion, deletion of T-cells occurs through apoptosis [23, 29] (Fig. 8.2). Like $CD8^+$ T-cells, $CD4^+$ T-cells also lose function during chronic infections; however, there is little information about the mechanisms of exhaustion in this T-cell subpopulation [23, 30].

Exhausted T-cells possess a molecular profile that is distinct from those of memory, effector, and anergic T-cells [12]. First, many membrane inhibitory receptors are upregulated, for instance, PD-1, LAG-3, and TIM-3. Second, transcription of gene encoding molecules involved in TCR signaling (such as Lck and NFAT), along with cytokine receptors (IL-7 and IL-15 receptors) is downregulated. Third, the pattern of genes involved in chemotaxis, migration, as well as adhesion is changed. Fourth, there is an altered pattern of differentiation compared with memory or effector T-cells. Finally, exhausted T-cells present deficiencies in translational, metabolic, and bioenergetic processes, such as the Krebs cycle [12].

8.4.1 Mechanisms for Inducing T-Cell Exhaustion

Coinhibitory receptors play a key role in many aspects of adaptive immunity, including self-tolerance, prevention of autoimmunity, as well as cancer. The mechanisms of regulation through coinhibitory receptors have not been characterized in detail; nevertheless, several studies suggest that these receptors attenuate T-cell responses in many ways. Accumulating evidence highlights the pivotal role of the PD-1/PD-L1 pathway in maintaining an immunosuppressive tumor microenvironment. This pathway has been proposed to be the most important coinhibitory signal involved in T-cell exhaustion [31, 32].

PD-1 (CD279) is a transmembrane receptor of the Ig superfamily, which is upregulated in mice chronically infected with lymphocytic choriomeningitis virus (LCMV) [31, 33]. PD-1 interacts with its ligands PD-L1 (B7-H1, CD274) or PD-L2 (B7-DC, CD273), which are members of the B7 family [32]. PD-1 is rapidly upregulated on activated T-cells; then, after antigen clearance, the expression of this receptor is reduced on effector T-cells. Upon subsequent antigen stimulation, effector T-cells show upregulated PD-1 expression. Thus, the continuous stimulation of T-cells during chronic infections induces the accumulation of $PD\text{-}1^+$ T-cells [23]. High levels of PD-L1 expression on APCs (or tumor cells) might sustain PD-1 expression on T-cells and impair T-cell effector maturation, which allows the progression of chronic infection [34–36]. PD-L1 interaction with PD-1 induces inhibition of the PI3K/AKT pathway, by increasing PTEN phosphatase activity [33]. Moreover, signaling through PD-1 inhibits glycolytic metabolism and promotes metabolism of lipids, which inhibits T-cell metabolic reprogramming, consequently preventing differentiation to effector T-cells [37].

Studies in mouse tumor models show that the inhibition of PD-L1 or PD-1 using blocking monoclonal antibodies (mAbs) increases the cytolytic activity of $CD8^+$ T-cells and reverses T-cell dysfunction [38, 39]. Subsequently, Barber et al. showed that the inhibition of PD-1 using anti-PD-1 mAbs in chronically infected mice enhances the proliferation, as well as effector functions of exhausted T-cells [31]. Since the publication of these seminal reports, many other studies have shown that the interaction of PD-1 with its ligand (PD-L1) is crucially involved in T-cell exhaustion in chronic human pathogen infections and cancer [25–27, 40–42].

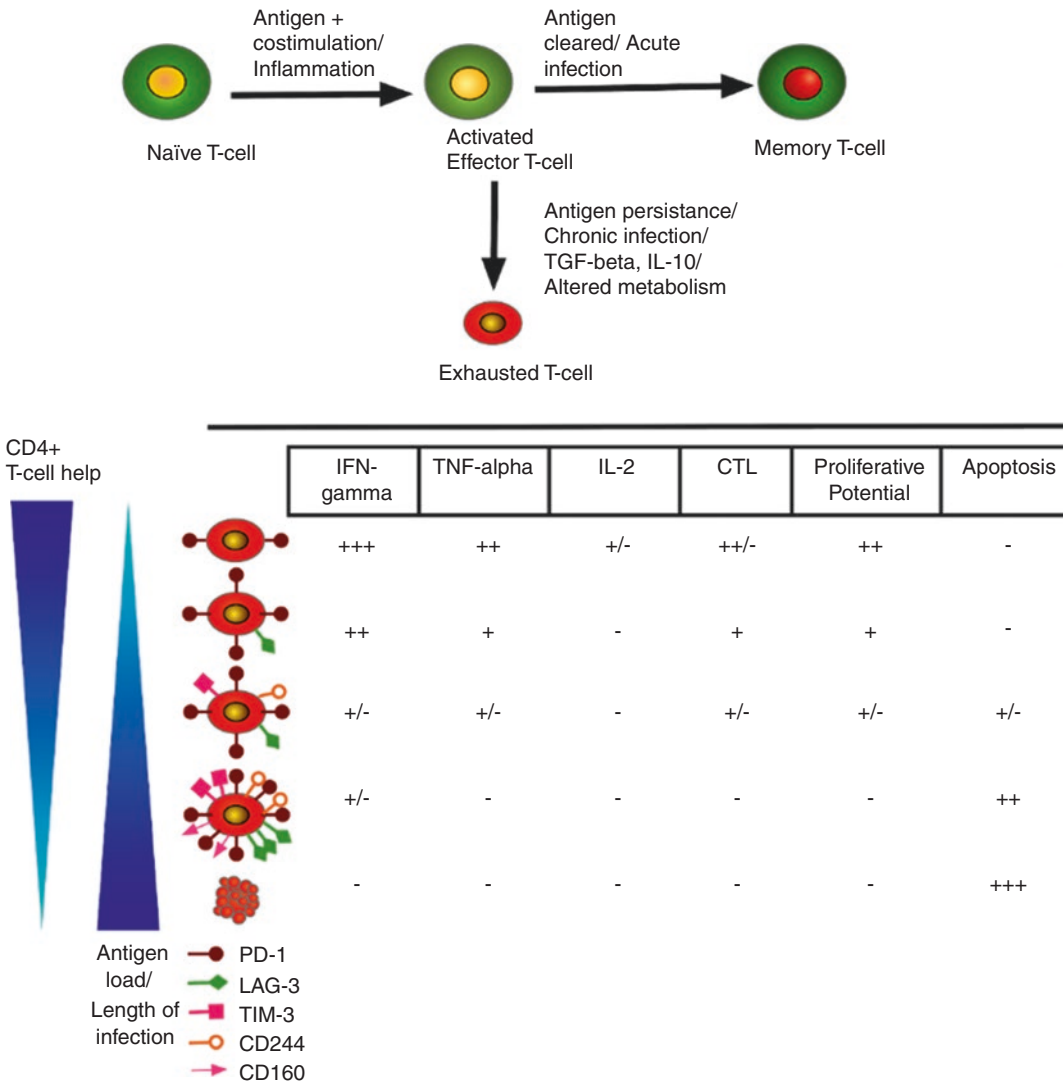

Fig. 8.2 T-cell exhaustion during chronic inflammation. In an acute inflammatory process, naïve T-cells are primed by an antigen, costimulatory molecules, and cytokines that promote differentiation into effector T-cells. After clearance of the antigen and once inflammation is resolved, a subset of effector T-cells differentiate to become memory cells. During chronic processes, such as viral infections, the antigen persists, and T-cells go through several stages of dysfunction, losing effector functions (cytolysis and secretion of cytokines) and proliferative potential in a hierarchical manner. Finally, deletion of T-cells by apoptosis occurs. As antigen load increases or $CD4^+$ T helper subpopulation decreases, T-cells become more exhausted. Expression of coinhibitory receptors is correlated with the level of exhaustion. The scale of each activity is presented from high (+++) to low (−)

In addition to PD-1, many other cell surface inhibitory receptors also participate in T-cell exhaustion. These coinhibitory receptors regulate distinct T-cell functions. For instance, PD-1 pathway affects survival, together with proliferation, whereas LAG-3 affects cell cycle progression, calcium flux, and cytokine production but has less influence on apoptosis [23]. LAG-3 is structurally analogous to the CD4 molecule, is upregulated on activated T-cells, and binds MHC class II molecules. In addition to activated effector $CD4^+$ T-cells, Tregs can also express LAG-3 and is involved in the suppressor function. Nevertheless, the pathway(s) involved for inducing T-cell inhibition are not clear [43].

TIM-3 is an inhibitory molecule that downregulates effector Th1 responses. This molecule has several ligands, such as galectin-9 and phosphatidylserine, along with the high mobility group protein B1 (HMGB1). The latter two molecules have more relevant roles in innate immune cells. The cytoplasmic tail of TIM-3 may interact with different TCR components. When galectin-9 binds to TIM-3, two sites of the cytoplasmic tail (Y256 and Y263) are phosphorylated, which favors the release of HLA-B-associated transcript 3 (Bat-3) from the cytoplasmic tail. This process promotes T-cell inhibition by allowing binding of SH2 domain-containing Src kinases to the tail and subsequent downregulation of TCR signaling [43]. TIM-3 targets signaling pathways involved in T-cell metabolism, such as PI3K/Akt/mTOR; thus, it has been hypothesized that, similar to PD-1, TIM-3 alters the metabolism of effector T-cells [44]. Upregulation of TIM-3 molecule has been found in HIV-specific and HCV-specific $CD8^+$ T-cells in patients with progressive HIV and HCV infections, respectively. Importantly, the co-expression of TIM-3, together with PD-1, has been associated with severe $CD8^+$ T-cell exhaustion in terms of the proliferation as well as secretion of effector cytokines, such as IFN-γ, TNF-α, and IL-2 [23]. Interestingly, $CD8^+$ T-cells expressing both coinhibitory receptors also produce the suppressive cytokine IL-10 [45]. Other receptors belonging to the tumor necrosis receptor family are upregulated in exhausted T-cells, such as Fas, TNF-R, as well as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors; hence, these death receptors have been implicated in the induction of exhaustion, as T-cells might become prone to activation-induced cell death (AICD) [23, 46, 47].

The increased and sustained expression of multiple coinhibitory receptors is a key feature of CD4+ and CD8+ T-cell exhaustion. However, exhausted T-cells do not necessarily co-express all of the coinhibitory molecules. The pattern as well as the level of expression of coinhibitory receptors simultaneously expressed in the same CD8+ T-cell might considerably influence the severity of dysfunction [48]. Remarkably, functional effector T-cells express coinhibitory receptors during activation [49].

Several factors, such as the duration of the infection, the level of antigen exposure, and the availability of $CD4^+$ T-cell help, in addition to the type of APCs that present the antigen, have been implicated in the severity of T-cell exhaustion. Metabolic pathways that depend on Akt/mTOR signaling, which is essential for the activation of T-cell metabolism, may be affected by coinhibitory molecules such as PD-1 or CTLA-4 [37, 50]. Ligand availability for coinhibitory receptors could also influence the degree of exhaustion, as well as environmental factors such as the presence of immunoregulatory cytokines or metabolites [30]. In chronic viral infections, IL-10 expression is associated with T-cell dysfunction [48, 51]. In addition, TGF-β has also been linked to exhaustion in chronic infections in humans [52, 53]; nevertheless, the mechanisms underlying IL-10 and TGF-β-mediated T-cell exhaustion are unclear. Remarkably, both cytokines are secreted by several human tumors [54, 55].

Dysfunction of T-cell metabolism has been associated with exhaustion [37, 56]. Recently, interferon regulatory factor 4 (Irf4), which is a TCR-responsive transcription factor, has been shown to be highly expressed in exhausted T-cells in a murine model of LCMV. IRF4 represses anabolic metabolism, as well as mitochondrial function; increases the expression of coinhibitory receptors, such as PD-1 and TIM-3; and impairs the effector functions of LCMV glycoprotein-derived epitope gp33-specific CD8+ T-cells [56]. Lysine/histone acetyltransferase 2B (KAT2B) is a transcriptional co-activator that has anti-apoptotic functions when cells are under metabolic stress. This molecule has been recently shown to be upregulated in activated Th1 cells and is a marker of patients with HCV, malaria, or influenza that responds to therapy [57]. Thus, expression of KAT2B may be downregulated in exhausted T-cells.

8.4.2 Identification of Exhausted T-Cells

Genomic studies support the notion that T-cell exhaustion represents a particular state of differentiation, different from that of effector or

memory T-cells; hence exhausted T-cells have a particular molecular and phenotypical profile [12, 30]. Exhausted T-cells show a poorly differentiated phenotype ($CD27^{hi}CD28^{lo}CD57^{lo}CD127^{lo}CCR7^{-}CD45RA^{+}$ or $CD27^{+}CD45RO^{+}$) that correlates with dysfunction. Although PD-1 upregulation in T-cells was initially considered as a hallmark of T-cell exhaustion, this molecule is upregulated along with activation markers, such as CD38 or HLA-DR [58]. In healthy adults, the percentage of PD-1^{+} cells varies from 40 to 80% of ($CCR7^{+/-}CD45RA^{-}$) memory T-cells; remarkably, these cells do not exhibit characteristics of exhaustion [59]. Thus, PD-1 is associated with T-cell activation in addition to T-cell differentiation [49].

Many cell surface coinhibitory receptors are expressed in exhausted T-cells. LAG-3, TIM-3, CD244 (2B4), CD160, CTLA-4, and B- and T-lymphocyte attenuator (BTLA) are co-expressed in antigen-specific $CD8^{+}$ T-cells during chronic infection. The pattern and the level of coinhibitory receptors simultaneously expressed in the same $CD8^{+}$ T-cell considerably influence the severity of dysfunction [48]. However, depending on the chronic infection or cancer, exhausted T-cells may express a different pattern of coinhibitory molecules.

Several transcriptional pathways have been associated with T-cell exhaustion. Basic leucine zipper transcriptional factor ATF-like (BATF), is upregulated upon ligation of PD-1. BATF has been shown to reduce proliferation and IL-2 secretion albeit it does not increase the expression of other coinhibitory receptors (CD244 or CD160) [60]. Also, increased expression of transcriptional repressor Blimp-1 is associated with upregulation of coinhibitory receptors such as PD-1, LAG-3, CD160, and CD244. In addition, the expression of transcription factor NFATc1 (NFAT2) is also increased, but shows a dysregulated function [12, 61]. On the other hand, the transcription factor T-bet plays a role in protection against T-cell exhaustion, as T-bet promotes terminal differentiation after acute infection. Besides, the increased expression of this transcription factor inhibits the expression of coinhibitory receptors during chronic viral infection. T-bet expression is downregulated through persistent antigenic stimulation, resulting in exhaustion [62]. Paley reported that two subsets of exhausted T-cells can be found in chronic LCMV infection, based on the expression of T-bet, Eomesodermin (Eomes), as well as PD-1. T-cells identified as $T\text{-}bet^{high}$ $Eomes^{low}$ $PD\text{-}1^{int}$ have some proliferative potential and produce IFN-γ along with TNF-α; these cells are located in the spleen and blood. On the other hand, cells that express the phenotype $T\text{-}bet^{low}$ $Eomes^{high}$ $PD\text{-}1^{high}$ have a lower proliferative potential, secrete lower levels of cytokines in comparison to the former subset, and express higher levels of other coinhibitory molecules; these cells have cytolytic activity and are located in peripheral tissues [63].

8.5 T-Cell Exhaustion in Cancer

Cancer and chronic viral infections have been thought to share similar mechanisms in establishing high antigen load together with an immunosuppressive environment. However, there is a fundamental difference between these two diseases: viral antigens are exogenous and extremely immunogenic, whereas tumor antigens are self-molecules that are weakly immunogenic. Thus, compared with tumor-specific T-cells, virus-specific T-cells are more frequent and easily detectable, facilitating identification, phenotypic characterization, as well as their isolation [14].

Some of the phenotypic, functional, and molecular changes that occur in T-cells during chronic infections are exhibited in TILs as well as peripheral blood T-cells from several cancer types. The initial aim of tumor immunotherapy was to prevent anergy, in addition to tolerance toward tumor antigens. However, the efficacy of this strategy is potentially limited by T-cell exhaustion [14]. Accordingly, Hailemichael et al. showed that in mice vaccinated with gp100 melanoma peptide, the persisting tumor antigen at vaccination sites induces the sequestration of $CD8^{+}$ T-cells, resulting in the dysfunction, as well as death of these cells [64].

In the tumor microenvironment, infiltrating T-cells become dysfunctional and show reduced effector functions. Several reports suggest that PD-L1 expression on tumor cells plays an important role in tumor-induced T-cell dysfunction. PD-L1 membrane expression has been observed using immunohistochemistry on many human tumors, such as melanoma, lung, larynx, colon, breast, cervix, and stomach [32]. In breast, esophageal, gastric, as well as renal carcinomas, the increased expression of PD-L1 on the surface of tumor cells is strongly associated with poor prognosis [32, 65]. Thus, T-cell exhaustion has been proposed as a mechanism for inducing dysfunction through the PD-L1/PD-1 pathway. However, as previously indicated, PD-1 expression cannot be viewed as the sole marker of T-cell exhaustion in chronic diseases and cancer; hence, other markers, as well as functional assays must be considered [66].

In metastatic melanoma lesions, TILs show upregulation of PD-1 expression, accompanied with reduced production of IFN-γ and TNF-α, along with IL-2. Both tumor-infiltrating $CD8^+$ T-cells, particularly MART-1-specific, and tumor-infiltrating $CD4^+$ T-cells show significantly higher levels of PD-1 expression than $CD8^+$ and $CD4^+$ T-cells from peripheral blood and normal tissues from cancer patients. In addition, a large proportion of $CD8^+$ T-cells from TILs were $PD\text{-}1^+CTLA\text{-}4^+$ cells compared with normal tissues, as well as blood. $PD\text{-}1^+CD8^+$ cells from TILs lacked CD25 together with CD127 expression, suggesting that these cells were unable to proliferate, produce effect or cytokines, and differentiate into memory cells [67]. $PD\text{-}1^+$NY-ESO-1-specific $CD8^+$ T-cells, from patients with advanced melanoma, upregulate TIM-3 expression and are more dysfunctional than $TIM\text{-}3^-PD\text{-}1^+$ or $TIM\text{-}3^-PD\text{-}1^-$NY-ESO-1-specific $CD8^+$ T-cells, producing less IFN-γ and TNF-α, along with IL-2 [68].

Derré et al. showed that tumor antigen (Melan-A/Mart-1)-specific $CD8^+$ T-cells express high levels of BTLA and are susceptible to functional inhibition through its ligand HVEM [69]. In addition, Baitsch et al. showed that in melanoma, tumor antigen-specific $CD8^+$ T-cells with effector phenotypes simultaneously express four or more of the coinhibitory receptors BTLA, TIM-3, LAG-3, KRLG-1, 2B4, CD160, PD-1, or CTLA-4 [70]. Moreover, tumor antigen-specific $CD8^+$ T-cells present a large variety of genes with a similar genetic profile to that of exhausted T-cells from chronic viral infections [71]. Taken together, these reports show that in melanoma patients tumor antigen-specific $CD8^+$ T-cells undergo exhaustion.

Additional evidence for T-cell exhaustion in other cancers comes from studies in patients with ovarian cancer. Matsusaki et al. reported that NY-ESO-1-specific $CD8^+$ T-cells from the peripheral blood of patients with ovarian cancer show impaired effector functions, along with co-expression of the inhibitory molecules LAG-3 and PD-1. The expression of LAG-3 and PD-1 on the surface of $CD8^+$ T-cells is upregulated through IL-10, IL-6, as well as tumor-derived APCs. In addition, $LAG\text{-}3^+PD\text{-}1^+CD8^+$ T-cells are deficient in IFN-γ/TNF-α secretion compared with $LAG\text{-}3^+PD\text{-}1^-$ or $LAG\text{-}3^-PD\text{-}1^-$ subsets [72].

PD-L1 expression is upregulated in Hodgkin's lymphoma (HL), as well as several T-cell lymphomas, but not in B-cell lymphomas. In addition, PD-1 is upregulated in TILs, as well as peripheral blood T-cells from HL patients, and the blockade of the PD-1 pathway restores IFN-γ production in T-cells [73]. Moreover, LAG-3 is expressed on TILs from patients with this malignancy [74]. Hence, these reports suggest that TILs from patients with HL are exhausted.

In patients with chronic lymphocytic leukemia (CLL), $CD8^+$ and $CD4^+$ effector T-cells show increased expression of CD244, CD160, and PD-1 molecules; in addition to expansion of the $PD\text{-}1^+$ $Blimp^{hi}$ subset $CD8^+$ T-cells from CLL patients show defects in proliferation and cytotoxicity, but with increased production of IFN-γ, as well as TNF-α, normal production of IL-2, and increased expression of T-bet. Thus, although $CD8^+$ T-cells show features of T-cell exhaustion, these cells retain the ability to produce cytokines [75]. In addition, it has been shown that, in B-cell non-Hodgkin's lymphoma, TGF-β upregulates

the expression of the costimulatory molecule CD70 on memory, as well as effector T-cells, which in turn increases the expression of PD-1 and TIM-3 molecules in these T-cell subsets. These $CD70^+$ T-cells are deficient in cytokine production and are prone to apoptosis. Also, a higher frequency of $CD70^+$ T-cells is associated with a poor outcome in patients with follicular B-cell lymphoma [76].

On the other hand, head and neck cancers that are positive for human papillomavirus (HPV) present a high infiltration of $PD\text{-}1^+$ T-cells, and their number is positively associated with a favorable clinical outcome. These $PD\text{-}1^+$ T-cells express activation markers, and 50% of this population lack TIM-3 expression and are functional after the blockade of the PD-1/PD-L1 pathway, suggesting that $PD\text{-}1^+$ T-cells are activated rather than exhausted [77]. Accordingly, Lechner et al. showed that PD-1, PD-L1, and CTLA-4 are highly expressed on T-cells from head and neck squamous cell carcinoma tumor tissue [78], which would reflect an immunosuppressive microenvironment, but not exhaustion.

In hepatocellular carcinoma, the frequency of $PD\text{-}1^+CD8^+$ T-cells is higher in tumor tissues than in non-tumor tissues, presenting decreased proliferative capacity, in addition to diminished effector functions, as demonstrated by reduced granule and cytokine expression compared with $PD\text{-}1^-CD8^+$ T-cells, although no other marker of T-cell exhaustion was analyzed in this study [79]. Interestingly, low tumor expression of PD-L1, as well as galectin-9, which is a ligand for TIM-3, is a predictor of poor hepatocellular carcinoma survival. Also, low $CD8^+$ TIL count has been shown to be a poor predictor of survival for this type of cancer [80]. Thus, $CD8^+$ T-cells from hepatocellular carcinoma are not rendered exhausted; instead, they may be activated in those patients that show a better survival.

In colorectal cancer, PD-1 expression has been shown to be upregulated on $CD8^+$ T-cells from tumor-draining lymph nodes and tumor tissue. $PD\text{-}1^+CD8^+$ T-cells are dysfunctional in tumors but not in tumor-free lymph nodes, because the former present a lower percentage of cytokine-producing cells [81]. However, this phenomenon would reflect an adverse microenvironment instead of exhaustion; moreover, as in other reports, the study of Wu et al. [81] only analyzed PD-1 expression as the marker of exhaustion. In another study, it was shown that TILs from colorectal patients present higher frequencies of $TIM\text{-}3^+CD8^+$ T-cells compared with para-cancerous tissues; this subset also produces lower levels of IFN-γ [82]. Even though the authors suggest that CD8+ T-cells are exhausted, neither other coinhibitory receptors nor functional capacity were evaluated, and blockade of PD-1 pathway did not show a response in patients with this type of cancer. The latter phenomenon might be explained to the fact that PD-L1 expression has been reported in approximately 10% of tumors, mostly microsatellite unstable [83]. Prall and Hüns also reported that colorectal tumors with an immunoreactive microenvironment, which is characterized by a dense immune infiltrate, show a high number of $PD\text{-}1^+CD8^+$ cells as shown by sequential immunohistochemistry [83]. The authors suggest that PD-1 expression is a consequence of T-cell exhaustion; however, since only the PD-1 molecule was identified in this study, and no functional analysis was done, PD-1 expression might be the consequence of activation rather than exhaustion.

Interestingly, Haymaker et al. proposed that $PD\text{-}1^{high}$ $CD8^+$ T-cells in cancer patients are not exhausted [84]. This hypothesis is based on the observation that $CD8^+$ T-cells from the TILs of melanoma patients recover their proliferative potential ex vivo, despite expressing high levels of PD-1. These TILs mediate antitumor responses upon adoptive transfer into patients [85, 86]. Under this premise, infiltrating and peripheral blood $CD8^+$ T-cells, expressing PD-1, BTLA, and other coinhibitory receptors, are not exhausted. Instead, these cells are highly activated effector memory T-cells that can be stimulated through immunotherapy [84]. Nevertheless, these observations have been primarily achieved in melanomas. In other cancers, the reduced proliferative and effector capacities persist, even after stimulation, and immunotherapeutic strategies have failed to induce potent antitumoral responses [64, 75, 87].

8.5.1 A Particular Case: T-Cell Exhaustion in Lung Cancer Patients

Lung cancer is the leading cause of cancer-related mortality in developed countries and the second leading cause of death in countries with emerging economies. This disease is one of the most commonly diagnosed cancers worldwide, representing 13% of all cancer cases and approximately 18% of all cancer deaths [88]. Some reports indicate that the presence of TILs with memory phenotype is predictive of a favorable clinical outcome in lung cancer patients [89–91].

$CD8^+$ T-cells have been found in both TIL and the pleural compartment in lung cancer patients. These cells are functionally impaired and are poorly responsive or unresponsive to several T-cell-activating stimuli, even though memory cells infiltrate lung tumors. $CD8^+$ T-cells present low proliferation rate and diminished production of some Th1 cytokines, in addition to reduced cytotoxic potential, reviewed in [87]. Pleural effusion $CD8^+$ T-cells from lung cancer patients express cell markers associated with a memory phenotype ($CD45RA^-CD45RO^+CD27^+$granzyme-A^{low}perforin$^-$), similar to those markers found in $CD8^+$ T-cells from chronic viral infections. These phenotypical and functional dysfunctions suggest that $CD8^+$ T-cells have been rendered exhausted.

Zhang et al. reported that tumor-infiltrating $CD8^+$ T-cells from patients with non-small cell lung carcinoma (NSCLC) express increased levels of PD-1. These $CD8^+$ T-cells are impaired in cytokine production, as well as proliferative potential, which are partially restored after blockade of the PD-1/PD-L1 pathway [92]. In a study by Gao et al., TIM-3 was found to be highly upregulated on both $CD4^+$ and $CD8^+$ T-cells from lung tumor tissues, but almost undetectable on T-cells from peripheral blood samples. However, TIM-3 expression on $CD8^+$ T-cells was not associated with any clinical pathological parameter in lung cancer patients (e.g., tumor size, lymph node metastasis, or tumor stage) [93]. Recently, Thommen showed that cumulative expression of inhibitory receptors (PD-1, TIM-3, CTLA-4, LAG-3, in addition to BTLA) on T-cells from tumor tissues correlated with a progressively impaired capacity to respond to polyclonal activation and with a progression of NSCLC. Interestingly, $PD\text{-}1^+CD8^+$ T-cells were found to co-express low percentages of other inhibitory receptors analyzed, whereas $BTLA^+CD8^+$ T-cells expressed high levels of these receptors [94].

In the previous edition of the present chapter, PD-1 expression was reported to be higher in pleural effusion T-cells from lung cancer patients, compared to those from nonmalignant origin [95]. This observation was later confirmed by a more detailed study from our group; remarkably, a total of $CD8^+$, as well as $CD4^+$ T-cells, do not show co-expression of the coinhibitory receptors TIM-3 and LAG-3. To analyze whether tumor-specific $CD8^+$ T-cells show an exhausted phenotype, we used the surrogate marker CD137 for identifying T-cells responding to tumor antigens MAGE-3A or WT-1. With this strategy, it was shown that most tumor-responding T-cells showing a memory phenotype ($CD45RA^-CD27^+$) express PD-1 molecule but do not co-express TIM-3 (see Fig. 8.3 for representative data and [96]). Of note, the use of anti-PD-L1 blocking antibody increased the expression of granzyme-B along with perforin on polyclonal- and tumor-specific CD8+ T-cells [96]. Taken together, these studies suggest that T-cells from lung cancer patients are not exhausted; instead they are primed, but incompletely differentiated, leading to a deficiency in their effector functions of cytotoxic activity, as well as cytokine secretion.

Interestingly, the administration of PD-1 antibody as a blocking agent against PD-1 pathway has shown durable partial tumor regression in patients with lung cancer, which was long thought to be a "non-immunogenic" tumor [97]. Thus, reactivation of immune responses in lung cancer patients, via blocking PD-1, TIM-3, or other regulatory pathways, in combination with other therapeutic modalities, such as radiotherapy or chemotherapy, will provide major clinical benefits to patients with this disease.

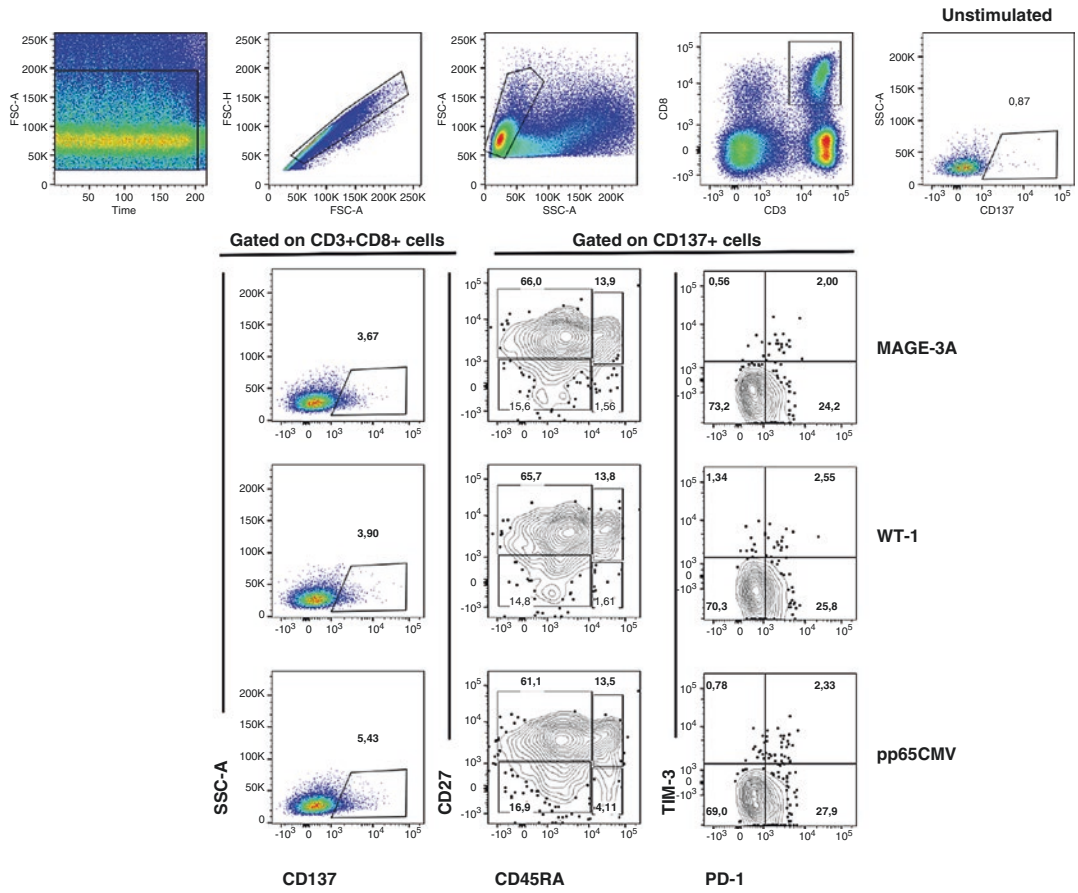

Fig. 8.3 Representative flow cytometric analysis for identification of markers of exhaustion in antigen-specific CD8+ T-cells of a lung cancer patient. Pleural effusion mononuclear cells were stimulated overnight with tumor peptides from MAGE-3A, WT-1, or CMV pp65 peptides (used as control), and cells were then immunophenotypified. Upper panel from left to right, time vs FSC-A graph was done to exclude artifacts (bubbles or clumps), then single cells were gated from FSC-A vs FSC-H graph, next lymphocytes were selected from a SSC-A vs FSC-A graph, and a CD3 vs CD8 graph was done to select CD3+CD8+ T-cells. To analyze antigen-responding CD8+T-cells, surrogate marker CD137 was used, and unstimulated CD8+ T-cells are shown. Lower panel, from CD137 vs SSC-A graphs, the frequency of CD137+ cells was quantified and further identified with CD45RA, CD27, PD-1, and TIM-3 markers. Most antigen-responding cells have a memory phenotype (CD45RA+CD27- and CD45RA-CD27-); however, tumor-responding cells do not co-express PD-1 and TIM-3; for details see Ref. [96]

8.6 Concluding Remarks

T-cell exhaustion is a stage of differentiation where T-cells show poor effector functions, sustained coinhibitory receptor expression, as well as a transcriptional state distinct from memory, effector, and even anergic T-cells. Some types of cancer have been shown to induce T-cell exhaustion, because the tumor microenvironment provides and maintains the required conditions for inducing this phenomenon. Among other conditions, the tumor mass is a source of antigens that chronically stimulate infiltrating T-cells. In most cancers, tumor cells expressing PD-L1 have been associated with a negative disease outcome. Many tumors also secrete IL-10 along with TGF-β, immunosuppressive cytokines that are associated with exhaustion in chronic viral infections. Also, metabolic reprogramming of tumor cells favors a protumoral microenvironment that nonetheless has deleteri-

ous effects on T-cell metabolism. From a temporal perspective, T-cell anergy possibly occurs during the early stages of tumor progression, whereas exhaustion might play a crucial role in T-cell dysfunction during the late stages of cancer.

Because the terms anergy and exhaustion have been used to describe dysfunctional T-cells, it is necessary to carefully evaluate not only surface markers but also the functionality of T-cells (proliferation, cytokine production, along with cytotoxic functions) in order to assign such terms. On the other hand, studies on T-cells from cancer patients are limited by the amount and quality of the sample, and it is not always feasible to make a detailed characterization; thus, the term "dysfunctional" should be used when describing T-cells with poor functional activity until further data is obtained.

The reduced functions of T-cell observed in vitro, the correlation of the clinical prognosis of cancer patients with the expression of PD-L1 in tumor cells, and the limited success of T-cell-based immunotherapy provide evidence that exhaustion plays an important role as a tumor evasion mechanism from the host immune system in some types of cancer.

Understanding the mechanisms of tumor-induced T-cell exhaustion will conduce to the development of vaccine-induced T-cells aimed at promoting tumor rejection. Clinical findings with blockers of immune-regulatory pathways, such as the PD-1/PD-L1 pathway, suggest that this strategy is promising for enhancing antitumor immunity with the potential to produce long-lasting clinical responses.

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The Role of NK Cells in Cancer

9

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9.1 Introduction

Human NK cells were defined and shown to be important effectors of the innate immune system with a unique ability to directly lyse transformed, virus infected cells as well as cells that have undergone physical or chemical injures, i.e., stressed cells, without prior sensitization or MHC class restriction. Human NK cells were initially described as non-adherent, non-phagocytic,

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CD16 (low affinity FcγRc^{+}), large granular lymphocytes [1]. Today, human NK cells are defined as CD3^{-}16^{+}CD56^{+} and NKp46 natural cytotoxicity receptor (NCR) positive lymphocytes [2]. According to the density of expression of these receptors NK cells are divided into two subsets, one of which is cytotoxic (CD56^{dim}CD16^{bright}), while the other is regulatory (CD56 ^{bright}CD16b$^{dim/-}$) and produces abundant cytokines (IFN-γ, TNF-α, IL-10, IL-13, and GM-CSF) [3–5].

To date human NK cells are subject to intense study because of their ability to directly lysis tumor cells as well as to participate in the immunomodulation of the tumor response [6, 7].

9.2 Discovery and Basic Characteristics

NK cells were first described in 1975 as a peripheral blood lymphocyte subset capable of cytotoxicity against mouse Moloney leukemia cells by Kiessling who named them natural killer (NK) cells [8] and in parallel by Herberman [9]. Although initially regarded as an "experimental artifact" in T-cell cytotoxicity with the identification of the NK1.1 receptors [10] it became possible to define the murine NK cells and describe them as large granular lymphocytes distinct from T and B-cells. Moreover, recently NK cells have been designated as first members of the novel innate immune cell family (ILC) with distinct patterns of cytokine production that closely resembles the heterogeneity of T helper cell subsets. According to cytokine production and lineage specific master transcription factors ILC are grouped into three functionally distinct groups and NK cells are the main population of ILC group 1 (ILC1) [11]. Furthermore, although NK cells are innate immune cells, novel evidence also indicates that NK cells might mediate long-lived memory-like response after reactivation in some viral infections and following cytokine stimulation, an attribute considered to be inherent to adaptive immune system [12].

9.2.1 Origin and Maturation of NK Cells

NK cells are derived from CD34^{+} hematopoietic progenitor cells (HPCs) within the bone marrow still considered as the primary site for NK cell development, while recent studies show that common lymphoid progenitor cells with NK cell commitment potential traffic from BM to other tissues, mainly secondary lymphoid organs (SLOs), which also serve as putative sites of human NK cell development [13] indicating that maturation from NK progenitors, as well as shaping of NK cell function is guided by a particular environment with its unique combinations of developmental conditions [14–16].

It has recently been shown that NK cells develop through five successive stages, from "stage I" or pro-NK cells characterized as CD34^{+} and c-Kit cytokine receptor positive that acquire IL-2/15Rβ cytokine receptor [14, 17] and are able to give rise to IL-15 responsive, "stage II" pre-NK cells and differentiate from pre-NK cells to "stage III" immature NK cells (iNK) that represent committed NK cell lineage. Stage III iNK cells no longer express CD34 and are c-Kit$^{+/-}$ but lack features of mature NK cells as they are not able to produce interferon γ (IFN-γ) and lack cytotoxicity. iNK cells subsequently differentiate to "stage IV" NK cells, also defined as CD56^{bright} NK cells [14, 17, 18] that represents immunoregulatory NK cell subset capable of producing abundant cytokines (IFN-γ, TNF), but expresses very low amounts of cytolytic granules and displays poor cytotoxic capability. These CD56^{bright} NK cells show low expression of cytotoxic CD16 receptor and are positive for inhibitory CD94/NKG2A receptor, while still low or negative for the main inhibitory KIR receptors. Finally, CD56^{bright} NK cells downregulate the expression of CD94 inhibitory receptor while upregulating the expression of KIRs, CD16 receptor as well as cytotoxic molecules (perforin and granzymes) and differentiate into "stage V" mature cytotoxic CD56^{dim} NK cells. These terminally differentiated NK cells are accompanied by the progressive loss of their proliferative capacity and the acquisition of more efficient cytolytic activity characterized by CD57 expression [19, 20].

The functional maturation of NK cells includes a process of education, also referred to as licencing, arming, or tuning, by which NK cells acquire effector functions [21]. In addition to its well-known role in the regulation of NK cell effector functions MHC I recognition by NK cell inhibitory receptors is also involved in NK cell education. This model conceptualizes that signaling from inhibitory receptors licenses or arms functional activation of NK cells, which are by default unresponsive or unlicenced. It also relies on an instructive role for inhibitory receptors and implies that inhibitory receptor signaling might trigger the activation signals that are needed for stimulating NK cells. Therefore, in the process of NK cell maturation interaction of NK cell inhibitory receptors with self MHC class I molecules renders NK cells not only tolerant to self but licenses them for functional activation due to activating receptor movement into plasma membrane nanodomains that represents their optimal localization for interaction with target cells with stress-induced NK cell stimulating ligands [22].

In case of MHC class I deficient organisms or in case that NK cells do not express inhibitory receptors for particular self MHC class I molecules, that occurs in a small percent in normal organisms, NK cells do not undergo the process of education and they are hyporesponsive to generate cytotoxicity or cytokine production. In spite of this, hyporesponsive NK cells might also play important roles to viral infections and in neuroblastoma because self-inhibitory receptor-deficient NK cells respond more strongly than inhibitory receptor-positive NK cells owing to their recognition of specific activating ligands on target cells that in the absence of inhibitory signals favors activation signaling. Cytokines at sites of infection and tumors may functionally activate hyporesponsive NK cells to respond even toward MHC I-expressing target cells [23]. Furthermore, it has recently been shown that NK cells can switch from a hyporesponsive to a competent status upon recognition of cognate MHC in a different setting, i.e., reeducation, which indicates the possibility of NK cell adjustment to its surrounding environment [24, 25].

Following NK cell education for their full activation they need to undergo "priming" with dendritic cells (DC), CD4+ T-cells, and neutrophils, as well as in the presence of cytokines such as IL-2, IL-12, IL-15, IL-18, or IL-21 [26]. The most important NK cell priming occurs with DCs and includes transpresentation of IL-15 by DC IL-15 receptors.

NK cell-mediated antitumor response is regulated by the balance of signals mediated by various activating and inhibitory receptors and their ligands on tumor cells [27]. However, in malignancies tumor-derived immunosuppressive factors often affect NK cell receptor expression that together with cytolitic molecule dysregulation lead to inhibition of NK cell function [28–31] (Fig. 9.1). In this sense, better understanding of NK cell biology would give better insight into the interaction of NK and tumor cells in order to better harness NK cell antitumor potential.

9.2.2 NK Cell Receptors

NK cell expresses various activating and inhibitory receptors that bind cognate ligands on tumor cells that allow NK cell-mediated antitumor response [27]. NK cell inhibitory receptors include the killer cell inhibitory, i.e., immunoglobulin, receptors (KIR) that are type I membrane glycoproteins responsible for the inhibition of NK cell-mediated lysis of normal cells in the organism that express MHC class I molecules. Another NK cell inhibitory receptor is a c-type lectin that consists of CD94-NKG2A heterodimer. Furthermore, leukocyte Ig-like receptors, LIR, is another family of NK cell inhibitory receptors.

On the other hand, NK cell activating receptors include natural cytotoxicity receptors family (NCR), the activating C-type lectin family receptors a homodimeric NKG2D, and heterodimeric CD94/NKG2C, CD94/NKG2E, CD94/NKG2H, FcγRc IIIA (CD16), activating killer immunoglobulin receptors, KIR2DS1, KIR2DS4, and KIR2DL4 that belong to the KIR family and costimulatory receptor DNAM1 that cooperate with other cytotoxic and inhibitory receptors to determine NK cell cytotoxicity against transformed cells [32].

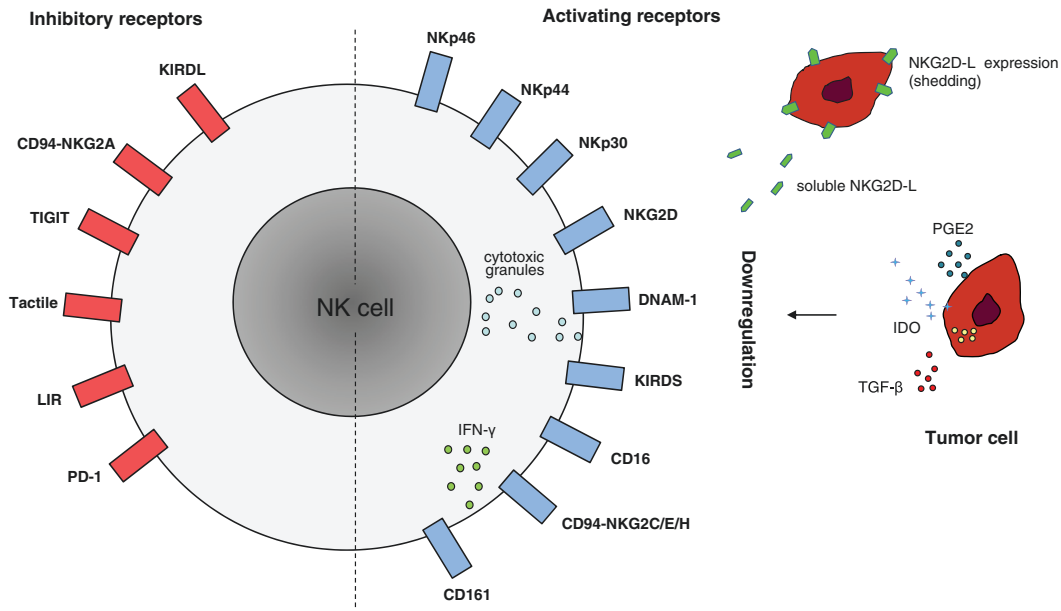

Fig. 9.1 NK cell interaction with tumor cells. Expression of major activating (blue) and inhibitory (red) receptors by NK cells determines NK cell cytotoxicity and IFN-γ production directed against tumor cells. NK cell activating receptors are downregulated in tumors by suppressive factors such as immunosuppressive cytokines, TGF-β, enzymes, IDO, and mediators of inflammation, PGE2. Chronic engagement of NK cell activating receptors with either tumor cell surface-expressed or shed NK cell ligands, e.g. NKG2D-L, represents another most common mechanism of inhibition of NK cell activity. These alterations lead to anergic NK cells and allow tumor immune escape

9.2.3 Killer Cell Immunoglobulin-Like Receptors (KIR)

The human killer cell immunoglobulin-like receptor (KIR) family comprises polymorphic molecules expressed on NK cells and a small subset of αβ and γδ T-cells. They are type I membrane glycoproteins that may express in their extracellular domain two (KIR2D) or three (KIR3D) immunoglobulin domains, while based on the length of their intracellular domain they are designated as activating with short (S) intracellular domains and inhibitory KIRs with long (L) intracellular domains [33].

There are 15 KIR genes that reside in a single complex on chromosome 19.

KIR receptors are specific for MHC class I antigens and are divided into haplotype A and B, with A being more frequent and including inhibitory receptors compared to B that includes both types of receptors with predominance of activating receptors. NK cell KIR repertoire depends on both KIR and HLA polymorphisms [34]. Consequently, differential expression of these inhibitory receptors by subsets of human NK cells allows them to carefully monitor self (and foreign) MHC molecules and uniquely regulate cytotoxicicity when pathological processes perturb MHC expression [35, 36].

Each KIR has a subgroup of HLA class I allotype ligands with HLA-C being dominant HLA class I locus. All HLA-C allotypes carry valine at positions 76, while position 80 displays dimorphism, either asparagine or lysine. Nearly half of the HLA-C allotypes (Cw2, 4–6 and 15) carry lysine at position 80 (conventionally termed C2 epitope) that binds inhibitory and activating receptors KIR2DL1/KIR2DS1. The remaining HLA-C allotypes (Cw1, 3, 7, 8) carry asparagines at the position 80 (termed C1 epitope) and bind inhibitory and activating receptors KIR2DL2/3 and KIR2DS2/3 [34–37].

Activating and inhibitory KIRs may have identical or different HLA, although inhibitory KIRs have a higher binding affinity. In this sense, inhib-

itory KIRs suppress NK cell activity through a receptor-associated immune tyrosine based inhibitory motif (ITIM) by recruiting protein tyrosine phosphatases (SHP-1 and SHP-2) responsible for dephosphorylation of tyrosine kinases associated with NK cell activating receptors. Contrary to this, activating KIRs associate with unique ITAM containing adaptor originally designated as DAP12 that are phosphoryled by Src family kinases and deliver activation signals through recruitment of Syk/ZAP-70 tyrosine kinases to mediate downstream activation signaling [38].

Clinical studies have correlated KIR gene content with infection, cancer, autoimmunity, pregnancy syndromes, and transplant outcome [35, 39, 40]. Regarding cancer, upregulation of inhibitory KIRs may dampen NK cell-mediated antitumor response and are associated with disease progression in different malignancies. Our studies of CD158a (KIR2DL1) and CD158b (KIR2DL2/3) KIR receptor expression on NK cells in MM patients [5] show that they have an increase in the expression of CD158b receptor that shows negative correlation with NK cell cytotoxicity [41].

9.2.4 NKG2D Receptors

Calcium-dependent lectin-like receptor, NKG2D, is an activating receptor expressed on NK cells and most NKT, γδ, and CD8+ T-cells. It belongs to NK group 2 receptors (NKG2) as member D. Contrary to the other members of NKG2 family that form heterodimers, NKG2D forms only homodimers and recognizes a number of MHC-class-I-related molecules, MICA/MICB and UL16-binding protein (ULBP) [42] expressed on cells in dangerous situations such as transformation, infection, heat shock, or genotoxic stress [43, 44].

NKG2D as pivotal activating receptor that upon binding stress-induced ligands and phosphorylation of intracellular domain (YINM) of DAP10 induces cytotoxicity by recruiting, p85 subunit of PI3K and Grb-2-Vav1 that can activate MAPK and Jak/STAT signaling pathways [45].

It has been shown that downregulation of NKG2D receptor on NK cells impairs NK cell-mediated antitumor cytotoxicity and is associated with breast, lung, colorectal, cerviacval, pancreatic, gastric cancer, and melanoma [5, 30, 41]. Decreased expression of NKG2D is mediated by immunosuppressive cytokines (TGFβ and IL-10) produced by tumors and different immunosuppressive cells (MDSC and Tregs). NKG2D downregulation on NK cells has been observed in experimental settings with tumor cells that have high expression of NKG2D specific ligands such as MICA/B [46]. For this reason, activating NKG2D receptor has a role in tumor immunosurveillance, as well as in immune-mediated rejection of tumor cells to prevent tumor progression [47].

9.2.5 NKG2 C-Type Lectin Heterodimers

This family of C-type lectin NK cell receptors includes inhibitory CD94-NKG2A heterodimer that downregulates NK cell activity, as well as several NK cell activating CD94-C/E/H heterodimer receptors. The ligands for these receptors are non-classical MHC class I molecules, HLA-E. The expression of HLA-E in malignant cells reflects aberrant overall biogenesis of MHC class I proteins as it is formed from peptides derived from the leader peptides of HLA-A, B, C, and G that cannot be properly assembled [48]. In humans the expression of these receptors may be related to *KIR* gene expression, as NK cell clones lacking expression of inhibitory KIR were shown to express an inhibitory CD94-NKG2 heterodimer, inhibit NK cell activation. CD94-NKG2A NK cell inhibitor is expressed early, in stage 3, of NK cell development prior to inhibitory KIR expression and in the absence of inhibitory KIRs they may overtake their role in NK cell education [49].

The inhibitory function of CD94/NKG2A is mediated by intracellular ITIM (immunoreceptor tyrosine-based inhibition) domain [50] that recruits protein tyrosine phosphatases and dephosphorylates surrounding tyrosine kinases and adaptor proteins.

Activating CD94-NKG2C and E heterodimer receptors that also bind HLA-E have been shown to associate with DAP12 and have a role in NK cell antitumor response [51].

In breast and colorectal cancer the increased expression of inhibitory activity of NKG2A receptor is associated with poor disease prognosis [52]. Contrary to this, decreased expression of NKG2C activating receptor in acute myeloid leukemia (AML) results in NK cell dysfunction [53].

9.2.6 Natural Cytotoxic Receptors: NCR

The NCRs are NK cell activation receptors that have immunoglobulin structural motives and associate with ITAM-bearing adaptor molecule, DAP12. They were identified on NK cells and are present on T-cells and NK-like cells. NCR include NKp46, NKp30, and NKp44 [54, 55]. NKp46 and NKp30 are constitutively expressed on all activated and resting NK cells thus making them the only NK-specific markers known today, whereas NKp44 expression is restricted only to activated NK cells.

The reported ligand for NKp46 is haemagglutinins, while ligands for NKp30 are BAT3 (the nuclear factor HLA-B-associated transcript 3) and B7-H6 from the B7-family [56, 57]. Three different isoforms of NKp30, NKp30a, b, and c were described. The final outcome of NKp30 activation depends on the NKp30 isoform expressed on the surface of NK cells that results in quantitatively and qualitatively different antitumor response [58].

Another member of the NCR family, NKp44, was primarily defined as an activating receptor that binds haemagglutinins and a recently defined ligand expressed on cancer cells named mixed lineage leukemia-5 (MLL5) [59]. However, recently, Rosenthal et al. reported its inhibitory properties regarding NK cells upon binding proliferating cell nuclear antigen (PCNA) on tumor cells [60]. Expression of PCNA was associated with shorter overall survival of breast cancer patients [61] and might be involved in tumor immune evasion.

Upon ligation NKp46 and p44 activate ITAM containing adaptor proteins. NKp46 binds CD3 FcγR heterodimers, NKp30 to CD3ζ homodimer, and NKp44 binds directly to DAP12. Upon ligation the tyrosinases in the ITAM domain become phosphorylated and recruit SH2 domains of Syc/ZAP70 kinase. Activation of Syc/ZAP70 ultimately leads to ERK activation and granule mobilization [62]. The full effector cytotoxic potential of NK cells is mainly achieved upon simultaneous engagement of several activating NCRs [63].

NCR can also mediate the production of proinflammatory cytokines by NK cells. Early experiments showed that cross-linking of NKp46, NKp44, and NKp30a and b resulted in the production of IFNγ and TNFα and potentiation of adaptive immune antitumor responses. In particular binding of NKp30 expressed on NK cells to its ligands on the surface of immature DCs by producing high amounts of these cytokines trigger autologous DC maturation. Contrary to this, NKp30c induces production of IL-10 and is associated with reducing NK cell effector functions [58].

NCR expression on NK cells is negatively regulated by inhibitory cytokines including TGF-β [64] and IL-10, as well as by metabolites such as L-kynurenine a product of tryptophane degradation induced by tumor-derived indolamin-2,3-dioxygenase (IDO). Decreased expression of NKp46, NKp44, and NKp30 has been reported in various hematological and solid malignancies [7, 65]. NKp44 activating receptor expressing ILC3 cells have been shown to have tumor protective role [11].

9.3 Nectin and Nectin-Like Binding Receptors

This is another family of NK cell receptors that are adhesion molecules and members of Ig-superfamily that has recently been shown to have a role in the recognition of tumor cells and NK cell-mediated responses to tumors. These receptors have similar ligands that have been recently identified on the surface of target cells as crucial regulators of NK cell function, i.e., nec-

tin-2 (CD112) and poliovirus receptors (CD155), although they may have activating or inhibitory functions [66]. This is an important family of receptors that aside from best characterized CD226 (DNAM1-DNAX Accessory Molecule-1) also includes CD96 (TACTILE—T-cell-activated increased late expression) and TIGIT (T cell immunoreceptor with Ig) and ITIM domains receptors. These receptors are important in settings in which the tumor is mainly non-immunogenic, as it does not express stress ligands or costimulatory molecules, which is a common situation for many epithelial cell malignancies.

The most prominent member of this family DNAM1 is an NK cell activating receptor that by binding to PVR and nectin-2 on tumor cells recruits the tyrosine kinase Fyn and PKC that transduce activating signals. Moreover, DNAM1 upon interaction with its ligands induces actin polymerization and together with activation of other surface receptors contributes to more stable NK cell and target cell interaction. Aside from its involvement in the NK cell-mediated responses to tumors DNAM-1 has a role in migration of NK cells into secondary tumor deposits [48, 67].

The importance of DNAM1 in NK cell antitumor recognition has been shown in solid tumors and AML [7, 53, 68, 69] and illustrated in melanoma by inhibition of NK cell function after DNAM1 downmodulation by tumor associated fibroblasts (TAF) [28]. Also, DNAM1 ligand CD155 upregulation on multiple myeloma (MM) cells has been reported and resulted in increased sensitivity to NK cell-mediated lysis [70].

However, the other two members of this family, TIGIT and TACTILE receptors counteract DNAM-mediated activation as they contain an ITIM motive and inhibit NK cell antitumor activity [71]. TIGIT is strongly expressed on tumor-infiltrating lymphocytes in a wide range of tumors. For this reason, these molecules may be promising therapeutic targets for antibody therapy directed against these and similar inhibitory receptors, i.e., immune checkpoint inhibitors, for the treatment of malignancies [66].

9.3.1 Leukocyte Ig-Like Receptors (LIR)

The family of leukocyte Ig-like receptors (LILR/LIR/ILT) or CD85 includes members that are primarily inhibitory (LILRB), although some are activating (LILRA), as well as soluble receptors that regulate a broad range of cells in the immune response. These receptors bind a broad spectrum of MHC class I molecules, HLA-A, B, C, the non-classical HLA-G, and MHC-like molecules including human cytomegalovirus (HCMV) encoded protein UL-18. Inhibitory LIRs (CD85d and j) contain ITIM motives in their cytoplasmic region, while activating LIRs (CD85i, h and e) with short cytoplasmic tail were proved to recruit the ITAM motive of FcεRIγ. However, the effects, mechanisms, and structure of many activating LIR receptors remain unknown [72].

LIR1 (ILT2), CD85j, is the primary type of inhibitory receptor expressed on mature NK cells. LIR1 has been identified in the context of human cytomegalovirus (HCMV) infection as a receptor for viral encoded protein UL-18 and may lead to the suppression of NK cell-mediated antiviral responses [72]. LIR1 represses the activation of NK cells and plays a key role in immune escape in gastric cancer and MM upon binding its ligands on tumor cells, that include classical and non-classical MHC class I molecules, especially membrane-bound and soluble HLA-G, overexpressed in cancer patients [73]. Although LIR1 inhibits NK cell activation, inhibitory KIR and CD94/NKG2 receptors are thought to be more dominant.

9.3.2 CD16 (Fc γ Receptor IIIA)

This activating NK cell receptor is involved in antibody-dependent cellular cytotoxicity (ADCC) and direct cytotoxicity against tumor cells [74, 75], proliferation and post-activational NK cells apoptotic death, as well as in cytokine production [76]. CD16 receptor contains a cytoplasmatic domain consisting of ITAM structural motive that is comprised of FcεRIγ or TCRζ chains that upon ligand-binding become phos-

phorylated and induce signal transduction by activation of non-receptor tyrosine kineses Syk and ZAP-70 [77].

The expression CD16 receptor has been reported to be decreased on NK cells in breast cancer and MM patients [68, 78] due to post-activational receptor internalization and MMP-mediated receptor shedding following contact with target cells [79, 80]. In this sense, as CD16 defines the two functionally different NK cell subsets its decreased expression leads to the loss $CD16^{bright}$ cytotoxic subset, a finding that has been detected in numerous malignancies such as breast cancer, MM, and melanoma [78, 81, 82].

9.3.3 Natural Killer Receptor-P1 (NKR-P1)

This is a human homologue of rodent NK1.1 receptor which is a prototypical NK cell marker. CD161 is encoded by NKR-P1 gene family and is type 2 membrane glycoprotein receptor that belongs to the C-type lectin family. Five receptors NKR-P1A, -B, -C, -D, and -F have been identified in which NKR-P1-B and D both contain an ITIM suggesting inhibitory function; however, it was found that NKR-P1-C, analogous to rodent NK1.1 receptor, associates with ITAM containing FcεRI to induce NK cell activation although the biological relevance of this remains unclear [30, 41, 81]. NKR-P1A (CD161) receptor has been shown to appear early during NK cell development and its activating function may be confined to immature NK cells. The signaling of NKR-P1A has not been fully characterized, although it may activate acid sphingomyelinase which was suggested to result in NK cell resistance to apoptosis.

Ligands for NKR-P1-B and D have been identified as Ocil-Clr-b, glycoprotein expressed on hematopoetic cells and Clr-g, a c-type lectin expressed on activated NK cells. These receptors may be involved in NK cell antitumor responses, as expression of Ocil-Clr-b can be downregulated on tumor cells in some form of "missing self" recognition of target cells. Cross-linking NKR-P1A (CD161) receptor to lectin-like transcript 1 (LLT1) inhibits NK cell cytotoxicity [83, 84].

However, the biological relevance of CD161 expression in malignant tumors remains unclear, as its detected decreased expression does not correlate with decrease NK cell cytotoxicity in metastatic melanoma [5].

9.4 NK Cell Effector Functions

Unlike adaptive T and B lymphocytes, NK cells do not rearrange their receptor genes somatically, but rather rely on fixed number of inhibitory and activating NK cell receptors that are capable of recognizing MHC class I (HLA-C) and MHC class I-like molecules (MICA/B, ULBP), as well as other ligands. Downregulation of MHC class I molecules or loss of its expression during viral infection or carcinogenesis releases the inhibitory signal to NK cells and permits their activation. NK cell effector function can be triggered by the engagement of activating NK cell receptors with stress-induced cell surface ligands expressed by transformed or infected cells. NK cell triggering is the result of a complex balance between inhibitory and activating signals and requires not only the deficient MHC I expression on target cells, but also the expression of inducible ligands of activating NK cell receptors [85, 86]. NK cells exert their biological functions by various means that includes cytotoxicity and cytokine and chemokine production [29].

9.4.1 Cytotoxic NK Cell Function

NK cells exert their cytotoxic function using two main pathways: direct cytotoxicity (receptor-independent) based on degranulation of lytic granules containing perforin and granzymes and indirect cytotoxicity (receptor-dependent) based on expression of death ligands, such as FAS ligand (FasL) or tumor necrosis factor related apoptosis-inducing ligand (TRAIL).

9.4.2 Lytic Granule Cytotoxicity

Upon recognition of the NK cells with its target via specific receptor ligand interaction, subse-

quent adhesion molecule interaction (LFA-1, ICAM-1) initiate immunological synapse formation. At this point within the activated NK cell, lytic granules are mobilized toward the immune synapse via polarization of microtubule organizing center (MTOC) and drastic cytoskeletal rearrangement. Upon fusion of granular vesicles with the plasma membrane, the lytic components, namely the pore forming protein perforin and serine proteases or granzymes are released into intercellular space. Perforin monomers are exposed to extracellular levels of free Ca ions (Ca^{++}) in the synaptic space, which elicits simultaneous unfolding, polymerization and insertion into opposing target cell plasma membrane. The resulting perforin pores lead to transient Ca^{2+} influx and endocytosis of granzymes and other lytic granule components. The most important effect of granzymes is ignition of programmed cell death by both caspase-dependent and caspase-independent pathways. Aside from this perforin mediated membrane damage leads to colloid osmotic lysis due to the presence of pores in the membrane [87, 88].

Perforin was first characterized as a component present in dense cytoplasmic granules of both NK and CTL [89] that causes rapid killing upon granule exocytosis. NK cells have been shown to have preformed perforin [15, 90]. Upon cytokine stimulation transcription of perforin gene in NK cells is regulated by STAT1, 4 and 5 [15, 91–99]. Impaired STAT1 signaling is associated with low NK cell cytotoxicity and low production of IFNγ in patients with malignancies [98, 100]. Loss-of-function mutations in the gene coding perforin (PRF1) markedly reduce the ability of NK cells to kill target cells, causing immunosuppression and impairing immune regulation as seen in melanoma, lymphoma, colorectal carcinoma or ovarian cancer [101, 102].

Five granzymes have been identified in humans: A, B, H, M and tryptase-2/granzyme 3 [103] that belong to the family of serine proteases with a wide range of substrate specificities. While granzyme B induces apoptotic death in a caspase-like fashion [104], granzyme A, aside from inducing non-apoptotic cell death, targets nuclear proteins and directly in a noncaspase-like fashion induces DNA fragmentation [105].

9.4.3 Death Receptor Mediated Cytotoxicity

Death ligands, Fas ligand (FasL) or TRAIL can induce target cell apoptosis by interacting with their respective receptors on target tumor cells [106, 107]. FasL is a type II membrane protein and it is expressed constitutively by NK cells. FasL recognizes and causes aggregation of death domains in cytoplasmyc region of its receptor on target cells. Following endocytosis of the receptor ligand complex the adaptor molecule FADD (Fas associated death domain) binds the death domains of the complex and the classical apoptotic signaling cascade is initiated. In an apparent mechanism to thwart the antitumor response, a variety of tumor cells also express FasL which by counter-attack has been shown to result in NK cell apoptosis and depletion [108]. TRAILs on NK cells induce signaling through their functional receptors on target tumor cells and also integrate on the FADD-dependent signaling apoptotic cascade. This apoptotic mechanism is slower (several hours) and often less efficient than granule-mediated cytotoxicity.

9.5 Regulatory NK Cell Function

Beside their spontaneous capacity to mediate natural cytotoxicity towards tumor or virus infected cells, NK cells are also able to produce cytokines, such as Th1 cytokine and IFN, as well as both proinflamatory and immunosuppressive cytokines, such as TNFα and IL-10, respectively, and growth factors such as GM-CSF and IL-3. NK cells also secrete many chemokines including CCL2 (MCP-1), CCL3 (MIP1-α), CCL4 (MIP1-β), CCL5 (RANTES), and IL-8 which are key for NK cell co-localization and interaction with other cells of the innate and adaptive immune system in areas of inflammation and tumors [109].

Although, it was considered that regulatory CD3-CD56^{bright} NK cells are the main producers of IFNγ, it has recently been shown that after target recognition and cytokine activation cytotoxic CD3-CD56^{dim} NK cells show after short stimulation, 4–6 h, rapid production of IFNγ that is transient and not detectable after 24 h [7, 97]. It is after this period that the regulatory CD3-CD56^{bright} NK cell subset continues to consistently produce IFNγ [110].

These beneficial primary biological activities of IFNγ in the tumors present in early inflammatory response, has recently been shown to change to pro-tumorigenic effects in chronic tumor-related inflammation. This immunosuppressive effect of IFNγ is based on its ability to adversely affect the protective role of neutrophils and myeloid cells and to induce mediators (prostaglandine E2) and enzymes (indoleamine 2,3-dioxygenase-IDO) that suppresses NK cell function by downregulation of their activating receptors [111]. Furthermore, NK cell produced IFNγ induces non-classical, HLA-G expression on tumor cells that by binding inhibitory NK cell receptors (KIR, LIR) negatively affect not only NK, but T-cell responses. Moreover, IFNγ by the induction of PD-L1 on tumor cells that bind inhibitory checkpoint PD-1 receptors on NK and CTLs, further suppresses their function [112].

9.6 Conclusion

The comprehension of NK cells function has extremely grown in last period and involved better characterization of activating and inhibitory NK cell receptors, their ligands and signaling pathways, as well as identification of novel NK cell receptors. However, in malignancies many immunossuppressive mediators and cytokines contributes to functionally impaired NK cells by affecting receptor repertoire that eventually leads to tumor immune evasion and disease progression. Better understanding of alterations associated with diminished NK cell activity in cancer patients may represent useful biomarkers of the course of disease and may assist in selection of immunotherapy to restore and sustain NK cell antitumor response.

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Role of Plasmacytoid Dendritic Cells in Cancer

10

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10.1 Introduction

Dendritic cells (DCs) are highly specialized antigen-presenting cells (APCs) essential in order to generate immune responses [1], recognizing, processing, and presenting "danger signals" to the adaptive immune system. It is clear that DCs are not a unique homogeneous cell population, but rather a pool of subsets with different origins, phenotypes, and functions [2, 3]. However, two of these are of greater importance: myeloid-derived dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs). mDCs reside in an immature state in peripheral tissues where they behave as sentinels to actively capture and process antigens (Ags). Following exposure to proinflammatory cytokines or pathogen-derived products (pathogen-associated molecular patterns, PAMPs), they undergo a maturation process and migrate to draining local lymph nodes via the afferent lymphatics [4]. In contrast, pDCs do not reside in peripheral tissues during homeostasis, but are encountered in the peripheral blood and lymphoid organs [1, 5]. They were first identified in human blood and tonsils [6]. Unlike mDCs, they do not express myeloid antigens (i.e., CD11c); rather they are characterized by CD123; CLEC9A, the receptor for actin exposed during cell necrosis [7]; the cell adhesion molecule CADM1 (NECL2); the antigen BTLA; and high levels of intracellular indoleamine 2,3-dioxygenase (IDO) [8]. The hallmark of pDCs is their unique capability to produce large amounts of interferon-α and interferon-β (type I IFN) in response to viruses [9]. Furthermore, pDCs can differentiate into mature DCs when stimulated by viruses [10, 11]. Thus, pDCs are key effectors in innate immunity and act as the ideal cell population in connecting innate and adaptive immunity [9]. This discovery dates back to more than 50 years ago when Lennert and Remmele [12] identified a previously unrecognized rare cell type with plasma cell-like morphology in the paracortical area of reactive lymph nodes. Later data revealed that these cells express both T-cell and monocyte markers and were therefore designated as plasmacytoid T-cells or plasmacytoid monocytes [2, 3, 13]. In the 1980s, pathologists became increasingly aware of this enigmatic cell, and its tissue accumulation was shown to be restricted to lymphoid organs afflicted by reactive or neoplastic disorders [3, 4], as well as skin-associated lymphoid tissue [14, 15]. However, despite an increasing interest in these cells, their functional significance has still remained enigmatic.

10.2 Localization and Trafficking Patterns of Plasmacytoid Dendritic Cells (pDCs)

The development and molecular regulation of pDCs is still under investigation. FMS-like tyrosine kinase 3 ligand (Flt3L) is the main growth factor that induces the differentiation of common myeloid progenitor cells into both mDCs and pDCs [16]; however, the E2-2 transcription factor is uniquely required for pDC differentiation [17]. During steady-state conditions, mouse pDCs reside in lymphoid organs and blood, as well as the liver, lung, and skin; nonetheless, their proliferation rate is very low [18]. Human pDCs reside in primary, secondary, and tertiary lymphoid organs (aggregates/follicles—lymph nodes (LNs), tonsils, spleen, thymus, bone marrow, and Peyer's patches) [19], in addition to the liver and blood [20]. They can migrate from lymphoid organs toward T-cell-rich areas of secondary lymphoid tissues through high endothelial venules (HEV) and toward the marginal zone of the spleen [21]. In contrast, during pathological conditions, pDCs leave the bone marrow or the circulation and infiltrate inflamed tissues where they can "sense" danger signals, both PAMPs and endogenous danger signals (danger-associated molecular patterns, DAMPs), leading to the release of large amounts of type I IFNs [19, 21]. In this scenario they generate protective immunity as type I IFNs can activate mDCs, B, T, and NK cells [19, 21]. In particular, pDCs accumulate in inflammatory sites, e.g., lymphoid hyperplasia of the skin [14], cutaneous systemic lupus erythematosus (SLE), psoriasis vulgaris (basal epidermis and papillary dermis, but not normal skin), contact dermatitis, and allergic mucosa

[22]. pDCs also infiltrate ascites associated with primary and malignant melanoma [23, 24], head and neck carcinoma [25], and ovarian carcinoma [26]. Recruitment into these sites suggests that pDCs may contribute to the ongoing inflammatory response through the release of cytokines and chemokines and lead to the activation of lymphocytes [27] or, alternatively, to the induction of tolerogenic responses [28].

An intriguing question is how do pDCs enter LNs and inflammatory sites? Chemokines are important regulators of DC trafficking in vivo. Similar to mDCs, blood pre-pDCs (an immediate precursor of pDCs) undergo maturation and upregulate functional CCR7 after activation with microbial stimuli or CD40 ligation, thereby acquiring responsiveness toward CCL19 and CCL21 expressed by HEVs and LN constituents [29, 30]. Furthermore, pDCs express L-selectin (CD62L), which recognizes corresponding ligands (peripheral lymph node addressin [PNAd]) on HEVs [21]. These observations may account for the localization of pDCs around HEVs and in T-cell-rich areas of LNs during pathological conditions. pDCs also express ligands for VCAM-1, an inducible molecule on endothelial cells which may enhance migration to draining LNs [28]. Pre-pDCs express several additional chemokine receptors, e.g., CCR2, CCR5, and CXCR3 [31, 32]. Nevertheless, unlike mDCs, they marginally respond to the corresponding ligands (MCP-1; RANTES, MIP-1α, and MIP-1β; Mig [CXCL9], IP-10 [CXCL10], and I-TAC [CXCL11], respectively). Instead, they migrate efficiently following the recognition of CXCR4 ligand SDF-1/CXCL12, which is expressed on dermal endothelial cells, in LN-derived HEVs, and in malignant cells [28]. Although relatively inactive on their own, CXCR3 ligands produced by Th1 cells can enhance the responsiveness of pre-pDCs to SDF-1 by 20- to 50-fold [29, 32]. During microbial infection or inflammation, the induction of CXCR3 ligands may drive the recruitment of immature pDCs to tissues responsible for SDF-1 production. In tonsils and in psoriatic skin, epithelial cells expressing SDF-1 have been associated with the expression of CXCR3 ligands [32]. However, pDCs lose their responsiveness to SDF-1 once differentiated [31]. Interestingly, pDCs express cutaneous lymphocyte-associated antigen (CLA), which binds to E-selectin on dermal endothelial cells and which may enhance their recruitment to cutaneous inflammatory lesions [33].

Adenosine has recently been identified as a potent chemotactic factor for immature pDCs via an A1 receptor-mediated mechanism [34]. Upon maturation, the receptor is downregulated, resulting in loss of migratory function. In turn, the A2a receptor is upregulated, through which adenosine reduces the production of proinflammatory cytokines [34]. Thus, adenosine, as a resultant of tissue injury from the degradation of the increased release of ATP, as well as SDF-1 and CXCR3 ligands, facilitates the recruitment of immature pDCs from blood to inflammatory sites, but subsequently limits their contribution to an inflammatory response upon maturation after an encounter with virus, bacteria, or activated T-cells [34].

"Local" maturation upregulates CCR7, allowing pDCs to migrate to LNs in response to CCL19 and CCL21 and resist apoptosis [35]. At this site, pDCs could potentially present peripherally acquired Ags to T-cells. Recently, IL-18 produced by mDCs in inflamed sites was shown to attract pre-pDCs and modulate their function to skew Th cells toward Th1 cells [36].

10.3 Plasmacytoid Dendritic Cells (pDCs) Phenotype

pDCs are a rare cell type representing only 0.5% of circulating cells in healthy individuals [19]. They are round-shaped cells characterized by a prominent endoplasmic reticulum [21]. Mouse pDCs manifest most of the morphological and phenotypical features of their human counterpart [19, 21, 37]. Human pDCs are $CD4^+$, $CD45RA^+$, IL-3αR $(CD123)^+$, immunoglobulin-like transcript factor (ILT)-3^+, ILT-$1^{low/-}$, Siglec-H^+, and $CD11c^{low/-}$ cells (Table 10.1) [21]. Two additional surface markers for human pDCs are represented by BDCA-2 and BDCA-4 that correspond to the murine mPDCA-1, restricted to the

Table 10.1 Markers currently identified on pDCs

Marker	Structure/function	Ligand	Effect of activation
BDCA-2/ BDCA-4	Associated with FcεRIγ to form a signaling receptor complex	ITAM	Upon ligation, they inhibit TLR activation and release of type I IFN
CD4	A glycoprotein expressed on the surface of T-helper cells, monocytes, macrophages, and dendritic cells	It recognizes the TCR-MHC class II complex and is required together with the CD3 zeta chain for the recognition of antigens	Activation of pDCs
CD123	The IL-3 receptor (70KD) is composed of a ligand-specific alpha subunit and a signal-transducing beta subunit shared by the receptors for interleukin 3 (IL-3), colony-stimulating factor 2 (CSF2/GM-CSF), and interleukin 5 (IL-5)	IL-3	Amplification of inflammation
IL-T3	Characterized by its cytoplasmic ITIM domain	Fc receptor	Tolerance induction
IL-T7	Characterized by its cytoplasmic ITIM domain and is also expressed on B, T, and NK cells	IFN-I	Inhibition of release of type I IFN (negative feedback)
CD11c	A heterodimeric integral membrane protein composed of an alpha chain and a beta chain. It is present only on mouse, but not human, pDCs	ICAM-2 and VCAM-1	Induces cell activation; it is an adhesion receptor that is implicated in phagocytosis of latex beads and bacteria in the absence of complement. It plays an important role in the inflammatory response and can lead to the production of proinflammatory cytokines after an APC response
TLR7	An intracellular endosomal pattern recognition receptor	Single-stranded RNA	Upregulation of CD40, CD80, CD86, and CCR7. Induction of high levels of type I IFN. Does not induce IL-12p70 production
TLR9	An intracellular endosomal pattern recognition receptor	Unmethylated CpG oligonucleotides from bacterial DNA	Upregulation of CD40, CD80, CD86, CD83, HLA-DR, and CCR7. Upregulation of type I IFN, IL-6, TNF-α, IL-8, and IP-10. Does not induce IL-10 secretion
PD-L1	Ligand of PD-1 (programmed cell death-1) receptor (also known as CD279) expressed on the surface of activated T-cells		PD-1 and PD-L1 belong to the family of immune checkpoint proteins that act as co-inhibitory factors, which can halt or limit the development of the T-cell response. PD-1/PD-L1 interaction ensures that the immune system is activated only at the appropriate time in order to minimize the possibility of chronic autoimmune inflammation
CD80	A type 1 transmembrane protein expressed mainly on APCs and members of the B7 co-signaling molecule family. It is constituted by two extracellular domains: a membrane distal variable-like domain (IgV) and a membrane proximal Ig constant-like domain (IgC) along with an intracellular domain	CTLA-4	CD80/CTLA-4 interaction has effect on the two major subsets of CD4+ T-cells: downmodulation of helper T-cell activity and enhancement of regulatory T-cells (Treg)

Table 10.1 (continued)

Marker	Structure/function	Ligand	Effect of activation
CLEC9A	The C-type lectin (CTL) 9A, also known as DC NK lectin group receptor-1 (DNGR-1), is a protein consisting of a single extracellular CTL domain connected to the transmembrane domain by a stalk region and an intracellular cytoplasmic tail with potential signaling motifs	Unknown	Recognizes necrotic cell-associated epitopes and plays an important role in cross-presentation of dead cell-derived antigens
CADM1	A protein that belongs to immunoglobulin superfamily; is a homophilic, transmembrane Ig domain-containing protein with intracellular PDZ protein-binding motifs. It is a ligand of class I-restricted T-cell-associated molecule (CRTAM)		Acts as a major receptor for the adhesion of mast cells (MCs) to fibroblasts, human airway smooth muscle cells (HASMCs), and neurons
BTLA	A type I transmembrane co-signaling receptor belonging to the CD28 Ig superfamily. BTLA binds to the TNFR family member herpes virus entry mediator (HVEM)		HVEM/BTLA interaction recruits the protein tyrosine phosphatases SHP-1 and SHP-2, thus inhibiting signaling cascade downstream of the TCR and BCR
CD2	A member of the immunoglobulin superfamily with two immunoglobulin-like domains in its extracellular portion; is a specific marker for T-cells and NK cells	CD58, CD48, CD59, and CD15	Its activation results in augmentation of factor tyrosine phosphorylation (as PLCγ1); increases specific signaling through the TCR; T-cell activation and release of cytokines and induction of apoptosis
CD5	A glycoprotein receptor expressed on the surface of all T-cells and at lower density on a minor population of murine and human B-cells	CD72	Ligation of CD5 modulates the TCR and BCR signaling pathway
CD81	A surface protein composed of four transmembrane (TM) and two extracellular (EC) domains; expressed on T- and B-cells, NK cells, monocytes, dendritic cells, thymocytes, endothelial cells, and fibroblasts	E2 envelope glycoprotein of HCV, bona fide HCV particles	Induces B-cell adhesion via VLA-4 integrin and is involved in early T-cell development. CD81/CD19 association regulates B-cell signaling; interactions with CD3 and ICAM-1 regulate the integrity of the immune synapse during T-cell activation. CD81 is involved in hepatitis C virus (HCV) and *Plasmodium* sporozoite invasion of hepatocytes and also contributes to the assembly and budding of human immunodeficiency virus and influenza A virus
AXL	A member of a receptor tyrosine kinase (RTK) family that shares a conserved intracellular tyrosine kinase domain and an extracellular domain similar to those seen in cell adhesion molecules	Gas6 (vitamin K-dependent protein growth-arrest-specific 6)	AXL/Gas6 interaction leads to PI3K activation and its downstream targets Akt and S6K, as well as NF-κB. Gas6/Axl signaling is involved in cell growth and survival in normal and cancer cell
SIGLEC	A type I transmembrane protein with N-terminal portion in the extracellular space and the C-terminal in the cytosol. Each SIGLEC contains an N-terminal V-type immunoglobulin domain (Ig domain) which acts as the binding receptor for sialic acid	Sialic acid	Cell adhesion and cell signaling

peripheral blood and bone marrow-derived pDCs [21]. BDCA-2 is a C-type lectin transmembrane glycoprotein which can internalize Ags for presenting to T-cells. Some data show that triggering BDCA-2 can potently inhibit in vitro induction of IFN-α/IFN-β expression in pDCs by viruses [38]. On the other hand, BDCA-4 does not have a substantial effect on pDC function, but can be used for the purification of pDCs by magnetic selection (Table 10.1). Recently, a previously unknown population of pDCs has been discovered in human blood, bone marrow, and tonsil, which are morphologically, phenotypically, and genetically distinct from most pDCs [39]. These cells express CD2, CD5, and CD81 on their surface and fail to produce type I IFN upon CpG oligonucleotide stimulation due to substantially less IRF7 mRNA expression. Higher levels of IRF5 than $CD5^{-}CD81^{-}$ pDCs induce transcription of proinflammatory cytokines, chemokines, and costimulatory molecules, as well as IFN-α/IFN-β, more abundant production of IL-6, and elevated expression of CD40, CD80, and CD86. The role of this subset of pDCs is to strongly activate B and T lymphocytes, playing an important role in the immune response in physiological conditions [39]. To date, recent findings have shown a new population named AS (AXL^{+} and $SIGLEC^{+}$) DCs characterized by the expression of *AXL*, *SIGLEC1*, and *SIGLEC6* antigens sharing properties with pDCs, but more potently activate T-cells. This finding suggests that pDCs, originally described as "natural" interferon-producing cells (IPCs), have lower capability to induce T-cell proliferation [40].

In addition, recent evidence has demonstrated that $CD9^{+}$ Siglec-H^{low} pDCs secrete IFN-α when stimulated with TLR agonists, induce CTLs, and promote protective antitumor immunity. By contrast, $CD9^{neg}$ Siglec-H^{high} pDCs secrete negligible amounts of IFN-α, induce $Foxp3^{+}$ $CD4^{+}$ T-cells, and fail to promote antitumor immunity [41]. Although newly formed pDCs in the bone marrow are $CD9^{+}$ and are capable of producing IFN-α after aggregating in peripheral tissues, they lose CD9 expression and the ability to produce IFN-α. Therefore, recognition of pDC surface markers is actually very important, not only to distinguish pDCs from mDCs and other myeloid cells but also to identify their function and to allow researchers to isolate them.

BDCA-2-DTR [42] and Siglec-H-DTR models [43] are the recently developed murine models used to study the role of pDCs in the pathogenesis of various diseases. These mouse models allow the study of pDCs in pathophysiological conditions through the depletion of pDCs by diphtheria toxin (DT) using the human diphtheria toxin receptor (DTR) that is driven by the BDCA-2 promoter, as the mouse receptor for DTR binds DT with a lower affinity. However, many studies have also been conducted by using specific depleting antibodies (Abs), such as 120G8 Ab [44], BST-2 Ab [45], and mPDCA-1 [46] in vivo. All these Abs bind to the same surface marker (BST-2 or CD317). Ab depletion models seem to be less specific than DTR models, but are still very efficient in pDC depletion, thus allowing the investigation of the role of pDCs during steady-state and pathological conditions. The limitation of Ab-mediated pDC depletion stands on the role of some molecules, such as BST-2, which is also expressed by stromal and other immune cells after an inflammatory stimulus [45].

10.4 Activation of pDCs

Plasmacytoid dendritic cells are highly specialized for sensing nucleic acids via the intracellular pattern recognition receptors, Toll-like receptors (TLR) 7 and TLR9 [19, 37]. pDCs and mDCs have a different repertoire of TLR expression [19, 21, 37]. Human and mouse mDCs can express TLR1, TLR2, TLR4, TLR5, TLR7, and TLR8, while pDCs selectively express high levels of TLR7 and TLR9 [47]. TLRs are a family of receptors associated with the innate immune response [48]. In particular, TLR7 recognizes single-stranded RNA enriched with guanosine or uridine from viruses, synthetic imidazoquinolines, and guanosine analogs [48]. On the other hand, TLR9 is activated by unmethylated CpG oligodeoxynucleotide (CpG-ODN) motifs typical of viruses and bacteria [48]. Interestingly,

the response of human pDCs is dependent upon the class of synthetic CpG-ODN used to stimulate them. Stimulation with CpG-A (D)/2216 ODN induces sustained high IFN-α production by pDCs, but minimal upregulation of cell surface maturation markers including CD80, CD86, and major histocompatibility complex class II (MHC-II) [49, 50]. CpG-A has no effect on B-cells (which also express TLR9). On the other hand, stimulation with CpG-B (K)/2006, a strong B-cell activator, results in increased expression of costimulatory and Ag-presenting molecules and higher IL-8 and TNF-α secretion, but lower levels of IFN-α production by pDCs. Two distinct pathways of IFN-α/IFN-β production have been identified regarding stimulation with CpG-A vs CpG-B [50]. pDCs constitutively express IRF7 and synthesize high levels of IFN-α in response to CpG-A, which also triggers an autocrine feedback loop involving the IFN receptor-dependent pathway [47]. In contrast, IFN-α/IFN-β induction by CpG-B is independent of the IFN-α/IFN-β receptor loop [50, 51]. Recently, CpG-C, a new class of CpG-ODN in which structural elements of CpG-A and CpG-B have been combined, has emerged. This sequence activates B-cells and induces IFN-α production by pDCs [52]. Furthermore, non-CpG-containing ODNs have been shown to bind human TLR9 [52, 53] and to stimulate pDCs [54].

TLR7 and TLR9 are very sensitive to different stimuli; the first triggers ssRNA viruses, and the latter responds to DNA viruses [55]. TLR7 and TLR9 activation recruits a cytoplasmic adaptor, myeloid differentiation primary response gene 88 (MyD88), which is able to assemble a multiprotein signal-transducing complex inducing interferon regulatory factor 7 (IRF7) activation [48]. MyD88 also leads to TRAF-6-mediated NF-κB and MAP-kinase (MAPK) activation, essential for the transcription of proinflammatory cytokines, chemokines, and costimulatory molecules [48, 56].

The exposure of pDCs to TLR7 or TLR9 ligands can lead to the production of type I IFN and proinflammatory cytokines, such as TNF-α, and chemokines, such as IL-8 (CXCL8) [1, 19, 21]. Constitutive expression of IRF7, which is different from mDCs in which induction is needed, renders pDCs high producers of type I IFN [1, 19, 21], regulating T-cell immunity and leading toward Th1 and cytotoxic T lymphocyte polarization and activation of mDCs, NK cells, and B-cells [1, 19, 21]. Remarkably, IFN-α modulates several aspects of the immune system, including pDC survival [57], mDC differentiation, modulation of Th1 and $CD8^+$ T-cell responses, cross-presentation, upregulation of MHC and costimulatory molecules, activation of NK cells, and induction of primary Ab responses [58]. However, a recent study found that type I IFN negatively controls pDC turnover, in that an overproduction of type I IFNs can lead to the death of pDCs during steady-state conditions and viral infections [55]. pDC activation can also lead to the production of IL-12p70, IL-1β, and IL-6 [59]. Furthermore, a recent discovery found that pDCs may mediate the release of IL-10 [29]; however, another group [60] showed that these cells do not directly produce IL-10 (Fig. 10.1).

Moreover, it has recently been demonstrated that pDCs produce high amounts of granzyme B [61], which is effective only in combination with perforins mainly produced by cytotoxic T lymphocytes (CTLs). This further connects pDCs to the adaptive immunity. Additionally, in the absence of an "efficient" adaptive CTL immunity, pDCs can behave as killing DCs due to the release of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and the induction of DR5 expression, a TRAIL receptor, on the cell target [42, 61].

A diversity of C-type lectin receptors (CLRs) has been identified on DC subsets, including DC-SIGN (CD209), DEC-205 (CD205), langerin (CD207), mannose receptor (CD206), BDCA-2, and dectin-1. CLRs typically recognize carbohydrate-rich structures on microbes and self-antigens [38]. They have been implicated in cell adhesion and regulation of signaling events (e.g., BDCA-2), migration and homing (e.g., DC-SIGN), Ag uptake and processing for MHC-II presentation to T-cells (e.g., DC-SIGN, BDCA-2, langerin, and mannose receptor), cell-cell transmission of pathogens (e.g., DC-SIGN), and tolerance to self-antigens (e.g., DEC-205). pDCs express BDCA-2 and BDCA-4, dectin-1, and possibly DEC-205

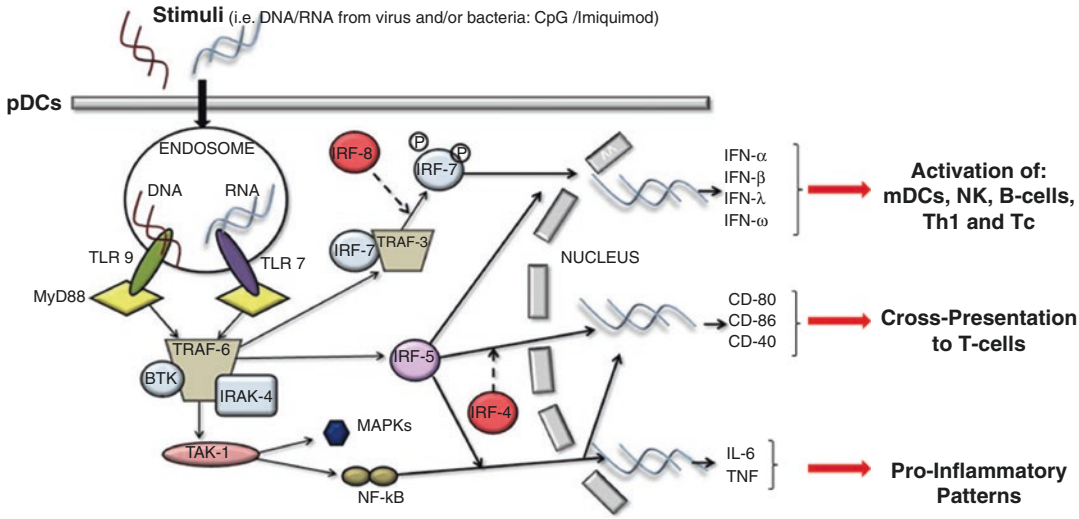

Fig. 10.1 The recognition of stimuli, such as DNA or RNA motifs from viruses and bacteria, by pDCs via TLR7 and/or TLR9, induces the activation of MyD88-dependent signaling pathways that lead to the expression of cytokines such as IL-6 and TNF-α, costimulatory molecules such as CD80, and the synthesis/release of type I IFN

but lack DC-SIGN and langerin, found on $CD34^+$ and monocyte-derived DCs and Langerhans cells (LCs), respectively [62]. The physiologic function of CLRs on pDCs remains unknown. Anti-BDCA-2 Abs are rapidly internalized and efficiently presented to T-cells, suggesting a role in Ag capture and presentation [38]. Interesting relationships between CLRs and TLRs have been documented. In mDCs, interaction of DC-SIGN with lipoarabinomannan secreted by mycobacteria inhibits lipopolysaccharide (LPS)-induced DC activation through TLR4 [63]. This mechanism may permit pathogens to evade immune responses and perpetuate tolerance to self-antigens in the face of TLR activation by microbes. On the other hand, it has been shown that dectin-1 collaborates with TLR2 in inducing proinflammatory cytokine secretion in murine macrophages and DCs [64]. Whether BDCA-2 has any connection to TLRs in pDCs remains to be elucidated. However, early reports have shown that secretion of type I IFNs by pDCs in response to the influenza virus (most likely triggering TLR7/TLR8) or to complexes of plasmid DNA and anti-DNA Abs (possibly stimulating both FcR and TLR9) is significantly inhibited by ligation of BDCA-2 with anti-BDCA-2 Ab [38]. It is worth noting that BDCA-2 is downregulated after pDCs' maturation and that mature pDCs secrete less IFN-α/IFN-β in response to viral stimuli than immature pDCs do [65, 66]. BDCA-2 has an intracellular domain of 21 amino acids without known motifs implicated in signal transduction; however, ligation induces Src family protein-tyrosine kinase-dependent intracellular calcium mobilization and protein-tyrosine phosphorylation of intracellular proteins [38]. BDCA-4 (neuropilin-1) is also upregulated in blood mDCs after overnight culture and may participate in DC-lymphocyte interactions [67].

Furthermore, a recent study shows that a microbial or cytokine stimulus can lead pDCs to multiple activated statuses, specialized in several innate and adaptive immune functions. In particular, Alculumbre et al. found that the stimulation of pDCs with influenza virus led to three stable pDC subpopulations: P1-pDCs ($PD\text{-}L1^+CD80^-$) which displayed a plasmacytoid morphology and were specialized for type I IFN production, P2-pDCs ($PD\text{-}L1^+CD80^+$) which displayed both innate and adaptive functions, and P3-pDCs ($PD\text{-}L1^-CD80^+$) which encountered a DC morphology and adaptive immune function in promoting T-cell activation and Th2 differentiation [68]. These results propose a new model of immune cell differentiation opening new possibilities for generating specialized cellular

populations through pharmacological manipulation. Moreover, these data further highlight that according to the environment they encounter, pDCs can behave differently and achieve differential immunological activity.

10.5 pDCs: Bridging the Gap Between Innate and Adaptive Immunity

The production of type I IFNs by pDCs represents the bridge between the innate and adaptive immune system. Type I IFN (IFN-α and IFN-β) is an important component of the innate immunity, especially during viral infections [19, 21]. In contrast to mDCs, pDCs produce high amounts of type I IFNs upon activation [19, 21], which both amplify their own production in an autocrine manner and induce the release of other proinflammatory cytokines such as IL-12p70 from mDCs and NK cells [69] (Fig. 10.2a). Activation of mDCs diverts the immune environment toward a Th1-like bias, during which IFN-γ production facilitates Th1 differentiation [19, 21, 69], long-term T-cell immunity [21, 69], and a CTL-mediated response [70], as well as proliferation and survival of T-cells [69, 70]. Moreover,

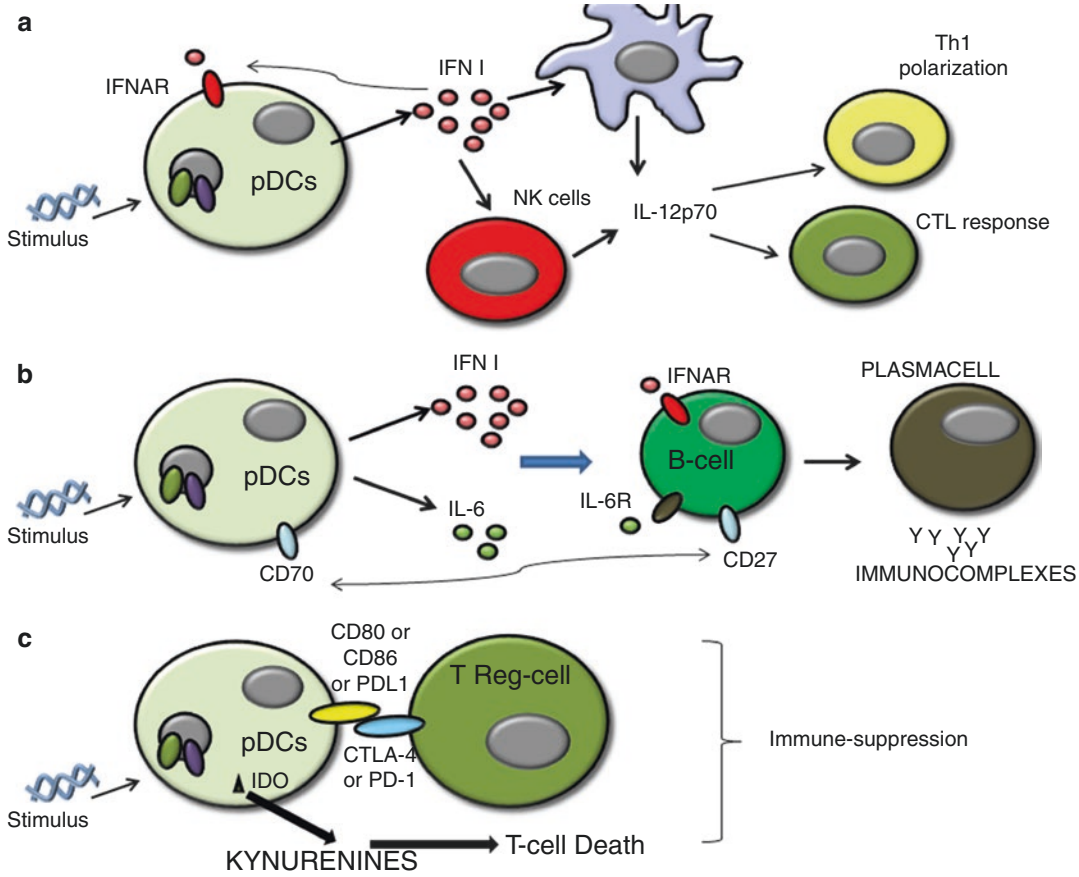

Fig. 10.2 (**a**) Activated pDCs produce high amounts of type I IFNs which both amplify their own production in an autocrine manner via the expression of IFNAR on themselves and induce the release of other proinflammatory cytokines such as IL-12p70 from mDCs and NK cells that lead to Th1 and CTL polarization; (**b**) pDCs induce B-cells to differentiate into plasma cells via the activation of IFNAR and IL-6R and the interaction of CD70-CD27 on B-cells; (**c**) pDCs can lead to immunosuppression via both direct interaction with Treg (CD80 or $CD86^{+}$CTLA-4 or $PD\text{-}L1^{+}$PD-1) and the release of IDO-induced kynurenine metabolites which induce Th1 cell death

through the production of IL-6 and type I IFNs, pDCs induce B-cells to differentiate into plasma cells which are immunoglobulin-producing cells (preferentially IgG and IgM) (Fig. 10.2b). In the process of B-cell activation, a key role is played by the CD70 receptor expressed on pDCs, as it can induce the differentiation and proliferation of IgG-producing B-cells [71] (Fig. 10.2b).

In addition, activated pDCs can undergo other important phenotypic changes that induce them to change their phenotype toward an mDC phenotype [1]. The upregulation of MHC and T-cell costimulatory molecules enables pDCs to engage and activate naïve T-cells [72–74]. There have been many controversies regarding the role of pDCs to prime T-cells and cross-present Ags [74]. The expression of MHC and T-cell costimulatory molecules is not as high as in mDCs, and that is why pDCs are less efficient than mDCs at priming T-cells [75]. Moreover, the repertoire of Ags that can be presented by pDC-derived MHC molecules is more restricted than that of mDCs, since not all of these Ags reach the endocytic compartment inside pDCs [74, 75]. However, some pDC receptors such as BDCA-2, Siglec-H, and DCIR are able to bind Ags, mediate endocytosis, and process and present to T-cells [74, 75].

Interestingly, activated pDCs can also promote Th2-like immune responses [69] underlining their functional plasticity. There is evidence that IFN-α stimulates the differentiation of pDCs into Th1-polarizing pDCs, whereas in the absence of IFN-α (but only in the presence of proinflammatory signals), pDCs can also stimulate Th2 polarization/differentiation [76]. Moreover, some authors reported that CpG-activated pDCs exert a strong immunosuppression and induce the differentiation of allogeneic $CD4^+CD25^+$ T-cells into $CD4^+CD25^+$ regulatory T-cells in tumor conditions [55, 60]. Interestingly, pDCs can directly or indirectly recruit Treg cells via the PD-L1/PD-1 axis [77] (Fig. 10.2c), the release of immunosuppressive cytokines, such as IL-10 [60, 77], and the membrane tolerogenic inducible costimulator ligand (ICOS-L) [8].

pDCs can also synthesize large amounts of functional IDO, which requires autocrine release of type I IFN, upon TLR9 and CD200R ligands' stimulation [19]. IDO-derived metabolites promote T-cell death [60, 78] and suppress T-cell immunity in normal and pathological settings. In the same manner, reduced tryptophan amounts can lead to the release of regulatory cytokines, such as IL-10 [79], associated with a tolerogenic environment.

In this scenario, it was recently reported that pDCs could have inflammatory activities eluding T-cell-dependent immune response. The accumulation of pDCs in the intestinal lamina propria amplified the intestinal inflammation by recruiting inflammatory monocytes/macrophages and cDCs into the inflamed colon, leading to the initiation and exacerbation of a T-cell-independent acute colitis [80].

Taken together as a whole, these data suggest that pDCs are a key effector cell in both innate and adaptive immunity regulation [1].

10.6 pDCs and Human Diseases

A wide spectrum of human diseases such as infection, autoimmunity, and cancer are associated with the accumulation of pDCs in lymphoid and peripheral tissues and had a strict correlation with the reduction of these cells in the peripheral blood [24]. For many of these diseases, compelling evidence supports a pathogenic role of pDCs, mainly related to either the increase or reduction of proinflammatory or anti-inflammatory functions of pDCs. Alternatively, pDC accumulation might exert an adjuvant immune function, as in viral infection and in imiquimod-treated cancers, where they seem to encounter an antiviral and antitumor activity. In many other pathologies, available information is still limited, and pDC biology is largely unknown.

10.6.1 Role of pDCs in Human Infections

pDCs have been most extensively studied during HIV and chronic viral hepatitis, particularly hepatitis C virus (HCV) infections. The emerging picture suggests an important role for pDCs in

these infections; however, the exact mechanism and consequences of pDC activity are controversial at present [81]. pDCs can respond to HCV and particularly to HCV-infected hepatocytes which induce pDCs to signal via an endocytosis- and IRF7-dependent mechanism, but not via the NF-κB pathway, implying a non-full functional response of pDCs that contribute to the evasion of immune responses by HCV [82]. In contrast, other studies demonstrated normal pDC functionality in chronic HCV infection [83]. The resolution of this controversy would establish pDCs either as a weak link of anti-HCV immune response or as a potentially powerful effector type that can be harnessed for immunotherapy of chronic HCV.

Similarly, pDC dichotomy is observed in HIV infection, in which some authors assume that pDCs can be infected with HIV and/or respond to it with robust IFN secretion [84], while others reported impaired activity of pDCs in HIV-infected patients [85, 86]. Interestingly, pDCs are progressively depleted from the blood of infected patients, either through infection-induced death or due to redistribution to lymphoid organs. The key unresolved question is whether HIV-induced pDC activation is beneficial or harmful for the host. On one hand, IFN secretion by pDCs was shown to inhibit viral replication in T-cells and promote pDC and cDC maturation, leading to the killing of infected T-cells. In this context, it is likely that HIV may have evolved mechanisms to suppress pDC activation, e.g., through BDCA-2 ligation [87], which disables pDC functions as APCs and type I IFN-producing cells. On the other hand, the same functions of pDCs may exacerbate T-cell depletion, e.g., by disseminating HIV to uninfected $CD4^+$ T-cells or by bystander T-cell killing. Most importantly, elevated IFN response by pDCs may contribute to chronic immune activation and faster T-cell depletion [88]. It is plausible that the function of pDCs in HIV infection changes from protective to pathogenic as the disease progresses. In the early stages of infection, IFN production and virus cross-presentation by pDCs may help limit virus spread and mount cytotoxic T lymphocyte responses, whereas as virus replication escapes control, IFN secretion may drive polyclonal T-cell hyperactivation and depletion [83]. The eventual loss, redistribution, or functional impairment of pDCs in the late stages of infection would contribute to immunodeficiency.

A recent study identified T-cell immunoglobulin (Ig) and mucin domain-containing molecule-3 (Tim-3) as a novel biomarker of pDC dysfunction in HIV infection. Tim-3 was upregulated on pDCs during HIV infection, a sign of pDC abnormalities, even after combined anti-retroviral therapy (cART). The frequency of Tim-3-expressing pDCs inversely correlated with $CD4^+$ T-cell counts and positively with HIV viral loads and disease progression, implying a favorable role of pDCs for HIV-related disease probably due to the lower expression of IRF7 [89]. In contrast, a lower frequency of Tim-3 on pDCs led to higher levels of IFN-α and TNF-α in response to imiquimod and Sendai virus, TLR7 agonists, and to CpG, a TLR9 agonist. Furthermore, intracellular Tim-3 colocalized with p85 and IRF7 within LAMP1+ lysosomes, suggestive of a role in degradation. Thus, the role of pDCs in HIV and HCV infections highlights the power and danger of pDC activation and reveals another strategy of immune system subversion by these viruses.

Another aspect to take into consideration is the antifungal activity of pDCs. Indeed, pDCs express receptors involved in fungal recognition, such as CTL receptors including dectin-1, dectin-2, dectin-3, and mannose receptor and TLR9 that are deputed to recognize, signal, and respond to a wide variety of fungal pathogens, including *Aspergillus fumigatus, Cryptococcus neoformans, Candida albicans*, and *Pneumocystis jirovecii* [90]. The cellular responses of pDCs to fungal recognition are still under investigation, but recent studies found that human pDCs directly inhibited the growth of *A. fumigatus* hyphae and were able to produce IFN-α and TNF-α in response to in vitro hyphal stimulation [91]. The depletion of pDCs in mice subjected to *A. fumigatus* infection led to decreased survival rate, implying a role for pDCs in the antifungal immune response. However, further studies are needed to better understand and clarify the response of pDCs against fungi.

10.6.2 Role of pDCs in Autoimmune Diseases

Several autoimmune diseases are associated with elevated levels of type I IFNs, implying a potential role for pDCs in cytokine production [92]. To date, the strongest evidence for pDC involvement has been accumulated from the study of two diseases: psoriasis and systemic lupus erythematosus (SLE) [93]. In psoriasis, early skin lesions are highly infiltrated by activated pDCs, corresponding with decreased numbers of circulating pDCs [94]. Blocking IFN production by pDCs using anti-BDCA-2 Ab inhibited the development of skin lesions in a xenograft mouse model, providing causal proof of pDC function in the disease [94]. Gilliet's group [95] identified the activating stimulus for pDCs as complexes of self-DNA with the antimicrobial peptide LL-37. This and possibly other homologous proteins promote the aggregation of released cellular DNA and RNA into large complexes that efficiently activate pDCs [95, 96]. Although the origin of these immunostimulatory complexes and the consequences of pDC activation remain to be elucidated, the major role of pDCs in psoriasis is well established. Interestingly, recently a role for pDCs has been described in "paradoxical psoriasis," a condition characterized by inflammatory skin lesions observed in 2–5% of patients receiving anti-TNF therapy. Anti-TNFs directly prolong the ability of pDCs to produce type I IFN. The resulting overexpression of type I IFNs is sufficient to drive the development of the psoriatic skin phenotype. Unlike classical psoriasis, which is a T-cell-mediated autoimmune disease, development of "paradoxical psoriasis" is independent of T-cells, and it is likely that it is due to a pDC-driven innate immune response [97].

Similarly to psoriasis, lupus patients show a decrease in circulating pDCs and the accumulation of activated, IFN-producing pDCs in affected tissues such as the skin [98]. The hallmark of lupus is the production of antinuclear Abs and immune complexes of such Abs with endogenous nucleic acids, which were shown to activate pDCs through TLR7/TLR9 [99, 100]. These complexes may be delivered into the endosomal compartment of pDCs via Fc receptor II (FcγRII) [99, 101], and their stimulatory capacity can be augmented by the nuclear DNA-binding protein HMGB1 [102]. In addition, self-DNA forms complexes with LL-37 and other antimicrobial peptides released by neutrophils, and the resulting complexes induce IFN secretion in pDCs through TLR9 [102]. Notably, TLR-activated pDCs become resistant to glucocorticoids, which could underlie the limited efficacy of these drugs in lupus [103, 104]. The direct causal relationship between pDC-derived IFN and lupus progression/severity is hard to establish in the human system and should await elucidation in animal models.

An important study carried on lupus-prone BXSB.DTR mice showed that pDCs control the early threshold activation of adaptive immune cells accelerating lupus progression. In this mouse model, the depletion of pDCs led to reduced autoimmunity, in that lower autoantibody production and less severe glomerulonephritis were found during the early stage of the disease, implying not only the crucial role of pDCs in this autoimmune disease but also that even a transient depletion of these cells was able/sufficient to ameliorate disease symptoms [105].

Nevertheless, the likely connection between the formation of nucleic acid-containing immune complexes, pDC activation, and IFN secretion and the pronounced IFN signature of the disease makes a strong case for pDC as a major player in lupus pathogenesis [83]. Overall, the aberrant conversion of self-nucleic acids into ligands for TLR7/TLR9 on pDCs (via immune complex formation, antimicrobial peptide binding, and other mechanisms to be discovered) may represent a common pathogenesis step in psoriasis, lupus, and possibly other autoimmune diseases such as Sjögren's syndrome [106].

The activity of pDCs in viral and autoimmune diseases may teach us how and why pDCs highly populate cancerous masses, playing a pivotal role for the tumor immune microenvironment.

10.6.3 Role of pDCs in Cancer

Recent studies have shown that the density and location of immune cells in primary tumors can predict patient survival [107], supporting the notion that monitoring local immune responses may represent a critical step in predicting patient prognosis and likely response to antitumor strategies [108]. pDCs have been found in a variety of neoplasms; nonetheless their function is still unknown. Solid tumors, such as head and neck, breast, ovarian, lung, and skin, are populated by non-active pDCs [108]. Clinical studies have suggested a direct correlation between reduced numbers of circulating pDCs and higher presence of these cells in malignant masses [1, 108]. Although the causal relationship is still under investigation, recent results from mouse models are starting to define the specific role(s) of pDCs in tumor masses. The mechanism that induces the recruitment of pDCs to the tumor site is not clear. Circulating pDCs express multiple chemotactic receptors such as CXCR4 and ChemR23, being the only biological active receptors in healthy donors [31]. CXCR4 binds CXCL12, widely expressed in tissues and which most likely represents the main axis for pDC accumulation in human tumors [28]. CXCL9, CXCL10, and CXCL11, which bind CXCR3, present on pDCs, are all IFN-inducible proteins and might be involved in pDC infiltration [109]. In addition, cytokines, such as CXCL10 and CXCL12, and chemokines, such as CCL2, are released by tumor and stromal tumor-associated cells, such as cancer-associated fibroblasts (CAFs), allowing pDCs to migrate from the circulation to the injured tissue [26]. Accordingly, Drobits et al. demonstrated that CCL2 produced in the inflamed skin of tumor-bearing mice facilitated pDC recruitment [61].

Once recruited, pDCs seem to be important players in cancer immunoediting due to their capacity to bring together the innate and the adaptive immunity. In particular, it seems that a critical role is played by type I IFNs. Endogenously produced IFN-α/IFN-β was required for the prevention of the growth of primary carcinogen-induced sarcoma [110]. In this study, host hematopoietic cells were critical targets of IFN-α/IFN-β during the development of protective antitumor responses [110]. pDCs have been widely described as professional type I IFN-producing cells; therefore, the higher presence of pDCs in the tumor mass might directly link pDCs to cancer immunoediting in that pDCs may behave as antitumor cells. However, other reports showed opposite activities of pDCs in cancer. Animal studies demonstrated that tumor-associated pDCs (TApDCs) are defective in type I IFN production but instead secrete immunosuppressive factors responsible for tumor progression [111, 112]. In support, we found that lung tumor masses are highly populated by pDCs [113, 114]. We found that tumor masses presented a higher percentage of pDCs than healthy tissues. In particular, pDCs were in their immunosuppressive phenotype, as determined by higher levels of CD33 and PD-L1. Despite higher HLA-A and HLA-D expression, cancerous pDCs did not exert cytotoxic activity against tumor cells but instead promoted their proliferation. In this scenario, cancerous pDCs were able to produce high levels of IL-1α, which was strictly correlated to the activation of the inflammasome absent in melanoma 2 (AIM2), which led to higher cytoplasmic calcium release responsible for calpain activation. The blockade of type I interferon receptors and of AIM2 via the addition of LL-37 significantly reduced the release of IL-1α. Our data demonstrated for the first time that lung tumor-associated pDCs are responsive to the activation of AIM2 inflammasome, facilitating tumor cell proliferation in the lung.

It is clear that similar to viral infections and autoimmune diseases, the dichotomy of pDCs in cancer might underlie their phenotype and maturation state and might teach us how to provide personalized therapy for patients.

10.6.3.1 Antitumor Activity of pDCs

Type I IFNs are pleiotropic cytokines with a demonstrated clinical benefit to cancer patients and have recently emerged as the connection bridge between tumor cells and the immune system

[115]. pDCs produce large amounts of type I IFNs upon TLR7 and TLR9 stimulation. Drobits et al. showed that the intratumoral stimulation of pDCs with imiquimod renders these cells cytotoxic and contributes to tumor regression independently from conventional adaptive immune mechanisms, via the production of TRAIL and granzyme B secretion by pDCs via IFNAR1 signaling [61]. A recent study showed that activated pDCs can kill HER2-/Neu-positive breast cancer cells, obtained from BALB/c mice, through the release of TRAIL and granzyme B, which are known to activate NK and $CD8^+$ T-cells. In this study the authors showed that CpG activates the TLR9 signaling pathway and inhibits tumor growth in breast cancer mouse models [116].

However, the role of TApDC-derived granzyme B in the absence of perforins not produced by pDCs still remains to be elucidated.

Another mechanism that may underlie the antitumor activity of TApDCs is their antigen-presenting activity. Although in their immature state, TApDCs are still capable to internalize Ags in vivo and to activate $CD4^+$ T-cells [117]. The immature state of pDCs is reflected in that they have altered cytokine production in response to TLR9 ligands in vitro while preserving unaltered response to TLR7 ligands [118], which instead seem to have potential antitumor activity. To date, imiquimod is in phase III clinical trial against melanoma. In contrast to these results, systemic administration of CpG favored pDC-induced lung tumor progression [119], as also observed in a mouse model of breast cancer [118]. Similar to the data shown by Drobits et al., Le Mercier et al. proved that, although CpG did not alter TApDC activity, the intratumoral administration of a TLR7 ligand led to TApDC activation and displayed a potent curative effect in a type I IFN-dependent manner [61, 118], which seems to underlie the subsequent inhibition of tumor metastasis [120]. In addition, Liu et al. [121] demonstrated that the intratumoral activation of pDCs via CpG could induce NK cell-dependent tumor regression in a melanoma animal model. It is remarkable that TLR9 expression and responsiveness is impaired by tumor-derived components [122]. Similarly, in vitro differentiated pDCs exhibit an increased capacity to induce NK cells to kill acute lymphoblastic leukemia cells due to the production of higher amounts of IFN-λ2, known to play a critical role in the induction of antitumoral NK cells [123].

ILT7 on pDCs binds BST-2 expressed by tumor cells, and their interaction inhibits type I IFN production by pDCs, disabling TLR9-dependent signaling pathways [124]. Moreover, tumor-derived TGF-β and TNF-α have been identified as the main in vivo mechanisms blocking type I IFN production by pDC in tumors through inhibition of the IRF7 signaling complex, leading to a negative impact of defective pDCs in breast cancer through Treg expansion [125].

Taken together, these data supported the rationale to use TLR7 ligands to restore TApDC activation in both breast and skin cancer. However, it still remains to be determined how the activation of TLR7 and TLR9, which are MyD88-dependent, on pDCs, can differ based on tissue specificity and on the route of administration.

10.6.3.2 Pro-tumor Activity of pDCs

Several evidences have shown the prevailing immunosuppressive activity of pDCs due to both the impairment in type I IFN production and the release of pro-tumor factors [1]. Stimulation of lung tumor-bearing mice with systemic CpG, a TLR9 ligand, did not lead to the same results as observed by Liu et al. [121]. Activation of pDCs through CpG had the opposite effect in that pDC activation increased the recruitment of Tregs and limited the inflammatory cell influx to the lung, thereby establishing an immunosuppressive environment enabling tumor growth [1, 119, 125]. The same was observed in another mouse model of breast cancer in which in vivo depletion of pDCs delayed tumor growth showing that TApDC provides an immune-subversive environment, most likely through Treg activation, thus favoring breast tumor progression [126]. The discrepancy in these data and the one from Liu et al. [121] could be a result of tissue specificity and route of CpG administration which is very important in determining the tumor microenvironment, which in turn strongly influences immune cell phenotype. Moreover, in the absence of a spe-

cific stimulus, pDCs in the tumor mass have been associated with the development and maintenance of the immunosuppressive microenvironment [127]. Similar to mice, human pDCs in tumor masses are in their immature phenotype; nonetheless, a thorough study has never been conducted on the role of these cells in human tumor microenvironment. Nevertheless, it is clear that pDCs play a fundamental role in the tumor microenvironment. The specific depletion of pDCs induced lung tumor regression with a concomitant Th1 polarization that arrested tumor progression [119]. On the other hand, stimulation of TLR7, rather than TLR9, can subvert the immunosuppressive activity of TApDCs. TLR7-dependent pathway induced melanoma regression in mice [61] through the transformation of pDCs into tumor-killing cells able to produce granzyme B and TRAIL. Likewise, another group revealed that human pDCs can kill melanoma cells in vitro under imiquimod and IFN-α stimulation [128]. While pDCs can produce high levels of granzyme B, their role as cytotoxic immune cells remains to be determined as they lack the pore-forming perforin [128]. On the other hand, it has been proposed that under IL-3 and IL-10 exposure, pDCs release abundant granzyme B, which in turn is capable of blocking T-cell proliferation, thus suggesting a new potential mechanism for tumor-immune evasion [128]. Moreover, recent studies have demonstrated that peripheral pDCs from patients with hepatocellular carcinoma (HCC) exposed to tumor-derived factors would enhance IL-10 production by $CD4^+$ Tregs, through upregulation of ICOSL, favoring tumor cells to escape the immune system [129].

Several mechanisms have been postulated for the immunosuppressive nature of tumor-associated pDCs: (1) release of tolerogenic factors, (2) ILT-7 expression, (3) PD-L1 expression, (4) Siglec-H activity, and (5) induction of a Th2-like environment. Tolerogenic factors produced by tumor cells, such as PGE2 [130] and TGF-β [125], can alter type I IFN signaling pathway. Tumor-derived PGE2 and TGF-β act synergistically to block IFN-α and TNF-α secretion by pDCs [19, 125]. Opposite to IFN-α and TNF-α, IL-6 and IL-8 production are enhanced in PGE2- and TGF-β-treated pDCs [131]. Both IL-6 and IL-8 promote immune cell survival and chemotaxis but also enhance tumor cell proliferation and angiogenesis [132, 133]. Moreover, PGE2 is crucial for the secretion of other immunomodulatory factors such as SDF-1, the ligand for CXCR4, which is upregulated on both human pDCs and tumor environment [134]. Thus, pDCs can be retained in the tumor tissue via PGE2-induced sensitization for SDF-1 [32]. In further support, PGE2- and TGF-β-mediated retention of pDCs in the tumor tissue is accompanied by the suppression of the lymph node-homing receptor, CCR7 [130]. PGE2-exposed pDCs are unlikely to present Ags and to prime T-cells in the regional LNs. Concomitantly, suppression of CD40 expression and the overexpression of CD80/CD86 on pDCs enhance and even promote Treg activation via the negative regulatory receptor cytotoxic T lymphocyte antigen-4 (CTLA-4) [135, 136] (Fig. 10.2c).

Another potential mechanism for pDCs favoring tumor immune escape is the release of IDO-derived metabolites [136] from both pDCs (Fig. 10.2c) and tumor cells, inducing Treg differentiation and Th1 cell apoptosis [60, 79]. Most human tumors overexpress IDO [137], explaining the elevated tryptophan catabolism in cancer patients. Interestingly, the activation of IDO in either cancerous cells or regulatory DCs can be sufficient to promote tumor immune escape [138]. Some cancer cells, such as lung cancer-derived cells, highly express ILT7L, which can bind to the ILT7 that is on pDCs [139]. ILT7L is induced by IFN-γ and inhibits IFN-α production by human pDCs, indicating that the ILT7L-ILT7 interaction between cancer cells and pDCs may cause impairment of pDCs in the tumor microenvironment, possibly leading to immunosuppression and poor prognosis of cancer patients as observed in preclinical studies [136]. Moreover, under tumoral conditions, pDCs can also direct mDC phenotype toward a more immature state, as already reported for human lung cancer [19, 76, 119]. However, the underlying mechanism is still not defined.

To date, pDCs can directly interact with Treg via the PD-1/PD-L1 axis [60] (Fig. 10.2c), paving the road to another mechanism of action

of the newly approved monoclonal Ab, anti-PD-1 for cancer immunotherapy. In addition, IDO inhibitors have been added to the actual immune checkpoint inhibitors (ICI) [140]. IDO and PD-L1 are highly expressed by pDCs, and the current success of the ICIs allows us to suppose that the putative inhibition of PD-L1/IDO signaling from pDCs underlies the multifaceted mechanisms of the therapeutical successes of ICIs in melanoma, prostate, and breast cancer (NCT01560923; NCT01042535-phase II part).

Moreover, Ag targeting to pDCs via Siglec-H inhibits Th1 cell-dependent immunity [117]. The administration of CpG increased Siglec-H expression on pDCs recruited to the lung of tumor-bearing mice, further supporting their implication in the inhibition of Th1 cell expansion [119].

pDCs activated by IL-3 and CD40 ligand (CD40L) promote the differentiation of naïve $CD4^+$ and $CD8^+$ T-cells into Th2 cells and anergic IL-10-producing $CD8^+$ regulatory T-cells, respectively [141]. This state of anergy is mediated by IL-10, either directly (by interaction with cytotoxic T lymphocytes, CTLs) or indirectly (by inhibition of DCs) [131]. Since the tumor microenvironment is Th2-like, pDCs participate in this scenario by further augmenting immunosuppression.

Another important result is demonstrated in our study published on human lung cancer samples. The higher presence of pDCs in the tumor masses was associated with AIM2 and IL-1α production, implying that the therapeutic modulation of AIM2 activity in TApDC in the tumor site might prove to be novel and effective to limit tumor cell proliferation in lung cancer, which represents a big killer [113].

Overall, these effects may allow pDCs to establish a reduced inflammatory pattern but, at the same time, to favor tumor progression/establishment, as observed in asthma [142], virus infection [143], and cigarette smoke exposure [76]. To note, the aforementioned studies describe the role of pDCs which are not activated by a specific stimulus; then, it seems obvious that the activation of pDCs at the tumor site is a limiting step in tumor regression. Therefore, the dichotomy of pDCs in cancer may rely on the stimulation/activation of pDCs with specific stimuli as in the case of imiquimod.

10.7 Potential Therapies: Clinical Significance

Secreted factors by tumor cells, such as TGF-β, VEGF, and IL-10, may inhibit pDC functions, with the resulting suppressive immune response dictated by the same pDCs and adaptive immune cells. On the contrary, other studies reported tumor-infiltrating pDCs as functional and fully competent APCs. Production of IFN-α renders TApDCs as antitumor cells. In this context, the activation of intratumoral pDCs by means of imiquimod (TLR7 ligand) and/or CpG (TLR9 ligand) has been successfully used in the clinic to treat basal cell carcinoma and melanoma [1]. TLR signaling on pDCs can be used to induce type I IFNs and possibly protect pDCs from tumor-derived inhibitory factors (such as TGF-β and IL-10), as well as to support T-cell-mediated antitumor immune response. However, this practice can only refer to the activation of TApDCs in loco, as mouse models showed that systemic administration of CpG rendered pDCs immunosuppressive, favoring lung and breast tumor progression [1, 112, 119, 125, 126].

Many therapeutic trials have been designed to potentiate CTL responses. Myeloid-derived dendritic cell-based vaccines succeeded in inducing specific T-cells in patients, but without sufficient clinical efficacy [144]. A potential explanation of this failure may underlie the role of pDCs in modulating tumor immune-environment and, more specifically, mDC activity [119]. Animal studies on several diseases, such as asthma, virus infection, and cigarette-exposed and lung cancer models, revealed that pDCs can hamper the activity of mDCs [119]. In particular, the presence of high levels of pDCs in tumor masses was associated with immature mDCs incapable of mounting an effective adaptive immune response against cancer. Specific ablation of pDCs rendered mDCs active and prone to induction of a CTL response against tumor cell proliferation [119]. Therefore,

we speculate that pharmacological manipulation of pDC phenotype could result in successful antitumor therapy together with conventional strategies. In support, our published data showed that doxorubicin or oxaliplatin—drugs that are highly used in the clinical antitumor practice—had a much effective activity against lung tumor progression due to the induction of proinflammatory pDCs, activated by tumor cell death [114]. This latter study was conducted on mouse models. Therefore, clinical correspondence could prove the potential antitumor activity of proinflammatory pDCs resulting in tumor regression. In addition, previous studies on the role of pDCs as antitumor cells only after intratumoral activation of these cells by means of imiquimod and CpG could underlie the same mechanism of action. In other words, several endogenous molecules (DAMPs) that participate in sterile inflammation have been described as potential TLR ligands. Similarly, we could speculate that tumor cell death can induce the release of DAMPs which activate pDCs in a TLR7- or TLR9-dependent manner leading to type I IFN production by pDCs. This prevails and allows the gap between the innate and the adaptive immunity to overcome tumor-mediated immunosuppression. In this scenario, Aspord et al. demonstrated that stimulation of PBMCs from HLA-A∗0201^{+} donors by HLA-A∗0201 matched allogeneic pDCs pulsed with tumor-derived peptides triggered high levels of antigen-specific and functional cytotoxic T lymphocyte responses; this resulted in melanoma regression in a humanized mouse model [145]. This semi-allogeneic pDC vaccine was more effective than conventional mDC-based vaccines, endowing a strong potential for clinical application in cancer treatment [145].

10.8 Concluding Remarks

In the last decade, several studies have provided evidence that pDCs actively participate in a wide spectrum of human diseases including infection, autoimmunity, and cancer. In particular, human neoplasms are populated by pDCs, whose presence is related to a poor prognosis. However, the role of tumor-associated pDCs (TApDCs) remains controversial. Various studies indicate that pDCs play an immunosuppressive role and facilitate tumor progression in both animal models and humans. In contrast, others found that the presence of activated pDCs results in tumor regression in mice. Given these findings, it is clear that pDC function plays a critical role in tumor biology. However, due to the great therapeutical success of anti-PD-1/PD-L1 inhibitors in melanoma, breast, and lung cancer, it is likely that the interference with pDC activity in the tumor microenvironment could be targeted in a more specific manner with a better clinical outcome. Nevertheless, pDC biology in cancer still needs further elucidation, especially to understand the controversial data in the literature.

We believe that understanding pDC biology represents an important necessity and will pave the road to novel therapeutic strategies to fight malignancies.

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The CD95/CD95L Signaling Pathway: A Role in Carcinogenesis

11

Amélie Fouqué and Patrick Legembre

Contents

11.1 Introduction

Apoptosis, or programmed cell death, plays a pivotal role in development, organ homeostasis, and immunosurveillance. The term apoptosis was coined by Kerr et al. in 1972 [1] to describe the process of cell death associated with morphological changes, including nucleus and cytoplasm condensation and protuberances from the plasma membrane producing apoptotic bodies, so-called blebs, which are rapidly phagocytosed [1, 2].

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Inhibition of this cellular process is observed in different pathologies, such as cancer and autoimmunity, while amplification of the apoptotic signal was reported in neurodegenerative disorders including Alzheimer's and Parkinson's diseases [3, 4], as well as infection by human immunodeficiency virus (HIV).

The origin of the apoptotic signal has been used to distinguish two main signaling pathways. The intrinsic pathway stems from accumulation of DNA damage, deregulation of mitochondrial function, or virus infection and induces the release of proapoptotic factors from the mitochondria, whereas extrinsic signals are transmitted by the binding of apoptotic ligands to death receptors present at the cell surface. Interconnections exist between these two signaling pathways: both leading to the activation of a family of cysteine proteases specific for aspartic acid residues, called caspases [5]. The apoptotic role of mitochondria is associated with reduction in its transmembrane potential and the loss of its extracellular membrane integrity, leading to the release of different apoptogenic factors in the cytosol. Among them, cytochrome c associates with the caspase-9/APAF-1 complex to form the apoptosome and trigger apoptosis [6].

These two signaling pathways share common features, and both require the aggregation of initiator caspases as their preliminary events. During interactions with respective ligands, members of the death receptor superfamily recruit adaptor proteins such as Fas-associated protein with a death domain (FADD) [7, 8] or tumor necrosis factor (TNF) receptor 1-associated death domain protein (TRADD) [9], resulting in the aggregation and activation of the initiators caspase-8 and caspase-10 to form the death-inducing signaling complex (DISC) [10]. In a similar manner, release of cytochrome c and ATP by mitochondria promotes the formation of the apoptosome with the cytosolic APAF-1, thereby aggregating and activating the initiator caspase-9, which in turn cleaves caspase-3 [11].

It should be kept in mind that death receptors CD95 [12], TNFR1 [13], DR4 [14], DR5 [15], and DR6 [16] have been cloned based on their ability to elicit apoptosis. Although studies have revealed the ability of Fas/CD95, DR4, and DR5 in triggering non-apoptotic signaling pathways even immediately after their cloning [17, 18], most, if not all, studies have been focused on characterizing the molecular events leading to cell death. Accordingly, several agonistic molecules were developed in order to kill cancer cells, neglecting the impact of non-apoptotic signals in pathophysiological contexts. More recent data changed this vision by evaluating the biological role of death receptor-mediated non-apoptotic signaling pathways in chronic inflammatory disorders and carcinogenesis.

In this chapter, apoptotic signaling pathways induced by death receptors are discussed. Moreover, recent evidences pointing to the non-apoptotic signals transmitted by the same receptors are brought up, which may imply their tremendous impact on tumor progression and the design of therapeutic tools.

11.2 TNF Receptor Family

Death receptors TNFR1, Fas, DR3, DR4, DR5, and DR6 belong to the tumor necrosis factor receptor (TNFR) superfamily. These type I transmembrane proteins share common features, such as extracellular amino-terminal cysteine-rich domains (CRDs) [19, 20], which contribute to ligand specificity [21], and pre-association of the receptor at the plasma membrane [22–24] and a conserved 80-amino acid sequence located in their cytoplasmic tail called death domain (DD), which is necessary for DISC formation and initiation of the apoptotic signal [25, 26].

11.2.1 TNFR1 Signaling Pathways

TNF-α exerts its effects by binding to two receptors, TNFR1 and TNFR2 [20]. Recently, progranulin was identified as a ligand of TNFR with a higher affinity than TNF-α. Progranulin antagonizes TNF-α signaling and plays a critical role in the pathogenesis of inflammatory arthritis in mice [27]. TNFR1, a 55 kDa protein expressed in almost all cell types, presents a DD in its

intracellular region, whereas TNFR2, a 75 kDa protein, is mainly detected in oligodendrocytes, astrocytes, T-cells, myocytes, thymocytes, endothelial cells, and human mesenchymal stem cells [28]. Uncertainty remains on the TNFR2 signaling pathway, which has been previously reviewed [28]. The CRD1 of CD95, TNFR1, and TNFR2 is involved in homotypic interactions, leading to pre-association of the receptor as a homotrimer in the absence of ligand [23, 24, 29]. This domain has been designated as the pre-ligand binding assembly domain (PLAD) [29]. Receptors of the TNFR superfamily do not possess any enzymatic activity on their own and rely on the recruitment of adaptor proteins for signaling. Among these adaptor proteins, TRADD or FADD is instrumental in the implementation of cell death processes [7–10].

TNF is synthesized as a 26 kDa transmembrane type II protein (m-TNF) of 233 amino acids [30] which can be cleaved by the metalloprotease TACE [31, 32] to release the 17 kDa soluble form of the cytokine (cl-TNF). In contrast to cl-TNF, which only activates TNFR1, m-TNF can bind and activate both TNFR1 and TNFR2 [33].

Activation of TNFR1 leads to the induction of cellular processes ranging from cell death (apoptosis or necroptosis) to cell proliferation, migration, and differentiation; the implementation of such different cellular responses reflects the formation of different molecular complexes after receptor activation [28]. Binding of TNF to TNFR1 causes the formation of two consecutive complexes. While the plasma membrane complex (complex I) elicits a non-apoptotic signaling pathway, a second, internalized complex (complex II or DISC) triggers cell death [2]. In the presence of TNF, the adaptor protein TRADD interacts with TNFR1 and recruits other proteins involved in the signaling of the receptor, such as TRAF2, cIAP1, cIAP2, and RIP1, to form complex I. At the plasma membrane, this complex activates the NF-κB signaling pathway, which in turn promotes the transcription of antiapoptotic genes such as cIAP1, cIAP2, and c-FLIP [34]. The linear ubiquitin chain assembly complex (LUBAC) is also recruited to complex I via cIAP-generated ubiquitin chains [35]. HOIL-1, HOIP, and sharpin constitute the LUBAC complex. HOIL-1 and HOIP add a linear ubiquitin chain by catalyzing the head-to-tail ligation of ubiquitin [36] to RIP1 and NEMO (IKK-γ) in complex I [37], thereby activating NF-κB.

TNF-induced caspase activation is mediated by a second, intracellular complex II, which is formed when complex I dissociates from the receptor, along with FADD and caspase-8 recruitment [2]. NF-κB activation leads to c-FLIP overexpression, preventing formation of complex II. Contrariwise, when NF-κB activation is blocked, c-FLIP, whose protein half-life is short [38], is absent, and cells experience death [2]. RIP1 is deubiquitinated by enzymes such as Cezanne [39] and CYLD [40], and the complex composed of TRADD and RIP1 moves to the cytosol to form complex II. FADD is recruited to TRADD by DD-DD interaction and binds caspase-8 [2]. Noteworthy, when caspase-8 activity is inhibited or its expression is extinguished, DISC is unable to trigger the apoptotic signaling pathway; but TNFR1 or CD95 stimulation leads to the activation of another cell death signal, namely, necroptosis [41, 42]. To prevent the induction of the necroptotic signal, caspase-8 cleaves and inactivates RIP1 and RIP3 [43]. The fine-tuned control of necroptosis by members of the apoptotic signaling pathway in the organism has been elegantly confirmed by experiments showing that the embryonic lethality of mice harboring the single KO of caspase-8 or FADD is rescued by an additional KO of the RIP3 gene [44–46].

11.2.2 TNF/TNFR: A Gold Mine for Therapeutic Tools

Many studies on TNF demonstrated its pivotal role in fueling inflammation, a multistep process that promotes autoimmunity (e.g., rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, psoriasis, and refractory asthma) and cancer. Many TNF inhibitors, such as neutralizing monoclonal antibodies (mAbs) (e.g., infliximab, adalimumab, and golimumab), have been developed to treat these chronic inflammatory disorders,

demonstrating that altering ligand/receptor interactions with neutralizing mAbs is an invaluable opportunity to treat certain chronic inflammatory disorders. Other TNF-α antagonists, such as etanercept, a TNFR2-immunoglobulin Fc fusion protein, can improve the clinical course of rheumatoid arthritis [47].

While findings accumulate to decipher the molecular mechanisms involved in the induction of apoptotic and non-apoptotic signaling pathways by TNFR1 and to elucidate how the receptor can switch from one signal to the other, the mechanistic links involved in the implementation of non-apoptotic signaling pathways by CD95 remain elusive. However, recent findings have revealed its proinflammatory effects [48–54].

11.3 CD95: A Death Receptor?

In 1989, identification of the mAb APO-1 by Peter Krammer et al. revealed the existence of a 52 kDa protein whose aggregation was able to transmit an apoptotic signal in cancer cells [55]. This receptor was identified in 1991 by Nagata and colleagues and called Fas (CD95 or APO-1) [12]. Its ligand, FasL, was cloned in 1993 by the same group and was found to be mainly expressed at the surface of activated T-lymphocytes [56] and natural killer (NK) cells [57]; however, its expression was also detected in different tissues in which the presence of acute or chronic inflammation is undesirable including the eyes [58] and testes [59]. In addition, two mouse models, in which either the level of CD95 expression was downregulated (due to an insertion of a retrotransposon in intron 2 of the receptor gene, these mice are called *lymphoproliferation* (Lpr) [60–62]) or the CD95L affinity for CD95 was reduced (due to the germ line mutation F273L in CD95L, called *generalized lymphoproliferative disease* (*gld*), which decreases CD95L binding to CD95 [63, 64]), have provided some insight into the pivotal role played by this interaction in immunosurveillance and immune tolerance [65].

11.3.1 Structure/Function

The CD95 gene (*APT-1*) consists of nine exons, with exon 6 encoding the transmembrane domain [66] (Fig. 11.1). CD95 can be resolved under denaturing conditions between 40 and 50 kDa by SDS-PAGE. The receptor is a type I transmembrane protein harboring three CRDs. Similar to the TNF receptor [29], CD95 is pre-associated at the plasma membrane as a homotrimer, and this quaternary structure is mandatory for transmission of the apoptotic signals in the presence of CD95L [23, 24]. Homotrimerization of CD95 occurs mainly through homotypic interactions of the CD95-CRD1 [22–24]. Binding of CD95L or agonistic anti-CD95 mAbs to CD95 alters both the conformation and the extent to which the receptor is multimerized at the plasma membrane. The intracellular region of CD95 encompasses an 80-amino acid stretch designated as the DD (Fig. 11.1), which consists of six antiparallel α-helices [67]. Upon addition of CD95L, CD95 undergoes conformational modification of its DD, which induces a shift of helix 6 and fusion with helix 5, promoting both oligomerization of the receptor and recruitment of the adaptor protein FADD [68]. A consequence of the opening of the globular structure of CD95 is that the receptor becomes connected through this bridge, which

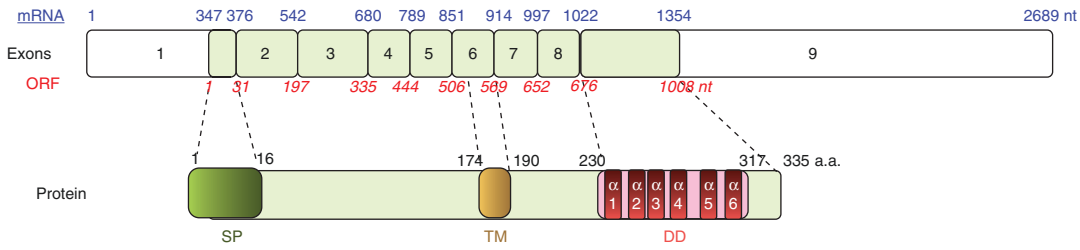

Fig. 11.1 CD95: mRNA to protein

increases the magnitude of its homo-aggregation. This long helix allows the stabilization of the complex by recruiting FADD. Overall, the CD95-DD/FADD-DD crystal structure provides some insights into the formation of the large CD95 clusters observed using imaging or biochemical methods in cells stimulated with CD95L. In addition, it also confirms that alteration in the CD95 conformation plays an instrumental role during signal induction [68]. However, this elongated C-terminal α-helix favoring the *cis*-dimerization of CD95-DD was challenged by Driscoll et al. who did not observe the fusion of the last two helices at a more neutral pH (pH 6.2), compared to the acidic condition (pH 4) used in the initial study to resolve the CD95-DD/FADD-DD structure [68]. Consequently, at pH 6.2, association of CD95 with FADD predominantly consisted of a 5:5 complex, which occurred via a polymerization mechanism involving three types of asymmetric interactions but without major alteration of the DD globular structure [69, 70]. It is likely that the low pH condition used in the study performed by Scott et al. altered CD95 conformation and resulted in the formation of nonphysiological CD95/FADD oligomers [68]. Nonetheless, it cannot be excluded that a local decrease in the intracellular pH affects the initial steps of the CD95 signaling pathway in vivo, through promoting the opening of the CD95-DD and eventually contributing to the formation of a complex eliciting a sequence of events different from the one occurring at physiologic pH.

Once docked on CD95-DD, FADD self-associates [71] and binds procaspase-8 and procaspase-10, which are auto-processed and released in the cytosol as active caspases, which cleave many substrates leading to the execution of the apoptotic program and cell death. The complex CD95/FADD/caspase-8/caspase-10 is called DISC (Fig. 11.2) [10]. Due to the importance of DISC formation in

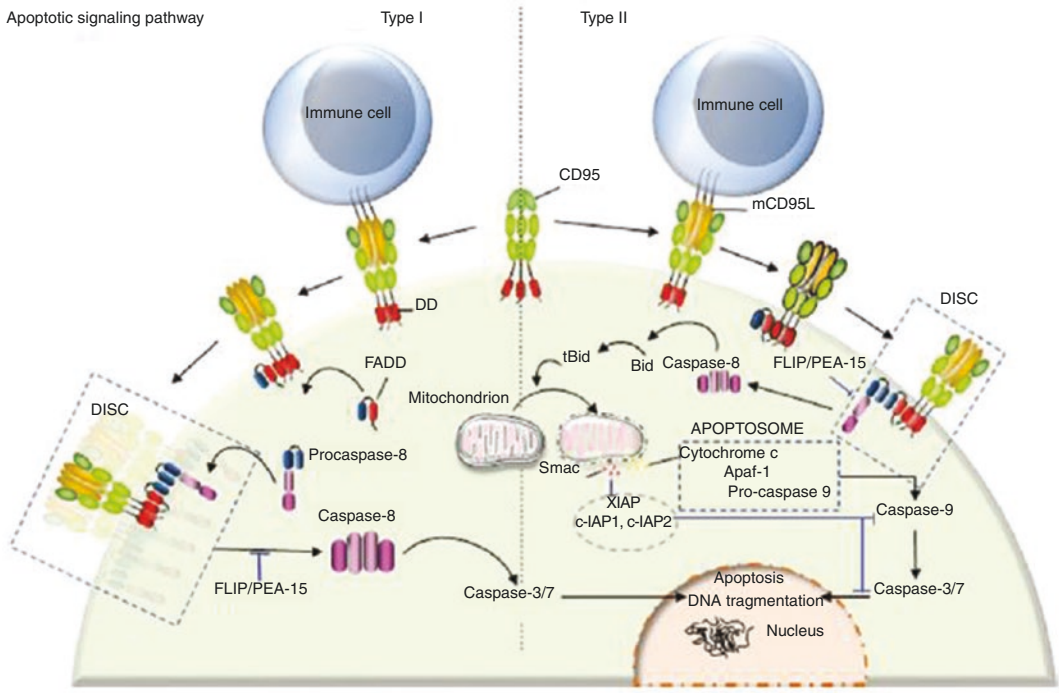

Fig. 11.2 Type I/II cells. Binding of transmembrane CD95L to CD95 leads to DISC formation. DISC consists of FADD and procaspase-8. c-FLIP and PEA-15 bind to FADD and prevent caspase-8 recruitment. At the DISC level, aggregation of procaspase-8 promotes its auto-cleavage and activation. Cleaved caspase-8 is then released in the cytosol where it promotes the cascade of caspase activation leading to apoptosis. Type I cells are characterized by an efficient DISC formation, which releases sufficient caspase-8 to directly activate caspase-3. By contrast, type II cells present a weak DISC formation, and the low amount of released caspase-8 activates the mitochondrion-dependent apoptotic pathway to amplify death signal

the fate of cells, it is not surprising that numerous cellular and viral proteins were reported to hamper the formation of this structure, such as FLIP [72, 73] and PED/PEA-15 [74], which interfere with the recruitment of caspase-8/caspase-10 (Fig. 11.2).

11.3.2 Type I/II Signaling Pathways

Following the discovery of CD95 and the first steps of its signaling pathway, Peter and colleagues described that cells can be divided in two groups with regard to the kinetics through which they respond to CD95-mediated apoptotic signals, the magnitude of DISC formation, and the role played by the mitochondrion in this pathway [75]. DISC formation occurs rapidly and efficiently in type I cells releasing a large amount of activated caspase-8 in the cytosol, while type II cells have difficulty forming this complex, and the amount of active caspase-8 is insufficient to directly activate the effectors caspase-3 and caspase-7 [75]. Nonetheless, type II cells experience cell death upon CD95 engagement and are even more sensitive to the CD95-mediated apoptotic signal compared to type I cells [75–77]. This discrepancy can be partly explained by the fact that the low amount of activated caspase-8 in type II cells is sufficient to cleave BID, a BH3-only protein, which constitutes the molecular link between caspase-8 activation and the apoptotic activity of mitochondria. Indeed, after cleavage by caspase-8, truncated BID (tBID) translocates to mitochondria, where it triggers the release of proapoptotic factors (Fig. 11.2) [78, 79]. Although CD95 stimulation activates the mitochondrion-dependent apoptotic signal in type I and type II cells, it seems that only type II cells are addicted to this signal as they display a higher amount of the caspase-3 inhibitor XIAP compared to type I cells [80]. Among the inhibitor of apoptosis protein (IAP) family, XIAP, cIAP1, and cIAP2 inhibit caspase-3, caspase-7 [81, 82], and procaspase-9 [83] activity by direct binding, thereby preventing access to substrates. Furthermore, XIAP can function as an E3 ligase whose activity is involved in the ubiquitination of active caspase-3 and its subsequent degradation through the proteasome [84]. To detach XIAP from caspase-3 and restore the apoptotic signal, cells require the release of SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI) by the mitochondrion [85, 86], explaining why type II cells are more addicted to this organelle compared to type I cells (Fig. 11.2).

To summarize, DISC formation and IAP amount are two cellular markers allowing a clear discrimination between type I and type II cells. Even though IAP overexpression can account for the mitochondrion dependency observed in type II cells, it remains unclear why DISC formation is hampered in type II cells and/or enhanced in their type I counterparts. Recently, high activity of the lipid kinase phosphoinositide 3-kinase (PI3K) or downregulation of its neutralizing phosphatase, phosphatase and tensin homologue on chromosome 10 (PTEN), was found in type II cells, while this signal is blocked in type I cell lines [87, 88]. The PI3K signaling pathway was reported to prevent the aggregation of CD95 [89], probably by retaining the receptor outside of lipid rafts [87, 90]. PEA-15, also known as PED, is a protein containing a death effector domain (DED) that has been shown to inhibit the CD95 and TNFR1 apoptotic signals (Fig. 11.2) [74]. Activation of PI3K and its downstream effector, serine-threonine kinase Akt, leads to phosphorylation of PEA-15 at serine 116 [87, 90]; this posttranslational modification promotes its interaction with FADD, ultimately inhibiting DISC formation [91, 92].

Notably, the existence of type I and type II cells is not only an in vitro observation, but has been identified physiologically in the human body. CD95-mediated apoptotic signal cannot be altered in thymocytes or activated T-cells expressing a Bcl-2 transgene, conferring to their type I nature [93], whereas hepatocytes expressing the same transgene resist CD95-induced apoptosis and thus behave as type II cells [94, 95].

11.3.3 What Can We Learn from CD95 Mutations?

Germinal mutations in *APT-1* have been reported in patients developing a syndrome termed autoimmune lymphoproliferative syndrome type Ia (ALPS, also called Canale-Smith syndrome) [96–98]. ALPS patients show chronic lymphadenopathy and splenomegaly, expanded populations of double-negative α/β-T-lymphocytes ($CD3^+CD4^-CD8^-$), and often develop autoimmunity [96, 97, 99, 100]. In agreement with the notion that CD95 behaves as a tumor suppressor, ALPS patients display an increased risk of Hodgkin and non-Hodgkin lymphoma [101]. Predominance of post-germinal center (GC) lymphomas in patients exhibiting either germ line or somatic CD95 mutations can be explained by the fact that, inside germinal centers of the secondary lymphoid follicles, the CD95 signal plays a pivotal role in the deletion of self-reactive maturating B-lymphocytes [102], in addition to the fact that *APT-1* belongs to a set of rare genes (i.e., PIM1, c-myc, PAX5, RhoH/TTF, and Bcl-6) subject to somatic hypermutation [103, 104], which may affect biological function. In addition to post-GC lymphomas, significant amounts of mutations in the CD95 gene were found in tumors of various histological origins (reviewed in [54]). Extensive analysis of CD95 mutations and their distribution in *APT-1* reveals that, with some exceptions, most are gathered in exons 8 and 9 encoding the CD95 intracellular region (Fig. 11.3) [105]. Remarkably, most of these mutations are heterozygous, mainly localized in CD95-DD, and lead to inhibition of the CD95-mediated apoptotic signal. Indeed, in agreement with the notion that CD95 is expressed at the plasma membrane as

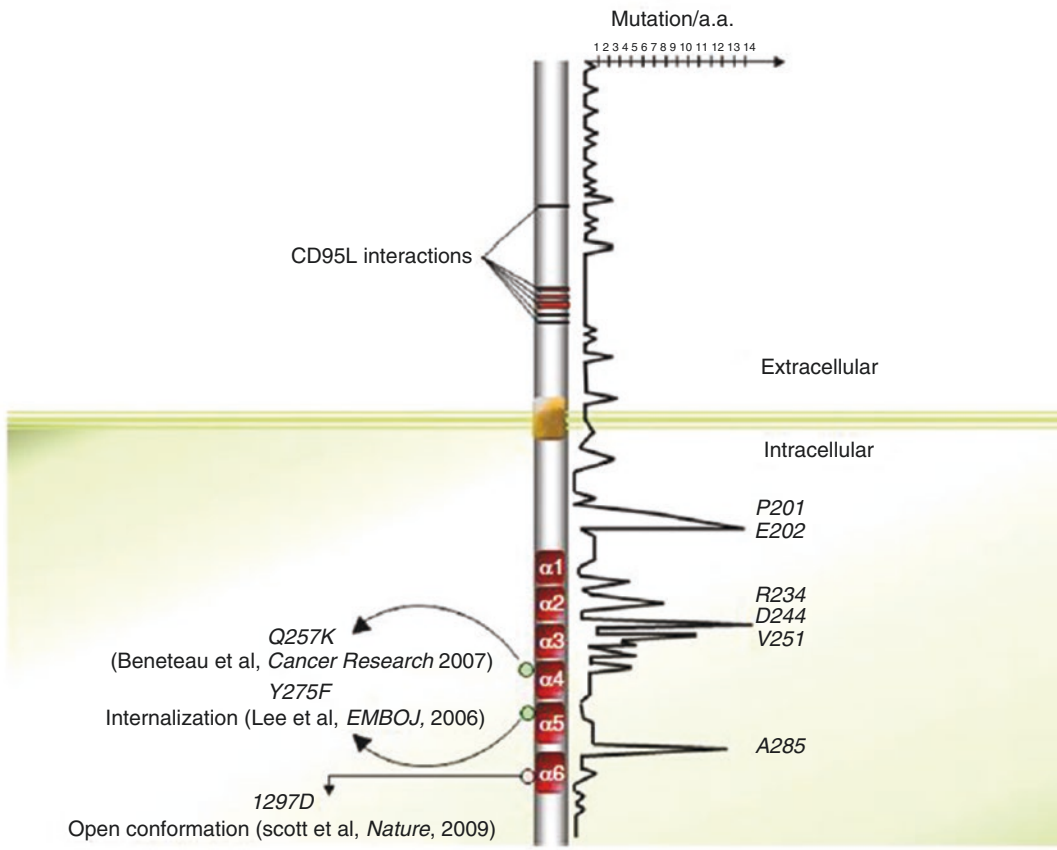

Fig. 11.3 Distribution of somatic and germinal mutations within CD95 protein sequence

a pre-associated homotrimer [23, 24], formation of heterocomplexes containing wild-type and mutated CD95 prevents FADD recruitment and abrogates the ignition of the apoptotic signal in a dominant manner.

Extensive analysis and positioning of various CD95 mutations described in the literature seem to highlight mutation "hot spots" in the CD95 sequence (Fig. 11.3). Among these hot spots, arginine 234, aspartic acid 244, and valine 251 account for a significant amount of the documented CD95 mutations. Indeed, among the 189 mutations annotated in the 335 amino acids of CD95, 30 (~16%) are localized on these three amino acids (Fig. 11.3). Strikingly, the pivotal role played by these amino acids in stabilization or formation of intra- and inter-bridges between CD95 and FADD may explain these hot spots. For instance, both R234 and D244 contribute to the homotypic aggregation of the receptor and FADD recruitment [67]. Nevertheless, the observation of death domain hot spots is in contradiction with the study of Scott and colleagues demonstrating that the region of the CD95-DD interacting with the FADD-DD extends over a disperse surface through weak binding affinity [68].

Most ALPS type Ia patients affected by malignancies do not undergo loss of heterozygosity (LOH), which formed the hypothesis that preservation of a wild-type allele may contribute to carcinogenesis [106, 107]. In the same line, it was demonstrated that expression of a unique mutated CD95 allele blocks the induction of apoptotic signals, while it fails to prevent non-apoptotic signals such as NF-κB and MAPK [106, 107], whose induction promotes invasiveness in tumor cells [105, 108]. In addition, mutations found in the intracellular CD95-DD exhibit a higher penetrance of ALPS phenotype features in mutation-bearing relatives compared to extracellular mutations. These results suggest that unlike DD mutations, CD95 mutations localized outside the DD somehow prevent the apoptotic signal but may fail to promote non-apoptotic pathways, which may contribute to disease aggressiveness.

11.3.4 Regulation of the Initial Steps of CD95-Mediated Signaling

11.3.4.1 Lipid Rafts

In addition to CD95 downregulation or expression of the mutated allele of the receptor, the plasma membrane distribution of CD95 represents an additional pathway for tumor cells to develop resistance to CD95L-expressing immune cells. Indeed, the plasma membrane is a heterogeneous lipid bilayer comprising compacted or liquid-ordered domains, called microdomains, lipid rafts, or detergent-resistant microdomains (DRMs). These domains are described as floating in a more fluid or liquid-disordered 2D lipid bilayer and are enriched in ceramides [109]. It has been elegantly shown that while CD95 is mostly excluded from lipid rafts in activated T-lymphocytes, TCR-dependent reactivation of these cells leads to rapid distribution of the death receptor into lipid rafts [110]. This CD95 compartmentalization contributes to reducing the apoptotic threshold leading to the clonotypic elimination of activated T-lymphocytes through activation of the CD95-mediated apoptotic signal [110]. Similarly, the reorganization of CD95 into DRMs can occur independent from ligand upon addition of certain chemotherapeutic drugs (e.g., rituximab [111], resveratrol [112, 113], edelfosine [87, 114, 115], aplidin [116], perifosine [115], cisplatin [117]). The molecular cascades that underlie this process remain elusive. Nevertheless, a growing body of evidence leads us to postulate that alteration of intracellular signaling pathway(s), such as the aforementioned PI3K signal [87, 90], may change biophysical properties of the plasma membrane, such as membrane fluidity, which in turn may facilitate CD95 clustering into large lipid raft-enriched platforms, favoring DISC formation and induction of the apoptotic program [118].

11.3.4.2 Posttranslational Modifications

Accumulation of CD95 mutations is not the only mechanism by which malignant cells inhibit the extrinsic signaling pathway. Posttranslational

modifications in the intracellular tail of CD95, such as reversible oxidation or covalent attachment of a palmitic acid, were reported to alter the plasma membrane distribution of CD95 and thereby its subsequent signaling pathway. For instance, S-glutathionylation of mouse CD95 at cysteine 294 promotes clustering of CD95 and its distribution into lipid rafts [119]. This amino acid is conserved in the human CD95 sequence and corresponds to cysteine 304 (or C288 when subtraction of the 16-amino acid signal peptide is taken into consideration [12, 120]). Interestingly, Janssen-Heininger and colleagues emphasize that death receptor glutathionylation occurs downstream of caspase-8 and caspase-3 activation whose catalytic activity damages the thiol-transferase glutaredoxin 1 (Grx1), an enzyme implicated in the denitrosylation of proteins [119]. The consequence of Grx1 inactivation is the accumulation of glutathionylated CD95, which clusters into lipid rafts, sensitizing cells to the CD95-mediated apoptotic signal. Based on these findings, caspase-8 activation occurs prior to aggregation of CD95 and redistribution into lipid rafts, both of which are requisite to form the DISC and subsequently activate larger amounts of caspase-8. In agreement with these observations, activation of caspase-8 was reported to occur in a two-step process. That is, an immediate and small amount of activated caspase-8 (<1%) is generated when CD95L interacts with CD95 that orchestrates acid sphingomyelinase (ASM) activation, ceramide production, and CD95 clustering, which in turn promote DISC formation and the outburst of caspase-8 processing essential to mount the apoptotic signal [121].

S-Glutathionylation consists in a bond between a reactive Cys-thiol and reduced glutathione (GSH), a tripeptide consisting of glycine, cysteine, and glutamate; its attachment to the protein will alter its structure and function in a manner similar to the addition of a phosphate [122]. S-Glutathionylation is not the only post-translational modification of CD95 on a cysteine. S-Nitrosylation of cysteine 199 (corresponding to C183 after subtraction of signal peptide sequence) and 304 (C288) in colon and breast tumor cells also promotes the redistribution of CD95 into DRMs, the formation of the DISC, and the transmission of the apoptotic signal [123].

Two reports have brought into light that covalent coupling of a 16-carbon fatty acid (palmitic acid) to cysteine 199 (C183) elicits the redistribution of CD95 into DRMs, the formation of SDS-stable CD95 microaggregates resistant to denaturing and reducing treatments, and the internalization of the receptor [124, 125]. Although their order remains to be fine-tuned, these molecular steps play a critical role in the implementation of apoptotic signals.

Of note, similar to S-nitrosylation, both the aforementioned S-glutathionylation at C304 (C288) and palmitoylation at C199 (C183) promote the partition of CD95 into lipid rafts and enhance the subsequent apoptotic signal. Further investigation is required to address whether these posttranslational modifications are redundant and occur simultaneously in dying cells or are elicited in a cell-specific and/or in a microenvironment-specific manner. Understanding the molecular mechanisms controlling these posttranslational modifications would be of great interest in order to identify the mechanism by which tumor cells block them, leading to their resistance to the extrinsic signaling pathway.

11.3.4.3 CD95 Internalization

Using a powerful magnetic method to isolate receptor-containing endocytic vesicles, it has been shown that CD95 promptly associates with endosomal and lysosomal markers when incubated with an agonistic anti-CD95 mAb [126]. In addition, expression of a CD95 mutant in which the DD-located tyrosine 291 (Y275) is changed to phenylalanine does not seem to alter the capacity to bind FADD but compromises CD95L-mediated CD95 internalization occurring through an AP2/clathrin-driven endocytic pathway [126]. More strikingly, expression of the internalization-defective CD95 mutant Y291F abrogates the transmission of apoptotic signals, but fails to alter the non-apoptotic signaling pathways (i.e., NF-κB and ERK), and even promotes them (Fig. 11.3). These findings provide insight into the presence of a region in the DD, interacting with AP2 and promoting a clathrin-dependent

endocytic pathway in a FADD-independent manner. Regarding the role of palmitoylation in receptor internalization, the interplay between lipid alteration and the AP2/clathrin-driven internalization of CD95 remains to be elucidated.

11.3.4.4 Ca^{2+} Response

It has been recently demonstrated that CD95 engagement evokes a rapid and transient Ca^{2+} signaling, which stimulates the recruitment of protein kinase C-β2 (PKC-β2) from the cytosol to the DISC [127]. This kinase transiently brakes DISC formation, providing a checkpoint before the irreversible commitment to cell death [128]. These findings raised the following questions: what are the Ca^{2+}-dependent molecular mechanisms transiently inhibiting DISC formation, and do tumor cells use this signal to escape the immune response and/or resist chemotherapy?

11.3.5 Programmed Necrosis also known as Necroptosis

In 1998, inhibition of caspase activity was shown to sensitize fibroblastic L929 cell line to TNF-mediated necrotic cell death [42]. With respect to CD95 signal, Tschopp et al. showed that FADD and RIP1 participate in the implementation of a non-apoptotic signaling pathway, which leads to a necrotic morphology without chromatin condensation and with loss of plasma membrane integrity [41]. Of note, BID cleavage was not observed in this necrotic signal. While FADD plays a crucial role in both apoptotic and necrotic pathways, RIP1 recruitment to CD95 occurs independently of this adaptor protein. Indeed, yeast two-hybrid experiments showed that RIP1 can bind directly to the CD95-DD, while this interaction is lost when a bait corresponding to mutated CD95-DD (replacement of Val 238 to Asn) is used [129]. In addition, RIP3 (RIPK3, a member of the RIP kinase family) is an indispensable factor for the induction of the necrotic signaling pathway [78–80]. A growing body of evidence supports the existence of necroptosis (programmed necrosis). In addition, identification of necrostatin, a chemical inhibitor of necroptosis [130], which specifically inhibits RIP1 kinase activity [131], has accelerated the pace of discovery in this field of cell death. Interplays exist between apoptosis and necroptosis; for instance, caspase-8, a potent inhibitor of necroptosis for both CD95 and TNFR1 [132], plays a critical role in necroptosis by its ability to process and inactivate RIP1 and RIP3 [133, 134]. At least for TNF signaling, the necrotic signal relies on the activity of CYLD, a deubiquitinating enzyme that is also cleaved and inactivated by caspase-8 [135].

Overall, these findings suggest that the apoptotic machinery controls the necrotic one. This concept has been recently established in vivo by double-KO experiments [44–46, 136]. The KO of FADD or caspase-8 is deleterious in mice mainly by the fact that these two apoptotic factors are beneficial in inhibiting a RIP1-/RIP3-dependent necrotic signal; thus, their loss unleashes the necroptotic program and leads to embryonic lethality. Yet, most studies on necroptosis have focused on the TNF signaling pathway, whereas the mechanism by which CD95 can elicit this cell death pathway, and how the switch in this receptor occurs between non-apoptotic, apoptotic, and necroptotic signals remains unclear. Importantly, the impact of each cell death on antigen presentation, and on the efficiency of immune response after elimination of infected or transformed cells, remains unclear.

11.3.6 CD95L, an Inflammatory/Oncogenic Cytokine?

11.3.6.1 A Ligand to Create Immune Privileges

The transmembrane CD95L (CD178/FasL) is present at the surface of activated lymphocytes [64] and NK cells [137] where it orchestrates the elimination of transformed and infected cells. In addition, CD95L is expressed on the surface of neurons [138], corneal epithelia and endothelia [58, 139], and Sertoli cells [59] to prevent the infiltration of immune cells and thus to prohibit the spread of inflammation in these sensitive organs (i.e., brain, eyes, and testis, respectively), commonly called “immune-privileged” sites.

The description of physiological immune privilege was followed by tumor-mediated immune privilege, since two groups reported that the ectopic expression of CD95L by malignant cells participated in the elimination of infiltrating T-lymphocytes and thus could play a role in the establishment of a tumor site whose access was denied to immune cells [140, 141]. However, these observations are controversial since ectopic expression of CD95L in allogenic transplant of β-islets [142, 143] and in tumor cell lines [144] led to a more rapid elimination of these cells than control cells, due to increased infiltration of neutrophils and macrophages endowed with antitumor activity.

11.3.6.2 At Least Two Different Ligands and Two Different Signals

Among the weapons at the disposal of immune cells, transmembrane CD95L contributes to the elimination of pre-tumor cells. Therefore, pre-tumor cells that escape the immunosurveillance will be shaped to develop resistance to CD95, a process termed immunoediting [145]. In other words, imprinting of the immune system on pre-tumor cells will select malignant cells with increased resistance toward the CD95L-induced signal. As previously mentioned, these alterations of the CD95 signal not only block the CD95-mediated apoptotic signal but also promote the transmission of non-apoptotic signals by CD95L, which may play a critical role in carcinogenesis [106–108, 146]. In agreement with this hypothesis, a complete loss of CD95 expression is rarely observed in malignant cells [147].

Accumulating evidence indicates that the apoptotic ligand CD95L behaves as a chemoattractant for neutrophils, macrophages [50, 143, 144], T-lymphocytes [53], and malignant cells in which the CD95-mediated apoptotic signal is nonproductive [108, 148]. Nonetheless, the biological role of CD95L has to be clarified due to the fact that pathophysiologically the ligand is present in at least two forms with different stoichiometries. Indeed, CD95L is a transmembrane cytokine whose ectodomain can be cleaved by metalloproteases such as MMP3 [149], MMP7 [150], MMP9 [151], and ADAM-10 (a disintegrin and metalloproteinase 10) [152, 153] and released as a soluble ligand in the bloodstream. Based on the data demonstrating that a hexameric CD95L represents the minimal level of self-association required to signal apoptosis [154] and that cleavage by metalloproteases releases an homotrimeric ligand [154, 155], this soluble ligand has long been considered as an inert ligand competing with its membrane-bound counterpart for CD95 binding, thus acting as an antagonist of the death signal [155, 156]. It has been recently demonstrated that this metalloprotease-cleaved CD95L (cl-CD95L) actively participates in the aggravation of inflammation and autoimmunity in patients affected by systemic lupus erythematosus (SLE) by inducing the non-apoptotic NF-κB and PI3K [51, 53] signaling pathways (Fig. 11.4). Unlike transmembrane CD95L, induction of the PI3K signaling pathway by its metalloprotease-cleaved counterpart occurs through the formation of a complex devoid of FADD and caspase-8 which recruits the src kinase c-yes instead [53, 148]; this unconventional receptosome was designated motility-inducing signaling complex (MISC) [53, 157] (Fig. 11.4). Even though experiments by the authors did not detect any trace of caspase-8 in the MISC, this enzyme has been shown to participate in cell migration. The protease activity of caspase-8 can be abolished by its phosphorylation at tyrosine 380 by src kinase [158]. This posttranslational modification was observed in cells stimulated with EGF and in colon cancer cells exhibiting constitutive activation of src; from a molecular standpoint, this modification does not alter caspase homodimerization or recruitment in DISC [158]. Moreover, the EGFR-driven phosphorylation of caspase-8 at Y380 turns out to be a potent inducer of the PI3K signaling pathway by recruiting the PI3K adaptor p85 alpha subunit [159]. Ultimately, caspase-8 phosphorylation triggers cell migration. Nonetheless, it is noteworthy that CD95-induced migration and invasion do not appear to require an intact DD (reviewed in [160]), suggesting that either the caspase-8-dependent mode of cell migration occurs as an alternative signal for death receptors or that it only participates in non-death

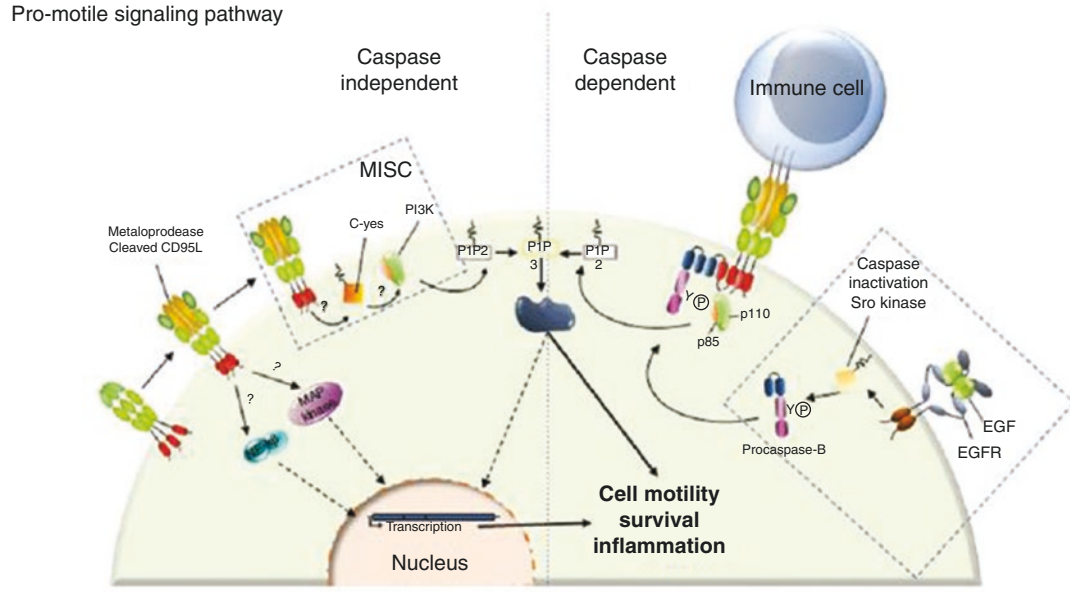

Fig. 11.4 CD95 triggers an unconventional PI3K signaling pathway. *Left panel*: In the presence of cl-CD95L, CD95 triggers MISC formation. This complex is devoid of FADD and caspase-8, but, instead, recruits the src kinase c-yes that implements the PI3K signaling pathway. CD95 engagement is also capable of NF-κB and MAPK activations through a yet unknown mechanism. *Right panel*: It was reported that procaspase-8 can be phosphorylated by the tyrosine kinase src upon EGFR stimulation. This posttranslational modification not only blocks the catalytic activity of caspase-8 but also promotes the recruitment of the p85 subunit of PI3K. We surmise that this caspase-8 phosphorylation may favor the non-apoptotic signals induced by CD95

receptor-induced cell motility. It would be interesting to address this question in the future. To date, it can only be surmised that phosphorylation of caspase-8 at Y380 upon EGFR stimulation may prime certain cancer cells to become unresponsive to the apoptotic signal triggered by cytotoxic CD95L and meanwhile promote cell migration, an essential event in the course of cancer cell metastasis (Fig. 11.4).

It is noteworthy that in a similar manner, a decrease in the plasma membrane level of CD95 or expression of a mutated CD95 allele, as observed in ALPS patients and malignant cells, inhibits the implementation of the apoptotic signal but does not affect the transmission of non-apoptotic signals, such as NF-κB, MAPK, and PI3K [106, 107, 147], suggesting that these signals may stem from a different domain than CD95-DD or rely on different thresholds to be elicited. In summary, although the CD95/CD95L interaction can eliminate malignant cells by implementation of the DISC or can promote carcinogenesis by sustaining inflammation and/or by inducing metastatic dissemination [50, 51, 53, 108, 147, 148, 161], the molecular mechanisms underlying the switch between these different signaling pathways remain enigmatic. An important question to be addressed is how the magnitude of CD95 aggregation controls the formation of "death"- vs. "motility"-ISCs. Addressing these questions will lead to the development of new therapeutic agents with the ability to contain the spread of inflammation or impede carcinogenesis at least in pathologies involving increased soluble CD95L such as cancers (e.g., pancreatic cancer [162], large granular lymphocytic leukemia, breast cancer [157], and NK cell lymphoma [163]) or autoimmune disorders (e.g., rheumatoid arthritis and osteoarthritis [164], graft-versus-host-disease (GVHD) [165, 166], or SLE [53, 167]). Altogether, these studies support the notion that the death function of CD95 may correspond to its "day job," while the receptor may act as "a night killer"

by fueling inflammation in certain pathophysiological contexts.

Strikingly, while the soluble form of CD95L generated by MMP7 (cleavage site inside the ^{113}ELR^{115} sequence, Fig. 11.5) induces apoptosis [150], its counterpart processed between serine 126 and leucine 127 does not [51, 53, 155]. To explain this discrepancy, one may speculate that the different quaternary structures of the naturally processed CD95L underlie the implementation of "death"- vs. "non-death"-inducing signaling complexes and downstream signals. In agreement with this notion, soluble CD95L bathed in the bronchoalveolar lavage (BALs) of patients suffering from acute respiratory distress syndrome (ARDS) undergoes oxidation at methionines 224 and 225 (Fig. 11.5), which enhances the aggregation level of the soluble ligand followed by its cytotoxic activity [168]. The same authors observed that the stalk region of CD95L, corresponding to amino acids 103–136 and encompassing the metalloprotease cleavage sites (Fig. 11.5), participates in the multimerization of CD95L, which accounts for the damage of the lung epithelium in ARDS [168]. Of note, in ARDS BALs, additional oxidation occurs at methionine 121 (Fig. 11.5), which in turn prevents the processing of CD95L by MMP7, and explains why this cytotoxic ligand keeps its stalk region [168]. Nonetheless, preservation of this region in soluble CD95L raises the question that whether an unidentified MMP7-independent cleavage site exists in the juxtamembrane region of CD95L, near the plasma membrane, or the ligand detected in ARDS patients corresponds to the full-length CD95L embedded in exosomes [169, 170]. Indeed, this peculiar exosome-bound CD95L can be expressed by human prostate cancer cells (i.e., LNCaP) and evokes apoptosis in activated T-lymphocytes [171].

Overall, these findings emphasize that it will be of great interest in the future to finely characterize the quaternary structure of the naturally processed CD95L from the sera of patients affected by cancers or chronic/acute inflammatory disorders, to better understand the molecular mechanisms implemented by this ligand and thus predict its subsequent biological functions.

11.4 Concluding Remarks

Apoptosis is a fundamental process contributing to tissue homeostasis, immune response, and development. CD95, also called Fas, is a member of the tumor necrosis factor receptor (TNFR) superfamily. Its ligand, CD95L, was initially detected at the plasma membrane of activated T-lymphocytes and natural killer (NK) cells where it contributes to the elimination of transformed and infected cells. Given its implication in immune homeostasis and immune surveillance combined with the fact that

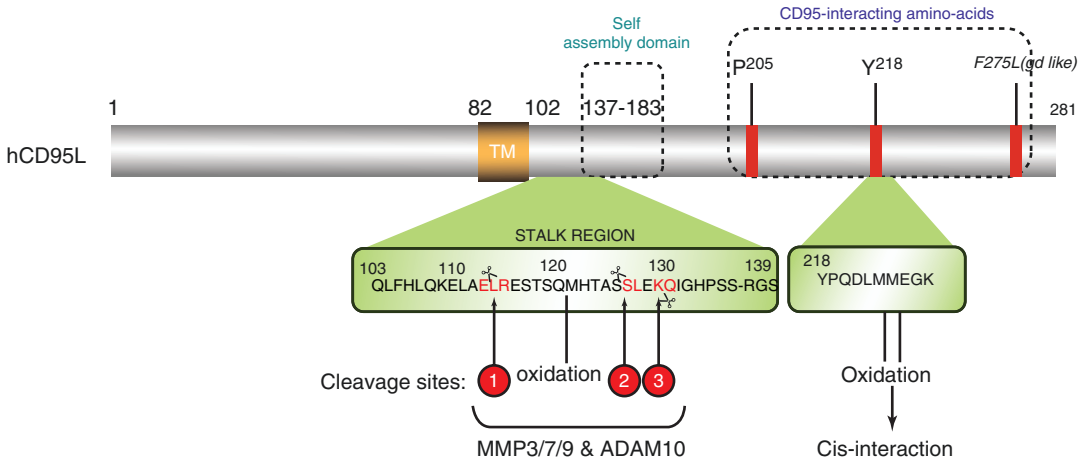

Fig. 11.5 CD95L: metalloprotease cleavage sites and domains

various lineages of malignant cells exhibit loss-of-function mutations, CD95 was initially classified as a tumor suppressor gene. Nonetheless, in different pathophysiological contexts, this receptor is able to transmit non-apoptotic signals and promote inflammation and carcinogenesis. Although the different non-apoptotic signaling pathways (NF-κB, MAPK, and PI3K) triggered by CD95 are known, the initial molecular events leading to these signals, the mechanisms by which the receptor switches from an apoptotic function to an inflammatory role, and, more importantly, the biological functions of these signals remain elusive.

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12 MHC Class I Molecules and Cancer Progression: Lessons Learned from Preclinical Mouse Models

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Contents

12.1 Introduction

The major histocompatibility complex (MHC) is composed of a set of molecules that play a pivotal role in the immune response against different pathogens and tumor cells. These molecules were described in mice for the first time by Gorer while performing transplantation studies with tumor cell lines injected in inbred strains of mice [1]. In the middle of the 1950s, Jean Dausset described the HLA system in humans which is equivalent to the mouse H-2 complex [2]. MHC class I (MHC-I)

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molecules comprise the classical (class Ia) HLA-A, HLA-B, and HLA-C antigens in humans and H-2 K, H-2 D, and H-2 L in mice and the nonclassical (class Ib) HLA-E, HLA-F, and HLA-G in humans and Qa and Tla antigens in mice [3]. Their structure is quite similar in humans and mice, forming a trimolecular complex consisting of a 45 kDa highly polymorphic heavy chain, a peptide antigen, and the nonpolymorphic 12 kDa β_2-microglobulin (β2m) light chain [4]. HLA/H-2 class I molecules are expressed on the surface of nucleated cells [5]. It is estimated that there are up to 250,000 of each MHC-I molecule on the surface of a somatic cell [6].

MHC-I molecules bind antigens in the form of peptides, generated from endogenous proteins, present on the cell surface to $CD8^+$ T-cells. In tumor cells, MHC-I molecules present tumor-associated antigens (TAAs) to cytotoxic T-lymphocytes (CTLs) activating cell proliferation, cytokine production, and target cell lysis. These TAAs are generated from degraded foreign endogenous proteins by the antigen presentation machinery (APM). This process is carried out by a large number of proteins and accessory molecules [7–9]. Correct functioning of these APM components gives rise to cells with normal surface expression of the MHC-I molecules [10, 11]. Any defect in these processes will lead to nonexpression of MHC-I molecules on the cell surface. These MHC-I-deficient tumor cells might be recognized by natural killer (NK) cells [12].

In this chapter, we will focus on analyzing the role of MHC-I antigens in cancer immunosurveillance in murine tumor models without obviating the great contributions done in human tumor models; the authors' laboratory is the reference to the findings described.

12.2 MHC-I Cell Surface Expression on Tumor Cells and Primary Tumor Growth

For over 30 years, our group of investigators has worked on human and mouse preclinical tumor models in an attempt to define the mechanisms through which tumor cells evade the immune system. We have found that tumor cells develop sophisticated molecular and biological mechanisms which allow them to escape immunosurveillance. Among the mechanisms studied, MHC alteration is one of the most important and frequent mechanisms, possibly playing a relevant role in the tumor-host scenario [13–15]. Any alteration affecting the surface expression of MHC-I molecules, the expression and function of APM components, and the expression of MHC-I heavy chains or β2m in tumor cells will have a profound effect on the recognition and killing of those tumor cells by T-lymphocytes [16, 17]. In this context, a new phase has been proposed into the tumor evolution, called the *immunoblindness* phase, which comes after the three phases of the immunoediting process [18]. During this phase, CTLs lose control over tumor cells, since losing MHC-I surface expression makes them invisible.

Our research group has a long and well-established history identifying and defining the HLA class I altered phenotypes present in human tumors. In fact, the data accumulated indicate that alterations in HLA class I expression are commonly found in most human tumors [19, 20]. Seven different altered HLA class I phenotypes have been defined in a large variety of human tumors, and the molecular mechanisms that have been found to underlie these alterations in MHC-I expression are multiple [21]. These defects can occur at any step required for MHC synthesis, assembly, transport, or expression on the cell surface. Only some of these defects can be recovered by cytokines or other agents, while others remain unrecovered. Thus, MHC alterations can be classified into two main groups: reversible defects (regulatory or soft) and irreversible defects (structural or hard) [22, 23].

Many studies in human and experimental tumors have reported variations in MHC-I antigen cell surface expression [24–27]. These variations have been associated with important changes in tumor behavior and metastatic colonization [28, 29]. The crucial role of MHC-I in local tumor growth and metastasis has also been demonstrated in many different murine tumor models. The first detection of MHC-I lack in mouse tumors was described in 1976; loss of

one H-2 K^k private specificity was reported in Gardener lymphoma derived from a C3H mouse [25]. Following these studies, different groups reported altered expression of MHC molecules in other tumors, i.e., the absence of some H-2^d molecules in a methylcholanthrene-induced sarcoma (MCG4) in a BALB/c mouse [30], loss of K^k antigen (Ag) expression in a particular AKR tumor cell line designated K36.16 (this tumor cell line showed resistance to killing by AKR anti-MuLV CTLs in vitro) [31], loss of the products of the H-2 L^d locus in a BALB/c fibrosarcoma [32], and absence of H-2 Ds Ags in SJL/J lymphomas [33].

Another field in the study of MHC-I Ags in murine tumors originates from transfection of MHC-I molecules in MHC-I-deficient murine tumors. The transfection and cell surface expression of one *H-2*^{k} gene product in the AKR lymphoma cell line K36.16, a subline of K36 (H-2 K^k-negative) lymphoma, inhibited the syngeneic growth of this tumor [34, 35]. Studies with the methylcholanthrene (MCA)-induced T10 sarcoma demonstrated that the transfection of K^k or K^b gene into H-2 K-negative parental cells reduced tumorigenicity and abolished the formation of metastasis in syngeneic mice [36]. Similar results were obtained in other experimental models [37]. In all these studies, absence of MHC-I molecules has been interpreted as a factor which selects immunodeficient variants and represents a major escape mechanism from T-cell recognition. The reconstitution of H-2 class I expression has demonstrated that even MHC-I molecules on tumor cells are responsible for regulation of NK susceptibility. Restoration of these molecules by transfection with *β2m* gene resulted in a strong decrease in susceptibility to NK lysis in the S3 cell line, a negative variant for H-2 D^b and K^b of the murine thymoma EL4 [38].

The differential expression of H-2 class I K, H-2 class I D, and H-2 class I L molecules is another event present in some tumors. Studies on AKR-derived B-cell lymphomas (H-2^k) have shown that D^k molecules are processed slower than K^k molecules, with a half-time of 4–5 h [39]. Other studies have shown that L^d Ags are expressed at levels three to four times lower than D^d or K^d Ags [40]. This is in line with the studies that show that in BALB/c S49 lymphoma sublines, there is a locus-specific regulation for K^d, D^d, and L^d surface molecules [41]. The differential expression of these molecules on the cell surface could be a mechanism used by the tumor cells to escape from immunosurveillance. Therefore, these studies all together could add to our knowledge about tumor biology [39]. Some examples of this locus-specific regulation have been documented in other tumor models. Green and coworkers have studied an MuLV-induced AKR tumor in which the expressed H-2 K and H-2 D Ags are differentially induced by IFN-γ [42]. In the spontaneous BALB/c line 1 murine carcinoma, it has been shown that the inductions of MHC-I antigen expression by IFN-γ and DMSO differ at the molecular level. A point mutation in the D1 region of the D^d promoter diminished IFN-γ responsiveness, but did not alter induction of D^d molecule by DMSO. Thus, DMSO appears to regulate MHC-I transcription through multiple regions of the MHC-I heavy-chain promoter by mechanisms distinct from IFN-γ [43]. Studies with mutant phenotypes have led to the description of factors controlling the folding, the intracellular transport, and the surface expression of class I molecules [44].

Components of APM are important elements in the MHC-I cell surface expression. Alteration in the Ag presentation pathway may serve as an evasive mechanism rendering tumors unrecognizable by host immunosurveillance mechanisms. Certain murine tumor cell lines, such as the chemical-induced CMS-5, EL4, MCA102, and MCA205 cells, with deficient expression and/or function of multiple APM components, in particular the peptide transporters (TAPs) and tapasin, show reduced levels of MHC-I surface expression accompanied by low immunogenicity, hence evading T-cell-mediated immune recognition in vivo [45]. In the B16 melanoma, MHC-I-deficient phenotype has been attributed to the downregulation or loss of the expression and function of multiple APM components [46]. In other studies, it has been shown that inoculation of C57BL/6 mice with a mixture of TAP-1-positive and TAP-1-negative tumor cell lines, generated from a transformed murine fibroblast

line, produced tumors exclusively composed of TAP-1-negative cells, indicating an in vivo selection for TAP-deficient cells. Thus, loss of TAP function can allow tumor cells to avoid T-cell immunity producing tumor cells with increased tumorigenicity [16]. In the APM-deficient mouse lung carcinoma cell line CMT.64, reexpression of TAP-1 after infection with TAP-1 adenovirus vector led to an increase of MHC-I cell surface expression and increased susceptibility to specific CTLs [47].

In addition, there are examples of tumor progression associated with increased expression of MHC Ags. For instance, one H-2 class I-deficient cell line from RBL-5 lymphoma (RMA-S), isolated after mutagenization and several cycles of selection by lysis of MHC-I-positive cells, was rejected in syngeneic C57BL/6 mice. In contrast, the H-2-positive wild-type cell line (RMA) was highly tumorigenic [48]. The transfection of this H-2 class I-deficient mutant (RMA-S) with *TAP-2* gene led to a marked increase in tumor outgrowth potential in vivo. This occurred despite restored antigen presentation and sensitivity to CTLs and was found to be due to escape from NK cell-mediated rejection. These data suggest that a defect in the machinery responsible for processing and loading of peptides into MHC-I molecules is sufficient to render cells sensitive to elimination by NK cells [49]. These data are in accordance with the *missing self-hypothesis* [12] in which NK cells are able to distinguish class I-expressing and class I-deficient tumor cells. These cells are able to kill TAP-deficient RMA-S cells (H-2 class I negative) more efficiently compared to RMA cells (MHC-I positive). NK cells refrain from killing when target cells express self-MHC-I molecules [50]. Similar results have been obtained after IFN-γ treatment in murine H-2-negative YAC-1 lymphoma cell line. In this case, reexpression of H-2 antigens abrogated NK lysis of the cells [51]. In other tumors including EL4 lymphoma [12, 48] and murine tumor cell lines expressing human papilloma virus (HPV) 16-derived E6/E7 oncoproteins TC-1 (MHC-I-positive) and MK16 (MHC-I-negative) variants, NK cells appear to be an effective tool against MHC-I-deficient cells [52, 53]. In this case, immunization with the MHC-I-negative (MK16), but not with TC-1 (MHC-I-positive), cell line inhibits the growth of MHC-I-negative tumors. NK cells are responsible for this immunity, although IFN-γ production by $CD4^+$ and $CD8^+$ T-cells cannot be excluded [54]. The heterogeneity of MHC-I expression in tumor cell population and the balance of the MHC-restricted CTL and MHC-unrestricted NK cell immune mechanisms determine the final outcome of the MHC-I expression in the primary tumor [55].

12.2.1 Studies in GR9 Tumor Model: H-2 Antigen Surface Expression and Tumorigenic Capacity

Since the generation of the GR9 tumor model in the 1980s, our knowledge about the role of MHC-I molecules in the tumor scene has increased dramatically [28, 29, 56, 57, 58]. GR9 tumor model is a subcutaneously induced methylcholanthrene (MCA) fibrosarcoma in BALB/c. The original tumor mass was directly adapted to tissue culture without any in vivo passage in syngeneic or allogeneic mice to avoid immunoselection [56]. Forty-three cell lines were obtained after cloning using a phase-contrast microscope and limiting dilution, adapted to tissue culture and cryopreserved. The GR9 fibrosarcoma tumor and the GR9-derived clones have been extensively studied and characterized by our group. The H-2 class I phenotype of the different cell lines was analyzed (Fig. 12.1) [13, 56, 59]. GR9 cell line presents surface expression of the three H-2 class I molecules (K^d, D^d, and L^d), and it is composed of tumor clones with a great heterogeneity in H-2 phenotype which could be classified in four groups: highly positive clones (D8, A7, G2), middle positive clones (B10, B7, B3), low positive clones (B6, C11, C5, G10), and very low/negative clones (B9, B11) (Fig. 12.1) [13, 56, 59]. Transcriptional analysis of the H-2 class I heavy chain, β2m, and APM component genes showed a correlation between the expression of these genes and the surface expression of MHC-I molecules [59]. A coordinated transcrip-

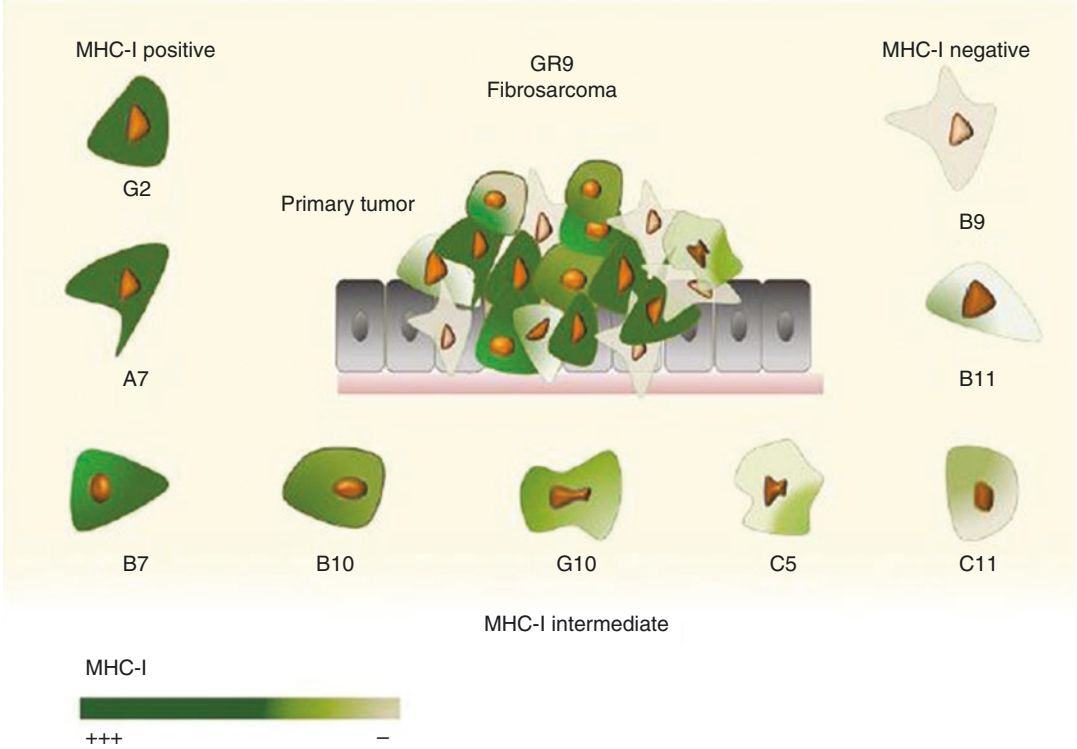

Fig. 12.1 GR9 fibrosarcoma tumor model. Cell clones are adapted to tissue culture from the primary tumor and classified according to MHC-I surface expression

tional downregulation of H-2 L^d heavy chain, calreticulin, LMP-2, and TAP-1 has been found in B11, B7, and C5 clones in comparison with A7 clone. In all instances, H-2 class I K^d, D^d, and L^d molecules of all tumor cell lines could be recovered after IFN-γ treatment [59]. This data indicates that tumor cells have reversible (soft) defects underlying MHC alterations [23, 60]. More recently, we have shown that the tumor suppressor gene *Fhit* is involved in the coordinated transcriptional regulation of various APM components and/or MHC-I heavy chains [58]. Transcriptional levels of Fhit are significantly lower in tumor clones with low expression of MHC-I molecules. Results have shown that the transcriptional level of *Fhit* in A7 clone is 1.4 higher than those found in B7 clones and 3.6 and 3.2 times higher than those expressed in C5 and B11 clones [59].

The intratumoral heterogeneity in H-2 class I expression presented in GR9 cell lines is not an unusual case since other MCA-induced tumors obtained in our laboratory (GRB7.1, GRB7.2, and GRIR5) presented similar levels of H-2 class I heterogeneity. These differences have a strong influence on in vivo tumor behavior in immunocompetent mice [13]. Local tumor growth of different clones of GR9 in syngeneic immunocompetent BALB/c mice showed an inverse correlation between the MHC-I phenotype of tumor clones and their local tumorigenic capacity [59, 61]. Comparing local tumor growth after subcutaneous injection of 6.25×10^5 cells of A7, B7, C5, and B11, we found that all cell lines grew in vivo locally. A7 and B7 showed similar growth rate, but different from C5 and B11. Thus, local tumors of mice injected with C5 and B11 cell clones began to grow at day 8 and were removed at days 23 and 28, respectively. In contrast, the other two clones, A7 and B7 cells, began to grow later at days 14 and 16 postinjection, respectively; the primary tumor was removed at day

39. Clones with high MHC-I expression are very immunogenic in local tumor growth experiments; in contrast, clones with decreased MHC-I expression grew rapidly in vivo when injected subcutaneously. The behavior is totally opposite in spontaneous metastatic capacity (see the following section). In brief, results clearly show that in this tumor model, an inverse correlation between MHC-I surface expression on tumor clones and local tumorigenic capacity exists. Moreover, these differences in local tumor growth were associated with an immune response, since the clones progressed similarly in irradiated syngeneic BALB/c mice [61].

12.3 MHC-I Expression and Metastatic Progression

Metastatic progression is a complex process during which cancer cells leave the heterogeneous primary tumor to spread to secondary sites. Thus, pathogenesis of cancer metastases involves a set of sequential events initiated when tumor cells acquire an invasive phenotype [62–64]. These invasive tumor cells detach from the matrix, invade the tissue, and migrate toward the blood or lymphatic vessels to finally get access to the systemic circulation. However, most tumor cells are destroyed after extravasation into circulation by the immune system or hemodynamic forces, and only a small proportion eventually extravasate and arrive at the new site [65, 66]. This last step requires complex interactions between tumor cells and distant tissue microenvironment [67, 68]. Some in vitro model systems have contributed to the study of individual steps of the metastatic cascade [69, 70]. However, the major limitation of these models is that they do not incorporate the complex interplay between the host and tumor cells; therefore, it is necessary to work with in vivo models. One of the most common problems about cancer research and treatment is difficulty reproducing metastatic human disease using in vivo models. Preclinical tumor models must mimic the fundamental steps associated with the metastatic cascade [71, 72]. Three main types of models in vivo have been employed to approximate the situation observed in patients with advanced metastatic disease: genetically engineered mouse models (GEMM), transplantable tumor model systems (GRAFT) or spontaneous metastasis assays, and experimental metastasis assays. At first, an oncogenic alteration is introduced (deletion or overexpression) in a specific tissue [63, 73–75]. The other alternative extensively used, GRAFTs, recapitulates all steps of secondary colonization by spontaneous visceral metastasis. In these models, tumors or tumor cell lines are transplanted into mouse, generating a primary tumor that will be excised to prolong survival of the host, thus increasing the possibility of distant spontaneous metastases [76–79]. Experimental metastasis assay also is the other common test to investigate biological behavior of tumor cells in vivo. In experimental metastasis assays, tumor cells are directly injected into blood circulation to spread to organs. We considered that spontaneous metastasis assay resembles all sequential steps associated with the metastatic cascade, from primary local tumor to secondary colonization. In contrast, experimental metastasis assay is a bypass in the metastatic cascade, evading the first steps: local primary tumor growth, migration, and extravasation into the blood and/or lymphatic vessels. Our research group has compared the behavior of different tumor cell lines in experimental and spontaneous metastasis assays, finding that it is opposite. Tumor cell lines with high spontaneous metastatic ability showed very low experimental metastatic capacity [59]. In consequence, we think that experimental metastasis assays should not be used as a model for studying metastatic advanced disease.

12.3.1 MHC Class I Expression on Primary Tumor Cells May Determine Spontaneous Metastatic Capacity

During the late 1970s, heterogeneity in metastatic potential of tumor populations was demonstrated by Fidler and Kripke, using a mouse malignant melanoma [80]. Great difference

between the abilities of clones from the B16 cell line was observed in terms of developing metastatic colonies in vivo. This fact suggests that a heterogeneous population composed the primary tumor where there were nonmetastatic and metastatic tumor cells. Later research on various cell lines including clones with different metastatic potentials isolated in tumor cell populations of BALB/cfC3H mammary adenocarcinoma or [81] methylcholanthrene- [82] or ultraviolet light-induced fibrosarcomas [83, 84] supported these findings. However, Haywood and McKhann were the first to suggest the possible influence of the MHC-I genes on metastatic capacities of tumor cell populations [85]. They compared metastatic capacity of five methylcholanthrene-induced sarcomas, finding that more metastatic tumors had quantitatively more H-2 surface expression. These results, as well as later evidences observed by other groups, showed that the level of MHC-I expression was implicated in the metastatic capacity of the tumor cells. Three different spontaneous tumors originated in mouse, Lewis lung carcinoma (3LL), B16 melanoma, and BW T lymphoma, have been used by Eisenbach's research group to show whether metastasis disease is influenced by MHC-associated mechanisms. They worked with different tumor cell variants of these tumors, finding that metastatic ability directly correlated with surface expression levels of the H-2 D Ags and inversely of the H-2 K Ags [86–89]. Moreover, H-2 K-negative/D-positive clones with high metastatic ability reverted their metastatic phenotype, inducing H-2 K-restricted CTLs when transfected with the *H-2 K* gene [87, 90, 91, 92]. In brief, these results support that the metastatic phenotype is associated with H-2 D surface expression and loss of H-2 K surface expression in primary tumor cells. In this context, Kazav et al. using T10 sarcoma (H-2 b × H-2 k) [induced by methylcholanthrene in a (C57BL/6J X C3HeB/-FeJ) mouse] reported that expression of MHC-I increased the metastatic capacity of tumor cells [93, 94]. Several clones of T10 sarcoma presented differential expression of H-2^b and H-2^k haplotypes: H-2^b × H-2^k positive and only H-2^b positive. Metastatic clones characterized to express both parental haplotypes and nonmetastatic clones only showed expression of H-2^b haplotype [95]. Furthermore, metastatic potential in this tumor system was only acquired when H-2 D^k-Ags were expressed on the surface of tumor clones. Moreover, T10 clones expressing only H-2 D^k-Ags were more metastatic than clones expressing both H-2 D^b and H-2 D^k-Ags, while clones merely expressing H-2 D^b Ag were nonmetastatic [95, 96].

12.3.2 Different MHC-I Surface Expressions on GR9 Tumor Clones Determine Their Spontaneous Metastatic Capacity

In our laboratory, the GR9 fibrosarcoma murine model was used to assess whether levels of MHC-I surface expression on primary tumor cells exert influence on their spontaneous metastatic capacity. Four cell clones (A7, B7, C5, and B11) with different MHC-I surface expressions were chosen for spontaneous metastasis assays (Fig. 12.1). Results showed significant differences in metastatic capacity between these clones [59]. For example, A7 clone with a strong H-2 class I surface expression was highly metastatic, generating metastases in 90% of the hosts and resulting in 1–50 metastases per animal. Clones with intermediate or low H-2 class I expression, as B7 or C5, presented lower metastatic capacity, 50 and 20%, respectively. In contrast, MHC-I-negative B11 clone did not present spontaneous metastatic capacity, and the B11 tumor-bearing mice remained free of overt metastasis at the end of the assays for more than 24 months. However, when these immunocompetent hosts were immunodepleted of T- or NK lymphocytes, overt pulmonary metastases appeared in the immunodepleted hosts. These data show that hosts injected with B11 clone presented micrometastases in permanent immunodormancy [97]. In brief, cell clones with high surface expression of H-2 class I molecules were also highly metastatic, but those clones with low or negative H-2 class I expression were weakly metastatic or nonmetastatic (Fig. 12.2). Our experimental evidences support

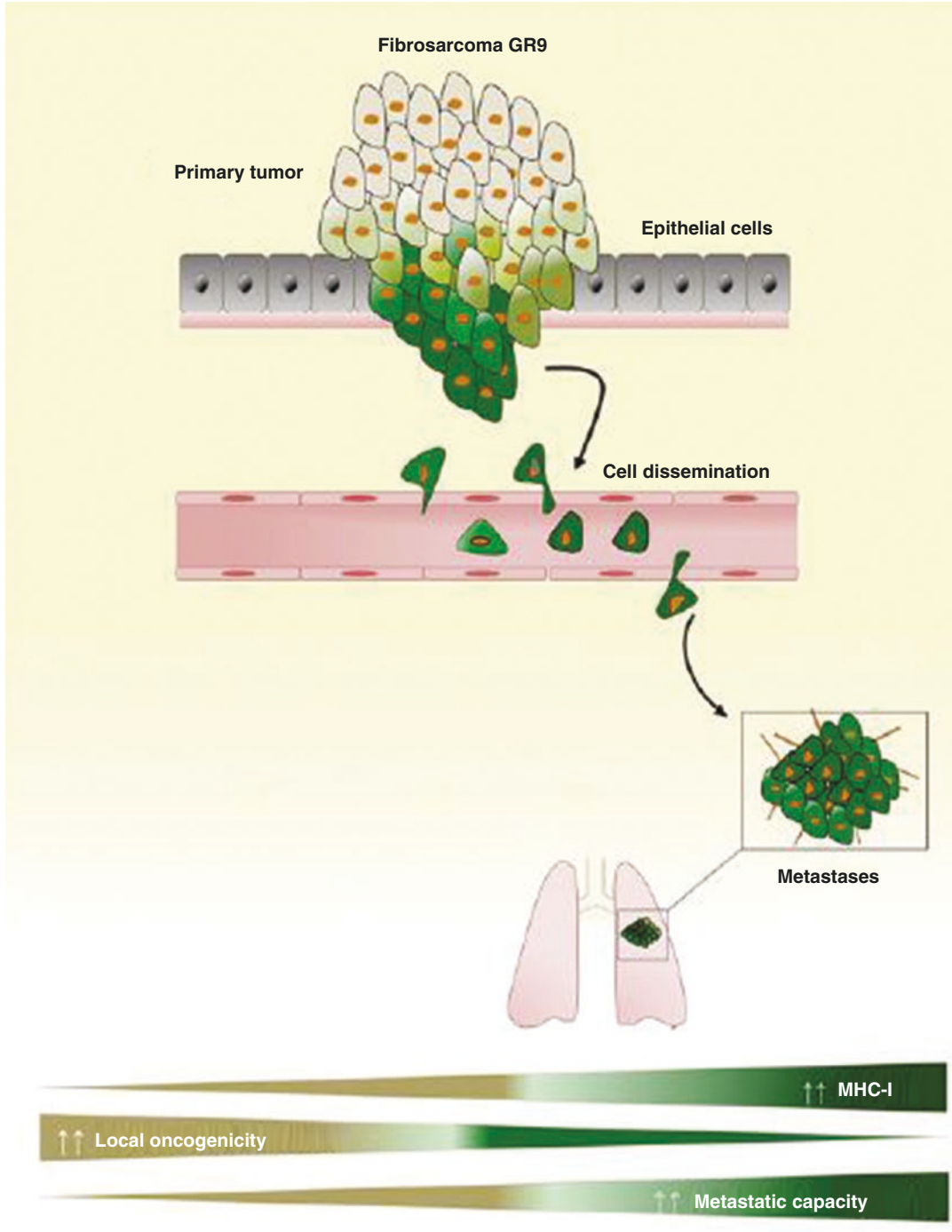

Fig. 12.2 Schematic representation of the dissemination and invasion of GR9 primary tumor cells. MHC-I-positive tumor cells from GR9 primary tumor presented a high spontaneous metastatic capacity, whereas MHC-I-negative tumor cells presented a weak spontaneous metastatic capacity

the idea that levels of MHC-I surface expression of primary tumor cells directly correlated with spontaneous metastasis ability and inversely with local oncogenicity, as it was shown above [59] (Fig. 12.2). Consequently, extrapolation of oncogenic and metastatic behavior of tumor cells in vivo is not always possible, because they may be completely opposite.

Analysis of MHC-I cell surface expression on spontaneous metastases derived from these fibrosarcoma clones displayed that in all cases the metastases presented the same or lower MHC-I surface expression than the original clone [59]. In consequence, metastatic progression promoted a downregulation in MHC-I surface expression. Analysis of leukocyte subpopulations in tumor-bearing mice revealed a distinct behavior among different clones. A7 and B7 produced immunosuppression characterized by decrease in T-lymphocytes and increase in Treg cells [29]. In contrast, B11 tumor-bearing mice developed a strong immunostimulation characterized by an increase in T-lymphocytes, dendritic cells, and macrophages cells [97]. In brief, A7 and B7 cells progressed to metastatic disease suppressing the immune response, whereas the B11 clone promoted an immune response which avoided metastatic progression. The other GR9 tumor clone studied was B9, with H-2-negative surface expression and with weak spontaneous metastatic capacity (zero to one metastasis per mouse). In contrast, this clone is highly metastatic using nu/nu BALB/c mice, ranging from five to seven per mouse [28, 98]. Moreover, metastases were H-2 class I negative in immunocompetent hosts and H-2 positive in immunodeficient hosts. Thus, we observed that H-2 phenotype of spontaneous metastases was influenced by the immunological state of the hosts.

The GR9 fibrosarcoma cell line, composed of different cell clones, presented intermediate levels of H-2 K^d, H-2 D^d, and H-2 L^d molecules. Analysis of spontaneous metastasis assay with GR9 tumor cells revealed that GR9 cells have high spontaneous metastatic capacity; 90% of tumor-bearing mice develop metastases, ranging from one to nine per animal. GR9 produced strong immunosuppression in tumor-bearing mice. Interestingly, 96% of metastases derived from GR9 clone showed downregulation of MHC-I surface expression. These results suggest that MHC-I-positive clones, as A7 or B7, produced immunosuppression, favoring the growth of MHC-I low or negative clones.

Other experimental evidences from our tumor model also support the idea that in GR9 fibrosarcoma tumor, the amount of MHC-I Ags also affects NK cell cytotoxicity [99]. Since NK cells have been recognized as one of the main host immunological mechanisms against metastasis disease, this notion seems imperative [100]. In our system, tumor clones with no or low expression of MHC-I molecules were found to be sensitive to NK-mediated lysis, while clones with high levels of MHC-I expression were relatively resistant [99].

12.4 Immunotherapy as a Treatment Against Cancers with Different MHC-I Surface Expressions

12.4.1 Immunotherapy as a Treatment Against Primary Tumors with Different Levels of MHC-I Expression

As mentioned above, MHC-I molecules present TAAs to CTLs; therefore, MHC-I surface expression on tumor cells may play an important role in the outcome of immunotherapies as anticancer treatments. During treatment with vaccines containing peptides derived from TAAs, MHC-I-positive surface expression on tumor cells presenting these TAAs is crucial to make this immunotherapy effective. As a consequence, before the application of immunotherapies, MHC-I surface expression on tumor cells must be analyzed. Furthermore, two immunosuppressive mechanisms have been described recently showing evasion of tumor cells from CTL attack, mediated by expression of noncognate MHC-I molecules or by myeloid-derived suppressor cells (MDSCs) [101, 102].

Several murine tumor models have been used to evaluate the application of different immunotherapies to recover MHC-I surface expression in MHC-I-deficient tumor cells, in order to promote an antitumor immune response. In MHC-I-negative B16 melanoma cells, intratumoral electroporation of IL-12 cDNA promoted an increase in their MHC-I surface expression, mediated by IFN-γ, leading to the eradication of established melanomas by activation of CTLs [103]. In cervical carcinoma cells, administration of synthetic oligodeoxynucleotide-bearing CpG motifs (CpG-ODNs) upregulated MHC-I surface expression causing tumor regression mediated by CTLs [104]. Other studies also have reported that CpG-ODN immunotherapies delayed the growth or inhibited minimal residual tumor disease of both MHC-deficient and MHC-positive tumors [105, 106]. Moreover, combination of dendritic cell-based vaccines with CpG generated inhibition of tumor growth in MHC-positive and MHC-negative tumors [107]. CpG-ODN 1585 only produced regression of MHC-deficient tumors, principally activating NK cells [106]. In other assays, depletion of T(reg) cells avoided the growth of recurrent tumors after surgery of MHC-negative and MHC-positive tumors [108]. In all these assays, the action against MHC-I-deficient tumors was mediated by NK or NK1.1$^+$ cells [109]. Previous to the application of immunotherapy, MHC-I-deficient tumor cells may be treated with agents to upregulate MHC-I surface expression. Epigenetic mechanisms are frequently implicated in MHC-I downregulation of tumor cells; as a result, application of agents as 5-azacytidine (5AC) or trichostatin A could increase MHC-I surface expression [110, 111]. Treatment of 5AC with CpG-ODN or with IL-12 showed additive effect against MHC-I-deficient tumors, being the immune response mediated by CD8$^+$ T-cells [112]. Other chemoimmunotherapies, based on ifosfamide derivative CBM-4A together with IL-12, also led to significant inhibition in the growth of MHC-I-deficient tumors [113].

12.4.2 Immunotherapy as a Treatment Against Metastatic Progression Derived from Primary Tumors with Different MHC-I Expressions

Immunotherapy has also been used as an antimetastatic treatment against spontaneous metastasis derived from primary tumors with different MHC-I expressions. As mentioned above, studies performed by Eisenbach et al. showed an inverse correlation between H-2 K tumor cell surface expression and spontaneous metastatic capacity [86, 89, 90, 114]. Tumor cell lines derived from H-2 K-low or H-2 K-deficient primary tumors presented high spontaneous metastatic capacity, which was reverted by transfection of tumor cells with *H-2 K* gene [86, 115, 116]. Moreover, injection of the H-2 K-transfected tumor cells that protect against metastatic disease originated from H-2 K-low or H-2 K-deficient tumors. Furthermore, therapy with IFN-γ-treated tumor cells or with H-2 K-transfected tumor cells promoted upregulation of H-2 K surface expression and protected against metastatic dissemination from parental tumor cells [114, 116]. An additional effect was reached when tumor cells were jointly transfected with IFN-γ and allogeneic *MHC class I* genes [117].

In GR9 murine tumor model, the influence of MHC-I cell surface expression on primary tumors has been investigated with respect to the success of immunotherapy as antimetastatic treatment. A7 is a fibrosarcoma clone with strong spontaneous metastatic capacity. Four treatments were used: two immunotherapies (CpG + irradiated autologous A7 cells and PSK) [118], one chemotherapy (docetaxel), and one chemoimmunotherapy (PSK + docetaxel). A7 tumor clone was injected subcutaneously in BALB/c mice, and the primary tumor was excised when the large tumor diameter reached 10 mm. Treatment began 1 week after tumor removal, on a weekly basis during 6 weeks; 1 week after the last dose, mice were euthanized, and autopsy

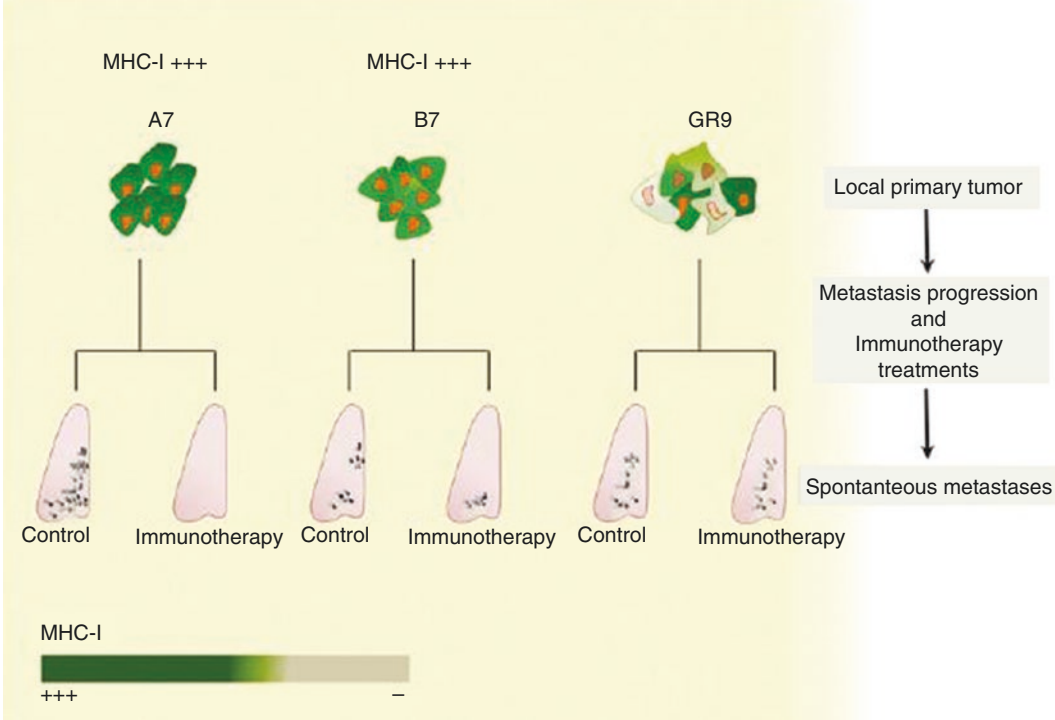

Fig. 12.3 Immunotherapy as an antimetastatic treatment against tumors with different MHC-I expressions. Immunotherapy was completely effective in inhibiting spontaneous metastatic progression in A7 tumor clone (MHC-I highly positive). For B7 tumor clone (intermediate level of MHC-I expression), immunotherapy accomplished partial reduction in the number of spontaneous metastases. In the case of GR9 fibrosarcoma, immunotherapy had no antimetastatic effect

was performed. Interestingly, all mice treated with each immunotherapy or chemo-immunotherapy appeared metastasis-free (Fig. 12.3) [29]. In contrast, partial reduction in the number of metastases occurred in the mice treated with chemotherapy. In the control group, mice injected with A7 tumor cells and treated with saline solution, a high number of spontaneous metastases in all mice were observed (Fig. 12.3) [29]. In brief, the two immunotherapy protocols and the one chemo-immunotherapy protocol eradicated metastasis completely and cured the mice, whereas chemotherapy treatment reduced the number of metastases partially. When the same four treatment protocols were applied against spontaneous metastases generated from B7 fibrosarcoma clone (intermediate MHC-I expression level and with lower spontaneous metastatic capacity than A7 clone), the antimetastatic effect was not as effective (Fig. 12.3). PSK, PSK + docetaxel, and docetaxel promoted partial reduction in the number of metastases, whereas that CpG + irradiated autologous B7 cell treatment did not produce any antimetastatic effect [119]. In the case of spontaneous metastases derived from GR9 fibrosarcoma, neither treatment had any antimetastatic effect [119]. Analysis of lymphocyte subpopulations in different assays showed that growth of local tumors promotes strong immunosuppression in the three cases. However, this immunosuppression was completely reverted by immunotherapies in the case of A7-injected mice, was partially reverted for B7-injected mice, and remained unchanged in GR9-injected mice [29, 119]. All these results suggest that immunotherapies may be potential antimetastatic treatments against primary tumors with high MHC-I cell surface expression.

12.5 Concluding Remarks

In tumor cells, MHC-I molecules may present peptides derived from tumor-associated antigens, which are new proteins expressed or overexpressed in tumor cells. Presentation of these new peptides may allow recognition and destruction of tumor cells by CD8+ T-lymphocytes. Loss of MHC-I expression on tumor cells is a widespread and frequent mechanism developed to escape from immunosurveillance. Alteration in MHC-I in both human and murine experimental tumors has been widely reported. Results show an inverse correlation between MHC-I expression on tumor cells and primary tumor growth, i.e., MHC-I-negative tumors grew more rapidly compared to MHC-I-positive tumors. In contrast, a direct correlation was found between MHC-I expression on primary tumors and spontaneous metastatic capacity. Immunotherapy as an antimetastatic treatment was completely effective against MHC-I highly positive tumors and was partially effective on tumors with an intermediate level of MHC-I expression.

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13 Role of Cytokines in Tumor Immunity and Immune Tolerance to Cancer

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13.1 Introduction

Strong evidence has been accumulated demonstrating that cancer cells in humans and animals are recognized in general as "non-self" by the immune system [1, 2]; both innate and adaptive immune reactions to cancer have been described. In cancer patients, many cases of spontaneous tumor have been reported following infection. Moreover, immunosuppressed patients are at increased risk for virally-induced tumors [3]. In fact, the presence of highly adaptive immune cell infiltrates within the tumors can be a positive prognostic indicator of patient survival [4]. Murine models of spontaneous and chemically-induced tumors have also been useful in demonstrating that the immune system naturally surveys for aberrant cells and has an important role in preventing tumor formation [2].

An antitumor immune response is initiated when innate immune cells are alerted to the presence of a growing tumor, at least in part owing to local tissue damage from stromal remodeling processes integral to basic solid tumor development [2, 5]. Once solid tumors reach a critical mass, they grow invasively and require an enhanced blood supply, which they induce via production of angiogenic proteins [6]. Invasive growth causes minor disruptions in surrounding tissues that induces inflammatory cytokines and chemokines, leading to the recruitment of innate immune cells. The innate response includes several cellular factors, such as natural killer (NK) cells, natural killer T (NKT) cells, γδ T-cells, macrophages, dendritic cells (DCs), and neutrophils [7]. These cells can reject tumors, either by directly killing tumor cells or by inhibiting angiogenesis. Innate immunity relies on pattern recognition receptors and other cell surface molecules to detect tumor cells. Cancer cells express families of stress-related genes, such as MHC class I-related stress-inducible surface glycoprotein A and B (MICA and MICB), which function as ligands for NKG2D receptors expressed on NK cells [8]. In addition, NK cells can be triggered for cytolytic activity by DCs depending on direct cell contact through their expression of cell surface molecules, such as CD48 and CD70, which are ligands for the NK cell-activating receptors, 2B4 and CD27, respectively [8]. DCs recruited to the tumor site become activated either by exposure to the cytokine milieu created during the ongoing attack by the innate immune system or by interacting with NK cells. Once activated, DCs can acquire tumor antigens directly by ingestion of tumor cell debris, or potentially through indirect mechanisms involving the transfer of tumor cell-derived heat shock protein/tumor antigen complexes [9]. Activated antigen-bearing DCs can then migrate to the draining lymph nodes, where they trigger the activation of tumor antigen-specific $CD4^+$ Th1 cells. In addition, DCs activate $CD8^+$ cytotoxic T lymphocytes (CTLs) via cross-presentation of tumor antigenic peptides on MHC class I molecules [10]. Activated tumor-specific $CD4^+$ and $CD8^+$ T-cells home to the tumor site where they kill tumor cells. Accordingly, mice lacking adaptive immunity (RAG-2 gene-deficient mice lacking T-cells) are more susceptible to carcinogen-induced and spontaneous primary tumor formation [2]. It appears adaptive immunity could possibly provide the host with the capacity to completely eliminate developing tumors. However, clinically evident cancers indicate that these innate and adaptive immune responses are not always suffi-

cient to prevent disease progression in patients, as cancer cells clearly manage to escape host-tumor immunity.

Tumors use several mechanisms to facilitate immune escape and avoid elimination, including impairment of antigen presentation, activation of negative co-stimulatory signals, and elaboration of immunosuppressive factors [11]. In addition, tumor cells may promote the expansion and/or recruitment of regulatory immune cell populations which can contribute to the immunosuppressive network; these populations include regulatory T-cells (Tregs), myeloid-derived suppressor cells (MDSCs), and distinct subsets of immature and mature regulatory DCs [12]. All of these host-derived immune cell populations can impair antitumor effector cell responses, both locally in the tumor microenvironment and systemically in the lymphoid organs [11]. In fact, both tumor-promoting and inhibiting immune cell populations can be seen in cancer patients [2]. Several recent studies have found correlations between particular immune cell infiltrates in tumors and patient prognoses. For example, infiltration of $CD8^+$ T-cells and mature DCs is associated with favorable outcomes [13, 14]. However, extensive macrophage infiltration correlates with poor patient prognoses in most cancers [15, 16]. The complexity of these tumor infiltrates, with both synergistic and oppositional effects, may influence tumor growth differentially, depending on their cytokine secretion. A number of immune-modulating cytokines have been shown to promote or inhibit antitumor immunity in multiple experimental models and in cancer patients. This chapter reviews the role of the antitumor cytokines (IL-12 and IL-27) in tumor immunity and immunotherapy while discussing the role of pro-tumor cytokines (TGF-β, IL-17, IL-23, IL-35, and IL-10) with pathogenic contributions to cancer progression.

13.2 Cytokine Regulation of Antitumor Immunity

Cytokines comprise a large family of intercellular communicating molecules that play important roles in immunity, inflammation, and repair, as well as general tissue homeostasis [17]. Cytokine functions also extend to many other aspects of biology, including cancer [17, 18]. In the tumor microenvironment, cytokines are produced by host stromal and immune cells, in response to molecules secreted by cancer cells [17]. In addition, cancer cells themselves can produce cytokines [18]. Increased levels of circulating cytokines and their receptors have been found in patients with various types of cancer, both at diagnosis of the primary disease and in metastases [17, 19, 20]. The cytokine repertoire present at the tumor site determines the type of host immune response directed against the tumor [18, 21]. Immunosuppressive cytokines secreted by tumor cells or tolerogenic tumor-infiltrating immune cells can impair the host antitumor response, whereas cytokines promoting T-cell-mediated immunity can induce or enhance antitumor responses [17, 21]. Studies using cytokine-deficient mice have revealed the dual role for the immune system in modulating tumor growth [17].

13.2.1 IL-12

13.2.1.1 Overview

IL-12 is a heterodimeric cytokine containing a 35 kD and a 40 kD subunit that signals through a receptor of the type I family of cytokine receptors [22]. The principal source of IL-12 are APCs, such as DCs and macrophages [22]. Secretion of IL-12 is generally activated via the physiological stimuli of CD40 along with toll-like receptors (TLRs), which recognize structurally conserved molecules derived from microbes [22]. IL-12 plays a major role in the development of antitumor immune responses [23]. Numerous studies report that IL-12 promotes effective destruction of cancer cells via induction of innate and adaptive arms of antitumor immunity [23–25]. In addition, IL-12 has potent antiangiogenic activity [23, 24]. Due to these features, IL-12 has been tried as a systemic cancer therapeutic agent, but clinical development has been hindered by its significant toxicity and disappointing antitumor effects observed in cancer patients [25]. However, emerging studies suggest that IL-12, in combination with other cytokines and checkpoint inhibi-

tors, could boost antitumor immunity and promote NK cells and CTLs with minimal toxic side effects [25, 26].

13.2.1.2 IL-12: Linking Innate and Adaptive Antitumor Immunity

IL-12 plays an essential role in the interaction between the innate and adaptive arms of antitumor immunity (Fig. 13.1). For example, it induces IFN-γ production by NK cells and T-cells. In fact, NK cells and T-cells were the first cells shown to express high-affinity receptors for IL-12 [27]. Tumor eradication via vaccination supported by IL-12 is dependent on NK cells in several animal models [28–30]. IL-12 enhances *in vitro* lysis of both NK cell-sensitive and NK cell-resistant tumor cells [31]. Consistent with animal studies, in patients with cancer, IL-12 enhances the cytolytic activity of NK cells and increases the expression of CD2, lymphocyte function-associated antigen-1 (LFA-1), and CD56, molecules which mediate NK cell migration [32]. Moreover, IL-12 has been shown to

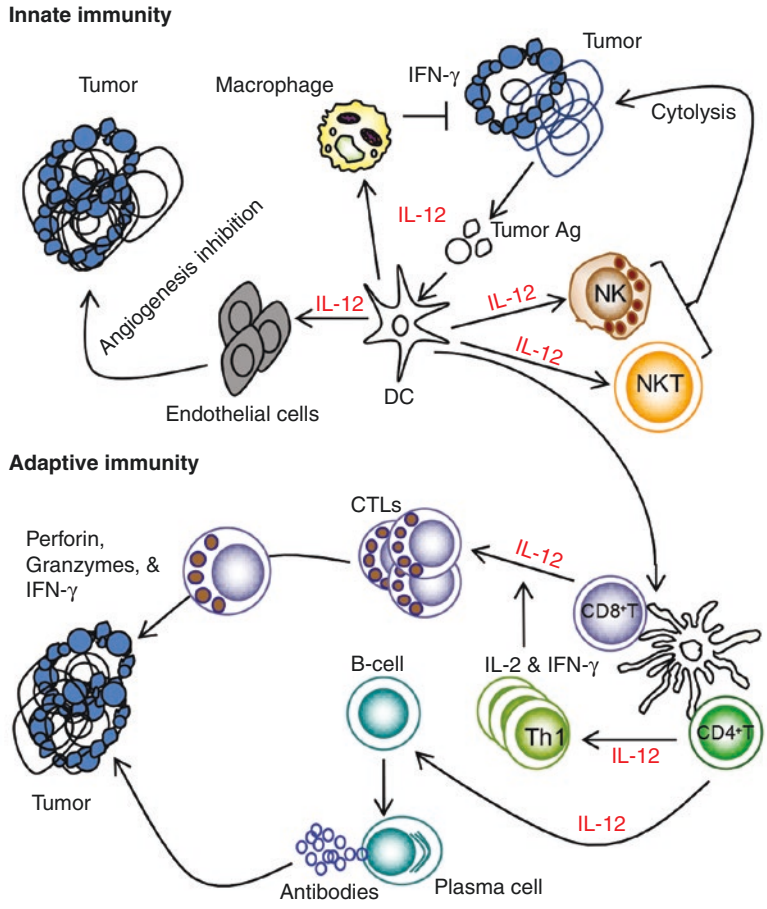

Fig. 13.1 IL-12 links innate and adaptive antitumor immunity. IL-12 utilizes several mechanisms to induce antitumor effects. IL-12 activates innate effectors, such as NK cells, NKT cells, and γδ T-cells, promoting their cytolytic activity and cytokine production. In macrophages, IL-12 induces IFN-γ production that can have cytotoxic effects on tumor cells. In endothelial cells, IL-12 induces the production of antiangiogenic molecules. In addition, IL-12 has a direct toxic effect on some tumor cells. Furthermore, IL-12 secretion by DCs can induce adaptive arms of antitumor immunity. For example, IL-12 can augment Th1 responses necessary for cellular immune responses. Il-12 also stimulates the differentiation and lytic capacity of CTLs and promotes immune memory. Finally, IL-12 can mediate antibody-mediated tumor clearance via B-cell activation

enhance the cytotoxicity mediated by NK cells from healthy donors against cancer cells derived from cancer patients [32].

In addition to its effect on NK cell cytotoxicity, IL-12 enhances $CD8^+$ T-cell-mediated cytotoxicity [33]. Crucial to this process are DCs, which facilitate the interaction between $CD4^+$ T-cells and antigen-specific $CD8^+$ T-cells. Priming of CTLs is enabled by the ligation of CD40 on DC and its ligand, CD154 or CD40L, on activated $CD4^+$ T-cells [34, 35]. The induction of IL-12 synthesis that occurs as a result of CD40 ligation suggests an important role for IL-12 in the molecular mechanisms responsible for CTL priming [36]. Indeed, it has been shown that IL-12, in the presence of antigen, acts directly on naive $CD8^+$ T-cells to promote clonal expansion and differentiation [37]. In fact, priming $CD8^+$ T-cells in the absence of IL-12 renders them unresponsive to the same antigen [38]. In murine models of T-cell-mediated immunity, agonistic CD40 antibodies (Abs) have been shown to substitute the function of $CD4^+$ T-cells, resulting in the rapid expansion of CTLs that can clear established lymphomas and provide long-term protection against tumor rechallenge [39, 40]. These observations provide an explanation for impaired tumor antigen-specific CTL activation in CD40-deficient mice and confirm a key role of the CD40-IL-12 pathway in the regulation of antitumor immunity. Moreover, a series of experiments, conducted by different groups, including ours, have indicated that injection of IL-12 systemically or directly into subcutaneous tumors results in CTL responses against the tumors in mice [41–43]. IL-12 also plays an important role in the establishment of memory $CD8^+$ T-cells [44]. After administration of IL-12, a strong antigen-specific CTL response has been observed in patients with advanced melanoma, including increased tumor-specific CTLs in circulation, and an influx of specific memory $CD8^+$ T-cells into metastasized lesions [45].

Tumor rejection requires $CD8^+$ T-cells, whose activation and maintenance depends on IL-12-mediated $CD4^+$ T-cells [35]. Upon stimulation, naïve $CD4^+$ T-cells differentiate into different lineages of T helper subsets, including Th1, Th2, Th17, and Tregs [46]. In the presence of IL-12, naïve $CD4^+$ T-cells differentiate into IFN-γ-secreting Th1 cells [22]. In contrast, IL-12 exhibits a strong inhibitory effect on Th2 differentiation [47]. These distinct $CD4^+$ T-cell subsets have varied impacts on tumor growth. While Th1 cells promote $CD8^+$ T-cell-mediated immunity to tumors, Th2 cells and Tregs negatively regulate $CD8^+$ T-cell function. Th1 cytokines, IL-2 and IFN-γ, stimulate the cytolytic activity of NK cells to clear tumor cells [8]. (Interestingly, autocrine IL-12 has been shown to promote DC activation and induce IFN-γ from DCs.) Th2 cytokines have been shown to accelerate tumor growth in multiple experimental models [48]. In fact, a shift from Th1 to Th2 cytokine production has been reported in progressive cancer patients, and a vaccine-inducing Th2 to Th1 shift in a murine tumor model has been shown to induce tumor rejection [49]. By altering the balance between Th1 and Th2 cytokines, IL-12 plays a critically important role in antitumor immune responses. Enhanced production of IFN-γ by $CD8^+$ T-cells, along with a Th2 to Th1 shift in the cytokine secretion profile of $CD4^+$ T-cells, has been observed in IL-12-treated mice [50]. Although IL-12 has been shown to inhibit Th2 differentiation, a major activator of B-cell responses, IL-12 also directly triggers a cascade of events that are known to activate B-cells and stimulate humoral immunity [51]. In a model of colon carcinoma, vaccination with IL-12-transduced tumor cells cures 40% of tumor-bearing mice. Favorable antitumor responses are related to the synthesis of Abs against tumor antigens, inducing tumor cell lysis in a complement-dependent cytotoxicity assay [52].

As discussed above, the ability of IL-12 to facilitate cell-mediated immune responses, including enhancement of NK cytotoxicity, generation of CTLs, and activation of DCs, suggests its role in both the innate and adaptive immunity resistance mechanisms against tumors [24, 23]. Experimental studies of systemic administration of the cytokine have indicated that IL-12 exerts potent antitumor activity against a variety of metastatic tumors, and can even prevent spontaneous tumor development in HER-2/

neu transgenic mice [53]. In addition, models based on intra-tumor cytokine delivery or *in vivo* transfer of cytokine-secreting tumors have indicated that IL-12 has significant dose-dependent antitumor activity against a wide spectrum of murine tumors, including melanoma, breast, ovarian, and bladder tumors [24, 54, 25]. All of these studies have demonstrated that IL-12 can inhibit tumor growth and improve survival of tumor-bearing animals, which are dependent on its ability to activate the innate and adaptive arms of antitumor immunity.

13.2.1.3 IL-12 in Angiogenesis Inhibition

Accumulating evidence indicates that the antitumor effects of IL-12 are mediated, at least in part, through mechanisms involving angiogenesis and its direct effects on tumors. Angiogenesis is an essential process for tumor growth and metastases; the balance between angiogenic and angiostatic molecules in the tumor microenvironment can determine tumor growth and progression. The antiangiogenic properties of IL-12 were first observed by Voest et al., who demonstrated that IL-12 treatment almost completely inhibits neovascularization in immunocompetent mice, severe combined immunodeficient mice, and T-cell-deficient nude mice [55]. This suppression of angiogenesis by IL-12 is dependent on its ability to induce IFN-γ expression. Accordingly, administration of IFN-γ reproduces the antiangiogenic effects of IL-12. Moreover, it has been shown that inhibition of tumor growth by IL-12 or IFN-γ requires intact signaling from IFN-γ receptors expressed by cancer cells [55]. This indicates that IL-12 can inhibit tumor growth by inducing cancer cells to produce antiangiogenic factors. Two of the most relevant factors identified are the IFN-γ-inducible chemokine genes, IFN-inducible protein 10 (IP-10) and monokine induced by interferon-γ [56]. Local and systemic treatment with IL-12 is associated with intratumoral expression of IFN-γ, IP-10, and MIG [57, 58]. In addition, subcutaneous intratumoral delivery of MIG in nude mice leads to tumor necrosis, associated with vascular damage [59]. Administration of neutralizing Abs to IP-10 and MIG substantially reduces the antitumor effects of IL-12 [59]. IP-10 and MIG interact with their receptor CXCR3 to mediate their angiostatic activity [59]. Together, these findings support that these CXCR3 ligands contribute to the antitumor effects of IL-12 via inhibition of tumor vasculature. In addition to IFN-γ stimulation, IL-12 promotes the expression of interferon regulatory factors 1 (IRF-1) and 4 (IRF-4), which are necessary for Th1 cell differentiation [60]. IRF-1 has tumor suppressor activities in cancer cells *in vitro* and decreases the tumorigenicity of cells inoculated into athymic nude mice [61, 62]. Similarly, IRF-4 suppresses c-Myc-induced leukemia in animal models and inhibits BCR/ABL-induced B-cell acute lymphoblastic leukemia [63, 64].

Emerging evidence indicates the involvement of lymphocyte-endothelial cell crosstalk at the beginning of angiogenesis inhibition by IL-12. It has been shown that neutralization of NK cell function reverses IL-12 inhibition of angiogenesis in athymic nude mice [65]. Neovascularization inhibited by IL-12 displays accumulation of NK cells and IP-10-positive cells. In addition, experimental Burkitt lymphomas treated locally with IL-12 present with tumor necrosis, vascular damage, and NK cell infiltration surrounding small vessels [65]. These studies document that NK cell cytotoxicity of endothelial cells is a potential mechanism by which IL-12 can suppress neovascularization. The antiangiogenic program activated in lymphocytes by IL-12 can also directly affect gene expression in neoplastic cells. In fact, upregulation of signal transducers and activators of transcription-1 (STAT-1) and angiopoietin 2, together with downmodulation of vascular endothelial growth factor (VEGF), has been observed in neoplastic cells exposed to soluble factors released by IL-12-stimulated lymphocytes [66]. In addition, IL-12 treatment reduces the production of metalloproteases, which play a role in matrix remodeling required for neoangiogenesis [67]. Moreover, the activation of integrin αVβ3 on endothelial cells is limited by IL-2-induced IFN-γ, which leads to decreased endothelial cell adhesion and survival [68]. IL-12-induced secretion of IFN-γ causes an increase in p53 activity, which subsequently results in tumor suppression due to apoptosis induction in cancer cells [69]. Furthermore, IL-12 dramatically decreases the

tumor-supportive activities of tumor-associated macrophages (TAMs), which are involved in tumor angiogenesis and metastasis [70]. The antiangiogenic mechanisms modulated by IL-12 are complex; they depend not only on direct effects on endothelial cells by the cytokines/chemokines induced by IL-12, but are also mediated via the recruitment of immune effector cells, such as NK and T-cells.

13.2.1.4 Regulation of IL-12 in the Tumor Microenvironment

Although controlled Th1 and CTL responses can exert significant antitumor immunity, the same responses, if exaggerated, may result in host-tissue destruction and autoimmunity. To maintain immune homeostasis, IL-12-mediated inflammatory responses need to be counter-regulated. Cancer manipulates these counter-regulatory mechanisms to limit the availability of IL-12 in the tumor microenvironment. In general, Tregs play a major role in controlling unwanted immune responses to self-antigens [71]. Treg functions are mediated in part through secretion of the immunosuppressive cytokines IL-10 and TGF-β. Both TGF-β and IL-10 can inhibit DC antigen presentation, IL-12 secretion, and effector functions in $CD4^+$ and $CD8^+$ T-cells [11]. Studies have revealed a significant role for Treg induction by cancer cells in immune tolerance to tumor antigens [72]. As an immunosuppressive tumor microenvironment develops, IL-12-secreting DCs may become scarce due to a variety of factors, including absence of DC activation signals, CD40, and inhibition of activated $CD4^+$ T-cells which could themselves activate DCs [73]. Consistent with the idea, we have shown that the CD40-CD40L interactions between DCs and T-cells lead to the induction of not only IL-12, but also IL-10, a pro-tumor cytokine that may act in an autocrine or a paracrine manner to down-regulate IL-12 secretion from DCs [74, 75]. Indeed, reduced CD40 expression on DCs or CD40L on T-cells from tumor-bearing hosts may explain the reduced levels of IL-12 observed in cancer patients [73]. Other factors present in the tumor microenvironment can also downregulate IL-12 production; for example, prostaglandin E2 (PGE2), produced by tumor cells or tumor-associated host cells (e.g., macrophages, endothelial cells, and stromal cells), is known to inhibit IL-12 [76]. Reduced expression of IL-12 has been observed in patients with advanced cancer types including glioblastoma, renal cell carcinoma, head and neck squamous cell carcinoma, gastric cancer, melanoma, colorectal cancer (CRC), hepatocellular carcinoma, and gastric cancer [20]. Moreover, IL-12 production by stimulated peripheral blood mononuclear cells decreases significantly in patients with advanced gastric or colorectal cancer [20].

13.2.1.5 IL-12 in Clinical Studies

Following provocative preclinical studies, IL-12 has been evaluated in patients with different malignancies. To date, more than 58 clinical trials testing IL-12-based therapies in various types of cancer have been reported, reviewed in [26]. Here, we outline some of the earliest and most recent studies. Early work with IL-12 administration in patients with advanced CRC, melanoma, and renal cell carcinoma resulted in only one partial response (renal cell carcinoma) and one transient complete response (melanoma), among 40 enrolled patients. Common signs and symptoms of toxicity such as fever/chills, nausea, vomiting, fatigue, and headache were also observed [77]. In another study, IL-12 resulted in stabilization of disease in several renal cancer patients, and partial regression of a metastatic lesion, but did not proceed in clinical development again due to toxicity [78]. In patients with B-cell non-Hodgkin's lymphoma (NHL), initial trials of IL-12 treatment in combination with rituximab did not result in a clinical response [79]. However, subsequent clinical studies have revealed positive results with IL-12 treatment. In one study, 21% of NHL patients had a partial or complete response without major side effects [80]. Similarly, subcutaneous IL-12 treatment resulted in complete response in 56% of patients with T-cell lymphoma with minor toxicity [81]. Furthermore, clinical trials on metastatic melanoma revealed that IL-12 administration induced tumor shrinkage, accompanied with increased frequency of circulating antitumor CTLs [45]. The poor efficacy of IL-12 in the abovemen-

tioned early clinical trials may be due to several factors, including an immunosuppressive microenvironment in advanced tumors. IL-12 may also self-limit its own therapeutic efficacy by inducing IL-10 and other suppressive factors. For example, IFN-γ induced by IL-12 can activate immunoregulatory molecules, such as programmed death ligand-1 (PD-L1) and indoleamine 2,3 dioxygenase (IDO), in a variety of cells (e.g., DCs, T-cells, and endothelial cells) [82]. Both PD-L1 and IDO can abrogate antitumor immunity through various mechanisms. Other factors, such as environment and diet, may also alter the effectiveness of IL-12-mediated antitumor immunity.

Multiple IL-12 treatment strategies are being pursued to overcome these limitations. Although systemic administration of IL-12 in patients is limited due to toxicity, emerging studies in animal models indicate that IL-12, in combination with other cytokines, can boost antitumor immunity without toxic side effects. Most recently, IL-12 and anti-PDL1 combination has been shown to be more effective to either monotherapy in preclinical tumor models [83]. Combining IL-12 with PD-1-blocking antibody is currently being tested in a phase II study in patients with melanoma (NCT03132675). To overcome toxic side effects, recent clinical trials have been performed with local intratumoral delivery of IL-12 [26]. Along this line, intratumoral injections of adenovirus expressing IL-12 have been tested in patients with advanced stage III and IV melanoma. Clinical responses were observed in 5 out of 7 patients (*NCT01397708*). Thus, selective targeted delivery of IL-12 to tumors, and/or reducing the dose of IL-12 while combining it with other therapeutics, may yield improved outcomes.

13.2.2 IL-27

13.2.2.1 Overview

IL-27 is a member of the IL-12 cytokine family that exhibits potent antitumor activity via different mechanisms, depending on the tumor [84]. Unlike IL-12, IL-27-mediated antitumor functions are more independent of IFN-γ, and IL-27-treated mice do not manifest potent toxic side effects. IL-27 is mainly produced by activated APCs, including DCs and macrophages. DCs secrete IL-27 upon exposure to physiological stimuli, such as type I and type II interferons (INF) and CD40 [85–87]. In addition, IL-27 expression is induced in APCs via stimulation by various TLR ligands, such as poly(I:C), lipopolysaccharide (LPS), and CpG-DNA, which are agonists of toll-like receptor (TLR)3, TLR4, and TLR9, respectively [88–90].

13.2.2.2 IL-27 in Antitumor Immunity

IL-27 performs a wide array of functions necessary for antitumor immune responses. For example, IL-27 has been shown to act on NK cells to enhance their cytotoxic activity both *in vitro* and *in vivo* as described below. Therapeutic administration of IL-27 increases NK cell susceptibility of tumors [91]. By activating NK cells, IL-27 might also enhance adaptive immunity to tumors. The killing of tumor targets by NK cells could in turn provide DCs with increased access to tumor antigens, thereby promoting T-cell responses. In addition to NK cell activation, IL-27 acts on $CD8^+$ T-cells and induces CTLs by enhancing the expression of cytotoxic effector molecules, such as granzyme B and perforin [92]. Similar to mice, IL-27 promotes IFN-γ and granzyme B production from human $CD8^+$ T-cells [93]. In highly immunogenic murine tumor cells, the overexpression of IL-27 facilitates CTL development with enhanced IFN-γ production [94, 95]. In line with these observations, $IL\text{-}27R^{-/-}$ mice fail to regulate tumor growth *in vivo*, reiterating the importance of IL-27 signaling in the generation of antitumor immunity [96]. Recently, DC-derived IL-27 has been shown to induce NK and NKT-cell-dependent antitumor immunity against methylcholanthrene-induced fibrosarcoma and transplanted B16 melanoma [97]. Moreover, IL-27 in combination with other cytokines, such as IL-2 and IL-12, further boosts antitumor immunity by contributing to the development of CTLs and NK cells [98].

In addition to the direct effect of IL-27 on $CD8^+$ T-cell activation, the influence of IL-27 on $CD4^+$ T-cell responses might provide further ther-

apeutic opportunities. Initial studies on IL-27 have indicated that it leads to the differentiation of Th1 cells [99]. IL-27 synergizes with IL-12 to enhance IFN-γ production [100]. Moreover, it has been shown that IL-27 inhibits Th2 polarization of naïve $CD4^+$ T-cells and suppresses Th2 cytokine production *in vitro* [101–103]. By altering the balance between Th1 and Th2 cytokines, IL-27 plays a critical role in antitumor immunity. Accordingly, a recent study has confirmed IL-27's ability to reverse Th2 polarization of *in vivo*-primed lymphocytes from pancreatic cancer patients [104]. IL-27-dependent enhancement of preexisting antigen-specific Th1 responses has also been demonstrated [101]. Furthermore, IL-27 may promote tumor regression through the inhibition of Tregs. IL-27 inhibits the generation of $Foxp3^+$ Tregs both *in vitro* and *in vivo*, possibly by inhibiting IL-2, a cytokine necessary for Treg development [105–107]. Correspondingly, in a murine model of neuroblastoma, IL-27 has been shown to limit IL-2-induced intratumoral Treg expansion, promoting antitumor immunity [95]. IL-27 also induces tumor-specific Ab responses which cooperatively elicit antibody-dependent cell-mediated cytotoxicity (ADCC) activity [108].

13.2.2.3 IL-27 in Angiogenesis Inhibition and Regulation of IL-27 in the Tumor Microenvironment

Similar to IL-12, IL-27 possesses multiple antitumor effects mediated by mechanisms involving angiogenesis and its direct effects on tumors. For example, IL-27 has been found to have antiproliferative activities which inhibit tumor growth and metastasis in murine melanoma [109]. A major antitumor role for IL-27 relies on its antiangiogenic effects in surrounding endothelial cells and fibroblasts. IL-27 significantly inhibits tumor growth in SCID mice through the induction of antiangiogenic factors, such as IP-10 and MIG, from endothelial cells [110]. Similarly, IL-27 has been shown to directly act on human umbilical cord endothelial cells and induce production of these antiangiogenic chemokines [111]. IL-27 also strongly inhibits tumor growth of primary multiple myeloma (MM) [112] cells through angiogenesis inhibition [113]. Along with a concomitant upregulation of the angiostatic chemokines, IP-10 and MIG, IL-27 has been shown to downregulate a wide panel of proangiogenic genes, including matrix metalloproteinase-9 (MMP-9), TGF-β, and VEGF [113]. IL-27 may further promote tumor regression through the inhibition of the proangiogenic cytokine, IL-17. IL-27 suppresses the Th17 key transcription factor, RAR-related orphan receptor gamma (RORγt), inhibiting expression of IL-17 by T-cells in both humans and mice [114]. Accordingly, mice which are deficient in either the IL-27 subunit, EBI3, or deficient in IL-27R, have increased levels of IL-17 [115]. Among the Th17-suppressive molecules found in the tumor microenvironment, IL-27 is one of the most potent inhibitors.

IL-27 can be induced in tumor-infiltrating DCs by galactin-1, IFN-γ, and apoptotic tumor cells in the tumor microenvironment [85, 116, 117]. However, the proangiogenic molecules which dominate the microenvironment in advanced tumors can limit the availability of IL-27. Osteopontin (OPN), a proinflammatory cytokine, inhibits the expression of IL-27 in DCs while inducing Th17 differentiation [85]. OPN promotes tumor growth through mechanisms involving angiogenesis and tumor metastasis [118], suggesting that OPN may release the brake on Th17 cell responses by suppressing IL-27 in DCs. Both OPN and IL-27 are expressed in DCs and macrophages; thus, Th17 accumulation in the tumor microenvironment may depend on the balance of these and other myeloid cell populations.

13.2.2.4 Advantages of IL-27 (over IL-12) in Antitumor Therapy

IL-27-mediated antitumor mechanisms are complex [84, 119]. Similar to IL-12, IL-27 utilizes effector mechanisms of innate and adaptive immunity to mediate antitumor immunity [84]. Specifically, IL-27 promotes tumor immunity through the induction of Th1 and CTL responses while inhibiting immunosuppressive Th2 cells and Tregs [84]. Unlike IL-12, IL-27-mediated antiangiogenic functions are more independent of IFN-γ. Therefore, IL-27-treated mice are not

observed with any toxic side effects [120]. The central role of IL-27 in orchestrating both innate and adaptive arms of immunity, together with multiple antiangiogenic functions, explains the essential contribution of this molecule to the development of antitumor immunity against both high and poor immunogenic tumors. Considering the lack of toxicity observed *in vivo* in preclinical trials with IL-27 treatment, there appears to be meaningful therapeutic potential for this approach. However, to the best of our knowledge, no clinical trials involving IL-27 have yet been pursued.

13.3 Cytokine Regulation of Immune Tolerance to Cancer

Although certain cytokines produced in the tumor microenvironment can function to inhibit tumor growth, others promote tumor progression [17]. Several cytokines have been found to serve as growth and survival factors that act on premalignant cells, stimulate angiogenesis and metastasis, and maintain tumor-promoting immunosuppression and inflammation [17].

13.3.1 TGF-β

13.3.1.1 Overview

Transforming growth factor-β (TGF-β) is a pleiotropic cytokine with broad tissue distribution that plays critical roles during embryonic development, normal tissue homeostasis, and cancer [121]. Elevated TGF-β serum concentrations have been observed in patients with different malignancies and associated with poor prognosis. TGF-β is released by a variety of cells in the tumor microenvironment, including T-cells, macrophages, and DCs, as well as tumor cells themselves [122]. Almost all human cell types are responsive to TGF-β, which signals through type I and type II TGF-β receptors. Upon binding of TGF-β to TGF-βRII, TGF-βRI is recruited and activated to phosphorylate the downstream mediators, SMAD2 and SMAD3. Phosphorylated SMAD2 and SMAD3 combine with SMAD4 to enter the nucleus and modulate gene transcription [123].

The function of TGF-β in cancer is complex; TGF-β can act as a tumor suppressor or promoter depending on the stage of tumor development. Initially, TGF-β acts as a tumor suppressor, since it induces apoptosis and inhibits the growth of normal and premalignant tumor cells [124]. At later stages of tumor progression, TGF-β acts as a tumor promoter. It has been proposed that cancer cells may protect themselves and acquire resistance to TGF-β inhibitory growth signals. In fact, cancer cells eventually start secreting nonphysiological levels of TGF-β in an autocrine and paracrine manner, which may affect the differentiation of tumor cells and the surrounding cellular environment, leading to tumor progression [124]. Notably, TGF-β induces epithelial–mesenchymal transition (EMT), whereby epithelial tumor cells acquire an invasive, mesenchymal-like phenotype accompanied by changes in the expression of cell–cell adhesion molecules and secretion of metalloproteinases, leading to metastasis [125, 126]. In addition to tumor cell-intrinsic growth promotion, the potent regulatory activity of TGF-β on immune cells represents an important mechanism of immune tolerance to tumors. The presence of TGF-β in the microenvironment of the developing tumor disables effective immunosurveillance by multiple mechanisms, most of which converge on the impairment of tumor cell-killing by innate and adaptive immune cells (Fig. 13.2).

13.3.1.2 TGF-β in Innate Immune Tolerance to Tumors

TGF-β is known to compromise antitumor immunity mediated by a variety of innate immune cells. For example, TGF-β is an important regulator of NK cell function, being a potent antagonist of IL-12-induced IFN-γ production by NK cells [127]. In addition, TGF-β inhibits NK cell activity by limiting expression of activating receptors, such as NKG2D, NKp30, and DNAM-1 [128]. In fact, reduced expression of NKG2D is associated with elevated levels of TGF-β in cancer patients [128]. Surface-bound TGF-β on MDSCs has also

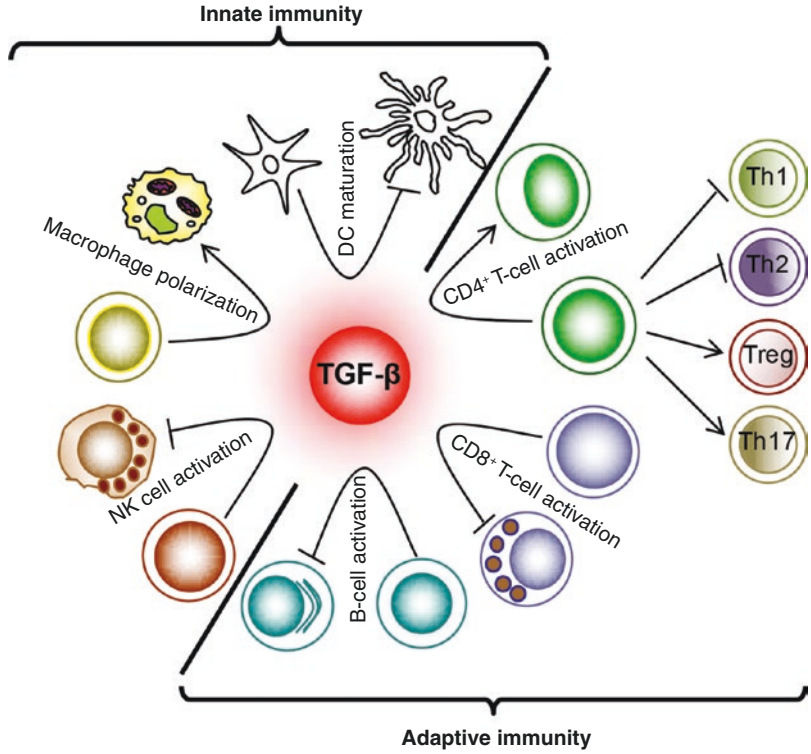

Fig. 13.2 TGF-β-mediated immunosuppression. TGF-β affects components of both innate and adaptive immune systems. TGF-β inhibits NK cell activation and effector functions. In addition, TGF-β inhibits DC maturation and antigen presentation while promoting polarization of M2-like macrophages. TGF-β inhibits $CD8^+$ T-cell-mediated antitumor immune responses. TGF-β also has a significant impact on $CD4^+$ T-cell differentiation and function; TGF-β induces Treg and Th17 differentiation while inhibiting Th1 and Th2 differentiation. Furthermore, TGF-β inhibits B-cell proliferation and antibody secretion

been found to inhibit NK cell cytolytic activity against mammary adenocarcinoma [129, 130]. Moreover, TGF-β suppresses MHC class I and MHC class II expression in a number of cell populations [131–133]. Importantly, the TGF-β-dependent decrease of MHC-I in tumor cells has been shown to result in reduced tumor cell lysis by NK cells [133].

Although NK cells are the major innate effectors, they also require activation by DCs. Numerous reports demonstrate TGF-β impairs DC function both *in vitro* and *in vivo*. Specifically, TGF-β inhibits upregulation of critical co-stimulatory molecules on the surface of DCs, and reduces cytokine production and antigen-presenting capacity [134, 135]. TGF-β can immobilize DCs, thereby interfering with their migration and the transport of antigen to draining lymph nodes for presentation to T-cells. TGF-β can also induce DC apoptosis [136]. In recent years, more correlative clinical data has supported the role of TGF-β and DCs in immunodefects in cancer. Increased serum TGF-β in human colorectal cancer correlates with reduced circulating DCs [137]. Moreover, tumor-infiltrating DCs both secrete and respond to TGF-β, in either an autocrine or a paracrine manner.

In addition to DCs, TGF-β can suppress or alter the activation and function of other innate immune cells such as NKT cells, neutrophils, and macrophages [122]. Macrophages, the predominant form of leukocytes, are key players in tumor growth; however, the role of tumor-associated macrophages (TAMs) in tumors is controversial [138]. TAMs originate from myeloid cells, such as blood monocytes or MDSCs, and are recruited

by a number of chemoattractants produced by tumor and stromal cells. The tumor-derived chemokine, CCL2, is particularly critical for this recruitment [139]. Macrophages can exhibit various phenotypes, characterized by differential cytokine production, when polarized under distinct conditions [140–142]. If stimulated with IFN-γ, M1 macrophages secrete high levels of IL-12, but low levels of IL-10 [140–142]. In contrast, M2 macrophages express high levels of IL-10, but low levels of IL-12 [140, 141]. Each possesses unique functions. For example, IL-12 produced by M1 macrophages can promote the differentiation of Th1 cells, which improves antigen phagocytosis and contributes to antitumor immunity. In M2 macrophages, IL-10 expression can promote the production of IL-4 and IL-13 by Th2 cells, both of which have been shown to impair antitumor T-cell responses [140, 141]. TGF-β pushes tumor-associated macrophage polarization toward an M2 vs. M1 phenotype, which further promotes TGF-β production and deepens immunosuppression [140]. In most tumors, infiltrating macrophages are considered to be of the M2 phenotype. These mostly tumor-supportive TAMs orchestrate various aspects of cancer, such as tumor progression, angiogenesis, metastasis, and immunosuppression. Other innate immune subsets, such as NKT cells, can suppress CTL responses through mechanisms involving TGF-β [122]. Therefore, blockade of TGF-β signaling not only enhances the frequency of antitumor CTLs, but also restores cytolytic machinery and prevents NKT-cell-mediated immunosuppression [143]. In addition, TGF-β has been shown to inhibit effector functions and induce regulatory phenotypes in γδ T-cells during cancer [122, 144]. Across various cell types, such TGF-β-mediated dampened innate immune responses lead to poor adaptive immunity, further resulting in tumor persistence.

13.3.1.3 TGF-β in Adaptive Immune Tolerance to Tumors

The presence of TGF-β in the tumor microenvironment can have a profound impact upon antitumor activity by T-cells. It has been shown that TGF-β can suppress CTL differentiation and CTL-mediated lysis of tumor cells [145, 146]. Specifically, TGF-β acts on CTLs to repress the expression of different cytolytic effector molecules, such as perforin, granzyme A, granzyme B, Fas ligand (FasL), and IFN-β, which are collectively responsible for tumor killing [147]. Blockade of TGF-β in tumor models has been shown to reduce tumor burden by improving $CD8^+$ T-cell-mediated tumor immunity [147]. Correspondingly, we have shown that anti-LAP antibody, which targets the LAP/TGF-β complex on a variety of immune cells, including $CD8^+$ T-cells, enhances antitumor immune responses and reduces tumor growth in multiple preclinical models [148]. Furthermore, TGF-β can suppress IL-2 production and IL-2-induced T-cell proliferation [149]. Tumor cells transfected with TGF-β have been found to attenuate the efficacy of DC-based tumor vaccines [134]. In addition, TGF-β functionally regulates the differentiation of T helper cell subpopulations both *in vitro* and *in vivo*. TGF-β inhibits Th1 and Th2 cells, whereas it promotes Treg and Th17 cell differentiation [150]. Recently, TGF-β has also been shown to play an important role in the development of IL-9-secreting Th9 cells [151, 152].

Although there are many sources of TGF-β in the tumor microenvironment, Tregs provide a significant source of the TGF-β responsible for attenuation of tumor antigen-expanded CTLs [122]. Tregs hamper the functions of Th1 cells, $CD8^+$ T-cells, NK cells, DCs, and other key effector cells of antitumor immunity [122]. Consequently, Treg accumulation and Treg-mediated immunosuppression have been proposed as key mechanisms underlying tumor immune evasion, and therefore are obstacles to successful immunotherapy [71]. The frequency of Tregs present in the peripheral blood of patients with various cancers is measurably higher than that of the normal population [153]. These Tregs, isolated from either peripheral blood or solid tumors, remain suppressive to T-cell activation *in vitro* [154]. Accordingly, Tregs from tumor-bearing mice inhibit tumor rejection, indicating that Treg cells suppress tumor-specific immunity and limit antitumor resistance. In contrast, depletion of Tregs with

anti-CD25 Ab in animal models enhances antitumor immunity and tumor regression. Furthermore, when tumor-specific $CD8^+$ T-cells are adoptively transferred with either Tregs or non-Tregs ($CD4^+CD25^-$ T-cells) into tumor-bearing hosts, $CD8^+$ T-cell-mediated immunity is abolished in those receiving Tregs, but not non-Tregs [72, 155]. Collectively, these studies provide strong evidence that the Treg-TGF-β axis can attenuate antitumor immunity by downregulating antitumor immune responses, ultimately facilitating the development of cancer.

13.3.1.4 TGF-β in Angiogenesis and Treg Promotion, and Treg Inhibition in Clinical Trials

Accumulating evidence suggests that TGF-β promotes tumor growth not only via mechanisms involving immunosuppression, but also angiogenesis. In fact, angiogenesis and tumor-associated immunosuppression are hallmarks of tumorigenesis [5]. This association is related to hypoxia, which induces both angiogenesis and immunosuppression via activation of hypoxia-induced factor 1 (HIF-1) [156]. HIF-1 induces vascular endothelial growth factor (VEGF), which then recruits various proangiogenic bone marrow-derived cells, including endothelial progenitors and myeloid cells [156]. Tregs also migrate to tumors from the periphery following hypoxia-induced chemokines in the tumor microenvironment, specifically chemokine CCL28 [157]. Consequently, forced expression of CCL28 in murine tumor cells results in accelerated tumor growth and Treg accumulation, associated with increased VEGF levels and angiogenesis. In addition, Tregs have been shown to express CCR4, the receptor for CCL22, and can therefore migrate to CCL22 present in the tumor microenvironment [158, 159]. Beyond recruitment of Tregs through chemokines, the TGF-β-enriched tumor microenvironment promotes the continued expansion of Tregs [72].

Within the tumor microenvironment, Tregs are one of multiple cell types with established roles in immunosuppression that have also been shown to promote angiogenesis. Treg accumulation in tumors has been correlated with VEGF overexpression and increased angiogenesis, providing evidence for a link between Tregs and angiogenesis [160, 161]. In fact, Tregs have been shown to contribute to tumor angiogenesis through different mechanisms. For example, they can promote angiogenesis indirectly by suppressing Th1 cells that release the angiostatic cytokine, IFN-γ, as well as interferon-induced chemokines, such as CXCL9 and CXCL10. Furthermore, Tregs can contribute to the direct promotion of tumor angiogenesis through the induction of VEGF and endothelial cell proliferation [161].

Additional therapeutic opportunities may be provided by accounting for these Treg abilities using well-planned manipulations, including Treg depletion, blocking Treg trafficking into tumors, and limiting Treg differentiation and suppressive mechanisms, in combination with current therapeutic approaches. For example, in an early phase I clinical trial in patients with metastatic breast cancer, the anti-CD25 Ab, daclizumab, significantly depleted Tregs and enhanced the immunogenicity of a cancer vaccine [162]. In addition, blocking Treg functions using Abs targeted against glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), and cytotoxic T-lymphocyte antigen 4 (CTLA-4), are under clinical evaluation in cancer patients [163]. In fact, CTLA-4 was one of the first immunotherapeutic antibodies to be approved for the treatment of cancer. Importantly, blocking PD-1-PDL1 interaction with Abs has been shown to promote antitumor immunity in part via Treg inhibition [164]. PD-1-PDL1 blocking antibodies have made great progress in cancer treatment; so far, at least five different monoclonal antibodies targeting PD-1-PDL1 pathway have been approved [165].

13.3.1.5 TGF-β in Clinical Trials

Because of the wide array of effects of TGF-β on tumorigenesis, blockade of TGF-β and its signaling pathways could be a potent approach to improve tumor immunity. Indeed, mice with fully or partially disrupted TGF-β function have severe self-reactive immune responses, suggesting such responses could be harnessed to promote tumor

reactivity [166, 167]. There are numerous TGF-β signaling antagonists under development in both preclinical and clinical stages, as reviewed in [168]. Ongoing clinical trials in cancer patients include monoclonal Abs against TGF-β molecules, or small molecule inhibitors that interfere with TGF-β receptor signaling. Fresolimumab, a monoclonal antibody that neutralizes all isoforms of TGF-β, including TGF-β1, TGF-β2, and TGF-β3, was one of the first monoclonal antibody tested in cancer patients. Fresolimumab was initially tested in malignant melanoma and subsequently in malignant pleural mesothelioma. Of the 29 melanoma patients included in the trial, one patient achieved a partial response and six presented with stable disease [169]. Of the 13 patients with malignant pleural mesothelioma, 3 had stable disease for 3 months [170]. In phase I/II clinical trials, intratumoral administration of AP-12009, an antisense oligonucleotide to TGF-β, resulted in a significant increase of survival time [171]. In addition, a vaccine containing allogeneic tumor cells modified to express antisense TGF-β has been tested in a phase I/II clinical trial. Using this approach, a meaningful response rate of 30% was reported in non-small cell lung carcinoma (NSCLC), with no serious toxicity observed [172]. Galunisertib (LY2157299), a small molecule inhibitor selective for the kinase domain of the type 1 TGF-β receptor, is currently being evaluated in patients with hepatocellular carcinoma and metastatic malignancies (NCT01246986). In addition, a combination therapy combining Galunisertib with anti-PDL1 is also being tested in metastatic pancreatic cancer (NCT02734160).

13.3.2 IL-17

13.3.2.1 Overview

IL-17 is a proinflammatory cytokine produced by Th17 cells [173]. In addition, IL-17 can also be produced by other populations, such as iNKT, $CD8^+$ T, γδ T-cells, and innate lymphoid cells (ILCs) [174–177]. Since Th17 cells produce large quantities of IL-17A, most Th17-mediated effects are attributed to this cytokine. Many factors are required for the induction and stabilization of Th17 cells. TGF-β and IL-6 are the most crucial cytokines for initial Th17 differentiation [178]. IL-6 induces production of IL-21, which subsequently favors Th17 differentiation in an autocrine manner [179]. To maintain the Th17 phenotype *in vivo*, Th17 cells require CD40 and/or TLR ligand-induced IL-23 [180]. Importantly, the differentiation of Th17 cells into IL-17-secreting cells requires the expression of the transcription factor ROR-γt [181]. Many factors released by tumor cells, and molecules secreted by tumor-infiltrating immune cells, such as TGF-β, IL-6, prostaglandin E2 (PGE2), IL-21, IL-23, osteopontin, IL-1β, and TNF-α, can play major roles in the induction of IL-17 [182–185]. Interestingly, Th17 cells are increased in the tumor microenvironment and have been found across patients with different tumors [180]. The association between elevated IL-17 and negative prognoses links increased systemic IL-17 concentrations with cancer development.

13.3.2.2 Th17 Differentiation in the Tumor Microenvironment

There are multiple sources of Th17 cells in the tumor microenvironment. Preexisting Th17 cells can either migrate from the periphery or differentiate from naïve T-cells under the influence of tumor microenvironmental factors. Th17 cells that traffic to tumors do so under the influence of tumor microenvironmental chemokines, such as regulated upon activation normal T-cell-expressed and secreted (RANTES) and monocyte chemotactic protein-1 (MCP-1) [186]. In addition, high levels of the chemokines, CXCL12 and CCL20, further facilitate Th17 cell migration to tumor sites [187]. Once present, Th17 cells can clonally expand following appropriate stimulation, including by tumor-associated macrophages [188]. Th17 cells can also be induced and differentiate in the tumor microenvironment [180]. It has become clear that IL-17-producing Th17 cells and Tregs have overlapping origins. Although TGF-β favors the differentiation of naïve T-cells into Tregs, the simultaneous presence of both TGF-β and IL-6 promotes the dif-

ferentiation of Th17 cells [178]. Given the tight association of TGF-β and IL-6 with tumor incidence and progression, naïve T-cells entering an established tumor are more likely to be exposed to conditions favoring Th17 differentiation [182–185]. Interestingly, upon stimulation with TGF-β and IL-6, $CD8^+$ T-cells not only lose their cytotoxic ability, but are also induced to secrete IL-17 [189]. In contrast to IL-17, IFN-γ expressed by Th1 or $CD8^+$ T-cells inhibits angiogenesis and induces MHC-I in tumor cells, favoring immune recognition and subsequent arrest of tumor growth [190]. Because IL-17 favors angiogenesis and tumor growth, replacing IFN-γ with IL-17 in the tumor microenvironment may have severe consequences for immune recognition and surveillance.

13.3.2.3 IL-17 in Tumor Promotion

Multiple functions of IL-17 contribute to tumor progression. A major tumorigenic role relies on its proangiogenic effects within tumor cells and surrounding endothelial cells and fibroblasts. IL-17-overexpressing human cervical cancer cells and NSCLC cells show greater tumor development in immunocompromised mice compared to control cells with no IL-17 expression, which is thought to be mediated by enhanced angiogenesis [191, 192]. IL-17 overexpression in fibrosarcoma cells also enhances tumorigenic growth in syngenic mice, primarily owing to the proangiogenic activity of IL-17. In fact, Th17 cells levels positively correlate with microvessel density in tumors [191]. By acting on stromal cells and fibroblasts, IL-17 induces a wide range of angiogenic mediators, including VEGF [193, 194]. IL-17 upregulation of VEGF production by fibroblasts promotes fibroblast-induced new vessel formation in the tumor microenvironment [195]. The IL-17-VEGF loop in turn induces TGF-β, another angiogenic factor, followed by additional VEGF-mediated angiogenesis [196]. TGF-β can enhance the VEGF receptivity of endothelial cells by increasing VEGF receptor expression [197]. IL-17 also induces IL-6 and PGE2, and enhances intercellular adhesion molecule (ICAM)-1 expression in fibroblasts, all of which are known to play a major role in angiogenesis and tumor invasion [195]. In addition, IL-17 appears to stimulate the production of IL-8 [198]. IL-8 signaling promotes angiogenic responses in endothelial cells, increases proliferation and survival of endothelial and cancer cells, and potentiates the migration of cancer cells and infiltrating neutrophils at the tumor site. Moreover, IL-17 has been found to induce IL-1β and TNF-α in macrophages, cytokines which can further synergize with IL-17 to activate neutrophil-specific chemokines, thereby recruiting neutrophils to the site of inflammation [199]. Chemokines can stimulate or inhibit proliferation and chemotaxis of the blood vessel endothelial cells which serve the tumor. IL-17 has been shown to selectively enhance the production of angiogenic chemokines such as CXCL1, CXCL5, CXCL6, and CXCL8 from tumor cells and epithelial cells [191, 200]. In addition, IL-17 is also known to inhibit angiostatic chemokine secretion by fibroblasts [191]. Thus, IL-17 may shift the balance between angiogenic and angiostatic chemokines toward a predominance of angiogenic chemokines, in order to enhance net angiogenic activity.

One of the most important mechanisms underlying IL-17 orchestration of inflammation in the tumor microenvironment is through NF-κB, the master regulator of inflammation [201]. IL-17R signaling results in the activation of NF-κB and regulates the activity of several mitogen activate protein kinases (MAPKs) [202], including extracellular-regulated kinase 1 (ERK1), ERK2, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinases [203]. Within tumor cells, IL-17 has been shown to modulate these pathways to promote tumor growth and survival. For example, IL-17R signaling within transformed colonic epithelial cells promotes colon cancer development by inducing NF-κB and MAPKs [204]. Accordingly, blocking IL-17 activity with Abs ameliorates both colitis-associated and sporadic colon cancer [205, 206]. While IL-17-mediated cytokine expression is regulated by NF-κB, the same cytokines can further stimulate NF-κB-mediated transcription themselves in tumor cells and tumor-associated stromal cells, thereby creating a sustained chronic

inflammatory state within the tumor microenvironment. In support of this notion, enhanced cervical cancer growth elicited by IL-17 has associated with increased IL-6 and macrophage recruitment to tumor sites [192]. Therefore, IL-17 might also function through IL-6 to promote tumor development. Correspondingly, IL-17-induced IL-6 has been shown to promote tumor growth via STAT-3 activation [207].

13.3.2.4 IL-17 in Antitumor Immunity

Although IL-17 seems to be a potential tumor-promoting cytokine, a considerable number of reports have described tumor-inhibitory effects of IL-17. Th17-polarized cells have been found to appear more effective than Th1 cells in eliminating large established tumors [208]. However, Th17-mediated antitumor responses are highly dependent on IFN-γ-based mechanisms; the effects of Th17-polarized cells are completely abrogated by the administration of IFN-γ-depleting Abs, but not by IL-17- or IL-23-depleting Abs. Nonetheless, more clear antitumor IL-17 functions have been documented. Adoptively transferred IL-17-secreting $CD8^+$ T-cells enhance antitumor immunity, resulting in regression of B16 melanoma [209]. In addition, IL-17 has been shown to inhibit the growth of hematopoietic tumors, such as mastocytoma and plasmacytoma, by enhancing CTL activity [210]. Different mechanisms have been proposed for the IL-17 enhancement of tumor-specific CTLs. IL-17 has been shown to induce IL-6 from a variety of cells. Moreover, IL-17 stimulation can induce IL-12 production from macrophages [211]. Both IL-6 and IL-12 have been associated with the induction of tumor-specific CTL [44, 212]. In addition, IL-17 promotes the maturation of DC progenitors, as indicated by increased expression of co-stimulatory molecules, MHC-II antigens, and allostimulatory capacity [213]. This may lead to further improvement in T-cell priming by tumor cells producing IL-17. Indeed, IL-17-transduced fibrosarcoma cells induce tumor-specific antitumor immunity by augmenting the expression of MHC class I and class II antigens [214]. Another recent demonstration studying IL-17-deficient mice, rather than exogenous IL-17 in established mouse lines, shows tumor growth in subcutaneous and lung tumor metastasis is enhanced by IL-17-deficiency [215]. This effect is accompanied by reduced IFN-γ levels in tumor-infiltrating NK cells and T-cells.

The evidence reviewed here demonstrates that IL-17-secreting cells can either stimulate or inhibit tumor growth and progression. The beneficial effects of IL-17 on upregulating host immune responses may be present early in inflammation, but appears to be eventually overcome by increasing tumor burden. This shift from beneficial inflammatory functions of IL-17 likely depends on the tumor type and inflammatory mediators in the tumor microenvironment.

13.3.3 IL-23

13.3.3.1 Overview

IL-23 is a heterodimeric protein composed of two subunits: IL-23p19 and IL-12p40 [216]. IL-23 is secreted by activated DCs and macrophages. Binding of IL-23 to the IL-23R complex, composed of IL-12Rβ1 and IL-23R, marks the beginning of the IL-23 signal-transduction cascade [217]. Because IL-23 plays an important role in bridging innate and adaptive responses, it has been described as a key cytokine promoting inflammation in peripheral tissues. The activity of IL-23 in the regulation of tumor immunity is just beginning to be elucidated [218].

13.3.3.2 IL-23 in Tumor Promotion and Inhibition

Despite belonging to the IL-12 family, IL-23 performs both pro- and antitumor functions. IL-23 is spontaneously produced by TAMs in several murine tumor models. Tumor-secreted PGE2 enhances the production of IL-23 and IL-1β in macrophages and DCs while downregulating IL-12 production [56, 219, 220]. Following this pattern, IL-12 production is decreased, and IL-23 increased, in tumors [221]. Together with IL-23, PGE2 favors the expansion of human Th17 cells

from PBMCs; PGE2 also enhances IL-17 production from memory $CD4^+$ cells induced by IL-23 [185]. Although IL-23 is not necessary in the initial differentiation of Th17 cells, it is crucial for the function, survival, and propagation of this T-cell population in the inflamed environment [173]. In contrast to the antitumor role of IL-12, IL-23 promotes inflammatory processes, including matrix metalloproteinase expression and angiogenesis, and reduces CTL infiltration and function, thus contributing to tumor growth [222]. Indeed, mice lacking IL-23/p19 are completely resistant to carcinogen-induced tumors. The lack of cancer in these mice correlates with the absence of various markers indicative of tumor-associated inflammation, including IL-17, GR-1^+, and $CD11b^+$ myeloid cells [222]. Recently, tumor-secreted lactic acid has been shown to activate the IL-23/Th17 pathway [183].

In contrast, IL-23-overexpressing tumors show reduced growth and metastasis [223–226]. These antitumor effects of IL-23 have been found to be mediated through enhancement of $CD8^+$ T-cell responses. In addition, intratumoral injection of IL-23-overexpressing DCs results in a similar phenotype [225]. Artificial overexpression of IL-23 could induce potent antitumor immunity through various mechanisms. IL-23 can mediate myeloid infiltration, including DCs, macrophages, and granulocytes, which instead may contribute to the inhibition of tumor growth and boost immune reactions to immune-sensitive tumors. In addition, overexpression of IL-23 is likely to increase systemic IL-23 levels that could in turn lead to the growth and survival of memory $CD8^+$ T-cells.

13.3.4 IL-35

13.3.4.1 Overview

IL-35 is a recently discovered IL-12 family cytokine composed of an IL-12 p35 subunit and an IL-12 p40-related protein subunit, EBI3 [216]. Not constitutively expressed in tissues, IL-35 is produced mainly by Tregs and DCs. IL-35 induces the transformation of $CD4^+$ effector T-cells into Tregs, which in turn express IL-35 (Treg35 cells) but lack the expression of conventional Treg marker, Foxp3 [227]. Treg35 cells generated *in vitro* can prevent the development of autoimmunity in various mouse models [228–231]. Most recently, it has been shown that human Tregs express and require IL-35 for maximal suppressive function. Substantial upregulation of EBI3 and IL-12A, but not IL-10 and TGF-β, has been observed in activated human Tregs compared with conventional T-cells [232].

13.3.4.2 IL-35 in Immune Tolerance to Tumors

Evidence of the role of IL-35 in immune tolerance to tumors is beginning to emerge. The IL-35 subunit, EBI3, is expressed in Hodgkin's lymphoma cells, acute myeloid leukemia cells, and lung cancer cells [233–235]. Small interfering RNA silencing of EBI3 in lung cancer cells inhibits cancer cell proliferation, whereas stable expression of EBI3 confers growth-promoting activity *in vitro* [235]. Accordingly, high EBI3 expression in human lung cancer cells is associated with poor prognosis [235]. Recently, IL-35-secreting Ag-specific Tregs have been observed in patients with prostate cancer [236]. A number of studies have demonstrated the functional role of Treg-derived IL-35 in limiting antitumor T-cell responses. For example, *in vitro*-generated Treg35 cells accelerate the development of B16 melanoma and prevent the generation of antitumor $CD8^+$ T-cell responses [227]. In addition, T-cells that secrete IL-35 and have suppressive functions can be induced in the tumor beds of melanoma and colorectal adenocarcinoma [237]. Blockade of this IL-35 relieves suppression mediated by Tregs [237]. Similarly, forced expression of IL-35 leads to significantly increased tumorigenesis in mice. This IL-35 upregulation increases the number of $CD11b^+Gr1^+$ myeloid cells in tumors, which promotes angiogenesis, and also renders tumor target cells more resistant to CTL destruction [237]. Most recently, neutralization with IL-35-specific Abs, or Treg restricted production of IL-35, has been shown to limit tumor growth in multiple models of human cancer [238].

13.3.5 IL-10

13.3.5.1 Overview

IL-10 is an important immunoregulatory cytokine produced by many cell populations. Due to its role in inhibiting the production of IL-2 and IFN-γ by murine and human Th1 cells, IL-10 was initially named as a cytokine synthesis inhibitory factor [239]. The function of IL-10 in cancer is enigmatic. Depending on the experimental model, IL-10 displays both immunosuppressive and immunostimulating activities. On the one hand, IL-10 promotes an antitumor CTL response leading to tumor regression; however, IL-10 can also induce immunosuppression and assist in escape from tumor immune surveillance, promoting tumor growth.

13.3.5.2 IL-10 in Immune Tolerance to Tumors

The cellular sources of IL-10 include Th2, Treg, Tr1, and Th17 cells; however, cytotoxic $CD8^+$ T-cells can also produce IL-10, as can some subsets of DCs, macrophages, B-cells, granulocytes, mast cells, keratinocytes, and epithelial cells. In addition, various cancer cells produce IL-10; among those are multiple myeloma, melanoma, human colon carcinoma, lung cancer, oral squamous cell carcinoma, renal cell carcinoma, non-Hodgkin's lymphoma, and chronic lymphocytic leukemia [240]. Circulating concentrations of IL-10 have been found to be raised in patients with different cancer types, and associated with adverse disease stages or with negative prognoses. For example, serum levels of IL-10 positively correlate with clinical disease progression in patients with metastatic melanoma, as well as colon cancer [240]. In addition, preoperative serum levels of IL-10 predict the likelihood of colon cancer recurrence [240, 241]. IL-10 can be induced and sustained in the tumor microenvironment by a variety of cytokines. Macrophage-derived IL-6 has been shown to induce production of IL-10 by cancer cells. Similarly, IL-6, in association with TGF-β, can induce IL-10 production in Th17 cells. However, TGF-β alone can induce IL-10, allowing IL-10 to enhance the expression of TGF-β in a positive feedback circuit. While promoting inflammatory responses in macrophages and monocytes, TNF-α also upregulates IL-10 as negative feedback, thereby terminating the inflammation [242]. In addition, IL-12 and IL-27 can induce IL-10 production from T-cells [114, 239].

IL-10 can act as a negative regulator in the crosstalk between innate and adaptive antitumor immunity (Fig. 13.3): One of the major mechanisms by which cancer cells escape immune attack is by avoiding detection [243]. IL-10 has been shown to prevent NK and $CD8^+$ T-cell detection of tumor antigens. IL-10 inhibits NKG2D ligand expression on tumor cells and suppresses cytotoxicity mediated by NK cells. In addition, IL-10 induces HLA-G molecules that prevent attack by NK cells [244]. IL-10 pretreatment can also convert tumor cells to a CTL-resistant phenotype by decreasing the expression of HLA class I molecules on their surface [245]. These changes allow tumor cells to survive from immunological attack by NK cells and to grow exponentially.

IL-10 acts on DCs and macrophages to inhibit the differentiation and antigen-presenting properties of these cells. Specifically, IL-10 inhibits essential steps required for immune detection, such as the expression of HLA-DR, and the costimulatory molecules, CD80 and CD86, on DCs. IL-10 also prevents the production of the Th1-polarizing cytokines, IL-12 and IFN-γ, from DCs [246]. Administration of IL-10 before and immediately after DC cancer vaccine results in immune suppression and tumor progression, in line with the predominantly inhibitory activity of IL-10 on DC-mediated antigen presentation [74]. Accordingly, we have shown that IL-10-deficient DCs are more effective at inducing protective antitumor immune response in mice [74]. Exposure of DCs to tumor cell lysates results in increased IL-10 production and expansion of regulatory Tr1 cells. Tr1 cells have been shown to downmodulate immune responses through the production of IL-10 [247]. In addition, IL-10 has been shown to mediate the immunosuppressive activity of Tregs. Therefore, DCs that encounter

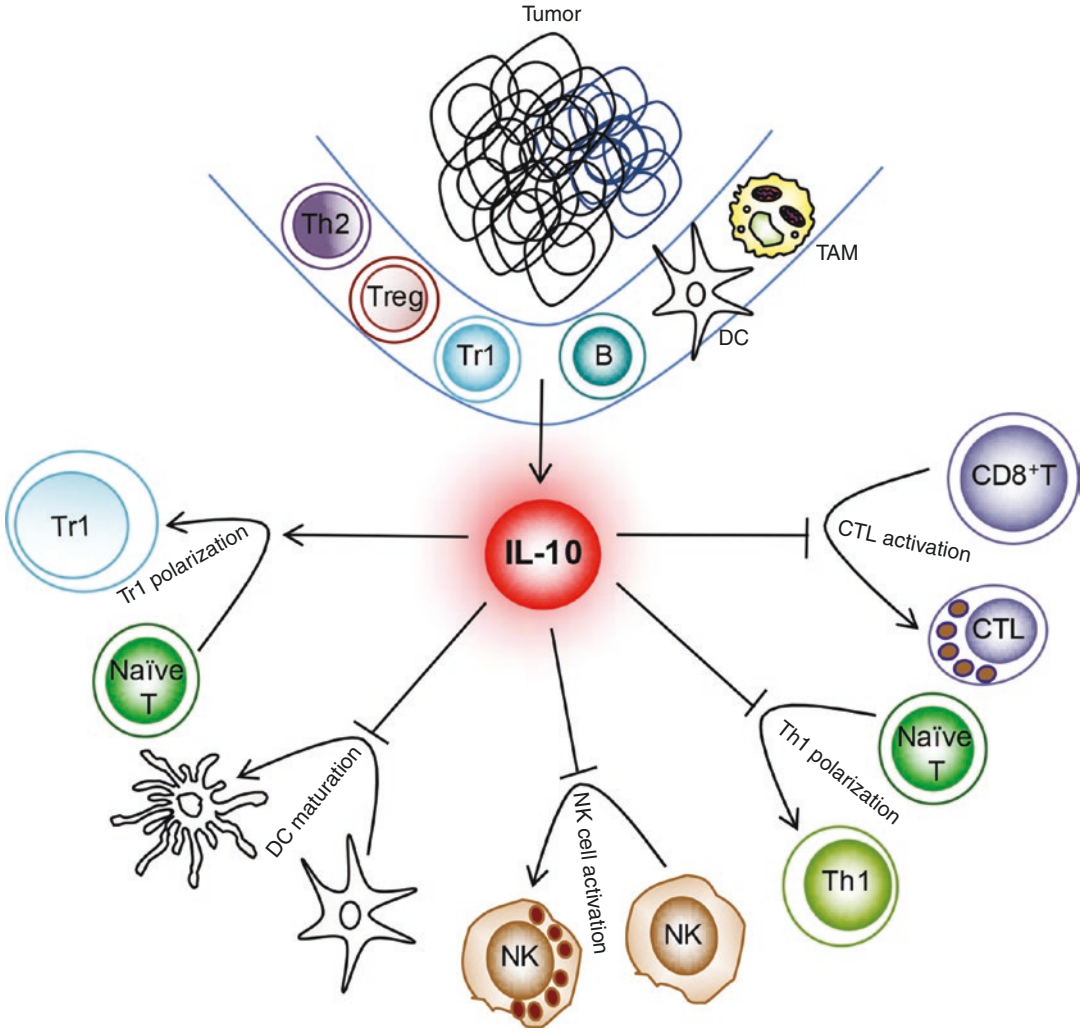

Fig. 13.3 IL-10-mediated tumor immunosuppression. IL-10 can be induced in the tumor microenvironment by many cell types, including Th2 cells, Tr1 cells, Tregs, DCs, TAMs, and tumor cells. IL-10 has a multitude of suppressive effects on the antitumor immune response. For example, IL-10 can inhibit the maturation of DCs and disrupt the differentiation of CTLs and Th1 cells. IL-10 can also inhibit the cytolytic activity of NK cells. On the other hand, IL-10 can promote tumor growth through the promotion of IL-10-producing Tr1 cells

tumor antigens in the presence of IL-10 *in vivo* acquire tolerogenic properties and subsequently induce T-cell tolerance to tumor antigens. In addition, IL-10 significantly suppresses other inflammatory cytokines such as IL-1β, IL-6, and TNFα, in DCs. Consistent with these observations, inhibition of IL-10 production by T-cells or malignant cells using anti-IL-10-/IL-10R-blocking Abs, or anti-IL-10 antisense oligonucleotides, improves antitumor immune responses in animal models [240].

13.3.5.3 IL-10 in Antitumor Immunity

Data from experimental models suggest that IL-10 may also possess some immunostimulating and antitumor properties. For example, overexpression of IL-10 in tumor cells leads to the loss of tumorigenicity, concomitant with increased immunogenicity accompanied by a strong antitumor immune response. IL-10 has been shown to increase $CD8^+$ T-cell numbers, IFN-γ secretion, and cytotoxicity in established tumors. Accordingly, overexpression of IL-10 in tumor

cells transplanted in mice leads to tumor rejection [246, 248]. Such observations suggest that IL-10 might maintain the number of antigen-specific CTLs. Therapeutic administration of recombinant IL-10 induces antitumor immunity against fibrosarcomas in mice [249]. However, as previously mentioned, higher expression of IL-10 correlates with tumor progression and metastasis in patients with cancer, indicating that IL-10 production in the clinical setting is detrimental [240]. To conclude, the pleiotropic activity of IL-10 on different immune cell populations and the variability of cancer models used to address the role of IL-10 in tumor immunity are likely responsible for the controversial findings reported in the literature.

13.4 Concluding Remarks

Coordinated, effective development of both innate and adaptive antitumor immune responses is necessary to keep cancer at bay. While certain cytokines produced in the tumor microenvironment can function to limit tumor growth, others can promote tumor progression. A more thorough understanding of tumor-cytokine and immune cell interactions in the tumor microenvironment, and thoughtful manipulation of the balance of pro- vs. anti-tumor cytokines, may pave the way for more effective cancer immunotherapeutic strategies.

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14

Role of Chemokines and Chemokine Receptors in Cancer

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14.1 Introduction

Living tissues are highly organized and dynamic structures at the cellular level. Tissue renewal, remodeling, and repair, immunosurveillance, and cell-to-cell interaction and communication are examples of physiological processes relying on the fine recruitment and displacement of numerous cell types. This equilibrium is strictly dependent on the principle of "recruiting the right cell at the right place and the right moment." One major component of this principle is the chemokine and chemokine receptor system. Chemokines (CKs) for chemoattractant cytokines are small, secreted molecules historically defined on the basis of their functional chemotactic activity [1–3]. They constitute a family of over 50 members which interact with about 20 defined corresponding/cognate receptors (CKRs). This discrepancy highlights the complexity of this system as several CKs can bind to a single receptor. Conversely, one receptor can bind several different CKs. This redundancy associated with differential avidity of the CK for their CKR and the specific expression by the different cell population contributes to the fine-tuning of cell migration (Fig. 14.1) and explains that a modest deregulation of the system can lead to severe pathological conditions. In addition, there is overwhelming evidence describing alternative functions of the CK/CKR couple in hematopoiesis, reproduction, angiogenesis, and immune-associated functions such as cell activation, proliferation, effector function, and

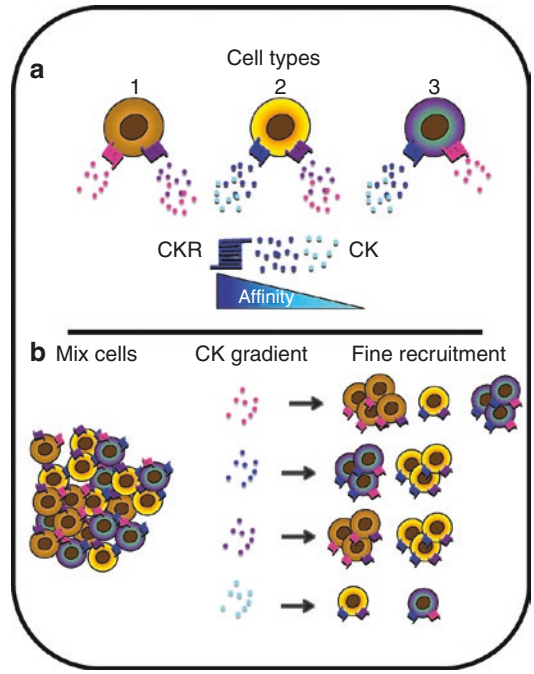

Fig. 14.1 Fine modulation of cellular recruitment by chemokines. The chemokine network is organized around several levels of complexity. (**a**) Most of the cell types (1, 2, 3) express several chemokine receptors, and a same receptor is found on several cell types. Moreover, different chemokines can bind to a same receptor, and most of the receptors can bind several chemokines with distinct affinity (color gradient represents differential affinity). This apparent complexity allows for the fine control of cell population recruitment. (**b**) The schematic representation illustrates the selective recruitment of cell populations according to the respective colored CK gradient. The number of cells recruited is related to the affinity of the respective CK for its receptor

survival [4, 5]. Numerous reports from the past two decades have validated the importance of the CK/CKR network with its diverse range of physiological properties and its involvement in various physiopathological disorders [6–8].

Cancer constitutes a very complex pathology in many aspects. Neoplastic cells result from the environmental, viral-induced, or inherited deregulation of genes known as "oncogenes" or "tumor suppressor genes." This primary modification often leads to uncontrolled expansion of undifferentiated cells for which the transcriptome and the proteome are highly modified in comparison with the original cell. Nevertheless, it is important to note that tumor development does not result from the simple expansion of neoplastic cell. Indeed, solid tumors (primary tumor as well as metastasis) are also constituted by a wide variety of stromal cells. The stroma is composed of nonhematopoietic cells, such as "healthy" cells of the affected tissue, fibroblasts, or endothelial cells, as well as hematopoietic cells. Hematopoietic cell populations are mainly composed of innate immune cells, such as tumor-associated macrophages (TAMs), dendritic cells (DCs), natural killer cells (NK cells), neutrophils, and partners of the adaptive immune response such as T- and B-lymphocytes.

The relative importance of the stroma compared to tumor cells depends on the type of cancer [9], but it is now well described that several stromal cells are important predictive markers of cancer evolution (macrophages, regulatory T-cells (Treg cells), and endothelial progenitor cells). Even though the stroma cannot be characterized properly in circulating hematological tumors, leukocytes will have an important impact on the expansion, survival, and potential homing of tumor cells to the specific tissue. This phenomenon is distinguishable from the metastatic process where the tumor cells need to cross the endothelial barrier from a primary tumor site and home to a distant tissue. The stroma contributes to the global organization and progression of the tumor known as "tumor microenvironment" through the production of growth factors, cytokines, and CKs, exchange of nutrients, and tissue remodeling and repair. In contrast, immune cells are responsible for the control of tumor growth. The concept of immunosurveillance proposed by Burnet et al. [10] in the early 1970s has been widely debated. Recently, Schreiber and colleagues provided experimental evidence for the clinical emergence of cancer as a result of strong selection and modeling of tumors by the immune system in a process termed as "tumor editing" [11]. In this process, neoplastic transformation occurs, and tumor cell expansion is detected by the innate and adaptive immune systems, which either succeed in complete tumor elimination or maintain a state of equilibrium between tumor cell expansion and elimination. This phase leads to the immune selection of tumor cell variants that develop immune resistance and immunosuppressive mechanisms resulting in tumor escape and cancer progression to a clinical outcome.

Cancer is a complex process whereby undifferentiated tumor cells expand locally in specialized tissues, migrate in an active manner by leaving the primary tumor site through the endothelial barrier, establish in a distant and different specialized tissue, and finally generate metastases. Inflammation generated by neoplastic transformation contributes to the recruitment of protumoral population and the production of growth factors as well as the recruitment of immune component with antitumor activity. Thus, tumorigenesis is a dynamic process involving important tissue remodeling and angiogenesis, recruitment and local migratory mechanisms, and survival and cell death for both tumor and stromal cells in which the CK/CKR network has major implication.

The CK/CKR network appears to be a promising target in cancer therapy and has already been used in standard therapeutic approaches, as well as in immunotherapy. Numerous basic and clinical interventions rely on the development of agonist or antagonist CKR in order to manipulate their critical biological function toward antitumor activity.

In this chapter, the role of the CK/CKR network in these aspects of cancer development, as well as its potential application in the improvement of cancer therapy, is described in detail.

14.2 Chemokines and Chemokine Receptors

Chemokines are small cytokines initially described for their chemotactic properties on leukocytes. During cell recruitment from the blood to inflamed tissues, CKs initiate the activation of circulating cells, promoting cell rolling, adhesion to activated endothelium, and extravasation (Fig. 14.2). In tissues, CKs determine cell directional migration, by establishing a concentration gradient (Fig. 14.3). Evidence from previous studies has shown that the control of cell mobility by CKs is implicated in developmental mechanisms and cell homeostasis, as well as in the induction and tuning of acute and chronic inflammation and control of the immune response. Numerous reviews have extensively described the CK classification, structural organization, and their associated biological properties [12, 13]. CKs are subdivided in four subfamilies based on the number of and spacing between conserved cysteines in the primary amino acid sequence [14]. CKRs are seven-transmembrane G-protein-coupled receptors classified according to the CK family they bind. As previously mentioned, most CKs bind to several receptors, and most of the receptors can bind several CKs with different affinities. Additionally, one cell subset can express different CKRs, and the

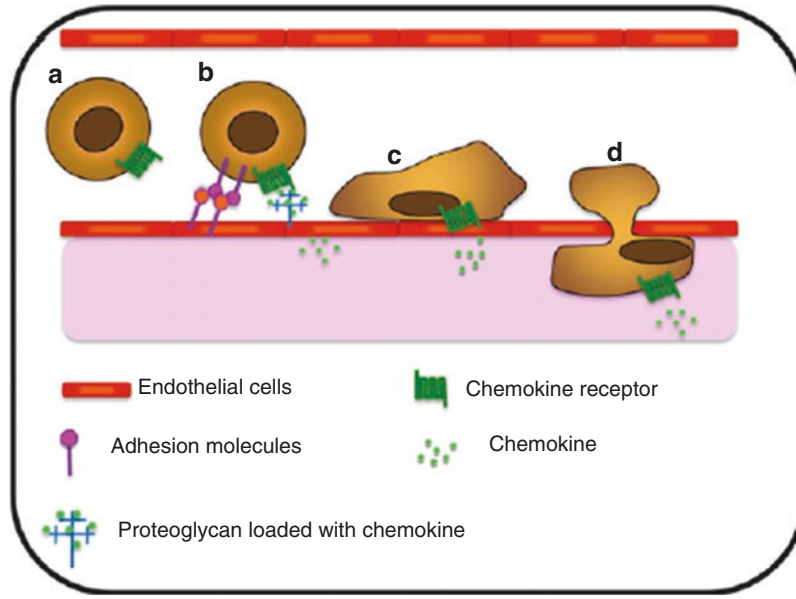

Fig. 14.2 Chemokine-associated extravasation process. (**a**) Circulating cell within the bloodstream. (**b**) Chemokine presented by proteoglycan on activated endothelial cells induces the expression of adhesion molecules implicated in the slow rolling and the capture process. (**c**) Once stuck to the endothelium, cell exerts crawling behavior on the luminal side of the blood vessel and (**d**) extravasates and migrates through the tissue toward a chemokine gradient

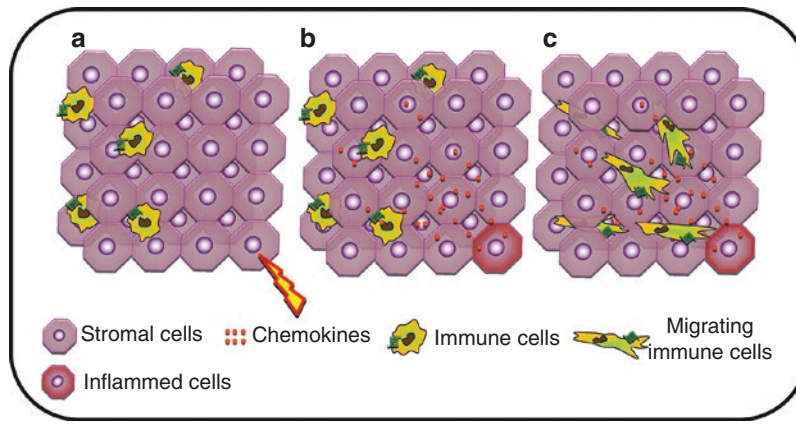

Fig. 14.3 Interstitial migration. (**a**) Upon activation, (**b**) stromal cells will produce chemokines forming a gradient within the tissue. (**c**) Tissue-infiltrated immune cells will migrate through the tissue toward the higher concentration of chemokine

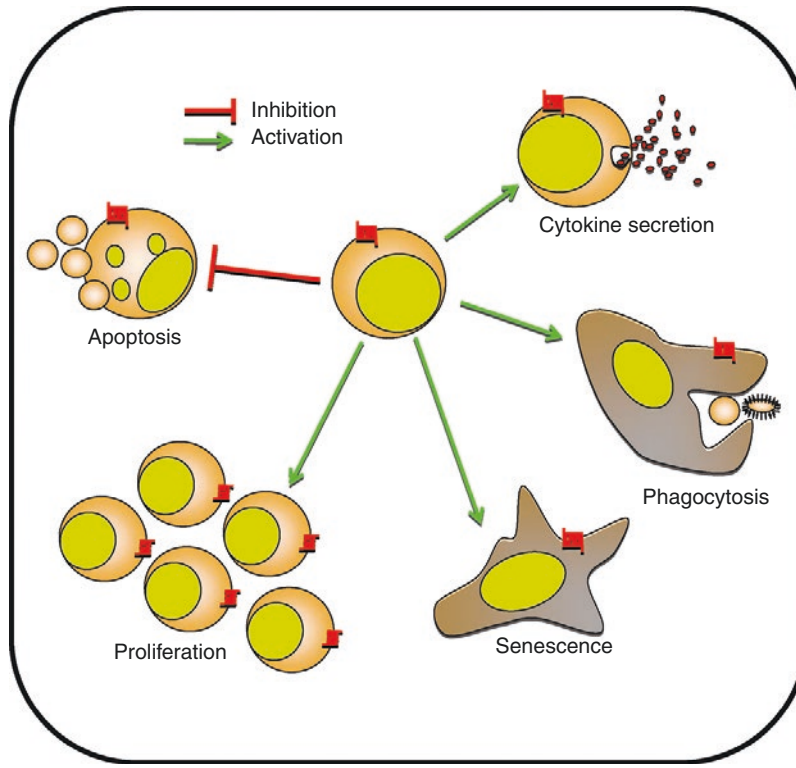

Fig. 14.4 Control of cell biology by chemokines. Besides cell migration, chemokines are implicated in multiple cellular functions including apoptosis, proliferation, and senescence. Chemokines are also directly implicated in cell activation, cytokine secretion, or phagocytosis

same CKR is expressed by different cell subsets. This apparent redundancy is in reality a tool to tightly regulate leukocyte, stem cell, and other cell types' migrations during physiological and pathological conditions.

It is now well established that CK function is not limited to cell migration. It has been clearly demonstrated that CKs directly control cell proliferation, survival, and senescence, as well as cytokine secretion and phagocytic properties (Fig. 14.4). It is the balance between these migratory, secretion, phagocytic, survival, and proliferation signals which explains the central roles of CK in development, tissue homeostasis, repair, inflammation, and immunity.

14.3 Control of Tumor Cell Behavior

The biological property controlled by the CK/CKR recognition system is not restricted to chemotactism. Several important processes involved in the behavior of tumor cells will be affected by these axes. In this section, the effect of CK/CKR expression on tumor cell behavior and cancer progression is discussed.

14.3.1 Chemokines and Chemokine Receptor Alterations During Neoplastic Transformation

Primary neoplastic transformation leads to strong modification of the transcriptome and proteome which is mainly shaped by immune selection of resistant tumor variants. CK and CKR are not oncogenes per se; however, modulation in the production of CKs or their receptors by tumor cells is often the result of oncogenic modifications and immune selection (Fig. 14.5). The first evidence came from a human papillary thyroid cancer. The authors showed that RET (rearranged during transfection) tyrosine kinase rearrangement promotes the secretion of numerous inflammatory cytokines, including CCL2, CCL20, and

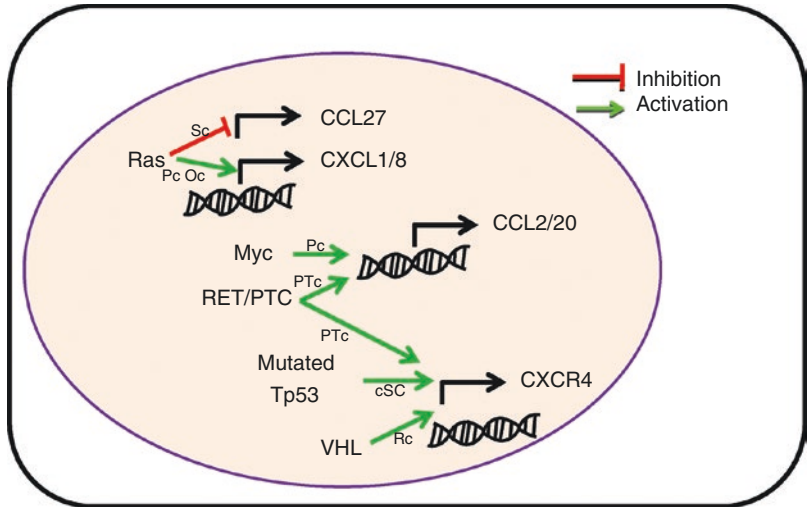

Fig. 14.5 Oncogenes induce altered chemokine and chemokine receptor expression by tumor cells. Common oncogene mutations are associated with modification of chemokine or chemokine receptor transcription, resulting in tumor promotion. *RET/PTC* rearranged RET tyrosine kinase, *VHL* Von Hippel-Lindau tumor suppressor gene, *Sc* skin cancer, *Pc* pancreatic cancer, *Oc* ovarian cancer, *HPTc* human papillary thyroid cancer, *cSC* cancer stem cell, *Rc* renal cancer

CXCL12, and increases the expression of CXCR4 [15]. Later studies have shown that Myc overexpression in pancreatic cancer has been associated with increased CK expression [16, 17]. Nevertheless, the predictive outcome of oncogenic modifications on the regulation of CK and CKR expression is difficult to assess. While RAS-RAF signaling pathway promotes CXCL8 and CXCL1 transcription in pancreatic and ovarian cancer, it inhibits CCL27 transcription in skin cancer [18–20]. Similarly, Von Hippel-Lindau tumor suppressor mutation in renal cancer [21] and TP53 mutation in cancer stem cells promote CXCR4 expression [22] while downregulating its expression in breast cancer cells [23].

Through modification in the profile of CKR expression, tumor cells will change their sensitivity to the microenvironment and acquire new migratory and homing capabilities.

14.3.2 Metastasis/Homing

The metastasis index is undoubtedly the major factor of prognosis and determines the therapeutic attitude. Metastasis defines the process through which tumor cells leave a primary site to settle in a distant location and create a new colony. This phenomenon is characteristic of tumor malignancy including tumor invasion, intravasation, and homing to different sites. This has to be distinguished from the potential secondary localization of circulating tumor cells which only involves the homing mechanism.

14.3.2.1 Tumor Invasion

The first step of metastasis spreading relies on either tumor cell- or stromal cell-mediated fibrosis activity and the ability of tumor cells to acquire migration and intravasation capabilities, in order to leave the primary tumor site and reach the bloodstream. Chemotaxis of tumor cells is well characterized [24]. This process requires a paracrine loop between tumor cells and stromal cells, such as macrophages shaping the microenvironment to favor metastasis [25]. Different chemical gradients may induce tumor cell chemotaxis, but the direct implication of CKs in this specific process is poorly documented. We can distinguish the indirect contribution of CK to the chemotaxis activity of cancer cells through angiogenesis, fibrogenesis, and matrix remodeling mediated by stromal cells.

CXCL12/CXCR4 is the major axis directly involved in tumor cell metastases. Overexpression of CXCR4 in rat mammary adenocarcinoma enhances the motility of tumor cells in the primary tumor [26]. This receptor is widely involved in the epithelial-to-mesenchymal transition (EMT) process, which is a major step leading to metastasis [27, 28]. Few studies have reported the implication of other CKs and CKRs such as CCL18, CCL2, or CXCR7 [29–31] through the activation of EMT-implicated signaling pathways. Interestingly, the integration of multiple CKR axes adds complexity to the tumor invasion process. Indeed, overexpression of CXCR4 promotes invasion. However, coexpression of CXCR7 which binds the same ligand CXCL12 impairs invasion but favors angiogenesis and primary tumor growth [26]. IL8/IL8R axis might also favor maintenance of the mesenchymal status of the tumor cell [32]. IL8 binding to CXCR1 or CXCR2 has been shown to promote tumor cell migration and EMT transition via inflammatory mediators and activation of MAPK/ERK-NF-kappaB in head and neck squamous carcinoma [33] and AKT signaling in renal cell carcinoma [34].

14.3.2.2 Homing

Once in the bloodstream, the tumor cell needs to migrate to a site that will allow its engraftment, survival, and proliferation. In 2001, Muller et al. demonstrated for the first time that the expression of specific CKRs by tumor cells could predict the implantation of malignant cells in tissues expressing high levels of the receptor ligands [35]. Since then, several other studies have established associations between metastases, CKR expression, and implantation sites for various cancer types (Table 14.1). Consistently with their homeostatic functions, CCR7 expression by tumor cells is associated with lymph node (LN) metastases; CCR10 with skin metastasis; CX3CR1 with brain, liver, and bone metastases; CCR9 with intestine metastases; and CXCR4 with bone and liver metastases [35–38].

Overall, these observations show that CK axes generate a complex relationship between tumor cell and the environment and deserve further attention in preclinical studies as it represents an important target with clinical application.

14.3.3 Senescence, Proliferation, and Survival

Tumor expansion results in the capacity of tumor cells to proliferate infinitely without developing senescent mechanisms. Several CKs have demonstrated the ability to activate signaling pathways in favor of this goal.

Cellular senescence is generally defined as an irreversible state of G1 cell cycle arrest in which the cell is refractory to growth factor stimulation. Activation of CXCR2 by either CXCL1 or CXCL8 can result in senescence induction [39]. CXCR2 activation is thus able to act as a suppressor of malignancy in prostate and breast cancer [40, 41].

Table 14.1 Metastasis implantation of various cancer types based on their chemokine receptor expression

		Primary tumor				
		Melanoma	Breast cancer	Non-small cell lung cancer (NSCLC)	Colon cancer	Leukemia
Metastases implantation site	Skin		CCR10/CCR7/CXCR4			
	Intestine	CCR9				
	Bone		CX3CR1			
	Lymph node		CXCR4/CCR7	CCR7	CXCR3	CCR7
	Liver		CXCR4	CX3CR1	CCR6	
	Brain	CCR4	CX3CR1	CX3CR1/CXCR4	CXCR4	CCR7
	Lung	CXCR4/CXCR2				

Inhibition of tumor proliferation by CXCR2 ligand is probably limited to tumor models and to early stages of tumor development. Indeed, the same CK axes display opposite effects in other tumor models. CXCR1 and CXCR2 activation by CXCL8 promotes the proliferation of gastric cancer, esophageal cancer, non-small cell lung cancer, and melanoma cell lines [42–45]. Other receptors of the CXC receptor family are involved in tumor cell proliferation. CXCR6 is involved in cell proliferation of pancreatic cancer cells [46], and CXCR4 is associated with tumor proliferation in numerous models, including ovarian, melanoma, glioma, renal, lung, and thyroid cancer cells [27, 47]. Few studies have investigated the implication of CCRs in the control of tumor cell proliferation. CCR6 favors colon tumor cell proliferation upon CCL20 activation [48], and CCR9 favors pancreatic cancer cell proliferations upon CCL25 activation [49].

Another role of CK in tumor cell biology is the ability to control tumor cell survival, essentially mediated through the CC receptor family. CCR10 activation promotes phosphatidylinositol-3-kinase-mediated protection from apoptosis of melanoma cells [50]. The same mechanisms are observed in squamous cell carcinoma of the head and neck after CCR7 activation [51]. CCR7 engagement by CCL21 is also implicated in the prevention of apoptosis in NSCLC, through ERK-dependent activation pathways [52].

CK direct promotion of tumor cell survival is not limited to CC chemokines; CXCL12 through CXCR4 activation promotes hepatoma, ovarian cancer, and chronic leukemia tumor cell survival [53], and CXCR7 activation increases cell survival by reducing apoptosis [54].

Overall, these observations highlight extended functional contributions of the CK system to tumor development and reveal that they are not merely restrained to chemotaxis.

14.4 Control of Immune Cell Behaviors

As described previously, the immune system is known to shape the tumor through the "tumor editing" phenomenon. In this context, CKs are directly or indirectly implicated in the control of immune cell activation, migration to the priming site, and immune response induction. It is now clear that in most cases, the CK network is shunted by the tumor, favoring its escape from immunosurveillance and tumor progression. Nevertheless, the production of some CKs promotes the antitumor immune response and has been associated with improved patient outcome, including lower recurrence rate or increased patient survival [55].

14.4.1 Chemokines Involved in T-Cell Antitumor Immune Response

Induction of antigen (Ag)-specific antitumor immune response requires the uptake of tumor Ag by professional antigen-presenting cells (APCs) and migration from the tumor site to the corresponding draining lymph node, in order to present the processed tumor Ag to T-lymphocytes. The same APCs represent key modulators of tumor-infiltrating lymphocyte (TIL) infiltration and activation within the tumor niche. These major immune functions can be divided into different steps for which the CKR network has important regulatory implications [56].

14.4.1.1 Regulation of CK and CKR Expression by Tumor APCs

In mouse and human tumors, cells capable of phagocytic activity, and subsequently presenting tumor antigen, include tumor-associated macrophages (TAMs), tumor dendritic cells (TuDCs), immature myeloid-derived suppressor cells (MDSCs), and monocytes [57–59]. TAMs and MDSCs have mostly been implicated in dampening the T-cell response during tumor progression as we will see below. TuDCs are composed of different subsets within tumors. Monocyte-derived TuDCs are prominent in tumor antigen uptake, but lack strong T-cell stimulatory capacity due to NO-mediated immunosuppression. Pre-cDC-derived TuDCs have lymph node migratory potential, whereby cDC1s ($CD11b^{-}$ $CD103^{+}$) efficiently activate $CD8^{+}$ T-cells and cDC2s ($CD11b^{+}$ $CD103^{-}$) induce Th17 cells [60]. cDC1 APCs are very low in number but are capable of

physically engaging T-cells in tumor distal regions and to a lesser extent in the tumor proximal regions, as shown by in vivo imaging. These DCs express CD103 (CD141 in humans) and are required for T-cell-mediated tumor rejection. Moreover, the expression of CD103 DC-related transcripts in human tumors predicts survival [61]. Encounter with tumor Ag induces maturation of APCs present in the tumor environment. One feature of this maturation is the downregulation of peripheral tissue-associated CKR like CCR1, CCR5, and CCR6 and the upregulation of CCR7. Due to the constitutive expression of CCR7 ligand, CCL19, and CCL21 by peripheral lymph nodes, this switch of CKR expression by APCs promotes their migration toward the priming site. Once in the draining lymph node, APCs will locate in the preferential area to present the tumor Ag to the CCR7-expressing naive lymphocytes. CD103+ DC or cDC1 bearing CCR7 has been subsequently demonstrated to play a critical role in tumor antigen trafficking to lymph nodes (LNs), priming of T-cell immunity, and induction of antitumor cytotoxic T-lymphocytes (CTLs) [60, 62]. These effects are dependent upon CCR7 expression by cDC1, which allows their migration to LN. Loss of CCR7 and of migratory properties of cDC1 results in defective LN T-cell priming and increased tumor outgrowth. CCR7 expression in humans correlates with the cDC1 signature and better clinical outcome. Tumor cDC1 production of the chemokines CXCL9 and CXCL10 is required for effector T-cell trafficking and adoptive T-cell therapy efficacy [63] (Fig. 14.6). Expansion and activation of $CD103^+$ DC progenitors at the tumor site also enhances tumor response to therapeutic PD-L1 and Braf inhibition [64]. Altogether, these data suggest that $CD103^+$ $CCR7^+$ cDC1 are at the forefront of anticancer immunity.

14.4.1.2 Ag Presentation to T-Lymphocytes

Despite the fact that APCs display low dynamic activity, naïve lymphocytes have a high basal mobility favoring scanning of thousand APCs per hour [65, 66]. This behavior requires CCR7 expression by T-lymphocytes [67]. An additional CKR-dependent mechanism favors the probability of encounter between APCs and T-lymphocytes. Encounter of Ag-specific $CD4^+$ or $CD8^+$ T-cells with an APC bearing their cognate Ag induces the secretion of CC chemokines by the conjugate, namely CCL19, CCL5, CCL3,

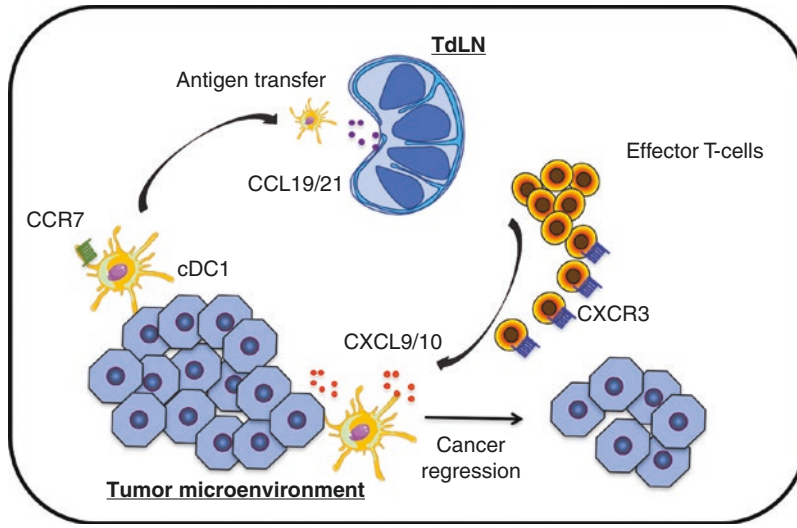

Fig. 14.6 Regulation of CK and CKR expression by tumor APCs. $CD11b^-$ $CD103^+$ cDC1 are a discrete subset of APCs usually located at the periphery of solid tumors. cDC1 expresses CCR7 which allows them to transfer tumor antigens to the tumor-draining lymph nodes (TdLNs) and to stimulate antitumor immunity. CCR7 expressing cDC1 in the tumor is capable of robust production of CXCL9/ CXCL10 which binds CXCR3 expressed on T-cells and participates in the efficacy of classical and immune-based anticancer therapies

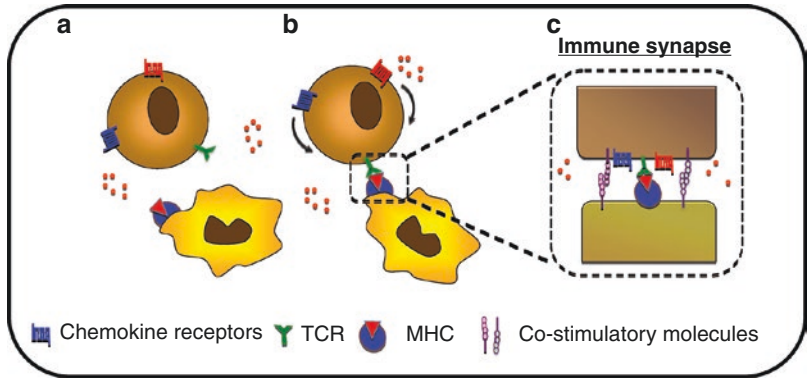

Fig. 14.7 Control of cell polarization toward immune synapse. (**a**) T-cell scan for their cognate antigen-presenting cell. (**b**) Upon recognition, T-cell will polarize chemokine receptors toward the immune synapse. (**c**) This sequestration of CKR leads to reduced sensitivity to distant CK gradient and may participate in the stabilization of the immune synapse

and CCL4. These CKs will promote naïve T-cell scanning behaviors and attraction toward the conjugate [57–59], which is known to favor the establishment of memory immune response, in addition to the induction of polyclonal responses against different tumor Ags [68].

CKs are also implicated in the improvement of APC/T-cell adhesion mechanism as well as in immunological synapse stabilization, promoting T-cell priming (Fig. 14.7). CCR7 ligands secreted in the lymph node promote immunological synapse formation by T-cells [69]. CXCR4 and CCR5 expressed by T-cells are recruited toward the immunological synapses made with the APC. This polarization results in desensitization of T-cells from external sources of CKs and improves synapse stability. A similar mechanism is observed during the interaction between tumor-infiltrating lymphocytes (TILs) and tumor cells. Indeed, the recruitment of CCR5 at the immune synapse formed between the TIL and the tumor cell results in defective responses to TIL toward a CCR5 gradient [70]. This mechanism allows for the modulation of the "GO" signals generated by CKs, competing with the "STOP" signals mediated by the TCR-MHC interaction [71].

14.4.1.3 Migration of Effector T-Lymphocytes to the Tumor

Naive T-cells, after clonal expansion and differentiation into effector T-cells, migrate toward the tumor site, implying that T-cells downregulate the expression of the CKRs implicated in the retention at the priming site like CCR7. In addition, they upregulate various CKRs including CCR1, CCR3, CCR5, and CXCR3 allowing their movement toward the tumor site [72]. Cytotoxic T-lymphocyte (CTL) recruitment to the tumor site is consistent with this pattern of CKR expression and is mainly mediated by CCL3, CCL5, CCL20, CXCL9, and CXCL10 [56]. Expression of membrane-anchored CKs such as CXCL16 and CX3CL1 has also been shown to correlate with greater numbers of tumor-infiltrating lymphocytes and improved prognosis in colorectal cancer [73, 74]. The antitumor effect of the membrane-bound CK form vs. the soluble form is yet to be clearly established.

The control of TIL localization within the tumor is ill-defined. It is obvious that in most cases, TILs are mainly found at the tumor periphery; however, the underlying mechanisms remain unclear. Several clues could help us speculate on the mechanism of trapping the TILs at the tumor periphery. The recent contribution of real-time imaging showed that dense peripheral extracellular matrix (ECM) might restrain TILs' access to the tumor parenchyma [75]. Whether specific niches of CKs are expressed on collagen fibers is unclear and needs further investigation. In addition, dynamic analysis showed that Ag-specific CTLs are trapped in the network of

tumor-associated APCs restraining their infiltration and probably favoring immunosuppression [76, 77]. The role of CKs in this trapping is not defined, but Ag expression by APC at least induces stable engagement between the CTL and the APC. In addition, experimental evidences showed that non-tumor Ag-specific TIL cannot infiltrate the tumor deeply without the prior tumor cells' destruction by Ag-specific CTL. These results suggest that deep infiltration of the tumor by TIL might be favored by chemotactic agents secreted upon tumor cell destruction by CTL or on extensive ECM remodeling to allow their interstitial migration [78].

Overall, considering the numerous CKs expressed by the various cell subsets of the tumor microenvironment, it is very difficult to address specific contributions of the CK/CKR couple in the interstitial migration and positioning of T-lymphocytes within the tumor parenchyma. The various properties of these molecules have demonstrated that this positioning is controlled by sensitivity to the chemotactic gradient and the subsequent desensitization upon polarization toward the synapse or the downregulation of the expression of CKRs.

14.4.2 Chemokines in Innate Immune Components

Innate immune cells constitute a first barrier against tumor development. However, due to their plasticity and capacity to produce a myriad of cytokines, chronically activated innate immune cells are key modulators of cell activation and survival, as well as regulators of the ECM metabolism. Several physiological processes necessary for tumor development, such as increased cell survival, tissue remodeling, angiogenesis, and suppression of antitumor adaptive immune responses, are regulated by innate immune cell infiltrates in the tumor.

Macrophages are the main stromal cell population present in the tumor parenchyma. They can account for more than 50% of the tumor mass. The role of TAMs in tumor development is critical, as these cells, depending on their state of activation, can display antitumor properties associated with the production of Th1 cytokine, high quantity of reactive oxygen species, phagocytosis and efficient Ag presentation; or they could display protumor properties mediated by the secretion of Th2 cytokine, proangiogenic factors, growth factors that support tumor survival, and proliferation and the secretion of MMP which promote tumor invasion and metastases. Consistently, the impact of TAMs on tumor development and metastases will depend on the balance between antitumor macrophages and protumor macrophages.

Tissue-resident macrophages originate from the self-renewal of yolk sac or fetal liver derived macrophages while a small proportion, depending on the tissue, may derive from the recruitment of circulating monocytes assuring immunosurveillance [79]. Within neoplastic tissues, it is suggested that TAMs are mostly recruited from the periphery [80]. Nonetheless, knowledge of the relative proportion of native resident macrophages within tumors remains poor in the field of oncology. CCL2, also called MCP-1 for monocyte chemoattractant protein-1, is probably the most frequently found CC-CK in tumors involving recruitment of circulating classical monocytes ($CCR2^{high}$ $Ly6C^{+}$ in mouse, $CCR2^{high}$ $CD14^{++}$ $CD16^{-}$ in human) [81]. A subpopulation of early-evolved cancer cells requires macrophages for early dissemination. CCL2 produced in the premalignant region attracts these macrophages which induce Wnt-1 upregulation that in turn downregulates E-cadherin junctions in early cancer cells, ultimately increasing metastatic burden at end stage of cancer progression [82]. TAMs are also capable of inducing the directional migration and the invasion of tumor cells. One described mechanism is that CSF1 produced by the tumor cells creates a feedback loop leading to EGF production by macrophages and subsequent accumulation of tumor cells around blood vessels [25]. TAMs are also capable of directly promoting the intravasation of tumor cells into the circulations [83]. Classical monocytes recruited via CCL2 promote perineural invasion of cancer cells via cathepsin B expression [84]. Recently, embryonically derived tissue-resident macrophages have been demon-

strated to co-exist with bone-marrow-derived macrophages recruited via CCR2 in pancreatic and brain malignancies and to display distinct functions [85, 86]. Similarly, in the context of lung carcinoma and breast cancer pulmonary metastases, both monocyte-derived macrophages and CCR2-independent tissue-resident interstitial macrophages densely colonize the tumors and promote their development. These two macrophage subsets display distinct transcriptomic signatures, anatomic distributions, and functions after anticancer therapies. On the other hand, alveolar macrophages, which are the most abundant tissue-resident macrophages in the alveolar lumen, decrease in number and seem to be excluded from pulmonary tumor nodules [87].

Tissue-resident macrophages are present in most tissues and have distinct transcriptional programs initiated in embryonic progenitors [88] compared to recruited macrophages. The relative proportion and specific features of tissue-resident macrophages might explain the heterogeneity of different tumor microenvironments according to the anatomical site of tumor development. One needs to further investigate whether it could serve as a prognostic factor of tumor growth and response to therapies. Moreover, one has to consider the co-existence within the tumor niches of TAMs from different ontogenies. This aspect is crucial considering the described roles of the CKRs in the accumulation of these subsets so far. Beyond their action in cell recruitment, their action in local proliferation and survival of resident macrophages should be considered.

Interestingly, in a melanoma system where tumorigenesis is dependent on an external growth factor CCL2, there is a biphasic effect depending on its secreted quantity. High amounts are associated with a massive recruitment of TAMs into the tumor with dominant antitumor activity, while lower amounts induce lower infiltration into the tumor resulting in tumor promotion through the secretion of growth factor by the macrophages [89]. Through CCR2 activation by CCL2, metastasis-associated macrophages (MAMs) can secrete CCL3 acting as an amplification loop to accumulate MAMs in a CCR1-dependent manner [90]. As we will see in Sect. 14.5.2, TAMs are important protagonists of tumor-associated fibrosis or ECM remodeling. These results point out the importance of the ratio between protumor and antitumor activities of macrophages of different origins within tumors.

Other CKRs implicated in TAM recruitment are CX3CR1 and CCR1. In human glioblastoma, the level of tumor infiltration by microglial cells is dependent on CX3CR1. Patients with a functional mutation in the CX3CR1 gene associated with impaired monocyte migration have a reduced TAM infiltration into the tumor [91]. Injection of a thymoma tumor cell line (EL4) with a liver tropism to mice results in an increased infiltration of the liver by immune cells, including macrophages. In CCR1 KO mice, this recruitment during the first stage of the tumor development is massively reduced [92].

CXC chemokine receptors could also be implicated in TAM recruitment. In humans, IL-4 and IL-13, two cytokines secreted in the tumor environment, sensitize monocytes to CXCL1 and CXCL8 by upregulating their receptors (CXCR1 and CXCR2). Thus, these cytokines indirectly promote the recruitment of TAM into the tumor through CXC chemokine receptors [93].

As previously discussed, CKs not only control leukocyte recruitment into the tumor but also organize their localization within the tumor. Lack of proper vascularization at the center of the tumor induces the secretion of several hypoxic factors like hypoxia-inducible factors (HIFs). HIFs promote the expression of CXCR4 by macrophages, favoring their recruitment toward tumor hypoxic areas [94].On the other hand, the tumor environment decreases CKR expression on monocytes. Indeed, macrophages from tumor sites express low levels of CKR [95]. Time-lapse imaging of TAMs in the experimental murine model revealed that TAMs display reduced displacement but intense protrusive activity [76, 77]. Downregulation of CKR might explain this retention at the tumor site.

CKs do not only act on leukocyte attraction but are also implicated in their activation. Induction of copper/zinc superoxide dismutase by CCL5/CCR5 activation causes tumor necrosis factor-alpha and reactive oxygen species pro-

duction by macrophages [96], promoting tumor destruction. Inversely, in human monocytes, CC chemokines induce the transcription of metalloproteinase, implicated in tumor invasion and spreading. The fact that both TAM recruitment and activation are regulated by CK increases the potential interest of targeting TAM for antitumor therapies. Nonclassical or patrolling monocytes ($CX3CR1^{high}$ $Ly6C^{low}$ in mouse, $CX3CR1^{high}$ $CD14^{+}CD16^{+}$ in human) are localized in the capillaries of different organs where they patrol and scavenge cellular debris via the CX3CR1-CX3CL1 axis [97]. These cells have been associated with antitumor functions in different models of murine metastasis. Patrolling monocytes establish early interactions with metastasizing tumor cells and scavenge tumor material from the lung vasculature. The detection of tumor material by patrolling monocytes is dependent upon CX3CR1 and promotes the recruitment and activation of NK cells to the lung tumor environment [98]. Exosomes from melanoma cells or from patients with nonmetastatic melanomas have the capacity to stimulate the expansion of patrolling monocytes via induction of the Nr4a1 transcription factor, causing cancer cell clearance [99].

NK cells represent another component of the innate immune system highly involved in antitumor immune responses. NK cell recruitment to the tumor is mainly mediated through the CXCL10-CXCR3, CX3CL1/CX3CR1, and CCL3-CCL4-CCL5/CCR5 axes. High CX3CL1 quantity is associated with increased NK cell recruitment into the tumor in both humans and mice [100, 101].

A similar phenomenon is observed with increased CCL5 and CCL3 expression by tumor cells in mouse models [102, 103]. CXCR3 is implicated in the recruitment of human NK cells to breast cancer tumor, which is mediated by CXCL10 secretion from tumor cells in response to IFN-γ produced by the NK cells themselves [104, 105]. Thus, CKs not only control NK cell recruitment but also regulate their antitumor properties. CX3CR1 activation by CX3CL1 results in improved antitumor cytotoxicity of NK cells [106, 107]. CCL3, CCL4, and CCL5 have been shown to activate NK cytotoxicity through induction of degranulation [108, 109].

14.4.3 Chemokine and Tumor-Induced Tolerance

Recruitment of tolerogenic cells such as regulatory T-cells or immunosuppressive myeloid subsets is a feature of immune escape. Tumor cells secrete ligands of CKRs expressed by immature, regulatory, or Th2 polarized cells. CCL22 and CCL17 produced by tumor cells recruit monocytes, as well as Th2 lymphocytes and regulatory T-cells (Treg cells) through CCR4 signaling [110]. This strategy of immune escape has been also selected in the viral-induced oncogenesis process. HHV8 virus, the pathogen of Kaposi's sarcoma, encodes three viral CKs which bind to CCR3, CCR4, and CCR8 involved in the recruitment of Th2 and Treg cells [111]. Treg cells recruited through CCL22/CCR4 are selectively activated by mature DCs though tumor-associated antigen presentations and lead to an adverse clinical outcome in breast tumors [112]. The tumor cell-derived cytokine IL-1α has been identified as a major inducer of the Treg-attracting chemokine CCL22 in human cancer cells. Silencing IL-1α prevents CCL22 induction by tumor or myeloid cells, resulting in suppression of Treg migration toward the tumor [113]. Blockade of the CCL22/CCR4 axis is thus a promising therapeutic strategy to inhibit tumor-induced immunosuppression [114].

Stromal cells produce CKs which promote the recruitment of protumoral cells. Among others, TAM produces CCL18 which is induced by IL-10 [115]. CCL18 favors the recruitment of naïve T-cells through activation of an unknown receptor. It is proposed that these naïve T-cells acquired tolerogenic properties in contact with the tumor environment. $CCR6^{+}$ immature lymphoid DC recruitment into the tumor is favored by the secretion of CCL20 from both tumor cells and TAMs [116]. CCL5 recruits immature DCs as well by binding CCR1 and CCR5 [117]. Immature DCs acquire tolerogenic properties in the tumor environment and participate in the

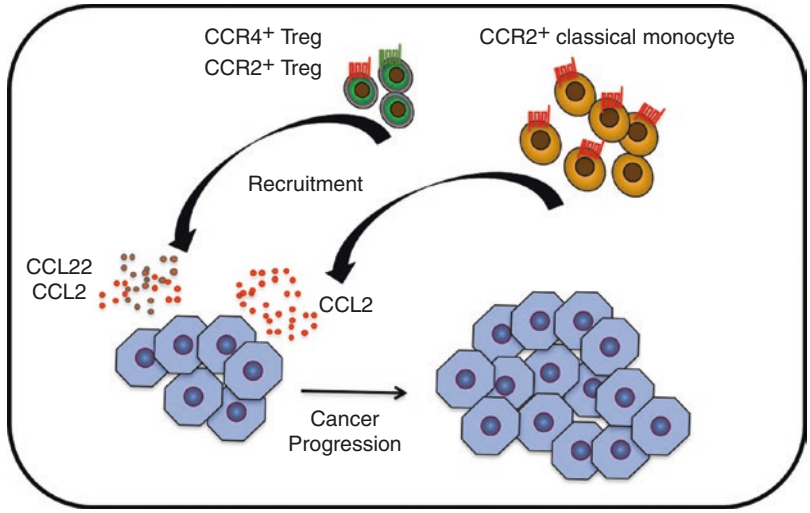

Fig. 14.8 Role of chemokines in the recruitment of immunosuppressive cells to tumors. The chemokines CCL2 and CCL22 are commonly secreted in both mouse and human tumors of different types and direct cancer immunity toward immune tolerance. CCL22 drives the enrichment of CCR4+ Treg cells, which can be targeted therapeutically. CCL2 production in tumor microenvironment is linked to the recruitment of CCR2+ classical monocytes and CCR2+ Treg cells. Monocytes can display immunosuppressive properties per se (MDSCs) but can also differentiate into TAMs upon extravasation into tumors. CCR2+ Treg cells represent a tumor antigen-specific and highly immunosuppressive subset

immune tolerance loops against tumor Ags [118]. CCR2, a chemokine receptor highly expressed by inflammatory monocytes, is crucial for the recruitment of the latter from the bone marrow to inflamed tissues but also displays chemotactic properties for T-cells. The major CCR2 ligand (CCL2) is highly secreted by both the tumor and tumor stromal cells in both human and mouse models. In cancer context, the CCR2-CCL2 axis has been largely implicated in the development and progression of cancer metastasis via the recruitment of MDSCs [119] and TAMs [90, 120]. MDSCs promote immune escape by limiting the activation of CD8 T-cell infiltration into tumors, whereas macrophages promote metastatic seeding of cancer cells, thereby amplifying the pathology. Beyond myeloid cells, CCR2 is also expressed by a subset of activated tumor antigen-specific, suppressive Treg cells in the LN. Given the lower frequency of CCR2-expressing Th cells, CCL2 production during tumor development represents a nonredundant mechanism of preferential recruitment of CCR2+ Treg cells and contributes to immune escape (Fig. 14.8). Depletion of CCR2+ Treg or CCL2 blockade enhances priming of tumor-specific CD8+ T-cells [121] and augments cancer immunotherapy [122, 123]. The recruitment of both monocytes and Treg cells via CCR2-CCL2 could favor the co-localization of these immunosuppressive cells and further indicates that this CK axis is an important mechanistic marker of tumor development, predicting clinical benefit after cancer therapy.

Subversion of tumor immune component is a central point of tumor outcome. The above-described implication of CK in cellular mechanisms should provide the basis to better understanding the clinical implication of CK network in cancer pathology. The regulation of the balance between immunogenic and tolerogenic components has deserved major attention for a long time and is the basis of immunotherapy which represents an apparent inexhaustible field of innovative anticancer strategies. Targeting the CK system in this goal is in the course of important investigation through the development of pharmaceutical compounds able to stimulate or antagonize CKR axes.

14.5 Alternative Tumor-Associated Physiological Functions of Chemokines

14.5.1 Angiogenesis

One of the features of CKs is their dual role in the angiogenic process. In the tumor environment, there is increased production of proangiogenic CK, while angiostatic CKs are downregulated. In addition to a direct angiogenic effect of CKs, this activity is indirectly potentialized by the CK-induced recruitment of leukocytes displaying angiogenic properties such as neutrophils or macrophages [124].

CKs from the CXC family are probably the most described for their direct implication in tumor-associated angiogenesis. CXCLs 1, 2, 3, 5, 6, 7, and 8 display angiogenic properties. All these CKs contain a specific amino acid sequence of glutamic acid-leucine-arginine (or ELR for short) immediately before the first cysteine of the CXC motif (ELR positive). This ELR sequence absence from the other CXC chemokines is responsible for the proangiogenic properties of most of the CXC chemokines [125].

ELR^+ chemokines mediate angiogenesis through binding to the CXCR2 receptor.

ELR^+ chemokines are able to recruit endothelial precursor cells, induce cell proliferation, and promote maturation. These mechanisms could be negatively regulated by a decoy CKR expressed by endothelial cells called duffy antigen receptor for CK (DARC). Unlike most of the other CKRs, DARC is not linked to G-protein, and its activation does not induce calcium flux. DARC reduces angiogenesis by sequestering all the ELR^+ CKs.

One specificity within ELR^- chemokines is attributed to CXCL12 which is the only ELR chemokine with proangiogenic activity. CXCL12 mediates its proangiogenic effect by directly promoting the recruitment of endothelial progenitor cells [126, 127] or indirectly by promoting tumor angiogenesis through the recruitment of $CXCR4^+$ $TIE2^+$ proangiogenic monocyte [94, 128] and through the secretion of vascular endothelial growth factor (VEGF) after CXCR7 activation [129] or WNT7B signaling [130]. Interfering pharmacologically with the CXCL12/CXCR4 axis or depleting $TIE2^+$ monocytes/macrophages increases the efficacy of vascular-disrupting agent based therapies [131]. Chemotherapy induces the upregulation of HMOX-1 which in turn upregulates CXCL12 in perivascular area of tumors. In this context, CXCL12 subsequently induces the clustering of tumor-relapse-promoting TAM population around tumor blood vessels. Perivascular TAMs promote tumor revascularization and relapse partly via their production of VEGF [132]. Signaling via VEGF-R1 in metastasis-associated macrophages regulates a set of inflammatory response and macrophage regulator genes which promote breast tumor metastases [133]. Moreover, anti-angiogenic therapy targeting VEGF-R2 has been reported to upregulate both CXCL12 and CXCR4, leading to the recruitment of neutrophils and $Ly6C^{low}$ monocytes with immunosuppressive action in experimental murine models of colorectal cancer [134].

In contrast, ELR^- chemokine secretion is often associated with attenuation of angiogenesis. ELR^- CXC chemokines are described by their angiostatic properties. ELR^- CXC chemokine secretion is induced by IFN-α and IFN-β. Through CXCR3 binding, these CKs mediate their angiostatic properties by inhibition of ELR^+ chemokine, VEGF-α, and β-FGF proangiogenic effects in vitro [135]. Interestingly, the expression of CXCR3 is dependent on the cell cycle phase, limiting the angiostatic properties of ELR^- CXC chemokines to the S/G2-M phase [136].

This important association of CKs and angiogenesis within the tumor environment sets the inhibition of ELR^+ chemokine as a robust antitumor therapy.

14.5.2 Fibrosis and Extracellular Matrix Remodeling

The association of CKs with EMT leading to fibrosis activity has been previously suggested by studies; however, there is no clear evidence that CKs play a direct role in this process.

Fibrosis and extracellular matrix remodeling are continuous processes present in the tumor

parenchyma reflecting the intense dynamic and migratory activity of the neoplastic tissue. Two different types of migratory activity are defined, namely the amoeboid and mesenchymal migrations. The amoeboid migration does not require extracellular matrix (ECM) remodeling through matrix metalloproteinase (MMP) activity due to the ability of the cell to squeeze through the ECM. The mesenchymal migration relies on previous proteolysis and degradation of the ECM to generate sufficient space for cell displacement. CK-mediated induction of MMP is mostly mediated by CC chemokines; CCL5 and CCL9 produced by mesenchymal stem cells promote tumor cell invasion in a MMP-dependent manner [137, 138]. CCL25 promotes MMP secretion in ovarian cancer cells through CCR9 binding and favors tumor cell invasion [139]. CCL21/CCR7 interaction favors MMP-9 secretion, tumor invasion, and metastases in colon cancer cells and in B-cell chronic lymphocytic leukemia cells [140, 141]. At least, one CXC chemokine has been related to MMP activity; thus, CXCL12 is implicated in increased MMP-2 activation and increased cell invasion in a pancreatic cancer cell line [142].

Studies have suggested that the extracellular matrix promotes tumor escape from the immune system by trapping antitumor leukocytes at a distance from tumor cell niches [143]. However, tumor progression and metastases require degradation of this extracellular matrix surrounding the tumor. Mesenchymal stem cell (MSC)-derived cell populations and TAMs are important protagonists of these physiological activities. CXCL12 is implicated in the recruitment of mesenchymal stem cells (MSCs) from the bone marrow. Bone marrow-derived MSCs can account for up to 25% of cancer-associated fibroblasts, the main source of fibrosis within the tumor [144]. Cancer-associated fibroblasts and MSCs have been shown to share protumorigenic activities with MSCs in neuroblastomas [145]. TAMs have also been shown to actively participate in the construction and assembly of the tumor-associated ECM by secreting collagen types I, IV, and XVI and factors enhancing fibroblast activities [146]. Production of proteases such as cathepsin by macrophages or MMP can also liberate growth factors sequestered in the ECM [147]. Overexpression of CCL2 in the mammary epithelium was associated with increased expression of matrix remodeling enzymes and higher density of the stroma and collagen without directly affecting mammary epithelial cell proliferation or death. Nevertheless, this CCL2-driven inflammation contributed to the increased risk of breast cancer in both mouse models and humans [148]. Comparison of TAM functions according to their ontogeny suggests that while tissue-resident macrophages directly promote tumor progression, partly via their pro-fibrotic activities [85, 86], TAMs derived from circulating $CCR2^{+}$ monocytes degrade collagen though cellular uptake [149].

There is ongoing evidence that targeting proteolysis activity in combination with chemotaxis would provide promising results in the strategy to inhibit tumor cell invasion and metastasis.

14.6 Clinical Aspect

CKs are implicated in several aspects of tumor development. Due to these pivotal roles in tumor biology, CKs have been frequently associated with tumor evolution and clinical outcomes and have been highlighted for their potential use as prognostic or diagnostic markers. Therefore, they represent a promising target with a potential for a diverse range of therapeutic strategies.

14.6.1 Prognosis

Due to its importance across a wide range of physiological mechanisms, CK/CKR network alteration could impact tumor development. Correlative studies using genetic polymorphisms provide essential information for prognosis. Several functional polymorphisms in CKs or CKRs have been studied in order to establish correlation between functional variants and tumor risk or progression (Table 14.2).

The paragraphs below focus on the most commonly described polymorphisms, their functional

Table 14.2 Association between chemokines and chemokine receptor polymorphisms and tumor risk and/or progression

		CCL2-2518 A<G	CCL5-403 G>A	CXCL8-251T>A	CXCL12 801G<A	CCR2 64I	CCR5 Delta 32	CX3CR1 V249I
Breast	Risk	–		–∗	=/–∗	–		
	Prog	–		–				
Hepatocellular	Risk	=			=/–	=/–	=	=
	Prog	=			=/–	=	=	
Gastric	Risk	–	+	–∗/=∗				
	Prog							
Glioblastoma	Risk	=						=
	Prog	=						+
Prostate	Risk	=	–	+∗	–	–	–	
	Prog							
Oro-/nasopharyngeal	Risk			–∗	–	–∗		
	Prog	–		–				
Melanoma	Risk	=					=	=
	Prog	–					=/–	=
Pancreatic	Risk		–					
	Prog							
Leukemia	Risk		+					
	Prog				–			
Colorectal	Risk			+/=				–
	Prog			=	–			
Bladder	Risk			=		–∗	–	
	Prog			=				
Lung	Risk			=	–∗			
	Prog							
Cervix	Risk					–∗		
	Prog					–		

Prog prognosis, + good indicator, – poor indicator, = no association, ∗ meta-analysis

relevancies, and their subsequent prognostic value in tumor risk and/or progression.

14.6.2 CC Chemokines/Chemokine Receptors

14.6.2.1 CCL2

A single-nucleotide polymorphism (SNP) in the CCL2 promoter, based on the substitution of an adenine by a guanine in position −2518 (A < −2518 < G), is associated with increased CCL2 secretion [150]. This polymorphism with an allelic frequency close to 30% is associated with an increased susceptibility to the development of breast, gastric, and oral squamous cancer. However, it is not associated with an increased risk of developing hepatocellular and prostate cancer, glioblastoma, and melanoma. Despite this lack of association with the development of melanoma, CCL2 polymorphism is associated with increased Breslow index, suggesting its link with melanoma progression [151]. CCL2-2518G variant is also associated with increased metastasis development in nasopharyngeal and breast cancer. In the former case, the deleterious effect of the polymorphism is observed only after radiotherapy [152]. Overall, the deleterious effect of the CCL2-2518G allele-associated increase of CCL2 expression is consistent with the protumoral effect of TAM in most tumors, as previously described above.

14.6.2.2 CCL5

Conflicting data arise from the study of the CCL5 G < −403 < A polymorphism on cancer

risk. This mutation is thought to be responsible for the decreased secretion of CCL5 and is associated with decreased risk of leukemia and gastric cancer in women [153], as well as an increased risk of prostate and pancreatic cancer [154]. This discrepancy could reflect the balance between the antitumor effects of CCL5 through recruitment of CTL and the protumoral effect of CCL5 through recruitment of immature DC. Nonetheless, there is no evidence supporting an association between CCL5 polymorphism and tumor progression.

14.6.2.3 CCR5

CCL5 main receptor (*CCR5*) is also subject to another relevant polymorphism. A deletion of 32 base pairs named CCR5 delta 32 results in a reading frame shift, associated with complete defect in receptor expression. The impact of the polymorphism in tumor risk and progression is not well documented. Most studies conclude a lack of association; however, one report suggests that CCR5Δ32 could be associated with higher risks of the development of gallbladder cancer [155]. In melanoma, CCR5Δ32 is associated with reduced survival of patients with grade 4 tumor treated by immunotherapy strategies [156]. These observations might reflect the role of CCR5 in the induction of T-cell priming and memory.

14.6.2.4 CCR2

CCR2 V64I polymorphism has also been studied for its implication in tumor risk and progression. There is no known effect of the genetic variation on the CCR2/CCL2-signaling pathway, but it is associated with CCR5 instability, which could be explained by stability alteration of the CCR2/CCR5 dimer. Most of the studies conclude that there is an increased risk for people carrying the rare variant. This is the case for cervical, oral, bladder, prostate, and endometrial cancer. A recent meta-analysis with 2661 cancer patients and 5801 healthy controls found an overall significant association between the CCR2-V64I polymorphism and cancer risk [157]. In the subgroup analysis stratified by cancer types, there was a significant association between this polymorphism and the risk of bladder, cervical, and oral cancer.

14.6.3 CXC Chemokines

Two CXC chemokines, CXCL8 (also referred as interleukin-8) and CXCL12 (SDF-1), have been intensively investigated for their association between polymorphisms and tumor risk and development.

14.6.3.1 CXCL8

CXCL8 T < −251 < A polymorphism is probably one of the most studied CK polymorphisms in cancer. Its physiological effect and its impact on CXCL8 expression remain to be elucidated. There is an apparent discrepancy between studies on these effects; however, this may reflect specificity depending on the cell type or the cell activation status. The implication of CXCL8 polymorphism in cancer risk and outcome remains unclear. Unfortunately, controversies in the literature make any interpretation challenging. Several meta-analyses have been performed in order to gain some clarity, and despite some variation in the conclusion, it appears likely that the rare variant of CXCL8 promoter region is associated with increased risk of gastric and oral cancer [158–160].

14.6.3.2 CXCL12

CXCL12 is subject to a polymorphism in a 3′ untranslated region named CXCL12 3′ G801A. The rare variant is associated with increased secretion of CXCL12. Consistent with the protumoral effect of CXCL12 mentioned above, studies essentially report that CXCL12 801A variant is associated with an increased risk of several cancers (lung, breast, oral, prostate, hepatocellular, and colorectal cancers). It is also thought to favor tumor progression or metastases in lung cancer, hepatocellular carcinoma, colorectal cancer, and myeloid leukemia. The only three meta-analyses performed to date conclude that there is an increased risk of breast and lung cancer, without any significant effect on other cancer types [161–163].

14.6.4 CX3C Chemokine Receptors

The only receptor for the CX3C chemokine family is CX3CR1, which is also subject to polymorphisms associated with cancer outcome. Substitution of a valine by an isoleucine in position 249 results in increased adhesion of the couple CX3CR1/CX3CL1 and defective migration of $CX3CR1^+$ cells. The rare variant is associated with increased risk of colorectal cancer, but not hepatocellular cancer, melanoma, and glioblastoma. In this last case, the rare variant is associated with improved patient survival after tumor biopsies and decreased infiltration of the tumor by microglial cells [91]. This is consistent with the promotion of glioblastoma invasion by microglial cells [164].

14.6.5 Chemokine Circulating Expression

CK circulating levels have also been related to cancer progression. A high concentration of CCL17 is associated with the progression of Hodgkin's lymphoma (HL) after treatment [165]. Interestingly, opposite effects are observed in melanoma, where high *CCL17* expression is associated with progression-free survival in patients with immunotherapeutic treatment [166]. Elevated concentrations of CXCL10 in the serum before treatment (monoclonal antibody therapy together with combination chemotherapy) are associated with an increased likelihood of clinical relapse and an inferior survival in patients with diffuse large B-cell lymphoma [167]. Elevated serum levels of CCL2 have been described in patients with breast, colon, gastric, prostate, ovarian, or skin cancers [168–171]; and a meta-analysis of gene expression databases identified CCL2 as an independent factor favoring the development of prostate cancer [172].

Despite numerous promising results, CK and CKR genes and molecules are not currently used in clinical settings to evaluate a patient's risk of developing cancer or to predict tumor progression. This could be explained in part by the nonhomogeneous distribution of the polymorphism variants among ethnic communities. Additionally, in most cases, *CK* and *CKR* gene polymorphisms are not singularly powerful predictive tools. Their clinical utility is most likely to be dependent on their association with other markers.

14.6.6 Therapeutic Strategies

As discussed throughout this chapter, CKs are implicated in all steps of the tumor development, invasion, and dissemination. Several tools have been developed to target CKs or CKRs as innovative strategies in cancer treatment. To date, there is no molecule targeting macrophage release; however, multiple clinical trials from phase I to phase III are recorded at clinicaltrials.gov (Table 14.3). Some strategies aim to promote the production of CKs implicated in the recruitment of immune-competent cells to the tumor by injection of IFN, "celecoxib," and "rintatolimod" (NCT01545141). In another trial, patients with lung adenocarcinoma were directly injected with CKs implicated in the recruitment of antitumor effector T-cells, in combination with vaccination approach (NCT01433172). Inversely, another trial aimed to inhibit the recruitment of protumoral leukocyte using an Ab against CCL2 (carlumab) in order to control metastatic castrate-resistant prostate cancer (MCRPC) (NCT00992186). However, this strategy failed as all the patients were removed from the study, due to progression of the tumor despite anti-CCL2 treatment. Although well tolerated, treatment with carlumab only inhibits CCL2 initially, but the levels of CCL2 in patients are rapidly increased above those seen before treatment (NCT00537368). It was concluded that carlumab is not efficient for a long-term inhibition of CCL2 in patients, but other strategies have been used to target the CCR2/CCL2 axis in clinical trials. An antibody against CCR2 (MLN1202) has been demonstrated to be safe and well tolerated in patients with different types of cancers with bone metastasis (NCT01015560). Finally, other inhibitors of CCR2 (BMS-681 and CCR2-RA[R]) have been developed [173, 174] occupying different orthosteric and allosteric pockets of

Table 14.3 Clinical trials evaluating the benefits of targeting chemokine or chemokine receptor cancer therapies

Inclusion criteria	Phase	Treatment
Colorectal cancer	Phase I/II	Chemokine-modulatory regimen
Stage IV adenocarcinoma of the lung	Phase I/II	GM.CD40L and CCL21
Metastatic castrate-resistant prostate cancer	Phase II	Anti-CCL2 carlumab
Solid tumors	Phase I	Human monoclonal antibody against CCL2 (CNTO 888)
Patients with bone metastasis	Phase II	Anti-CCR2 monoclonal antibody (MLN1202)
Colorectal cancer patients with hepatic liver metastases	Phase I	CCR5 antagonist (maraviroc)
Previously treated peripheral T-cell lymphoma	Phase II	Anti-CCR4 monoclonal antibody KW-0761 (mogamulizumab)
CCR4-positive adult T-cell leukemia-lymphoma	Phase II	Anti-CCR4 (KW-0761)
Solid tumors	Phase I	Anti-CCR4 (KW-0761)
High-grade glioma	Phase I	CXCR4 antagonist (plerixafor/AMD3100) and bevacizumab
Multiple myeloma previously treated with lenalidomide	Phase III	Filgrastim with or without CXCR4 antagonist (plerixafor/AMD3100)
Non-Hodgkin's lymphoma	Phase III	CXCR4 antagonist (plerixafor/AMD3100) and G-CSF
Multiple myeloma	Phase Ib	Anti-CXCR4 (BMS-936564) alone or plus lenalidomide/dexamethasone or bortezomib/dexamethasone
Multiple myeloma	Phase I/IIa	CXCR4 antagonist (BKT-140)
Solid tumors	Phase I	CXCR4 antagonist (plerixafor/AMD3100)

the receptor. These two inhibitors also present different mechanisms of CCR2 inhibition, suggesting that combined inhibition could potentiate therapeutic efficacy [175].

Multiple preclinical models have demonstrated the induction of antitumor immunity by targeting Treg cells via CCR4 inhibition in preclinical models of solid tumors [176–178] and patients [179]. As a result, the immunoregulatory activities of KW-0761 are now being evaluated in patients with advanced and/or metastatic solid tumors (NCT02281409).

Another approach aimed to directly target *CKR* expressed by neoplastic cells in order to control tumor or metastasis development. The CCR5 antagonist, named "maraviroc," originally commercialized for AIDS treatment, is under evaluation for its antitumor property in colorectal cancer (NCT01736813). Promising results have been obtained with an anti-CCR4 Ab named "KW-0761." Injection of KW-0761 in subjects with CCR4-positive adult T-cell leukemia-lymphoma resulted in the stabilization of tumor progression in half of them. This molecule is now under evaluation in cutaneous T-cell lymphoma (NCT01728805) and in second-phase treatment for peripheral T-cell lymphoma (NCT01611142).

CXCR4 antagonists are probably the most widely used molecules in trials targeting the CK network. "Plerixafor" is an FDA-approved CXCR4 antagonist for use in patients with non-Hodgkin's lymphoma (NHL) and multiple myeloma. It is used as a preconditioning regimen for its ability to mobilize bone marrow resident hematopoietic stem cells and tumor stem cells toward circulation before chemotherapy. Plerixafor and other molecules targeting CXCR4 are now evaluated in several clinical trials from grades I to III in combination with other treatments, in various forms of leukemia and myeloma. Evaluation of CXCR4 targeting in cancer therapies is not limited to blood tumors. Plerixafor is currently being evaluated in a phase I trial in conjunction with "bevacizumab" for patients with high-grade glioma (NCT01339039). Plerixafor is also studied by continuous adminis-

tration in patients with advanced pancreatic, ovarian, and colorectal cancers (NCT02179970).

14.7 Concluding Remarks

The advantages of targeting the CK network, through distinct strategies, have already been demonstrated as well as its limitations. A new generation of clinical trials based on a combination of approaches from standard chemotherapies to innovative immunotherapies offer new perspectives in CK network targeting strategies.

The 10 years following the discovery of the majority of CKs were characterized by extensive investigations in the involvement of these molecules in the control of cellular trafficking, specifically leukocytes. Later on, scientists demonstrated that CKs do not only control cell migration but also cell proliferation, survival, and activation state. It is now obvious that CKs act on a wider range of cell types rather than only leukocytes for which they were primarily characterized. The complex physiological processes in which CKs are involved such as tissue homeostasis, immune system maturation and surveillance, and tissue remodeling functions like angiogenesis or fibrosis are shunted in most cases toward tumor promotion. The central role of the CK network in these processes positions the CK system as an attractive target against tumor development, progression, and dissemination. Clinically, *CK* and *CKR* polymorphisms or serum levels are already associated with susceptibility or prognostic markers. Current investigations aiming at controlling tumor development by targeting the CK network are not limited to the direct effect on tumor cells. For instance, it is proposed that CKs could modulate the involvement of TAMs in tumor eradication or protection after chemotherapy suggesting that chemoattractant molecules could be used in combination with standard chemical chemotherapies to favor tumor eradication through modulation of the TAM activity. Despite numerous promising results, few molecules targeting CKRs have received FDA approval. The CXCL12 antagonism is already being used in patients with leukemia or myeloma to promote tumor cell mobilization toward the bloodstream before treatment, and the CCR5 antagonist maraviroc is currently being evaluated in colorectal cancer. These low numbers of molecules targeting CKs in the market could be explained by the relatively recent discovery and characterization of the CKs. In addition, the central role of CKs in most biological functions would lead to potential numerous side effects. Given the phenomenal amount of progress made by the scientific and the medical community, it is most likely that these challenges will be overcome. Several innovative technologies allowing for more efficient and specific delivery of chemical compounds have been proposed and optimized during the last few years, such as Ab-coupled treatment and encapsulated or viral delivered constructs. Targeting the CK network using these tools will probably constitute the next step in the development of a cancer therapy with minimal side effect.

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Role of the Inflammasome in Cancer

15

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15.1 Introduction

Inflammation is the seventh hallmark for cancer establishment and progression and represents the link between intrinsic (oncogenes, genome instability) and extrinsic (immune and stromal components) factors [1]. Essential to the development of cancer is the accumulation of genetic lesions in cells [2]. However, while these autonomous cell properties are necessary for tumorigenesis, they are not sufficient. Research over the last two decades has solidified the concept that tumor development and malignancy is the result of processes involving both cancer cells themselves and non-cancer cells, many of which compose the heterocellular tumor compartment [1–3]. Many tumors are associated with the infiltration

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of inflammatory cells that in most cases, due to their immune-suppressive nature, are related to a bad prognosis [4, 5].

Cancer-associated inflammatory responses play roles in many aspects of cancer biology including tumor initiation, progression, metastasis, and treatment [4, 6, 7].

Inflammation is a physiological response to protect the host against pathogen-associated molecular patterns (PAMPs) derived by invading microorganisms, and damage-associated molecular patterns (DAMPs) that derive by "sterile" endogenous threats [7]. Inflammation can be characterized by acute and chronic responses. Acute inflammation can be induced by tissue damage due to trauma, noxious, and/or microbial insults which facilitate the recruitment of immune cells to the inflamed site where they cooperate in order to isolate and eradicate the damage. In contrast, chronic inflammation represents an ongoing inflammatory response, during which immune cells are recruited, but the inflammatory stimulus is not eradicated, rather, it keeps on inducing tissue damage and destruction, manifesting as tissue fibrosis. In both cases, damaged tissues represent the alarm to restore homeostasis. In the case of a tumor mass establishment and progression, the inflammatory pattern plays a key role in that it can on one side "control," limiting the neoplastic development, but on the other the immune failure to eliminate the danger signal may result in ongoing inflammation or persistent damage that can promote the development of chronic inflammation, highly associated to cancer [8].

Epidemiology studies relate the incidence of tumors to chronic infections, dietary factors, obesity, inhaled pollutants, tobacco, and autoimmunity [3, 9, 10]. In support, higher incidence of tumor development is reported in tissues/organs exposed to both external and commensal pathogens, such as the lung, the intestine, and, to a lower extent, the liver [3, 11]. In particular, the exposure to air pollution, tobacco [12, 13], and chronic infections [14] induces the activation of inflammatory processes that render the subjects higher susceptible to malignancies. In this context, in our laboratory we proved that the stimulation of cells with air pollutants made smokers higher susceptible to the release of IL-1-like cytokines (i.e., IL-1α, IL-18) [12]. Of note, pro-inflammatory cytokines, such as IL-1β and IL-18, are detected at high levels in cancer patients, and while their pathophysiological role is still elusive, a number of studies document their ability to promote an immune-suppressive tumor microenvironment that facilitates tumor establishment and progression [1, 15, 16]. Moreover, according to the "sterile inflammation" theory, noninfectious insults, such as reactive oxygen species (ROS), oxidized and/or methylated DNA, high-mobility group box 1 (HMGB1), heat-shock proteins (HSPs), and adenosine triphosphate (ATP), generally identified as DAMPs, can independently induce chronic inflammation [17, 18]. Such endogenous stimuli, induced after the exposure to noxious as well as after microbial stimuli, can behave as tumor promoters via the induction of chronic inflammation that, rather than providing a protective response to loss of tissue homeostasis, can aberrantly facilitate tumor development. All these insults are sensed by the intracellular multimeric complex called inflammasome [19].

The inflammasome is composed of several proteins that promote caspase-1 activation (Fig. 15.1). Its activation follows engagement of Toll-like receptors (TLRs) and NOD-like receptors (NLRs), two classes of sentinel receptors that are pivotal for the detection of PAMPs and DAMPs (Table 15.1) [19]. The cooperation between these two systems allows to "sense" and respond to a large number of infectious and sterile insults. While most TLRs, except for TLR3, TLR7, and TLR9, are membrane receptors, NLRs are intracellular and, together with the adapter protein, apoptosis-associated speck-like protein containing a CARD (ASC), can assemble to form the active components of the inflammasome complexes. The recognition of PAMPs or DAMPs by TLRs can accompany or strengthen the activation of inflammasome complexes composed of specific NLRs, depending on the stimulus, leading to the activation of caspase-1 [20] (Fig. 15.1). Initially, NLRs were proposed to regulate inflammation through apoptosis, but nowadays this concept has been modified in that, while NLRs may serve as sentinels for cellular distress, their activity in the inflammasome com-

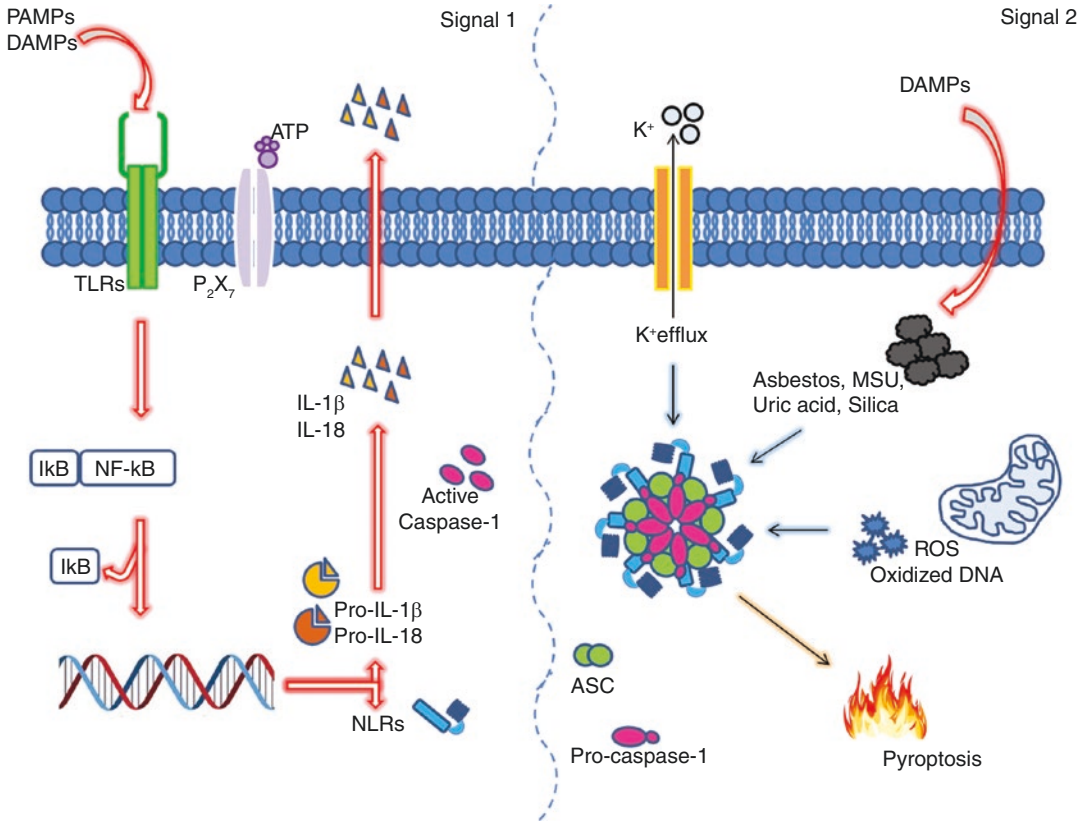

Fig. 15.1 Two-signal model of inflammasome activation. The recognition of PAMPs and/or DAMPs by extracellular or cytoplasmic TLRs leads to the activation of NF-κB (signal 1), which in turn promotes the transcription of pro-IL-1β/IL-18 or some NLRs (e.g., NLRP3). NLRs assemble into the inflammasome complex which via ASC can recruit pro-caspase-1 and promote its autocatalytic cleavage (signal 2). Caspase-1 can lead to a cascade of pro-inflammatory events via the activation of pro-IL-1β and pro-IL-18, which then interact with their own membrane receptors amplifying the inflammatory response. Furthermore, ROS, potassium efflux, changes in cell volume, calcium signaling, and lysosomal disruption have all been proposed as critical upstream signals required for inflammasome activation (signal 2). On the other hand, active caspase-1 can lead to cell pyroptosis with the consequence of membrane rupture and release of such alarmins as IL-1α and HMGB1. *PAMPs* pathogen-associated molecular patterns, *DAMPs* damage-associated molecular patterns, *TLRs* Toll-like receptors, *NLRs* Nod-like receptors, *ASC* apoptosis-associated speck-like protein containing, caspase recruitment domain (CARD), *MSU* monosodium urate, *ROS* reactive oxygen species

plex is not necessarily conducive to cell death [19, 21]. Several NLRs have so far been identified in both humans and mice, i.e., NLRP1, NLRP3, NLRP6, NLRC4, and the HIN200 protein AIM2 [22]. These proteins recognize distinct signals (Table 15.1) and, most importantly, are expressed at different levels in hematopoietic and stromal cell lineages. The expression of some NLRs is induced after the recognition of an insult (e.g., LPS) that triggers NF-κB-dependent gene expression (Fig. 15.1). In contrast, NLRC4 and AIM2 are constitutively expressed in hematopoietic cells and are directly activated by flagellin-like molecules and double-stranded DNA (dsDNA), respectively [22]. NLRP1 can sense muramyl dipeptide, *Toxoplasma gondii* and *Bacillus anthracis* lethal toxin; the identity of the ligand for NLRP6 was elusive until a recent study identified taurine, a microbial metabolite, as NLRP6 activator [23]. NLRC4, constitutively expressed in hematopoietic cells, is activated by flagellin and by the PrgJ protein from *Salmonella typhimurium*, *Pseudomonas*, *Legionella pneumophila*, and bacterial type III secretion apparatus from Gram-negative bacteria such as *Salmonella typhimurium* [24]. NLRP12 recog-

Table 15.1 The NLR species and the HIN200 protein, AIM2, are activated by specific exogenous (PAMPs) and endogenous (DAMPs) stimuli

	NLRP1	NLRP3	NLRC4	NLRP6	NLRP12	AIM2
PAMPs	*Bacillus Anthracis Toxoplasma gondii*	Fungi *Candida albicans* *Aspergillus fumigatus* *Saccharomyces cerevisiae* Viruses *Influenza, Sendai Adenovirus, Varicella zoster* Bacteria *Staphylococcus aureus* *Vibrio cholerae* *Streptococcus pyogenes, Chlamydia pneumonia* *Neisseria gonorrhea* *M. Tuberculosis, Listeria monocytogenes, Salmonella typhimurium, Shigella flexneri* *Escherichia coli*	Bacteria *Mycobacterium Tuberculosis* *Listeria monocytogenes, Salmonella typhimurium* *Shigella flexneri, Pseudomonas aeruginosa* *L. pneumophila*	Taurine	*Yersinia pestis* *Plasmodium*	Bacteria *Listeria monocytogenes, Francisella tularensis* Viruses *Cytomegalovirus* *Vaccinia virus*
DAMPs	?	Extracellular ATP Ions: K^+ and Ca^{2+} Lysosomal cathepsins Mitochondrial DNA ROS Cholesterol Ox-LDL Hyaluronan acid Uric acid Monosodium urate (MSU) Amyloid β protein Cardiolipin Asbestos and silica	?	?	?	DNA

The exposure of cells to fungi, bacteria, and viruses leads to common cellular responses that alter ion fluxes and promotes the cytosolic release of lysosomal proteases, mitochondrial dysfunction, and the generation of ROS, which have all been reported as NLRP3 stimuli. In addition, endogenous noninfectious stimuli, such as cholesterol, oxidized LDL, urate crystals, asbestos, and silica, can lead to NLRP3 inflammasome assembly. NLRP1 is activated by the cytosolic *Bacillus anthracis* lethal toxin and *Toxoplasma gondii*. NLRC4 is activated by flagellin and by the PrgJ protein from *Salmonella typhimurium*, *Pseudomonas*, and *Legionella pneumophila*, bacteria that follow type III secretion system. NLRP12 recognizes PAMPs from *Yersinia pestis* and *Plasmodium*. AIM2 binds double-stranded DNA of cells infected with *Listeria* and *Francisella* or viruses such as *Cytomegalovirus* and *Vaccinia*. NLRP6 ligand is still elusive; however, a recent study identifies taurine as a microbial metabolite NLRP6 activator

nizes PAMPs from *Yersinia* and *Plasmodium* infection [25, 26]. AIM2 is constitutively expressed in hematopoietic cells and is directly activated by dsDNA of cells infected with *Listeria* and *Francisella* or viruses such as *Cytomegalovirus* and *Vaccinia* or endogenous DNA released during cellular damage [27]. IFI16 is able to sense DNA from Kaposi

sarcoma-associated herpes virus [27]. NLRP3 inflammasome is the most characterized member of NLR and responds to various activators, a broad spectrum of microorganisms as well as their derived products, endogenous danger signals, and environmental insults. NLRP3 can be exogenously activated by *Sendai virus*, *Influenza virus*, *Adenovirus*, *Candida albicans*, *Saccharomyces cerevisiae*, *Staphylococcus aureus*, and even bacterial pore-forming toxins [28]. DAMPs like extracellular ATP (eATP), hyaluronan, monosodium urate (MSU), amyloid-β, and environmental crystalline pollutants like silica and asbestos endogenously induce NLRP3 activation [7, 28]. ROS, potassium efflux, changes in cell volume, calcium signaling, and lysosomal disruption have all been proposed as critical upstream signals required for NLRP3 activation (Table 15.1) [29]. NLRP3 inflammasome contains the adapter protein ASC, which, acting as a zipper, binds NLRP3 with pro-caspase-1, which in turn undergoes self-cleavage to form an active form of caspase-1, able to activate pro-IL-1β and pro-IL-18 into their active forms [7, 30]. So far, the activation of the canonical pathway that involved the inflammasome has proposed a two-signal model: the first signal induces the expression of NLRs, e.g., NLRP3, along with the synthesis of pro-IL-1β/IL-18 [19, 21, 22]. The first signal, defined as *priming*, mediates NF-κB activation in a TLR-dependent but also TNF receptor (TNFR)-, IL-1 receptor (IL-1R)-, and P_2X_7-dependent manner upon PAMPs or DAMPs sensing [7, 22]. The second signal involves the intracellular recognition of PAMPs or DAMPs by NLRs themselves and their assembly with ASC, which, through its CARD domain, mediates the recruitment of pro-caspase-1 and its autocatalytic cleavage (Fig. 15.1) [7, 22]. Differences exist in the activation and function of distinct NLRs and among species. In particular, it was proved that the two-signal model does not occur in human monocytes compared to murine macrophages [31]. In addition, the two-signal model seems to occur in the case of NLRP3, but not for all other NLRs, such as NLRC4 and AIM2, which do not require the *priming* for their gene expression [22].

The common function of all NLRs is the activation of caspase-1 (canonical pathway), which converts pro-IL-1β and pro-IL-18 into their active isoforms (Fig. 15.1). The third effector mechanism of the activated inflammasome, besides the release of active IL-1β and IL-18, is the induction of pyroptosis, a cell death process that requires the activity of caspase-1 and a critical mechanism by which inflammasomes contribute to host responses against Gram-negative and Gram-positive bacteria [21]. Pyroptotic cell death has been well described in cells of the hematopoietic lineage but can also occur in stromal cells, as shown in the central nervous system and in the cardiovascular system in response to ischemic and autoimmune insults [32]. The main executor of inflammasome-induced cell death is Gasdermin D (GSDMD), which can promote the formation of pores into the membrane leading to the release of intracellular content to the extracellular matrix, amplifying the inflammatory response characterized by immune infiltrates (Fig. 15.2) [33].

Alternative, noncanonical inflammasomes have also been described. This pathway engages caspase-11 (known as caspase-4 in humans) [34] or caspase-8 [35]. The activation of caspase-11 induces inflammasome-dependent caspase-1 activation and inflammasome-independent, pyroptosis-like cell death, via the release of such "alarmins" as IL-1α and HMGB1 [36]. Caspase-8, conversely, critically contributes to inhibiting receptor-interacting protein kinase 3 (RIP3)-dependent necroptosis [37] and can be involved in both apoptosis and cell survival, depending on the levels of the long and short segment of FLICE-like inhibitory protein (c-FLIP) [38]. Recent evidence suggests that noncanonical caspase-8-dependent activation of the inflammasome is required for caspase-1 function and release of IL-1β from LPS-primed macrophages [39] and dendritic cells (DCs) [40].

Because IL-1α, IL-1β, IL-18, and pyroptosis have the potential to damage the host and are strictly correlated to poor prognosis of cancer patients [41], tight control of these effector path-

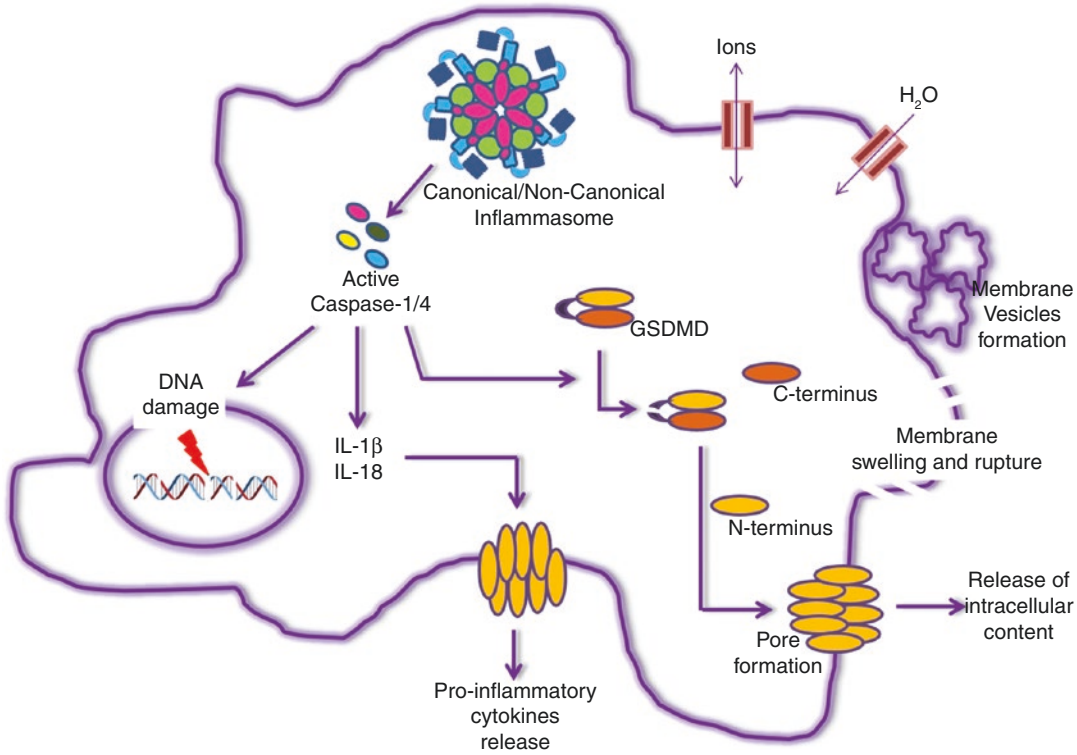

Fig. 15.2 Inflammasome-dependent pyroptotic cell death**.** Pyroptosis is induced by the canonical caspase-1 inflammasomes or by activation of caspase-4, caspase-5, and caspase-11. The activation of the above caspases cleaves gasdermin D (GSDMD) in its middle linker to release its gasdermin-N domain, which executes pyroptosis via its pore-forming activity. Caspase-1-dependent plasma-membrane pores dissipate cellular ionic gradients, producing a net increased osmotic pressure, water influx, cell swelling, and, eventually, osmotic lysis and release of inflammatory intracellular contents. DNA damage also occurs during pyroptosis

ways is critical for the prevention of chronic inflammation. These processes are key steps for the regulation of programmed cell death, differentiation, and proliferation [32], three aspects that in the context of cancer represent the rheostat for tumor proliferation versus tumor arrest/regression.

15.2 Pro-tumorigenic Role of NF-κB and STAT-3 and Their Link to the Inflammasome Complex in Cancer

NF-κB and signal transducer and activator of transcription 3 (STAT-3) have been widely described as over-activated in cancer and, more importantly, as the main transcription factors involved in tumor progression [3].

Constitutive activation of NF-κB exerts a pro-tumorigenic effect; indeed, patients with chronic inflammatory diseases have higher risk to develop cancer [42]. The continuous activation of NF-κB in the tumor microenvironment is related to the higher levels of cytokines released by both infiltrated and resident immune cells in the tumor mass [42]. In particular, IL-1β, as well as IL-1α, binds to IL-1R, which signaling pathway leads to NF-κB activation [43]. In support, conditional ablation of NF-κB in a colon and liver carcinoma mouse model resulted in reduced tumor size [44]. According to the two-signal model of inflammasome induction, the first signal primes NF-κB activation that leads to NLRs and pro-IL-1β expres-

sion (Fig. 15.1). The second signal is required for the activation of caspase-1 and IL-1β/IL-18 and for the regulation of transcriptional and posttranscriptional mechanisms that underlie NF-κB activity. While on one side the activation of NF-κB can amplify IL-1-like cytokine release, on the other it can also fuel the activity of oncogenes, such as K-Ras, in an IL-1α-, IL-1R-, and MyD88-dependent manner [45]. Besides activating NF-κB, pro-inflammatory cytokines involved in inflammation-driven carcinogenesis and tumor progression converge at the level of the transcription factor STAT-3 [3]. STAT3 and NF-κB work together in a network, by regulating a set of genes encoding chemokines and cytokines and by controlling various target genes including cell cycle control and antiapoptotic genes [3]. Active phospho-STAT-3 is detected in both mouse models and human samples of gastric, colon, liver, lung, and pancreatic cancers [3, 9, 46, 47]. Activation of STAT-3 by IL-6, TNF-α, IL-10, IL-22, IL-23, and epidermal growth factor (EGF) increases malignant cell proliferation by upregulating the expression of cell cycle regulators cyclin D1, cyclin D2, and cyclin B, of the proto-oncogene MYC, of the antiapoptotic genes, BCL-2 and BCL2-like 1, which encodes BCL-xl [3], K-Ras, the proto-oncogene tyrosine-protein (Src), and Abelson murine leukemia viral oncogene homolog 1 (Abl1) [28]. IL-6 release can be induced by IL-1-like cytokines [48]. In addition, STAT-3 signaling in epithelial cells drives K-Ras-dependent neoplastic development in a mouse model of pancreatic adenocarcinoma, even in the absence of an inflammatory insult [46]. Therefore, because NF-κB activation and the amplification of the NF-κB-IL-6-STAT-3 signaling cascade occur in most malignancies and facilitate pro-inflammatory and pro-survival gene expression, and because NF-κB activity is a critical first signal for inflammasome activation, the inflammasome complex may represent the rheostat for tumor-associated chronic inflammation. In fact, the tumor microenvironment is an important source of pro-tumorigenic inflammatory cytokines, such as IL-1-like cytokines, that are induced by and are in turn potent inducers of NF-κB and STAT-3 in a direct and indirect manner. Chronic inflammation can also increase cell susceptibility to genomic destabilization via the downregulation of DNA repair pathways and the accumulation of genetic mutations and instability [49].

As mentioned above, TLRs are the major sentinel receptors for the recognition of microbial PAMPs and endogenous DAMPs. Signaling through TLRs, except for TLR3, is MyD88-dependent and induces the activation of NF-κB, the first signal required for inflammasome activation [32]. MyD88, an adapter protein located downstream of TLR and IL-1R signaling, contributes to carcinogenesis in mouse models of skin, liver, pancreas, and colon cancers [3]. The genetic absence of MyD88 prevented dextran sodium sulfate (DSS)-azoxymethane (AOM)-induced colon [50], methylcolantrene (MCA)-induced skin [51], and diethylnitrosamine (DEN)-induced liver carcinogenesis [52]. The activation of a K-Ras/IL-1α autocrine loop has been shown to depend on MyD88 signaling [46]. However, divergent roles of MyD88 in carcinogenesis have been reported. In the DSS/AOM model of colitis-associated colon cancer, MyD88 has also been described as protective against tumor initiation/progression [50]. We have shown that TLR stimulation can result in either tumor cell survival or apoptosis depending on the activity of type I IFN, released via engagement of TIR-domain-containing adapter-inducing interferon-β (TRIF) subsequent to TLR4 and TLR3 signaling [5, 53, 54]. Hasan et al. reported that tumor cell proliferation is antagonized by type I IFN in favor of tumor cell apoptosis [55]. However, depending on the levels of type I IFN, both STAT-3 and STAT-1 can be induced, the former promoting a pro-carcinogenic, immune-suppressive environment and the latter inducing a Th1-biased, tumor-suppressive environment [56]. As an additional level of complexity, type I IFN is known to inhibit NLRP3 activity [21] but can also be the first signal for AIM2 and caspase-11 activation, which function in cancer is still under-investigated [32, 57, 58]. Type I IFN restricts NLRP3-

dependent inflammasome activity by both inducing STAT-3, leading to the release of the immune-suppressive cytokine IL-10, and hence favoring tumor immune escape by promoting, via STAT-1, the release of nitric oxide and NLRP3 nitrosylation [21]. It is likely then that STAT-1-dependent inhibition of the NLRP3 inflammasome could explain the anti-tumor activity of type I IFN. Other studies reported that IFN-γ mediated the anticancer activity of T lymphocytes that had been primed in a P_2X_7-NLRP3-ASC-caspase1-IL-1β-dependent fashion. In particular, the stimulation of P_2X_7 on DCs with ATP activated the NLRP3 inflammasome with the ensuing IL-1β release, which triggered IFN-γ-producing, tumor antigen-specific $CD8^+$ T-cells with consequent tumor regression/arrest [59]. On the other hand, though, oxidized DNA, produced during tumor initiation/progression, potentiates stimulator of IFN I genes (STING)-dependent signaling pathways [60], responsible for immune-regulatory/suppressive responses via the activity of indoleamine-2,3-dioxygenase (IDO) in DCs [60] and for the recruitment and differentiation of Treg in tumor masses [61]. Therefore, tumor cells interfere with anti-tumor immune editing and reprogram immune cells to a suppressive phenotype by tightly controlling anti-tumor inflammatory responses.

Given these premises, the production of IL-1-like cytokines via the canonical and noncanonical inflammasomes seems to be a critical step upstream of other inflammatory signaling pathways, including those depending on STAT-3 and NF-κB activation. Agents that specifically block inflammasome-dependent IL-1-like cytokines are deemed to be critically needed for cancer treatment and are under intensive investigation [41].

15.3 Role of NLRs in Carcinogenesis

NLRs play a crucial role in both promoting and dampening inflammation associated with tumors. While recent studies demonstrated that inflammasomes promote cancer development and progression in certain types of tumor, such as skin and breast cancer [27], others proved a protective effect in other cancers, such as colorectal cancer. Therefore, the role of NLRs, and thus of the inflammasome in cancer, is still ill defined. The conflicting findings reviewed here may be accounted for by specific tissue microenvironments and/or the differential involvement of NLRs in cancer initiation and progression.

Pro-tumorigenic Role of NLRP3 NLRP3 is certainly the most studied NLR. NLRP3 polymorphism is associated to higher susceptibility to melanoma [62] and to poor survival rate for colorectal cancer [63] and myeloma [64] patients. Similarly, NLRP1 genetic alterations were observed in patients exposed to asbestos that developed mesothelioma [65]. The alteration of NLRP3 and/or NLRP1 expression/activity is in line with reports of high levels of plasma and tissue IL-1β and IL-18 as bad prognostic biomarkers in cancer patients [66]. Several studies have focused on the pro-carcinogenic activity of NLRP3. It has been demonstrated that NLRP3 may suppress natural killer (NK) and T-cell-mediated anti-tumor actions and immune editing in a mouse model of carcinogen-induced sarcoma and metastatic melanoma [67]; in support, NLRP3, but not caspase-1/caspase-11 or IL-1R knockout (KO) mice, had a substantially reduced number of lung metastases compared with wild-type (wt) mice when injected intravenously with B16-F10 melanoma cells or RM-1 prostate carcinoma cells [67]. This phenomenon was mediated by IL-1β-dependent recruitment of immune suppressive cells, such as myeloid-derived suppressor cells (MDSCs) and Treg. Consistently, NLRP3-deficient mice had reduced pulmonary metastases in an orthotopic transplant mouse model of mammary adenocarcinoma [67] and reduced skin papilloma lesions [67]. One potential mechanism was associated to NLRP3-promoted expansion of immunosuppressive macrophages in pancreatic ductal adenocarcinoma (PDA), with the inhibition of an anti-tumor T-cell response [68]. Moreover, cellular distress, accompanied by higher fluxes of potassium and calcium ions, a higher level of oxidative stress

and related ROS, and mitochondrial dysfunction, is nowadays recognized as the main endogenous stimulus for NLRP3 activation [58] (Table 15.1). Release of ROS by neutrophils and other inflammatory cell types can be mutagenic and/or promote signaling events leading to proliferation and transformation of lung cells [69]. Together with other growth factors, ROS release can also contribute to promote oncogene activation, genomic instability, and matrix degradation. Inhalation of environmental pollutants, such as asbestos and silica, is at the basis of lung inflammation that can lead to both fibrosis and lung cancer [12, 13, 70]. The production of ROS triggered by the phagocytosis of asbestos and silica or other environmental pollutants by macrophages/monocytes leads to the activation of the NLRP3 inflammasome [71]. Exposure of a human monocyte cell line (THP-1) to asbestos and silica induced the release of IL-1β to levels comparable to those elicited by the addition of MSU crystals, an established inflammasome activator (Table 15.1). Similarly, smokers were more susceptible to IL-1-like cytokine production in a NLRP3-dependent manner [12, 72]. The genetic absence of NLRP3, ASC, and caspase-1 reduced IL-1β levels in the supernatants collected from asbestos- and silica-treated THP-1 cells. In contrast, the absence of MyD88-, TLR-, and IL-1R-dependent signaling did not affect IL-1β release, implying the exclusive involvement of the inflammasome in this model. The mechanism of inflammasome activation by ROS in particulate-activated monocytes is under investigation. ROS can induce cell distress subsequent to mitochondrial dysfunction and the oxidation of many cell targets, among which the mitochondrial (mt) DNA, which binds to and activates NLRP3 [73, 74]. These mechanisms underlie K-Ras-induced tumorigenicity in the lung [75]. In a model of *Chlamydia pneumoniae* infection, it was shown that the oxidation of mtDNA leads to apoptosis of bone-marrow-derived macrophages (BMDMs) via the induction of IL-1β and the reduction of BCL-2 levels [74]. In this model the induction of apoptosis was viewed as a protective host mechanism against bacteria dissemination. In contrast, the overproduction of ROS and thus of IL-1β facilitates the proliferation rate of tumor cells in a mouse model of melanoma and in human mesothelioma cells [76–78]. It can be inferred, then, that NLRP3 pro-carcinogenic activity is the result of combined effects on tumor cell proliferation and survival and the induction of apoptotic pathways in innate immune cells, which would prevent these cells from instructing the adaptive immunity on how to immune survey the tumor microenvironment.

Recent evidence suggests that NLRP3 inflammasome in tumor microenvironments support tumor growth and metastases in breast cancer [79]. In an orthotopic mammary gland tumor model with EO771 murine breast cancer cells, caspase-1 and NLRP3 KO mice had significantly fewer primary tumor growth, correlated to lower mature IL-1β and caspase-1 levels. This study highlighted that the NLRP3 inflammasome modulated the tumor microenvironment in that NLRP3 activation enhanced the infiltration of myeloid cells, including MDSCs and tumor-associated macrophages (TAMs) which facilitated tumor immune evasion [79]. Similarly, NLRP3 was involved in melanoma growth following P_2X_7 and PANX1, ATP-dependent iron channels, activation [28].

Anti-carcinogenic Role of NLRP3 Despite mounting clinical and experimental evidence of the pro-carcinogenic activities of NLRP3, its role in cancer is still controversial. The genetic absence of NLRP3 increased the susceptibility to cancer and the number of colon polyps in a DSS-AOM mouse model of colon carcinoma, suggesting that NLRP3 may have a protective role in tumor formation in the colon [80]. In support, Wei et al. found that NLRP3 inflammasome might suppress the development of human liver cancer as the expression of NLRP3 was significantly decreased or completely lost in cancerous samples of hepatocellular carcinoma (HCC) patients [81]. In a model of liver metastasis, the activation of NLRP3 inflammasome was able to suppress the metastasis by priming NK cells to enhance immunosurveillance [28]. However, despite it was shown an association of

increased NLRP3 with the promotion and metastasis of gastric cancer and colitis-associated colon cancer [82], recently it was observed that NLRP3 expression was heterogeneous in stromal, benign, and cancerous prostate tissues with no distinction between the adjacent benign and cancer tissues [83]. In an elegant study of epithelial skin carcinogenesis, ASC, a main component/adaptor of the inflammasomes, could behave as pro-inflammatory in infiltrating immune cells, favoring tumor development; in contrast, its activity in keratinocytes limits tumor cell proliferation through the activation of p53 [17]. It was shown that mice specifically deficient for ASC in keratinocytes developed more tumors than to WT mice [84]; however, the same authors demonstrated that the genetic absence of ASC in myeloid cells had protective action in cancer development, focusing on its potential as pro-carcinogenesis biomarker and potential therapeutic target.

It is feasible that NLRP3 exerts different functions in hematopoietic versus structural cells. Bone marrow chimera studies have identified that signaling through the NLRP3 inflammasome in the hematopoietic, but not in the stromal compartment, is essential for mediating protection against tumorigenesis [85]. The activation of NLRP3 in DCs induces IL-1β-dependent adaptive immunity against EG7 or EL4 cell-implanted thymoma [59]. This protective phenotype was observed after anthracycline treatment, which induced ATP release from dying tumor cells, "sensed" by the P_2X_7 purinergic receptor on DCs, triggering a downstream NLRP3/caspase-1/IL-1β-dependent anti-tumor mechanism. IL-1β and IL-18 released after ATP-induced NLRP3 inflammasome activation promoted γδT-cell-induced secretion of IL-17, which recruited $CD8^+$ αβ T-cells able to produce IFN-γ which damaged therapy-resistant tumor cells [28]. The administration of interleukin-1 receptor antagonist (IL-1RA) in this model restored tumor progression [59]. However, it was also demonstrated that NLRP3 dampened the effectiveness of a DC-based anti-tumor vaccine by promoting the recruitment of immune-suppressive MDSCs [86]. The depletion of MDSCs, which also express NLRP3, rescued WT mice but not NLRP3 KO mice [86], confirming that the protective activity of NLRP3 is strictly correlated to the cell lineage involved [87].

Taken together, these findings characterize NLRP3 as a problematic therapeutic target.

Pro- and Anti-carcinogenic Roles of Other NLRs NLRC4 recognizes a number of Gram-negative bacteria, including *Salmonella* and *Pseudomonas aeruginosa* [88] (Table 15.1). Apart from NLRP3, also NAIP/NLRC4 is crucial for the integrity of intestinal epithelium; indeed, NAIP/NLRC4/caspase-1 axis activation by intestinal epithelial cells increased the secretion of IL-18 to activate protective gut immune responses [28]. NLRC4- and caspase-1-deficient mice developed increased colonic inflammation, responsible for higher colon adenocarcinoma burden, in a DSS-AOM mouse model [24]. NLRC4 and caspase-1 were inferred to exert a protective function in that model via a direct effect on epithelial cell proliferation, which, in a non-hematopoietic compartment, is thought to play a more prominent role than colonic inflammation [24]. Consistently, EL4 thymoma and B16 cells expressing flagellin, recognized by NLRC4 and TLR5, were unable to induce tumor implantation and progression due to the activation of onco-suppressive pathways [89]. Interestingly, a recent study highlighted a causal link between obesity, inflammasome activation, and breast cancer progression [90]. The authors showed that the activation of the NLRC4 inflammasome by obesity, one of risk factors for tumor development, contributed to breast cancer progression and that tumor growth was depended on caspase-1. Caspase-1 KO mice had significantly reduced tumor growth under experimental obesity conditions, characterized by NLRC4-induced IL-1β production in myeloid cells, which augmented vascular endothelial growth factor (VEGF) levels and angiogenesis [90].

Other NLRs sensors, including NLRP12, NLRP1, and NLRP6, mediate protection against tumorigenesis [85]. NLRP12, like NLRP3,

NLRP6, and NLRC4, plays a protective role in the DSS-AOM mouse model [91]. The absence of NLRP12 in this model increased pro-inflammatory cytokine levels, extracellular signal-regulated kinase (ERK), NF-κB, and STAT-3 activation [92]. The expression of NLRP12 was significantly higher in malignant prostate cancer compared to adjacent benign tissues, while ASC and pro-caspase-1 were confined to aggressive prostate cancer cells, suggesting that an increased expression of these inflammasome sensors could underlie inflammation and pro-inflammatory cytokines release in prostate cancer [83].

NLRP6, required for the maintenance of both composition and distribution of commensal bacteria in the gut [27], confers protection against colon tumorigenesis; in fact, NLRP6 KO mice had increased propensity to develop colorectal cancer due to increased expression of pro-inflammatory cytokines (i.e., IL-18) after caspase-1 activation in response to AOM and DSS treatment [85]. Moreover, the deficiency of NLRP6 led to the alteration of microbiota compositions in the gut [28]. Nevertheless, although the expression of NLRP6 was higher in the intestinal epithelium than hematopoietic cells, its activity in the hematopoietic compartment was more important for host defense against the colitis-associated cancer (CAC) development [28].

Mutation in NLRP1, the most expressed inflammasome in human skin, increased susceptibility to skin cancer. Indeed, keratinocytes from patients with skin cancer displayed NLRP1 inflammasome activation and release of IL-1 family cytokines [93], contrary to what was observed in mice [94].

Pro- and Anti-carcinogenic Role of AIM2 in Cancer Despite its involvement in host defense against infections, the role of AIM2 in carcinogenesis is less clear. Controversial data have been reported for both tumor-suppressive and tumor-promoting functions of AIM2. The DNA-sensing inflammasome sensor AIM2, initially identified as a tumor suppressor in melanoma, suppressed colon cancer development inhibiting overproliferation of intestinal stem cells [95]. AIM2 can inhibit AOM-DSS-induced and spontaneous colorectal tumorigenesis via an inflammasome-independent mechanism and is associated with lower risk of colorectal cancer [95]. In contrast, AIM2 appears downregulated in hepatocarcinoma with an ensuing higher susceptibility to cancer progression [96].

Exogenous AIM2 expression was as well related to reduced breast cancer cell proliferation in humans [97]. Its activity was correlated to the inhibition of NF-κB transcriptional activity and to mammary tumor arrest in a mouse model [97].

Moreover, to evaluate the correlation between IFN type I signaling and AIM2 inflammasome, a research group studied the association of benign prostate hyperplasia (BPH) and prostate cancer, a type of tumor characterized by loss of type I IFN signaling [98]. The authors demonstrated that IFNs (α, β, or γ) induced AIM2 expression in human prostate epithelial cells (PrECs). The levels of AIM2 mRNA were higher in BPH than in normal prostate tissue, but significantly lower in clinical tumor specimens [98], implying that AIM2 may contribute earlier to tumor progression during the chronic inflammatory phase that leads to hyperplasia and then to the tumor mass.

More recently it was shown that AIM2 was specifically upregulated and involved in cutaneous squamous cell carcinoma (cSCC), the most common metastatic skin cancer [99]; indeed, AIM2 knockdown resulted in decreased cSCC cell viability and invasion, suppression of growth, and vascularization of cSCC xenografts in vivo.

In support to the pro-carcinogenic role of the AIM2 inflammasome, we found that lung tumor masses were highly populated by plasmacytoid dendritic cells (pDCs) [100, 101], able to produce high levels of IL-1α under AIM2 activation. In particular, we found that the activation of AIM2 in lung tumor-associated pDCs promoted calcium efflux leading to calpain activation and high levels of IL-1α, which facilitated tumor cell proliferation in the lung.

15.4 Role of Inflammasome-Dependent Cytokines in Cancer

High serum concentrations of inflammasome-related IL-1-like cytokines are found in malignancies with low-rate survival from the time of diagnosis [41]. These cytokines, directly or via the induction of TNF-α and IL-6 [102], are involved in cell proliferation and survival [103], as well as cell adhesion and migration [3, 24], all features of tumor progression and invasiveness.

In the tumor microenvironment, IL-1-like cytokines can be secreted by both malignant and infiltrated immune cells [104]. Tumorigenesis, tumor progression, dissemination, and tumor immune editing are affected by the presence or not of these cytokines which can mediate a variety of local and systemic activities.

IL-1β and IL-18 These cytokines represent the main effectors of inflammasome-mediated pathways. Both pro-IL-1β and pro-IL-18 are converted into their active forms mainly by caspase-1, and, in fact, caspase-1-deficient mice produce low IL-1β and IL-18 levels [22]. Moreover, caspase-11 may promote IL-1β and IL-18 maturation via the induction of caspase-1 activity [32, 57, 58]. However, pro-IL-1β can be processed by other enzymes, such as neutrophil serine proteinase-3 and granzyme A in humans [32, 105, 106]. In addition, caspase-8 can cleave pro-IL-1β and pro-IL-18 into their active forms following Fas ligand (FasL) activation in a caspase-1- and ASC-independent manner in LPS-primed macrophages [107]. In this study, the activation of Fas, a TNF family receptor, induced caspase-8 to cleave IL-1β and IL-18 independently of inflammasomes or RIP3, the latter involved in necrosis-induced cell death [35]. It has been recently shown that, following NLRP3 activation, caspase-8, rather than caspase-1, is mainly involved in IL-1β and IL-18 activation in DCs [40] and macrophages [39]. Hence, novel noncanonical inflammasome-dependent IL-1β/IL-18 activation pathways may exist in myeloid cells. While it is felt that these cytokine-dependent signaling pathways are crucial for inflammatory processes, their role in tumor immune surveillance is still debated.

IL-1β and IL-18 have contrasting functions in the tumor milieu. IL-1β induces fever, promotes T-cell survival, contributes to the polarization of Th1, Th2, and Th17 clones, and mediates leukocyte migration [108]. IL-18 can cooperate with IL-12 in Th1 polarization and the activation of NK cells and can promote Th17 responses in the presence of IL-23 [16]. In the absence of IL-12 and IL-23, IL-18 can vice versa promote a Th2-biased response [16]. Therefore, depending on the microenvironment, IL-1β and IL-18 can have contrasting effects on tumor-associated inflammation and tumor surveillance. These cytokines can exert a direct pro-carcinogenic activity via the release of trophic factors, such as fibroblast growth factor (FGF) 2 and VEGF, which allow malignant cells, cancer-associated fibroblasts (CAFs), and endothelial cells to fuel and foster tumor cell survival and invasiveness [1]. In addition, IL-1β induces IL-6, whose pro-tumorigenic activity is mediated through the activation of STAT-3 [109]. IL-1β can also induce the synthesis/release of TNF-α, which in some circumstances can act as an anti-tumor factor [110] but in others can participate to the recruitment of immune-suppressive cells, such as MDSCs, which favor neoplastic growth and progression [111]. In support of the pro-tumorigenic potential of IL-1β, the inoculation of lung cancer cells engineered to express higher levels of this cytokine resulted in higher aggressiveness and dissemination [112]. More importantly, elevated IL-1β levels are detected in human lung, colon breast carcinoma, stomach, and in melanoma [41]. Furthermore, reduced tumor growth is reported in mice given anakinra, an IL-1RA [41]. The association of anakinra and corticosteroids, these latter known to inhibit pro-IL-β gene expression and to upregulate the endogenous IL-1RA, resulted in lower-rate myeloma cell proliferation [113]. Therefore, IL-1R antagonists or neutralizing antibodies for IL-1β may represent novel anti-tumor therapies that can subvert both tumor

proliferation and tumor immune escape. Several clinical trials are actually being performed to prove the beneficial anti-tumor activity of anakinra [41]. In support, Guo et al. demonstrated that blocking IL-1R signaling with an IL-1RA or anti-IL-1R antibody inhibited breast tumor growth and metastasis accompanied by decreased myeloid cell recruitment [79]. Similarly, in our recent study we found that lung TAMs, critical components of tumor microenvironment, were able to release higher levels IL-1β than macrophages derived by the lung of naïve mice, implying that the release of IL-1β by TAMs favors lung carcinogenesis in a mouse model of carcinogen-induced lung cancer [114]. We proved that IL-1β release was caspase-11- and NLRP3/caspase-1-dependent and that IL-1β-producing TAMs were able to favor lung tumorigenesis after the activation of TLR4/caspase-1 and caspase-11 axis involved in NLRP3 inflammasome [114]. However, it has to be pointed out that in the same mouse model and in human samples of non-small cell lung cancer (NSCLC), we found that caspase-8 was involved in lung cancer in that its pharmacological inhibition by means of z-IETD-FMK significantly reduced lung tumor burden, accompanied by lower levels of IL-6, TNF-α, IL-18, IL-1α, IL-33, but not IL-1β, innate immune suppressive cells (i.e., MDSCs) [115]. These two latter studies confirm the involvement of the inflammasome and its related cytokines to tumor growth in the lung but point at the specific enzymes/cytokines according to the tissue/site of activation.

IL-18 levels correlate with cancer-related morbidity in patients with ovarian, head and neck, lung, and colon carcinoma [19]. Experimental mouse models of metastatic melanoma showed that IL-18 acts as an immunosuppressive cytokine by contrasting NK cell cytotoxic activity [116]. Further evidence of the pro-tumorigenic function of IL-18 come from studies of animals administered IL-18-binding protein (IL-18BP), an IL-18 soluble ligand that neutralizes its activity [117].

While clinical and experimental evidence strongly supports the pro-tumorigenic activities of IL-1β and IL-18, their established roles in Th1 and cytotoxic T lymphocytes (CTL) polarization would make these cytokines well suited to combat tumor immune evasion. Indeed, the anti-tumoral properties of IL-1β and IL-18 have been described in different stages of tumor progression [118]. In addition, the genetic absence of IL-18 increased the susceptibility to colitis and polyp formation in a mouse model of AOM-DSS-induced colon carcinoma [119]. In support to the importance of IL-18 in suppressing colorectal cancer development, studies suggested that IL-18 produced during inflammasome activation was critical for the homeostasis of the epithelial barrier in the intestinal tissue repair and remodeling [27]. Along this line, it is interesting to note that these cytokines are produced at high levels during classical chemotherapy protocols and are known to foster DC activity against tumor cells [59]. In sharp contrast, we found that human peripheral blood mononuclear cells (PBMCs) obtained from smokers and COPD patients, subjects at high risk for lung cancer were highly susceptible to IL-18 release under air pollution exposure [12, 13], implying that based on to the tissue/organ encountered, the inflammatory response could accordingly lead to differential phenomena.

IL-1α IL-1α is an alarmin that, like IL-1β and IL-18, can be activated from a precursor form [48]. However, while IL-1β/IL-18 maturation requires caspase-1 activation, the release of IL-1α is not strictly dependent on caspase-1 but can also be processed by caspase-11 and calpain [17, 32, 120, 121] and critically depends on the levels of the decoy receptor, IL-1R2 [120]. Active IL-1α can also be present on the plasma membrane to "instruct" the adaptive immunity and can be processed and activated in the extracellular milieu by granzyme B [122]. In addition, it has been recently discovered that the precursor form (previously referred to as non-active form) of IL-1α can also trigger sterile inflammation [48].

In models of DEN-induced liver carcinoma [52], skin papillomas [123], and gastric carcinoma [44], IL-1α is released by dying cells,

which stimulate oxidative stress pathways, responsible of local inflammation and in some cases of cell rescue from death to provide tissue regeneration and subsequent accumulation of mutations leading to tumor initiation/progression [64]. In support of these findings, IL-1α-induced IL-6 activates STAT-3 and promotes liver as well as gastric tumorigenesis [44, 52, 123]. An indirect evidence for IL-1α-dependent tumor outgrowth is provided in studies of IL-1R1- and MyD88-deficient mice, which are less prone to developing skin [51], colon [50], and liver [124] tumor lesions. Besides its role in tumor-associated inflammation, IL-1α activity was also associated to the activity of mutated K-Ras, one of the main oncogenes, that induces constitutive activation of NF-κB and AP-1, which on one side can promote an autocrine loop for further IL-1α expression/secretion and on the other increase tumor burden. These processes were well characterized in a mouse model of pancreatic carcinoma [125]. In contrast, in a model of MCA-induced fibrosarcoma, IL-1α KO mice had similar tumor lesions as WT mice [64]. This implied that IL-1α was not implicated in tumor outgrowth but that, rather, cell-membrane exposed IL-1α would promote anti-tumor surveillance via the activation of NK, $CD4^+$, and $CD8^+$ T-cells [64]. Of note, some cancer cells can express membrane IL-1α, which can increase their immunogenicity and promote anti-tumor immune surveillance and tumor regression. However, high levels of IL-1α in the tumor microenvironment can favor angiogenesis and invasiveness [120]. In support, IL-1RA administration inhibited IL-1α-induced angiogenesis in gastric cancer, suggesting that it may be a potential target in the clinical treatment of gastric cancer patients, possibly alone or in combination with an anti-VEGF antibody or with other chemotherapy agents [126].

Similarly, in our lab we found that IL-1α is one of the predominant cytokines in lung tumor microenvironment after inflammasome activation. Both human tumor-associated immunosuppressive plasmacytoid dendritic cells (TApDCs) and mouse TAMs produced high levels of IL-1α in an AIM2-dependent manner, favoring lung carcinogenesis [100, 114].

IL-33 Interleukin-33 (IL-33) is a member of the IL-1 family, which, in contrast to IL-1β and IL-18, is inactivated upon caspase cleavage but is biologically active as full-length IL-33. Additionally, its activity is enhanced approximately tenfold upon cleavage by neutrophil serine proteases cathepsin G and elastase [127]. Recent findings have revealed an important contribution of IL-33 to several cancers, where it may exert pro- and anti-tumorigenic functions [128]. It was demonstrated that CAFs in head and neck squamous cancer (HNSCC) microenvironment were able to release IL-33, which in turn triggered epithelial mesenchymal transition (EMT) of cancer cells, thereby supporting their ability for migration and invasion; in support to the protumor effect of IL-33, this cytokine in HNSCC patients was associated to lower survival rate [129].

Similarly, IL-33 is highly present in tumor lesions of NSCLC patients, associated with the disease clinical stage [130]. Moreover, the protumorigenic role of IL-33/ST2 signaling was also proved in breast cancer [127], in that the genetic absence of ST2, also known as interleukin-1 receptor-like 1 (IL1RL1), showed decreased tumor cell proliferation and reduced metastatic potential to the lung and liver in a syngeneic 4T1 breast cancer mouse model due to less accumulation of suppressor cells MDSCs and immunosuppressive TGF-β strictly correlated to reduced tumor growth [131]. Some data indicate that IL-33 is highly present in the serum of HCC patients [127] although another research group did not find differences in IL-33 serum levels in HCC compared to liver cirrhosis patients and healthy controls [132].

Furthermore, IL-33 appears to exert a procarcinogenic function in gastric cancer. A recent study reported a dose-dependent increase in cancer cell invasion and migration of human gastric cancer cell lines stimulated with IL-33; this effect, which was linked to ERK1/2 activation, a pathway known to be important for tumor invasion and metastasis, was abrogated by knocking down IL1RL1 [133].

Other Cytokines/Growth Factors Another mechanism by which the inflammasome may contribute to tumor immune escape is the secretion of other pro-inflammatory cytokines, such as HMGB1, and the induction of growth factors such as FGF2 [134], via as yet unclear biochemical mechanisms. Caspase-1 is essential for FGF2 secretion by macrophages [135]. However, unlike IL-1β and IL-18, HMGB1 and FGF2 are not processed by caspase-1 [66, 135, 136], suggesting an indirect mechanism of inflammasome-dependent regulation of these unconventional proteins. It is important to note, though, that secretion of these proteins might directly depend on caspase-1-mediated pyroptosis. In this context, an intriguing role was recently identified for caspase-11, which is engaged in noncanonical inflammasome molecular pathways [34]. While caspase-11 is not critical for caspase-1 activation upon LPS stimulation, it can mediate the activation of the NLRP3 inflammasome during endotoxemia [36, 57] and lead to caspase-1 maturation and IL-1β release from infected macrophages. The interplay between caspase-1 and caspase-11 in cancer is still underinvestigated, but it is felt that a more thorough investigation of these pathways in carcinogenesis may help reconcile some of the discrepancies in the field. Understanding the regulation and function of noncanonical inflammasome-dependent pathways, involving caspase-11 and caspase-8, may, therefore, help clarify the significance of the inflammasome and its effectors in cancer.

IL-27 is another cytokine with both pro- and anti-inflammatory properties associated to NLRP3 inflammasome activation in monocytes [137]. The effects of IL-27 on the immune response may be dual, resulting in tumor-promoting effects in vivo, as suggested by increased IL-27 expression in some human cancers [138]. Despite poor reports, IL-27 was found high in the serum of gastroesophageal cancer [139] and in breast cancer patients in correlation with VEGF and the clinical stage [140]. IL-27 was highly expressed in invasive cutaneous melanoma, particularly at advanced stages of progression, whereas no expression was found in benign nevi and in situ melanomas. Moreover, IL-27 expression was correlated with PD-L1 and IL-10 in melanoma samples [141]. Similarly, IL-27 was found to induce the expression of immune-regulatory molecules such as IL-18BP and PD-L1 and IDO in human ovarian cancer cells [138]. Evidence support a role of IL-27 in adult acute myeloid leukemia (AML), but not in pediatric AML, because it was able to promote the proliferation and survival of adult AML cell lines coexpressing IL-27Rα (WSX1) and gp130 [142]. IL-27-mediated signaling pathway activated STAT-1/-3 and ERK1/2 in leukemic cells [142].

Nevertheless, IL-27 has shown anti-tumor activity in several tumor models in vitro and in vivo, acting through multiple mechanisms such as activation of anti-tumor immune responses and direct inhibition of tumor cell proliferation, survival, and angiogenic and invasive properties [138].

IL-37 is another member of IL-1 family cytokines which has shown anti-inflammatory activities. It is able to suppress the production of pro-inflammatory cytokines such as IL-1α and TNF-α, without altering anti-inflammatory cytokines, such as IL-10; it blocks DCs activation, and is also involved in the adaptive immunity [143]. This cytokine is expressed by macrophages, epithelial cells, and PBMCs [144]. It was reported that caspase-1 is involved in IL-37 protein processing after LPS, TNF, other TLR agonists and IL-1 stimulation [144].

The anti-tumor effects of IL-37 have been studied in HCC [145], renal carcinoma [146], NSCLC [147], fibrosarcoma, and cervical and breast cancer [143]; these studies reported that IL-37 was well correlated to positive prognosis of patients.

15.5 Third Effector Mechanism of the Inflammasome: Pyroptosis

Besides cytokines, pyroptosis is the third effector mechanism following inflammasome activation. Although the molecular and cellular mechanisms

underlying pyroptosis induction still remain elusive, it is clear that this cell death process takes place independently of the secretion of IL-1β and IL-18 [32] (Fig. 15.2). Pyroptosis was first observed by Zychlinsky and his colleagues in macrophages infected with *Shigella flexneri* [148], but it can occur in several other cell types and be activated by a variety of stimuli other than the presence of infection [149]. It was initially recognized as apoptosis, but later, it was confirmed as a lytic form of cell death and revised as caspase-1-dependent cell death identified as pyroptosis. Pyroptosis is defined as a pro-inflammatory cell death process, critical for host defense against the invasion of pathogens. It differs from apoptosis in that it is characterized by cytoplasmic swelling and early plasma membrane rupture, as in the case of necrosis. Therefore, because of the release of the cytoplasmic content into the extracellular matrix, pyroptosis is considered a pro-inflammatory process. In particular, it can be induced by the canonical caspase-1 inflammasomes or by activation of caspase-4, caspase-5, and caspase-11 by cytosolic lipopolysaccharide. The activation of the above caspases cleaves GSDMD in its middle linker to release its gasdermin-N domain, which executes pyroptosis via its pore-forming activity (Fig. 15.2) [33]. Caspase-1-dependent plasma-membrane pores dissipate cellular ionic gradients, producing a net increased osmotic pressure, water influx, cell swelling, and, eventually, osmotic lysis and release of inflammatory intracellular contents. Indeed, cells dying by pyroptosis undergo a measurable size increase. Cleavage of chromosomal DNA is a fatal event that is often assumed to indicate apoptotic cell death; however, DNA damage also occurs during pyroptosis, and it is accompanied by marked nuclear condensation, but unlike apoptosis, nuclear integrity is maintained [150], and the cleavage of ICAD, the inhibitor of caspase-activated DNase, is not present. Like apoptosis, necrosis, and autophagy, pyroptosis results in the release of DAMPs, i.e., HMGB1, IL-1α, and ATP [151]. It is likely that these molecules, involved in several types of cancer, contribute to the tumorigenic potential of the inflammasome activation. On the other hand, the products of pyroptosis-induced cell death may limit malignant cell survival and sustain, via the immunogenic cell death-derived signals, the activation of the innate immune response against cancer development/progression [1]. In fact, increasing evidence highlights the role of pyroptosis in DC priming during conventional anti-tumor chemotherapy [59]. An emerging therapeutic area is exploring the ability of oncolytic viruses to induce cell death and the possibility to combine oncolytic virotherapies with further immunomodulation by cyclophosphamide and other immunotherapeutic agents, which can foster DC-mediated induction of anti-tumor immunity [152].

Recently, Wang and colleagues showed that chemotherapeutics induce pyroptosis through caspase-3 cleavage of gasdermin E (GSDME) [153]. Their findings are consistent with the idea that GSDME specifically requires caspase-3 to switch TNF-induced apoptosis to pyroptosis. This concept changes the understanding of programmed cell death, as caspase-3 has long been regarded as the hallmark of apoptosis. The explanation could be that the expression levels of GSDME determine the form of cell death in caspase-3-activated cells; in particular, $GSDME^{High}$ cells undergo pyroptosis upon "apoptotic stimulation" by chemotherapy, while cells lacking sufficient GSDME develop secondary necrosis after apoptosis [153].

The apparent inconsistencies in studies on the role of pyroptosis in cancer may reflect differences in the redox status of cells and specifically of molecules involved in this process. Given the role of the oxidative stress in the induction of the inflammasome [154, 155], the presence of oxidized DAMPs may discriminate pyroptosis-like cell death from apoptosis. For example, the reduced form of HMGB1, released from dying cells, triggers DCs via a TLR4-dependent pathway to induce an anti-tumor immune response [156]. In contrast, the oxidized form of HMGB1 released during apoptosis fails to activate immune responses [157]. While a strict relationship exists between caspase-1 activation and pyroptosis, it is still unclear if pyroptosis requires the same signal-1 that leads to inflammasome

activation. Nystrom and collaborators suggested that pyroptosis does not involve mitochondrial membrane depolarization as is the case of inflammasome-dependent caspase-1 activation. This implied that cell death may occur independently of mitochondrial dysfunction in non-primed, NLRC4-activated macrophages [158]. It still remains to be determined, though, if this model of pyroptosis involves other inflammasome complexes, such as those containing NLRP3 or AIM2.

In the context of carcinogenesis, one would be prompted to define pyroptosis as a protective mechanism. Impaired pyroptosis is currently proposed as a potential mechanism linking chronic inflammation to the development of colon carcinoma [3]. Colon epithelial cells from caspase-1-, NLRP3-, and NLRC4-KO mice are resistant to apoptosis and show greater rates of proliferation. On the other hand, oxidative stress, mitochondrial dysfunction, and alarmin release are all features of carcinogenesis, but, rather than tumor cell death and subsequent tumor arrest/regression, tumor progression occurs. Possibly, the role of pyroptosis in cancer progression would critically depend on the cell type undergoing this process. For instance, pyroptosis of innate immune cells, while being acknowledged as a host defense mechanism against pathogen infections, might have detrimental consequences in the context of tumor immunoediting. In addition, contrasting findings may be expected in different types of cancer.

To support the pyroptosis protective role, further studies demonstrate that GSDMD might protect against gastric cancer proliferation. The downregulation of GSDMD might contribute to the tumorigenesis and proliferation of cancerous cells by accelerating cell phase S/G2 transition, by activating ERK, STAT-3, and phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT) signaling pathways and regulating cell cycle-related proteins in gastric cancer [159].

Furthermore, it was also hypothesized that pyroptosis activation could represent a new anticancer mechanism in triple-negative breast cancer cells MDA-MB-231. In this study, the scientists demonstrated that docosahexaenoic acid (DHA) induced pyroptosis by activating some inflammasome-dependent pathways, associated to NF-κB translocation, caspase-1 and GSDMD activation, IL-1β secretion, HMGB1 translocation from the nucleus toward the cytoplasm, pore membrane formation, and loss of membrane integrity in MDA-MB-231 cells, shedding new light on the anticancer effect of DHA, which may have an important role in omega-3 supplementation in cancer therapy [149].

Further findings indicate that pyroptosis process is inactivated in human hepatocellular carcinoma (HCC) [160].

Further studies are needed to better define the role of pyroptosis and its correlation to the inflammasome activation. In particular, the protective or not role of pyroptosis in cancer may find scientific bases once reliable experimental tools to identify pyroptotic cells in vivo and in vitro will be developed.

15.6 Randomized Clinical Trials Targeting Inflammasome-Dependent Effectors

Despite the scientific progress on the role of the inflammasome in cancer, there is an urgent need to develop novel drugs that target inflammasome-related effectors.

One of the most important strategies to affect the inflammasome pathway in cancer treatment is to inhibit the IL-1β signaling activity by using monoclonal antibodies and recombinant derivatives of IL-1RN, which neutralizes both IL-1α and IL-1β [161]. As reported in ClinicalTrials.gov, Mayo Clinic in collaboration with National Cancer Institute (NCI) launched a Phase II study (NCT00635154) to evaluate the effect of anakinra with or without dexamethasone in treating patients with smoldering myeloma or indolent multiple myeloma (https://clinicaltrials.gov/ct2/show/NCT00635154?term=NCT00635154&rank=1). Anti-tumorigenic activity of anakinra may originate from blocking IL-1β-mediated production of IL-6, which is a key factor for inflammation-associated cancer. The results of

this study showed that treatment with IL-1 inhibitors triggered a decrease of IL-6 production and decreased myeloma proliferative rate and high-sensitivity C-reactive protein (hs-CRP) levels in responsive patients with enhanced progression-free survival (Table 15.2) (https://clinicaltrials.gov/ct2/show/NCT00635154?term=NCT00635154&rank=1). Nowadays, other clinical trials evaluating the efficacy of anakinra in different cancer types have been initiated, but their results are still not available.

Among anti-IL-1β antibodies, Novartis Pharmaceuticals launched (September 2016) a randomized, double-blind, placebo-controlled trial (NCT01327846) to evaluate the effect of canakinumab (50 mg, 150 mg, and 300 mg, subcutaneously every 3 months versus placebo) in lung cancer in patients with atherosclerosis in order to establish whether the inhibition of IL-1β might alter cancer incidence (Table 15.2) [162]. This study showed that lung cancer mortality was significantly less in the group of subjects treated with canakinumab (300 mg) than in the placebo group, suggesting canakinumab as a potential therapeutic tool to reduce lung cancer incidence and mortality.

Besides IL-1β, IL-1α is another effector correlated to the inflammasome activation. As reported in ClinicalTrials.gov from 2013 to 2017, XBiotech sponsored a Phase III (NCT01767857) double-blinded versus placebo study to determine if Xilonix (a human monoclonal antibody targeting IL-1α) could prolong the lifetime of colorectal carcinoma patients (Table 15.2) (https://clinicaltrials.gov/ct2/show/NCT01767857?term=NCT01767857&rank=1). Although the study is terminated, final results are not available yet.

Another study involved IL-18 (SB-485232 developed by GlaxoSmithKline; NCT00659178) (Table 15.2) [163]; the purpose of this Phase I dose escalation study was to assess safety, tolerability, and biological activity of SB-485232 administered by four infusions in combination with pegylated liposomal doxorubicin (Doxil) in patients with advanced-stage epithelial ovarian cancer [163]. The study reported no positive drug interactions; however, to date, no results were

Table 15.2 Randomized clinical trials targeting inflammasome-dependent effectors

Drug	Phase	NCT number	Results	References
Anakinra	2	NCT00635154	Decrease of IL-6 production, the myeloma proliferative rate and hs-CRP levels in smoldering myeloma or indolent multiple myeloma patients; increase of progress-free survival	ClinicalTrials.gov
Canakinumab	3	NCT01327846	Mortality was significantly lower in treated than placebo lung cancer patients	Ridker et al. [162]; ClinicalTrials.gov
Xilonix (a human monoclonal antibody targeting IL-1α)	3	NCT01767857	NA	ClinicalTrials.gov
SB-485232	1	NCT00659178	No positive drug interactions was observed in combination with Doxil in patients with advanced-stage epithelial ovarian cancer No final results were posted	Robertson et al. [163]; ClinicalTrials.gov
IFN-α, 13-cis-retinoic acid and paclitaxel combination	1	NCT00062010	After the combination these three drugs in patients with SCLC, values of OS, and progression-free survival are 6.2 and 2 months, respectively	ClinicalTrials.gov
5-FU and IFN combination	2	NCT01658813	Progression-free survival, in treated metastatic gastrointestinal, kidney, or lung cancer patients, is 2 months	ClinicalTrials.gov
Bortezomib	2	NCT01633645	Increase IL-1β release correlated to poor prognosis in lung cancer patients with advanced-stage (III-IV) NSCLC	ClinicalTrials.gov

posted. Based on the capability of IL-18 to induce IFN-γ, promoting Th1 cells, memory cytotoxic $CD8^+$ T lymphocytes, and NK cells activity [164], the investigation of recombinant IL-18 in the treatment of cancer may open news perspectives in that it may increase the activity of tumor-infiltrating immune cells.

Between 2013 and 2015, a Phase I study was registered on ClinicalTrials.gov (NCT00062010) evaluating the effect of co-treatment with IFN-α and isotretinoin (13-cis-retinoic acid) together with paclitaxel in patients with recurrent small cell lung cancer (SCLC) (Table 15.2) (https://clinicaltrials.gov/ct2/show/NCT00062010?term=NCT00062010&rank=1). The study reported that IFN-α and 13-cis-retinoic acid given on days 1 and 2, and paclitaxel given on day 2 for 6 weeks of an 8-week cycle, were associated to an OS (assessed every 3 months for 1 year and then every 6 months) and progression-free survival (assessed every 6 weeks) of 6.2 and 2 months, respectively.

Another Phase II trial (NCT01658813) was launched to test the combination of 5-fluorouracil (5-FU) and IFN-α2b in previously treated metastatic gastrointestinal, kidney, or lung cancer; this study reported that the value of progression-free survival, assessed up to 2 years, was only of 2 months (Table 15.2) (https://clinicaltrials.gov/ct2/show/NCT01658813?term=NCT01658813&rank=1).

As previously reported, because the amplification of the NF-κB-IL-6-STAT-3 signaling cascade and NF-κB activation occur in most malignancies, and its activity is a critical first signal for inflammasome activation, NF-κB may represent a possible therapeutic target to act on inflammasome pathway in cancer therapy. Nevertheless, nowadays the failure of drugs targeting NF-κB could be justified by the fact that myeloid-specific inhibition of NF-κB triggered the augmentation of pro-IL-1β processing by cathepsin G in neutrophils, leading to increased IL-1β and enhanced epithelial cell proliferation [165]. However, the combination of bortezomib, a proteasome inhibitor that blocks NF-κB activation, and anakinra reduced tumor formation and growth in vivo compared to monotherapy with bortezomib or anakinra which did not affect tumor growth. Moreover, in lung cancer patients with advanced-stage (III–IV) NSCLC (protocol NCT01633645), it was found that treatment with bortezomib significantly increased IL-1β release, but not IL-8, TNFα, or IL-6, and that plasma IL-1β levels were correlated with poor prognosis (Table 15.2). This evidence supports a causative role for neutrophil-derived IL-1β in lung tumorigenesis [165].

So far, no clinical trials are reported to inhibit the activity of NLRP3 and AIM2, further confirming that the role of these receptors in cancer may be pro-carcinogenic, rather than anti-tumor.

15.7 Conclusions

Carcinogenesis, tumor cell proliferation, and patient survival are strictly correlated to the presence of IL-1α, IL-1β, and IL-18. For instance, K-Ras-driven oncogenesis in pancreas, lung, and skin carcinoma is associated to the presence/activity of IL-1-like cytokines and MyD88-dependent signaling, which is in turn correlated to tumor proliferation without tumor cell death [118]. Besides the role of the inflammasome complex involved and of the cell type undergoing pyroptosis, a complex relationship exists between cancer establishment, progression, inflammation-induced pyroptosis, and oncogene activity. Inflammasome-dependent cell death may represent one of the potential therapeutic targets in cancer; however, cell death is a desirable achievement in structural tumor cells but not in innate immune cells, whose activity tightly regulates the anti-tumor adaptive response. While innate immune cell death induced by the activation of canonical and noncanonical inflammasomes is indispensable to defend the host against infections, the induction of pyroptosis in the context of systemic infections can contribute to sepsis-like disease and mortality [57].

Pharmacologists have long pursued anti-tumor agents able to induce tumor cell death; however, sterile insults from dying cells can contribute to further inflammasome activation in both structural and hematopoietic cells. Doxorubicin,

widely used in current anti-tumor protocols, induces tumor cell death but cannot be used alone and, more importantly, can induce inflammasome activation [166], possibly explaining why cumulative doses of glucocorticoids are needed for cancer patients who receive this treatment [167, 168].

The current assumption is that apoptosis is tolerogenic whereas necrosis is immunogenic. Therefore, the impact of dying cells on immune-competent cells depends on the type of cell death. The release of alarmins (immunogenic danger signals) from pyroptotic cells can fuel pro-inflammatory cascades that direct carcinogenesis and tumor progression.

Intriguingly, caspases are not only involved in cell death but can also coordinate pro-inflammatory signals delivered by cell death-derived alarmins. For example, caspase-1 and caspase-11-induced activation of IL-33 can polarize T-cells toward a Th2 phenotype [169], which in the context of the tumor microenvironment can facilitate malignant cell survival [5]. In contrast, the activity of IL-33 can be "neutralized" by caspase-3 and caspase-7 [170], as has been seen in the case of HMGB1, highly detected in tumor samples and implicated in cancer progression. Therefore, besides the immunogenic versus tolerogenic impact of caspases, the involvement of these caspases and of the upstream NLRs must be evaluated in the context of the specific microenvironment. The production of ROS and subsequent oxidation of cellular targets and the activation of proliferative K-Ras-dependent signaling pathways and pyroptosis are all processes that involve caspase-1, caspase-8, and caspase-11/4. These may serve as inflammation rheostats, acting as pro- or anti-inflammatory pathways that can impact on tumor development/progression. Therefore, to our opinion, the role of the inflammasome and its related cytokines in cancer is complex. Hence, it is not possible to make a general principle of the involvement of the inflammasome in oncogenesis, especially because it is strictly correlated to the nature/function of the tissue/organ that is affected by the malignancy.

Conflict of Interest Statement The authors declare that there are no conflicts of interest.

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16 Cancer Immunoediting: Immunosurveillance, Immune Equilibrium, and Immune Escape

Alka Bhatia and Yashwant Kumar

Contents

16.1 Introduction

The immune system's regulation of the cancerous process is a long-known fact. However, the role played by it in malignancies has been a matter of debate. The history of cancer immunity dates back to 1909 when Paul Ehrlich proposed the concept of immunosurveillance in cancers for the first time [1]. However, due to lack of experimental evidence, this concept fell into disrepute. In 1957 Burnet and Thomas argued that indeed the immune system fights and eliminates certain cancers and the frequency of malignancy would have been much higher if immunity was not there [2]. In the 1970s, several experiments were conducted in athymic mice to prove immunosurveillance in cancers; however, the results were not as expected, which was thought to be due to the presence of residual immunity in the animals used for these studies [3–5]. Consequently, the experiments done again on animal models with specific molecular immune defects revealed more frequent development of carcinogen-induced

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tumors in these immunodeficient animals [6]. However, more recently, the recognition of the dual nature of the part played by immune system in malignancies has led to the modern concept of cancer immunoediting. Since then, immunoediting in cancer has served as the foundation stone of most of the work being carried out in cancer immunity [7, 8].

16.2 Cancer Immunoediting with Its Three Es: Reflection of the Dual Role of Immunity in Cancer

The cancer immunoediting theory states that tumors are sculpted by the immune system, resulting in the selective growth of the variants which are better equipped to fight the immune system (Fig. 16.1). This selective growth advantage conferred on tumors is a consequence of a number of genetic and epigenetic events occurring within the tumors. The clue to the tumor-editing role of the immune system came from the experiments of Robert Schreiber's group on spontaneous and 3′-methylcholanthrene (MCA)-induced tumors in 129/SvEv mice (Fig. 16.2) [6]. The concept of immunoediting was introduced by Dunn et al. in 2002 to explain the antitumor as well as pro-tumor features of our immune response at different stages of cancer [3]. Since then, many studies conducted over a period of time have demonstrated the editing of tumors by host adoptive cells, leading to their complete reprogramming. A more recent study has linked processes such as epithelial mesenchymal transition in tumor cells, which result in an invasive phenotype, to the immunoediting process through the involvement of cytokines such as TNF-α and TGF-β [9]. Cancer immunoediting is a broad concept which includes three "Es" of elimination, equilibrium, and escape which together sum up to all the events occurring during an immune response to cancer [3].

16.2.1 Immune Elimination: Evidences for and Against

The immune elimination phase of cancer immunoediting is sine qua non of the original immunosurveillance process. It envisages the destruction or eradication of cancer by the host immune system and is believed to occur when a cell gets transformed by overcoming its intrinsic tumor suppressor mechanisms, before being able to establish a full-blown tumor. Although the existence of such a phenomenon has been hypothesized since long, the early experiments carried out on nude mice models which are only partially immunodeficient failed to prove it. The definitive experimental proof to its presence came from the work of Shankaran et al. in the last decade (Table 16.1, Fig. 16.2) [6]. However, despite the experimental evidence of its presence in mice, it has been difficult to demonstrate it in the clinical scenario. Still, the data obtained from various cancer registries wherein a higher cancer incidence especially of viral etiology has been observed in immunosuppressed transplant recipients suggests its existence in human subjects as well. Currently, a similar trend has been noticed in the setting of acquired immunodeficiency syndrome [13, 14]. The proponents of this stage in cancer immunity state that many of the cell transformation events occurring in our body may be removed quietly by the immune system without us ever being aware about it. Spontaneous regression has been reported in some tumors including cutaneous melanoma, retinoblastoma, osteosarcoma, etc., in humans [15]. Studies have shown that both innate and adaptive immune response contribute to fighting off the cancer from our body.

16.2.1.1 The Key Players in Anticancer Immunity

The key players responsible for launching an effective immune response against cancer include the immune cells and soluble molecules secreted into the tumor milieu (Fig. 16.3). In case the

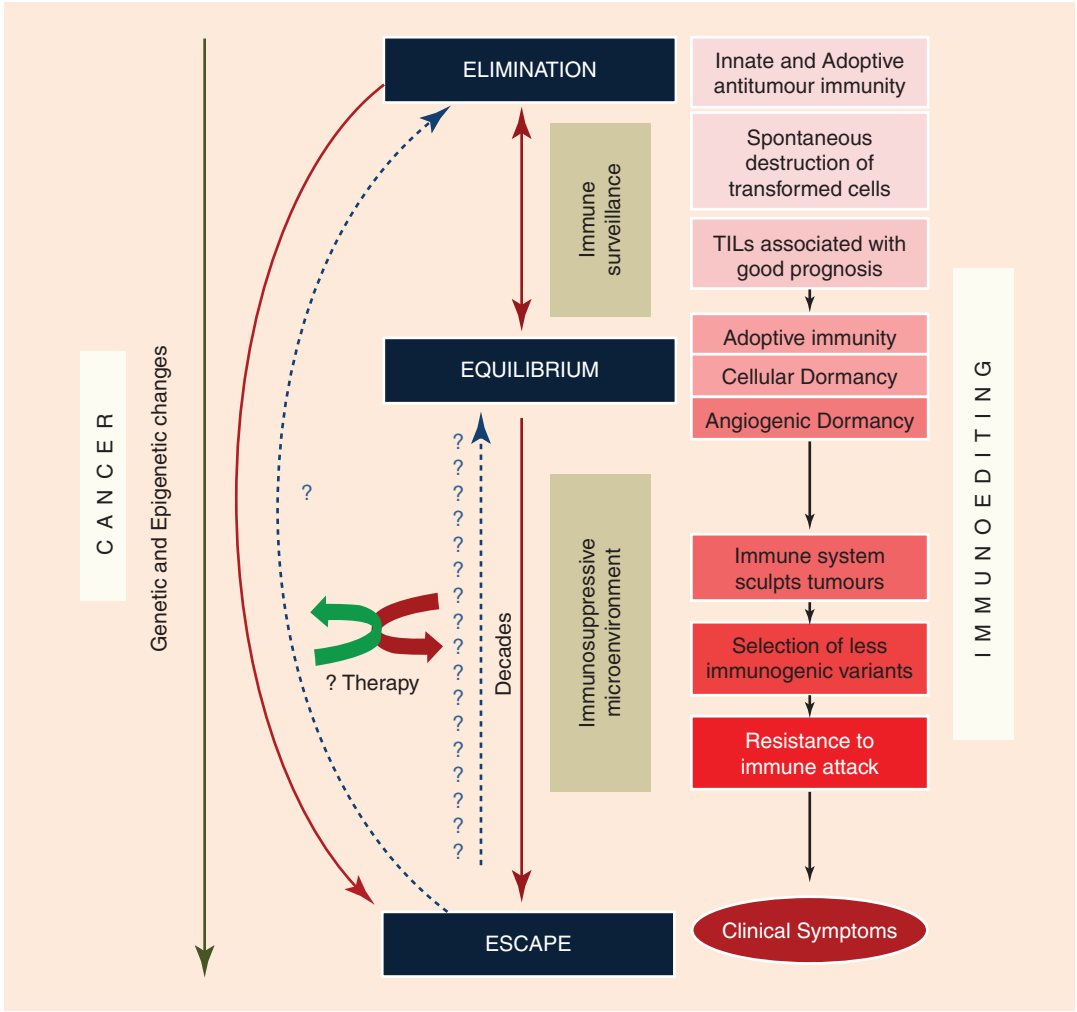

Fig. 16.1 Cancer immunoediting process with its three Es of elimination, equilibrium, and escape. Please note that although in many cases the sequence is followed, in others one or the other phase may be skipped. Although the events from equilibrium phase may proceed either toward escape or back to the elimination phase, the reversibility of the escape phase with or without therapy to other two phases is questionable

tumor exhibits high immunogenicity, a specific immune response occurs against it. However, if tumor immunogenicity is low, the nonspecific effector responses gain importance.

The major cell types involved in an antitumor immune response are adoptive T-cells, which not only kill tumor cells directly with the help of TNF-α but are also essential for the activation of other components of the immune machinery. The $CD8^+$ cytotoxic lymphocytes (CTLs) are able to directly recognize tumor cells which express MHC I and can also be activated by $CD4^+$ T-helper cells. They may cause lysis of the tumor cells via perforin- and granzyme-dependent mechanisms. The $CD4^+$ T-cells also secrete factors to induce proliferation of B-cells and to promote their differentiation to antibody (Ab)-secreting plasma cells. The latter may contribute

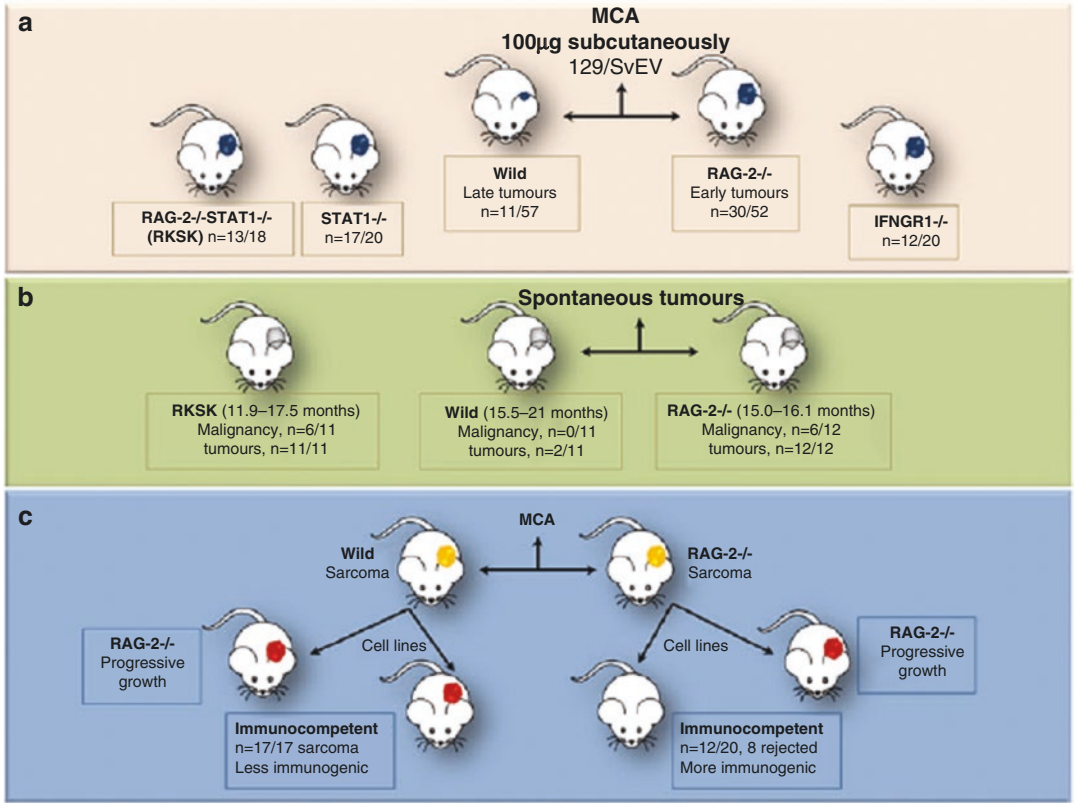

Fig. 16.2 Mice experiments by Shankaran et al. [6] demonstrating surveillance and sculpting roles of immune system. (**a**) Immunodeficient (RAG-2−/−/IFNGR1/STAT1−/− or combined RAG-2−/− STAT1−/−, RkSk) mice developed tumors earlier than wild type and with greater frequency on subcutaneous injection of MCA, thus necessitating the presence of intact T, NKT, and B-cells for prevention of chemically induced tumors. (**b**) Spontaneous tumor development was also observed to be higher in RAG-2−/− and RkSk mice as compared to unmanipulated 129/SvEv wild-type mice. Moreover, the latter merely developed benign tumors, and no malignancy was noted. (**c**) Furthermore, cells were taken from MCA-induced tumors in wild and RAG-2−/− mice and were injected into immunocompetent and RAG-2−/− mice. Progressive tumor growth was noted in immunodeficient mice transplanted with sarcoma cells derived from wild or RAG-2−/− mice. The immunocompetent mice transplanted with sarcoma cells from wild mice also showed progressive tumor growth; however, many mice transplanted with sarcoma cells derived from RAG-2−/− mice rejected the transplanted tumor cells. This occurred due to sculpting of sarcoma by the immune system in wild mice, thus rendering it less immunogenic. Tumors from the immunodeficient mice which were not edited were more immunogenic and thus were rejected by immunocompetent mice

to antitumor immunity by complement-mediated lysis or by antibody-dependent cellular cytotoxicity (ADCC). The $CD4^+$ T-helper cells also activate macrophages by secreting IFN-γ, TNF, IL-4, and granulocyte-macrophage colony-stimulating factor (GM-CSF). The activated macrophages may phagocytize tumor cells and kill them by releasing toxic free radicals including O_2^- and NO_2^- or by becoming antigen-presenting cells (APCs) which present tumor antigens to $CD4^+$ T-cells such as dendritic cells (DCs). Natural killer (NK) cells also have the potential to directly recognize and destroy tumor cells via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and IFN-γ-dependent mechanisms. Loss of MHC class I as commonly observed in tumors may be responsible for their increased susceptibility to NK-cell-mediated lysis. In addition, NK-cell activity may also be enhanced by IL-2 and IFN-γ produced by the $CD4^+$ T-helper

Table 16.1 Timeline of events depicting evolution of cancer immunity from immunosurveillance to immunoediting

Study	Hypothesis/observation/experimental evidence	Results
Coley [10]	Injected cultures of heat-inactivated bacteria or bacterial culture supernatants into cancer patients	Demonstrated marked regression of tumors and prolonged survival after the treatment
Paul Ehrlich [1]	Immune system protects the host from malignancy	Gave birth to the idea of immune control of malignancies
Burnet and Thomas [2]	Immune system must be removing the carcinogenic events arising out of ongoing evolutionary genetic remodeling taking place in an individual	Formal emergence of immunosurveillance hypothesis
Several groups (1965–1973)	Induced immunodeficiency by thymectomy or heterologous antilymphocyte serum or pharmacological agents. Immunodeficient animals are more prone to develop cancers	No consensus regarding immunosurveillance
Stutman [11]	The methylcholanthrene (MCA)-induced cancer incidence in immunodeficient nude athymic mice was not higher than the control mice	Rejection of immunosurveillance hypothesis
Kaplan et al. [12]	IFN-γ- and perforin-deficient animals were more prone to MCA-induced tumors as compared to controls	Resurrection of immunosurveillance in cancer
Shankaran et al. [6]	Experiments in RAG-2 null mice (lacking T, B, and NKT cells) revealed higher incidence of both MCA-induced sarcomas and spontaneous epithelial tumors in these animals	Definitive evidence of existence of cancer immunosurveillance
Dunn et al. [3]	Concept of cancer immunoediting to explain the tumor sculpting role of immune system	Coined the term immune elimination as a part of broader concept of cancer immunoediting with three Es of elimination, equilibrium, and escape

cells. NKT and γδ T-cells also recognize the danger signals released from the tumors and become activated. The NKT cells especially the invariant or the type I NKT, which are $CD4^-$ $CD8^-$ and mainly recognize the lipid/glycolipid antigens (Ags) via CD1d molecule, have been recognized to protect against certain cancers. The protective role is however supposed to be indirectly exerted via secretion of IFN-γ and subsequent activation of NK and $CD8^+$ T-cells. The γδ T-cells which represent 1–5% of peripheral blood T-cells are also reported to infiltrate and cause lysis of tumors, both in vitro and in vivo [16–20].

In various clinical studies on different cancers including colon, ovary, lung carcinomas, and melanoma, the tumor-infiltrating lymphocytes (TILs) have been associated with increased time to disease recurrence, an enhanced 5-year survival, and an overall good prognosis. Also, in a study on metastatic colorectal cancer, TIL density at the invasive margin was linked to a better chemotherapeutic response. Similarly, increased infiltration by $CD3^+$ and $CD8^+$ T-cells, NK cells, and γδT cells has been correlated with improved outcomes in epithelial ovarian cancers. Some of the above studies have done quantitative assessment of the TILs in tumors, thus impressed upon the need to have a scoring system for TILs in order to determine the exact tumor behavior [21, 22].

16.2.2 The Equilibrium Phase: The Most Controversial and the Least Understood Phase

This phase represents an intermediate stage of immune response in cancer. During this phase, the cancer and the immune system both coexist without allowing each other to dominate. The immune system cannot eliminate the cancer during this phase; however, it does not allow it to expand or metastasize. The cancer in turn is sculpted by the immune system, thus leading to the emergence of variants resistant to the immunological attack [3].

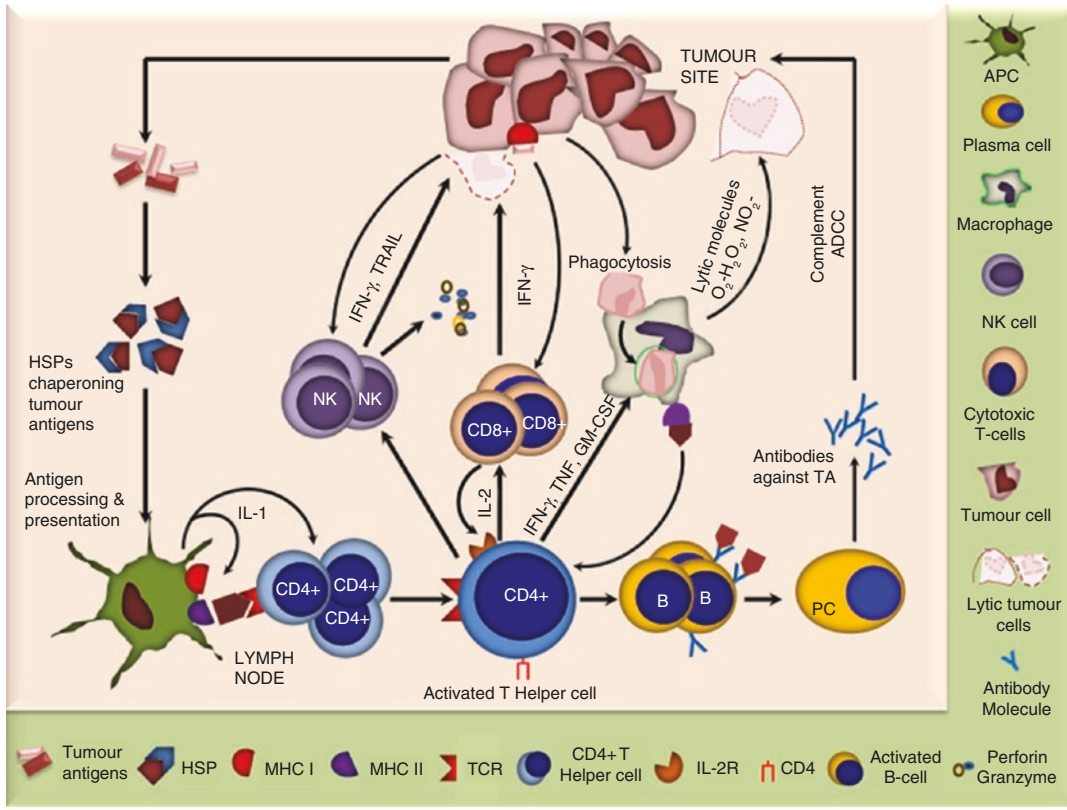

Fig. 16.3 Diagram showing key players involved in anti-tumor immune response. The tumor releases Ags which are chaperoned by heat-shock proteins and taken up by the APCs which process them and present to CD4⁺ T-cells. The latter being the central point of immune response activate various other cells including NK cells, CD8⁺ cells, macrophages, and B-cells which act in various ways to counteract the tumors. In addition, tumors may directly activate the cytotoxic cells including CD8⁺ and NK cells and phagocytic cells. While the former two can cause direct tumor lysis primarily via perforin and granzymes, the latter may engulf tumor cells and kill them by releasing lytic molecules or may process and present tumor Ags to CD4⁺ T-cells

Various studies in mice have pointed toward the occurrence of the equilibrium phase in cancer immunity. In experiments on MCA-induced tumors in mice, Koebel et al. demonstrated the presence of inert lesions in healthy mice, which grew when subjected to immunological oppression (Fig. 16.4) [23]. The study served to be an important milestone in proving the existence of the equilibrium phase in cancers. Likewise, the tumors have been observed to stay dormant for decades after remission in human cancer patients, which is believed to be due to the fact that the immune system keeps them in check. The immune system is believed to synergize with chemoradiotherapy in treatment-induced remission which renders the tumors silent. However, they relapse promptly after any kind of immune insult, thereby, further proving the presence of immune dormancy. The minimal residual disease commonly observed in hematological malignancies and the emerging donor-derived malignancies in immunosuppressed transplant recipients are considered two examples of the equilibrium phase in humans. Even though the immune system prevents monoclonal gammopathy of unknown significance (MGUS) from progressing to myeloma, it fails to eliminate the MGUS cells [24, 25].

Adoptive T-cells, both CD4⁺ and CD8⁺, have been observed to play a pivotal role in cancer immune equilibrium. Immune-sufficient mice with inert tumors are shown to develop into full-fledged tumors only upon depletion of T-cells/IFN-γ/IL-12. However, the depletion of innate immune cells was not found to result in the devel-

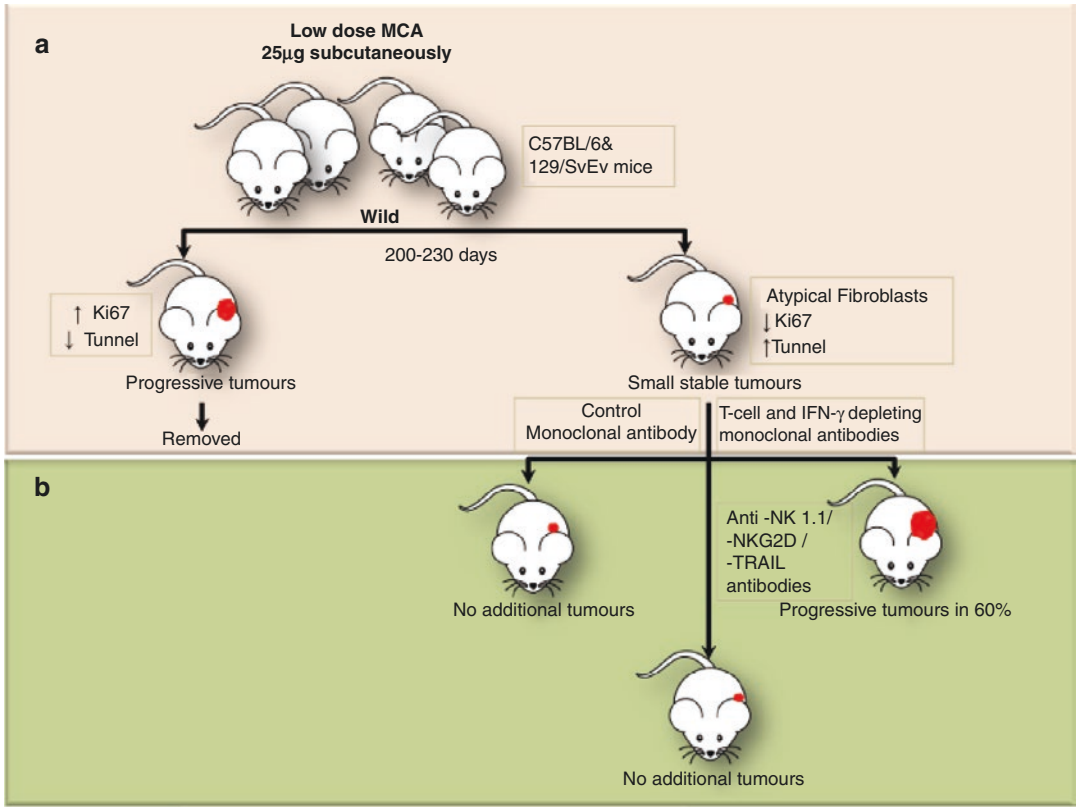

Fig. 16.4 Experiments conducted in mice by Koebel et al. demonstrating the presence of equilibrium phase in tumorigenesis. (**a**) Groups of wild-type C57BL/6 or 129/SvEv mice were injected with a single low dose of MCA. After monitoring for 200–230 days, the mice with rapidly growing sarcomas were set aside. (**b**) The remaining mice displaying small stable masses at injection site were injected with control Ab or mAbs depleting specific components of innate and adoptive immunity. The mice in former two groups did not develop any additional tumors; however, those in the last group (T-cell and IFN-γ depleted) showed rapid tumor growth. This could only be explained by cancer immune equilibrium in which the tumors were not removed but restricted by the immune process. However, on suppression of adoptive immunity, progressive tumor growth was observed

opment of tumors. Moreover, tumor cells were found to be highly immunogenic during the equilibrium phase, as they are unedited by the immune system and become less immunogenic at the end of this phase [23, 26, 27].

In addition, the mechanisms including cellular and angiogenic dormancy also complement the immune system in maintaining cancer cells in the dormant state. In the former, the tumor cells hide themselves in specialized niches, become quiescent, and wait for the opportunity to regrow. In the latter condition, expansion is not possible, due to the lack of adequate vascularization. When faced with favorable conditions, tumor cells come out of their slumber and undergo a series of genetic and epigenetic modifications which increase their immune resistance, eventually leading to the next phase of cancer immunity, known as immune escape. Studies are being conducted to identify the genetic and molecular signatures of dormant tumor cells which allow them to retain their dormant status or facilitate their escape [23, 26–29].

16.2.3 Immune Escape: The Best Studied Phase

The escape phase represents the final and most extensively studied phase of the immunoediting process. The unleashing of mechanisms underlying the escape phase has formed the basis for the development of various therapeutic agents with

the aim to stop the progress of the neoplastic process. Due to increasing genomic instability, cancer cells acquire various characteristics enabling them to ward off the immune process or to modify it in such a way which is beneficial to tumor cells. Tumors utilize a number of strategies to evade an effective immune response (Fig. 16.5). The basis of an effective immune response against any Ag is its recognition as a nonself and its presentation to immune effector cells. Tumors escape recognition by either presenting self Ags to which the immune system is already tolerized or by modulating their antigenicity. The latter involves the shedding of tumor Ags into the circulation from where they may be removed [30]. The next line of defense adopted by tumor cells is the modulation of APCs, rendering them incapable of effectively presenting cancer Ags to immune cells. The APCs like DCs are either deleted or functionally compromised in response to the factors secreted by malignant cells [31]. Tumor-induced co-inhibition of the second signal of the Ag presentation and consequent immunosuppression has now been recognized in several cancer types [32]. In addition, the tumors alter MHC molecules especially MHC class I and other components of Ag processing machinery in the APCs, so as to further incapacitate the presentation of its Ags to the immune system [33]. Besides, tumor cells plunge into an active battle against the immune process by attacking its adoptive and innate immune cells. Tumor cells subvert T-cells and render them anergic through co-inhibitory molecules including cytotoxic T-lymphocyte antigen-4 (CTLA-4) and PD-L1 [34]. Anergic T-cells are unable to produce cytokines such as IL-2 and IFN-γ. Therefore, the autocrine and paracrine activation of $CD4^+$ cells and other immune cells including B-cells, macrophages, and $CD8^+$ cells are blocked, leading to further suppression of the immune cascade [35]. Moreover, tumors also express Fas ligands on

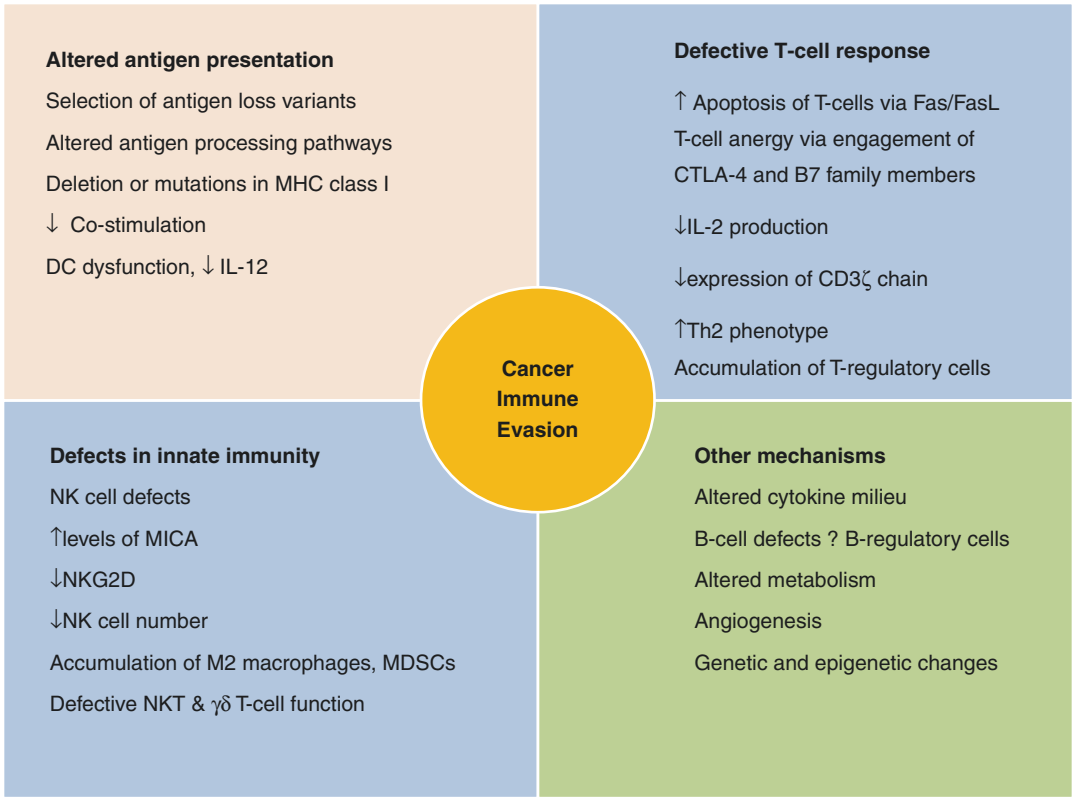

Fig. 16.5 Mechanisms of immune evasion by the cancer

Table 16.2 Mechanisms of immunosuppression induced by T-regulatory cells and myeloid-derived suppressor cells

T-regulatory cells
Secretion of immunosuppressive molecules like IL-10, IL-35, and TGFβ
Polarization of DCs toward tolerogenic phenotypes
Direct cytolysis of effector T-cells via granzyme B, TRAIL, and galectin-1
Metabolic changes like increased IDO in DCs and increased conversion of ATP to adenosine promoting immunosuppression
Stimulation of tumoral angiogenesis via VEGF secretion
Myeloid-derived suppressor cells
Inhibition of effector T-cell proliferation and function via L-arginine-dependent mechanisms
T-cell inhibition via production of ROS and TGFβ
Reduced T-cell homing via depletion of L-selectin
Promotion of Th2 and T-regulatory phenotypes via IL-10 secretion
Inhibition of DC function via IL-10
Promotion of angiogenesis via secretion of VEGF, basic fibroblast growth factor, HIF-1, etc.

T-cells, leading to lymphocyte apoptosis [36]. Not only do they suppress $CD4^+$ and $CD8^+$ cells but also promote the suppressor T-cell phenotype such as $CD25^+Foxp3^+$ T-regulatory cells. These cells secrete IL-10, TGF-β, and VEGF which suppress the antitumor response and promote tumoral angiogenesis (Table 16.2) [37]. Besides, tumors also inhibit innate immune response by induction of quantitative and qualitative defects in NK cells, macrophages, and neutrophils. NK cells have been found to exhibit decreased cytotoxic potentiality due to the presence of tumor-secreted factors including TGF-β in the tumor microenvironment (TME) [38]. The latter along with other cytokines (IL-4, IL-13, etc.) present in the tumor bed favors the accumulation of M2 macrophages, which also induce immunosuppression [39]. Recruitment of immature myeloid cells like myeloid-derived suppressor cells (MDSCs) further complements the tumor-immunodeficient environment by reducing T-cell and NK-cell activation and promoting neovascularization via factors like VEGF [40].

Other mechanisms such as anaerobic glycolysis, hypoxia, and acidity of the TME along with the existent defects in tryptophan metabolism induced by increased expression of the enzyme indoleamine 2,3-dioxygenase (IDO) further depress the antitumor immunity, thereby leading to cancer progression and metastasis [41–43].

16.3 Tumor Antigens and Cancer Immunoediting

Antigenicity of tumors has always been a matter of discussion. In the past, it was believed that since tumors are derived from self cells, the immune system is more receptive to their Ags. However, it was subsequently noticed that tumors may express Ags which are quantitatively or qualitatively different from self-Ags, thus rendering them sensitive to the immune attack. Quantitative differences include significantly increased expression of Ags, which are less expressed in normal or benign conditions or reexpression of Ags only expressed at a specific stage of embryonic development (Table 16.3). Moreover, the lineage-specific Ags expressed normally in specific tissues may be expressed aberrantly in tumor cells. Qualitative differences are produced due to mutational events occurring during carcinogenesis. Over the years, several efforts have been made for the identification and mapping of the Ags expressed on tumor cells; various nomenclatures have been used to characterize them such as tumor-associated Ags and tumor-specific Ags. Antigens capable of evoking a tumor-specific immune response have also been designated as tumor rejection Ags in some textbooks, e.g., tyrosinase, MUC-1, Her-2/neu, β-catenin, caspase-8, etc. [44]. Previous studies on tumor antigens (TAs) have mainly focused on the discovery of new Ags and their classification into two subclasses, a group which can evoke a protective immune response and another group serving as potential therapeutic targets. However, the advent of cancer immunoediting theory has changed our insight on TAs, as they are now considered to be one of the prime targets of the above process. Currently, ongoing studies are attempting to differentiate between the antigenicity of the original or unedited tumors and those sculpted by the immune system [17, 45,

Table 16.3 Examples of common categories of antigens present in tumors [44]

Antigen type	Antigen class	Antigen	Characteristics of antigens	Tumor
Tumor-associated antigens	Oncofetal antigens	CEA	Expressed in fetal tissues, reexpressed in tumors	Colon cancer
		AFP		Germ cell tumors, HCC
	Differentiation and lineage-specific antigens	CD5	Normally in T-cell but aberrantly in B-cells in CLL	CLL
		Melan A, tyrosinase	Melanocyte lineage	Melanoma
		Gp 100		Prostate carcinoma
		PSA		
	Cancer testes antigens	MAGE 1	Expressed in germinal tissues and reexpressed in malignancies	Melanoma
		NY-ESO-1		
	Heat-shock proteins	Gp 96		Fibrosarcoma, colon cancer
		HSP70		
	Gene amplification	Her-2/neu	Receptor tyrosine kinase	Breast cancer
				Ovarian cancer
	Aberrant posttranslational modification	MUC1	Under glycosylated mucin	Breast
				Pancreas
Tumor-specific antigens	Mutated oncogenes or proteins	Mutated p53	Point mutations	Many tumors
		BCR-ABL	Translocation 9;22	CML
		β-Catenin	Signal transduction pathway	Melanoma
		Caspase-8	Apoptosis regulation	Squamous cell carcinoma
Oncoviral proteins	HPV 16, E6, and E7 proteins		Viral transforming gene products	Carcinoma cervix

46]. Differences between the immunogenicity of tumors derived from carcinogen MCA (more immunogenic) and those arising spontaneously (less immunogenic) in mice have been described by DuPage et al. [47]. They also showed that primary sarcomas are edited by the immune system and, hence, become less immunogenic in order to escape the T-cell response. In the same line, Matsushita et al. obtained similar results in their study on tumor exomes [48]. A recent study has revealed the presence of anti-inflammatory antibodies to tumor-associated Ags like NY-ESO-1, thereby suggesting the importance of humoral immune system in cancer immunoediting [49]. Novel genetic-based approaches including exome sequencing, in silico analysis, and $CD8^+$ T-cell cloning are likely to further help in understanding the alterations in tumor antigenicity occurring during different phases of cancer immunity [48].

CEA carcinoembryonic antigen, *AFP* alpha fetoprotein, *Gp* glycoprotein, *PSA* prostate-specific antigen, *MAGE-1* melanoma-associated antigen 1, *NY-ESO-1* New York-ESO-1, *BCR-ABL* breakpoint cluster region-Abelson, *HPV* human papilloma virus

16.4 The Tumor Microenvironment During Cancer Immunoediting

The microenvironment surrounding the tumor plays a critical role in determining cancer behavior. TME is composed of cells (tumor as well as immune), various factors secreted by them, and the stroma. The TME is a dynamic system switching from host protective to tumor friendly during different phases of the immunoediting process. During the elimination phase, the milieu of the tumor comprises of factors which promote its eradication. Collaboration of factors including IFN-γ and lymphocytes has been found to help in regulating the development of tumors. In different studies, IFN-γ- and perforin-deficient mice together with T-cell and NK-cell defects are

found to exhibit a greater propensity for tumor development. Cytokines like IL-2, IL-12, and IL-7 have been found to promote antitumor immunity, suppress recruitment of suppressor cells, and inhibit tumor angiogenesis.

During the equilibrium phase, TME assumes the role of a niche, concealing relatively dormant cancer cells. The niche environment allows cancer cells to thrive without progression by maintaining a balance between the cytostasis and cytolysis. However, molecules which precisely maintain this balance during the immune equilibrium state remain to be defined.

During the escape phase, the tumor bed gets packed with factors and cells which promote immune suppression. Factors like IL-6, TGF-β, IL-8, and IL-10 help in generalized subversion of an effective anticancer immune response. Growth factors like VEGF not only promote angiogenesis but also facilitate the recruitment of T-regulatory cells and MDSCs to the tumor site. Besides, tumor cells induce downregulation of antitumor cytokines including IL-12 and IFN-γ. In addition, the abundant presence of other factors within the TME including prostaglandin E2, reactive oxygen and nitrogen species, phosphatidylserine, etc. aids cancer cells to evade the immune response. Furthermore, the stroma including cancer-associated fibroblasts, chemokines, matrix metalloproteinases, and adhesion molecules also participates in cancer's conquest over antitumor immunity.

Although the above few paragraphs have tried to provide a simplified view of the events occurring during various phases of the immunoediting process, there are several paradoxes involved. One set of factors may play an immunostimulatory and antitumor role under particular conditions, whereas they may exert an immune inhibitory and pro-tumor role under other circumstances. For example, IFN-γ which is a potent cytokine responsible for antitumor immunity is now emerging as an important player in cancer immune evasion. The pro-tumor effects of IFN-γ are believed to be related to an increase in T-regulatory cells and MDSCs and a decrease in neutrophilic infiltrate in the TME [50–53].

16.5 Clinical Relevance of the Immunoediting Process in Cancer

The introduction of immunoediting concept has added a new insight to understanding of cancer immunity. A clear understanding of the mechanisms underlying the three phases of cancer immunity is vital for designing the immunotherapeutic strategies to prevent, stop the progression, or treat cancers. In addition, it has contributed to the development of new markers for the diagnosis and prognostication of malignancies. Identification and manipulation of various molecules involved in different phases of the immune response to cancer has emerged as a promising approach for the development of novel immunotherapeutic strategies for cancer treatment and eradication. Table 16.4 provides examples of the immunotherapeutic approaches directed toward the three phases of the immunoediting process.

PBMCs peripheral blood mononuclear cells, *APCs* antigen-presenting cells, *DC* dendritic cell, *GM-CSF* granulocyte macrophage colony-stimulating factor, *EGFR* epidermal growth factor receptor, *CTLA-4* cytotoxic T-lymphocyte antigen-4, *mAb* monoclonal antibody, *MDSCs* myeloid-derived suppressor cells, *IDO* indoleamine-2, 3 dioxygenase, *VEGF* vascular endothelial growth factor

Deciphering the nature of the cellular infiltrate and secretory molecules produced in response to the transformation events and characterization of the mechanisms involved in the elimination of tumor cells at early stages has led to the development of novel cancer therapeutics. Moreover, quantitative as well as qualitative assessment of the immune cells present in TME may contribute to the development of algorithms demonstrating tumors' response to chemoradiotherapy. In vivo or in vitro expansion of tumor-specific effector cells is being applied as a strategy to boost up the antitumor immune response. Recognition of TAs which evoke an effective antitumor immune response has served as the basis for the development of different types of cancer vaccines. Monoclonal antibodies (mAbs) targeting diverse

Table 16.4 Examples of therapeutic approaches targeting different phases of cancer immunoediting

Phases of immunoediting	Approaches	Outcome
Elimination	In vivo or in vitro expansion of immune effector cells and using them for therapy	Sipuleucel T (autologous PBMCs, APCs, and recombinant fusion protein, i.e., PA2024, PA, PAP fused to a GM-CSF), FDA approved for prostate cancer [54]
	DC-based approaches	
	Tumor antigen-based vaccines	
	Tumor-specific monoclonal antibodies	Trastuzumab (Her2/neu), rituximab (CD20), cetuximab (EGFR) [55–57]
	Immunostimulatory cytokines	IL-2, IL-7, IL-15 [58–60]
Equilibrium	Adoptive transfer of cancer-reactive T-cells	Monitored for establishment of equilibrium phase [61]
Escape	Anti-CTLA-4	Ipilimumab approved for melanoma [62]
	Blockade of T-cell co-inhibition	mAb against B7-H1 [63]
	Depletion of T-regulatory cells	Denileukin diftitox [64]
		Lenalidomide [65]
	Inhibition of MDSCs	Sunitinib [66]
	Inhibition of IDO	1-methyl tryptophan [67]
	Blockade of VEGF	Bevacizumab [68]

TAs have entered clinical trials for several cancer types. Besides, TAs such as CEA have also been used as biomarkers for early detection and for determining tumor prognosis. The concept of immunogenic chemotherapy which stimulates adaptive immunity is also gaining impetus in recent years.

The equilibrium phase has also emerged as a potential target to immunotherapists, as maintaining cancer cells in the equilibrium phase indicates prevention or delay in cancer progression and fatality. In cases treated with mAbs which exert their effect via NK cells, an adoptive T-cell response was also found to be evoked, leading to the maintenance of tumors in equilibrium phase [69]. Furthermore, development of sensitive techniques to seek out the occult tumor cells in various organs may help in their specific targeting, resulting in their complete eradication. Identification and targeting of immune or nonimmune events shifting the balance from equilibrium to the elimination or to the escape phase may lead to tumor removal or at least progression restriction.

As discussed in earlier sections, tumor cells apply a variety of tactics to combat with the host immune system. The assessment of factors involved in the escape mechanism served as the mainstay for the discovery of many anticancer immunotherapeutic agents. Some developed agents like ipilimumab (anti-CTLA-4) are now being used clinically along with other forms of therapy, whereas many other agents have entered different phases of clinical trials, and a large number are still in experimental stages (Table 16.4).

16.6 Concluding Remarks

In conclusion, it could be stated that enough proof is available to establish the presence of cancer immunoediting in animals as well as in humans. Understanding the sequence of events occurring during the immunoediting process and recognition of the cellular and molecular mechanisms underlying its different phases has led to a spurt in cancer immunotherapeutic approaches. Further knowledge on the genetic and epigenetic features characterizing the three Es of cancer immunoediting are warranted for the development of more precise cancer immunotherapeutic approaches in the future.

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Apoptosis and Cancer

17

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Contents

17.1 Introduction

Life and death are essential parts of a natural cycle of all multicellular organisms. Cell division, cell death, shape modification, and cell rearrangements form critical processes on which tissues are shaped and organs are made. Cell death, in particular, plays an important role in the development and homeostasis of normal tissues [1, 2]. Cell death phenomenon was first reported

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in 1842 by Carl Vogt [3, 4]. Subsequently, the term programmed cell death (PCD) was coined by Lockshin and Williams in 1965 to describe the phenomenon of coordinated deaths of certain larval muscles during transformation into adult moths [5]. Kerr and coworkers later described a series of similar morphological characteristics following the death of a variety of tissue sources, which was named as "apoptosis" [6]. About the same time, Horvitz and colleagues started a systematic search for genes controlling PCD in the nematode worms, *Caenorhabditis elegans*. The discovery of cell death defective genes such as *ced-3*, *ced-4*, and *ced-9* suggests that PCD is a process with strict genetic program [7]. This was quickly followed by the identification of substrates and homologous genes in mammals and realization that mutations of some of these cell death genes were contributing factors in various cancers. The 2002 Nobel Prize in Physiology or Medicine was awarded jointly to Sydney Brenner, H. Robert Horvitz, and John E. Sulston for their extensive work and discoveries on genetic regulation of organ development and PCD.

An imbalance between cell growth and cell death is implicated in a variety of human diseases including cancer, autoimmune diseases, neurodegenerative disorders, viral infections, and AIDS [8–12]. Cell death has a profound effect on cancer growth and progression [13–15]. Malfunction of the cell death machinery, as a direct consequence of mutations of the signaling molecules involved either directly or indirectly in the cell death pathways, has long been identified as an important contributing factor in cancer. Continuous efforts in deciphering the mechanisms and signaling pathways of these cell deaths have also brought forward a new paradigm of which cancer may be efficiently targeted. Novel and specific cancer therapeutics and techniques directed at members of the cell death signaling pathways have been developed, and newer generation of drugs are currently being tested in clinical trials.

Based on the cell death classifications by the Nomenclature Committee on Cell Death (NCCD), the use of the term "programmed" is now limited to regulated cell death which occurs in the absence of any exogenous environmental perturbation and irrespective of the modality by which they are executed and in the context of embryonic and post-embryonic development and tissue homeostasis [16, 17]. On the other hand, other types of regulated cell death indicate cases of cell death whose initiation and/or execution is mediated by a dedicated molecular machinery and can be inhibited by targeted pharmacologic and/or genetic interventions [16]. Regulated cell deaths can originate from perturbations of the intracellular or extracellular environment and when such perturbations are too intense or prolonged for adaptive responses to cope with stress or restore cellular homeostasis [17]. Apoptosis is a form of regulated cell death and is implicated in the pathogenesis of cancer [17]. Its roles in tumorigenesis and some of the novel antitumor strategies and therapeutics will be discussed in this chapter.

17.2 Mechanisms of Apoptosis

The term "apoptosis" was introduced by Kerr and coworkers in 1972 and derived from a Greek term meaning "dropping off" of leaves or petals from trees or flowers [6]. Earlier methods to define cell death rely much on morphological criteria and the use of microscopes [4]. The earliest recognized morphological changes in apoptosis involve compaction and segregation of nuclear chromatin and condensation of the cytoplasm [6, 18]. The process is followed by the convolution of the plasma membrane and cell blebbing in a florid manner, producing fragments of cells known as apoptotic bodies. These fragments are membrane bounded and contain nuclear components [18, 19]. Apoptotic bodies are quickly taken up by nearby cells, and degraded within their lysosomes, usually with no associated inflammation [6, 18].

Biochemically, apoptosis is universally characterized by the double-stranded cleavage at the linker regions between nucleosomes, resulting in the formation of multiple DNA fragments [19], phosphatidylserine externalization [20], and is accompanied by a series of genes and proteins

expressions. According to the NCCD, apoptosis is functionally classified into extrinsic or intrinsic apoptosis [16, 21]. Extrinsic apoptosis is categorized depending on source of trigger, whereas intrinsic apoptosis is characterized by widespread mitochondrial outer membrane permeabilization (MOMP).

17.2.1 Extrinsic Apoptosis Pathway

Extrinsic apoptosis is a regulated cell death modality initiated by perturbations of the extracellular microenvironment [17]. It is essentially caspase dependent and is induced by extracellular stress signals which are mediated by specific transmembrane receptors such as death receptors or dependence receptors. In extrinsic apoptosis induced by death receptors, the signaling pathway is mediated by receptors belonging to the tumor necrosis factor (TNF) receptor superfamily which is characterized by extracellular cysteine-rich domains (CRDs) and intracellular death domain (DD). Ligands such as TNF ligand, TNF ligand superfamily member 10 (TNFSF10), FAS ligand, and TNF-related apoptosis-inducing ligand (TRAIL) interact with their respective death receptors [FAS/CD95, TNF-α receptor 1 (TNFR1), or TRAIL receptor (TRAIL-R1 or TRAIL-R2)], recruit Fas-associated death domain adapter protein (FADD), and form the death inducing signaling complex (DISC) [22, 23]. This complex recruits pro-caspase-8 and pro-caspase-10, leading to the activation of the executioner caspase-3, caspase-6, and caspase-7 [24, 25]. The molecular mechanisms regulating caspase-8 activity upon death receptor stimulation involves a cascade of events initiated by the binding of caspase-8 to FADD at the DISC [17]. The homodimerization and consequent activation by autoproteolytic cleavage of caspase-8 are thought to be mediated by c-FLIP. Both c-FLIP isoforms and caspase-8 are recruited at the DISC, and there are evidences that suggest that c-FLIP isoforms can either inhibit or activate caspase-8 and modulate its oligomerization [26]. The enzymatic activity of caspase-8 may be controlled by additional posttranslational mechanisms including phosphorylation at Y380 which inhibits the autoproteolytic activity of caspase-8, phosphorylation at T273 which promotes caspase-8 apoptotic functions, and deubiquitination which decreases caspase-8 activity and interrupts extrinsic activity [17].

Extrinsic apoptotic signals can be alternatively mediated by dependence receptors such as UNC-5 homolog family receptors (UNC-5A, UNC-B, UNC-C, and UNC-D) and deleted in colorectal cancer (DCC) family receptors. These receptors are activated by netrins, a family of extracellular proteins that direct cell and axon migration during embryogenesis [27]. Netrins are members of the laminin superfamily and contribute to the regulation of cell-cell adhesion and tissue organization [28]. Netrin-1 has been identified to be an anti-apoptotic survival factor in tumorigenesis [29]. DCC and UNC-5 homologs mediate cell death in the absence of netrin-1, and the binding of the ligand to these receptors switches between a pro-apoptotic signal and the promotion of survival and motility [29]. UNC-5B (also known as UNC-5H2) complex responds to the withdrawal of netrin-1 by recruiting a signaling complex consisting of protein phosphatase 2A (PP2A) and death-associated protein kinase 1 (DAPK1) [30]. In the presence of netrin-1, the PP2A complex is repressed by the recruitment of cancerous inhibitor of PP2A (CIP2A) into the UNC-5B/DAPK1 complex, of which DAPK1 is autophosphorylated and remained inactive. Conversely, netrin-1 withdrawal is associated with a conformational change in UNC-5B, resulting in the exposure of the death domain, releasing of CIP2A, and the recruitment of PP2A to the UNC-5B-DAPK1 complex. PP2A-mediated dephosphorylation of DAPK1 results in the activation of downstream apoptotic pathway. PP2A-like activity has been linked to the formation of DISC and is known to inhibit B-cell lymphoma 2 (Bcl-2) phosphorylation, leading to apoptotic cell death [31, 32]. In certain cell types, where the extrinsic apoptotic pathway is triggered but lower levels of DISC followed by lower levels of active caspase-8 are formed, amplification of the death signal is possible through the cleavage of Bid by caspase-8, which directly mediates Bak/Bax

oligomerization and triggers the release of cytochrome (Cyt) *c* [33, 34].

Another signaling pathway mediated by dependence receptors are the DCC and the Patched dependence receptor (Ptc). DCC encodes an approximately 200 kDa type I membrane protein, which displays homology with cell adhesion molecules in its extracellular domain, suggesting that DCC may play a role in cell-cell or cell-matrix interactions [35, 36]. DCC appears to drive apoptosis independent of both mitochondrial-dependent and death receptor/caspase-8 pathways. DCC interacts and drives the activation of caspase-3 through caspase-9 without requiring Cyt *c* or Apaf-1 [37]. Ptc, identified as a tumor suppressor, induces apoptosis but is suppressed by its ligand, sonic hedgehog (Shh) [38, 39]. Ptc interacts with the adapter protein DRAL/FHL2 in the absence of Shh and recruits a protein complex that includes DRAL/FHL2, the CARD-containing domain protein TUCAN, and apical caspase-9. It triggers caspase-9 activation and enhances cell death via a caspase-9-dependent mechanism [40, 41].

The death receptor and dependence receptor pathways converge at the activation of caspase-3, followed by cleavage and activation of downstream caspases. Caspases or cysteine aspartic acid-specific proteases are synthesized as inactive zymogens (or proenzymes) and are usually cleaved to form active enzymes or undergo autoproteolysis in a cascade manner. Initiator caspases such as caspase-8, caspase-9, and caspase-10 couple cell death stimuli to the downstream effector caspases such as caspase-3, caspase-6, and caspase-7. The major proteolysis activity that takes place during apoptosis is carried out by effector caspases. Caspase-3 appears to be the major executioner caspase during the demolition phase of apoptosis [42, 43]. Caspase-3 cleaves a number of structural proteins such as fodrin, gelsolin, rabaptin, nuclear lamin B, and vimentin [43–45]. On the other hand, caspase-6 appears to merely cleave the nuclear lamin A during apoptosis [43]. Caspase-3 also cleaves diverse regulatory proteins and enzymes, including focal adhesion kinase (FAK), protein kinase C delta, retinoblastoma protein (Rb) (a protein involved in cell survival), p21-activated kinase (PAK), U1 small nuclear ribonucleoprotein (U1snRNP), DNA fragmentation factor 45 (DFF45), inhibitor of caspase-activated DNase (ICAD), receptor interacting protein (RIP), X-linked inhibitor of apoptosis protein (X-IAP), signal transducer and activator of transcription-1 (STAT1), and topoisomerase I [43, 44, 46]. Initially, poly (ADP-ribose) polymerase (PARP) is reported to be an exclusive substrate for caspase-7 [43], but a later study proved that it is cleaved by both caspase-3 and caspase-7 [47].

Caspase-mediated cleavage of structural proteins is essential for the apoptosis-associated morphological changes. For example, cleavage of gelsolin in multiple cell types causes cells to round up, detach from the plate, and undergo nuclear fragmentation [48]. Inactivation of rabaptin-5 causes fragmentation of endosomes during the execution phase of apoptosis [49]. Fodrin is a major component of the cortical cytoskeleton of most eukaryotic cells; it has binding sites for actin, calmodulin, and microtubules [50]. Its proteolysis contributes to structural rearrangements including blebbing during apoptosis [51]. FAK is a tyrosine kinase of which its phosphorylation state and activity are linked to cell adhesion to the extracellular matrix through integrin receptors. It has a direct influence on the cytoskeleton, structures of cell adhesion sites, and membrane protrusions, leading to regulation of cell movement [52, 53]. Caspase-mediated cleavage of FAK is known to contribute to the morphological changes in apoptosis. On the other hand, PAK, a serine-threonine kinase, regulates morphological and cytoskeletal changes in a variety of cell types [54, 55]. Blocking PAK function during Fas-induced apoptosis inhibits the morphological changes but accelerates the phosphatidylserine externalization in the membrane. Stable Jurkat cell lines that express a dominant-negative PAK mutant are resistant to Fas-induced formation of apoptotic bodies and cleavage of PAK [56].

PARP cleavage is believed to attenuate the cell's ability to carry out DNA repair [44, 57]. Caspase-8 is also found to cleave PARP-2, a member of the PARP family involved in DNA

repair, suggesting that caspase-8 is both an initiator and effector caspase [58]. Active caspase-3 or caspase-7 proteolytically cleaves DFF45, which subsequently releases active DFF40, the inhibitor's associated endonuclease. It is responsible for the degradation of chromosomes into nucleosomal fragments, producing the characteristic hallmark of apoptosis [59, 60]. Cleavage of both structural and regulatory proteins is essential for the apoptotic-associated chromatin condensation, DNA fragmentation, nuclear collapse and morphological changes such as cell shrinkage and detachment, membrane blebbing, and formation of apoptotic bodies. Figure 17.1 illustrates the extrinsic apoptosis signaling pathway.

17.2.2 Intrinsic Apoptosis Pathway

Intrinsic apoptosis is a form of regulated cell death which is centrally mediated by the mitochondria. Intrinsic apoptosis can be triggered by DNA damage, γ-irradiation, oxidative stress, cytosolic Ca^{2+} overload, serum deprivation, and many other intracellular stress conditions. Upon stimulation, various molecules are released into the cytoplasm including Cyt *c* [25, 61], second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI (Smac/DIABLO) [62, 63], apoptosis-inducing factor (AIF, promotes chromatin condensation) [64], endonuclease G (EndoG, facilitates chromatin degradation) [65, 66], and high-temperature requirement protein A2 (HtrA2/Omi) [67]. Cyt *c* binds to and activates Apaf-1 protein in the cytoplasm, inducing the formation of apoptosome which subsequently recruits the initiator procaspase-9, yielding activated caspase-9 and finally mediating the activation of caspase-3 and caspase-7 [34]. Loss of Cyt *c* from the mitochondria also results in the inhibition of the respiratory chain. The condition elicits and aggravates reactive oxygen species (ROS) overproduction and is thought to activate a feed-forward circuit for the amplification of the apoptotic signal [68]. The function of Cyt *c* and its role in apoptosis is widely reviewed and discussed elsewhere [69–71].

Bcl-2 family of proteins plays an important role in the regulation of mitochondrial-linked apoptosis [72]. Bcl-2 subfamilies such as Bax, Bak, and Bcl-2 homolog (BH)3-only subfamily proteins (e.g., Bid) play a pro-apoptotic role, while Bcl-2 and Bcl-X_L are functionally anti-apoptotic. Activated Bax and Bak form homo-oligomer which creates pores on the mitochondrial membrane and releases toxic proteins from the mitochondria. Bcl-2 and Bcl-X_L inhibit the action by blocking the activation of Bax and Bak and prevent the release of pro-apoptotic proteins [73]. Nevertheless, the activation of Bax and Bak can be restored with the presence of pro-apoptotic BH3-only proteins. BH3-only proteins function as antagonists of specific subsets of their pro-survival relatives [74, 75]. The pore-forming activities of Bax and Bak trigger a condition known as mitochondrial outer membrane permeabilization (MOMP). MOMP can also be triggered by the opening of a multiprotein complex known as permeability transition pore complex (PTPC) [76, 77]. MOMP causes generalized and irreversible inner mitochondrial transmembrane potential (ΔΨm) dissipation. In the inner mitochondrial membrane (IM) of a healthy cell, the frontier between the intermembrane/intercristae space and the matrix is nearly impermeable to all ions, including protons which help create the proton gradient required for oxidative phosphorylation [68]. The charge imbalance that results from the generation of an electrochemical gradient across the IM forms the basis of the ΔΨm [68]. A loss of the ΔΨm or long-lasting or permanent ΔΨm dissipation can lead to cell death [78]. Pro-apoptotic Bcl-2 proteins appear to cause the release of Cyt *c*, Smac/DIABLO, and HtrA2/Omi but not EndoG and AIF [79]. On the other hand, BH3-only protein Bid cleavage by caspase-8 serves to engage a mitochondrial amplification loop during extrinsic apoptosis. Caspase-8 cleaves Bid, generating a truncated fragment known as truncated Bid (tBid) that can permeabilize the mitochondrion, resulting in MOMP [80].

Inhibitors of apoptosis proteins (IAPs) play an important role in the regulation of apoptosis. Eight human IAPs have been identified consisting of X-IAP, IAP-like protein-2 (ILP-2), cIAP-1,

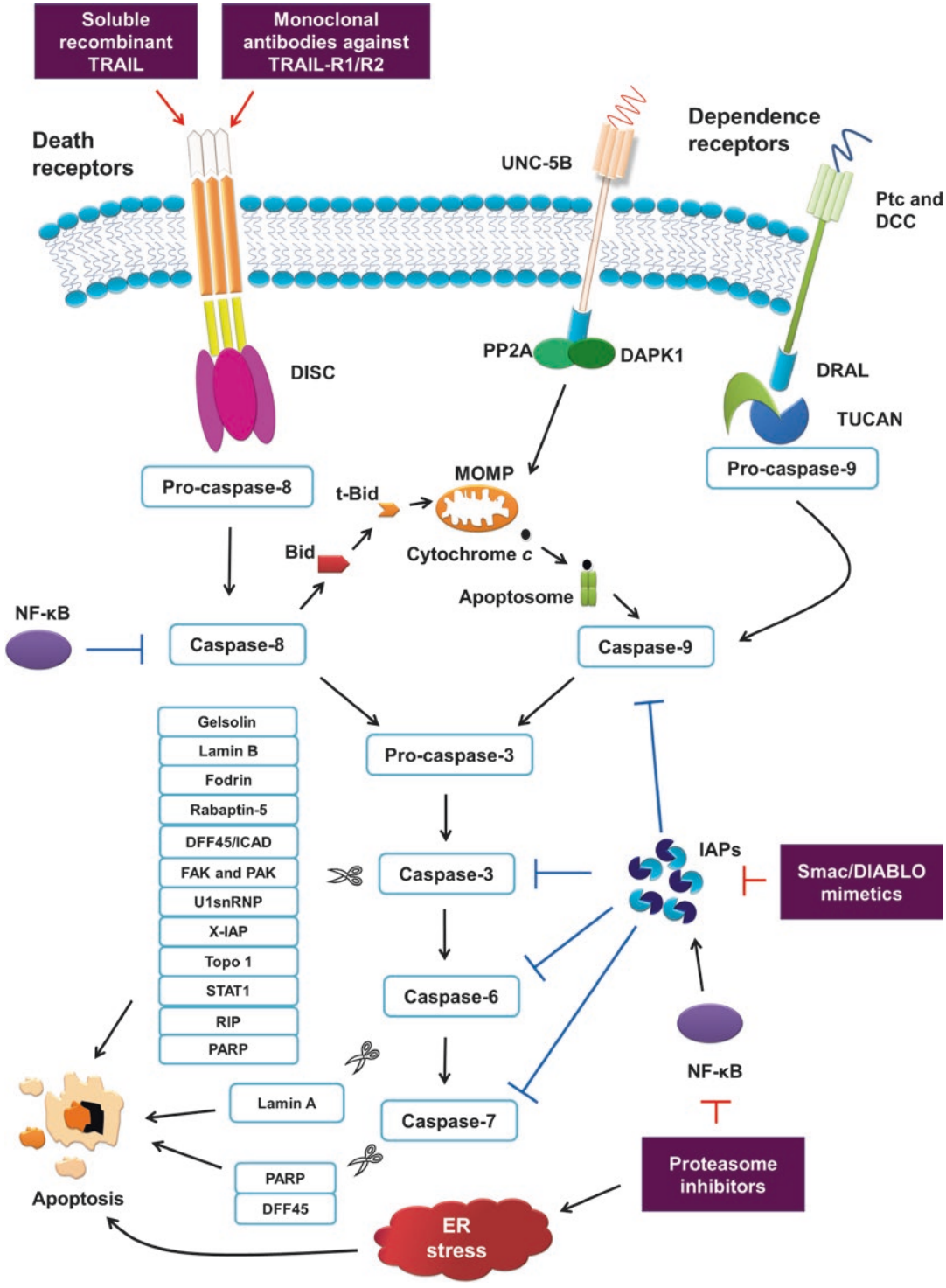

Fig. 17.1 Extrinsic apoptosis signaling pathway and therapeutic targets

cIAP-2, melanoma inhibitor of apoptosis protein (ML-IAP), neuronal apoptosis inhibitory protein (NAIP), survivin, and apollon [81]. Human IAP family members such as X-IAP, cIAP-1, and cIAP-2 are potent caspase inhibitors [82, 83]. X-IAP, cIAP-1, and cIAP-2 block Cyt *c*-induced activation of caspase-9, thus preventing the activation of caspase-3, caspase-6, and caspase-7. Furthermore, these IAPs bind to and inhibit the enzymatic activity of caspase-3 following its activation by caspase-8, thereby arresting the proteolytic cascade initiated by the initiator caspase [84]. X-IAP primarily inhibits caspase by disrupting the conformation of the active caspase and masking the substrate binding active site [81].

Smac/DIABLO and HtrA2/Omi inhibit the anti-apoptotic function of several members of the IAP family [85, 86]. Smac/DIABLO and HtrA2/Omi are two nuclear-encoded mitochondrial proteins functioning as IAP antagonists, identified in mammals [67, 87–90]. After their release into the cytosol stimulated by apoptotic triggers, Smac/DIABLO and HtrA2/Omi competitively bind to the BIR domains of IAPs via the IAP-binding motif, so that the BIR-bound caspases are released and reactivated [91–93]. Smac/DIABLO and HtrA2/Omi manifest distinct physical characteristics and biochemical activities, of which the active Smac/DIABLO is a homodimer, whereas HtrA2/Omi is a homotrimer [85, 94]. HtrA2/Omi is a mitochondrial serine protease and has diverse roles, including maintenance of mitochondrial homeostasis and regulation of cellular apoptosis [95–97]. A comprehensive proteome-wide analysis of Jurkat cell lysates leads to the identification of potential HtrA2/Omi substrates, for example, the cytoskeleton-associated proteins such as actin, α- and β-tubulin, and vimentin further suggest its role in the caspase-independent pathway [98].

EndoG and AIF function in a caspase-independent manner by relocating to the nucleus, where they mediate large-scale DNA fragmentation, independent of caspases [99, 100]. Mammalian EndoG is a nuclear-encoded protein targeted to mitochondria, compartmentalized in the intermembrane space (IMS), and is known to possess DNase/RNase activity [101]. It is implicated in the mitochondrial DNA replication and is shown to be involved in apoptotic DNA degradation [100]. In isolated non-apoptotic nuclei, EndoG first generates large fragments of DNA (> 50 kb) and then cleaves at inter- and intra-nucleosomal sites [102]. Although EndoG apoptotic activity appears to occur in the absence of caspase activation, the pathway leading to EndoG-dependent DNA damage remains controversial [103, 104].

AIF was originally discovered as an IMS component capable of inducing chromatin condensation and DNA loss in the nuclei isolated from healthy cells [102, 105]. AIF is a flavoprotein, which was first proposed to act as a protease or protease activator [106] and its apoptogenic activity is not affected by z-VAD-fmk [107]. Contribution of AIF to apoptosis depends on the cell types and death triggers [102]. Both endogenous and recombinant AIF are found to trigger peripheral chromatin condensation and large-scale DNA fragmentation in a caspase-independent manner [108, 109]. AIF is not known to possess nuclease activity; thus, AIF is postulated to directly interact with DNA and disrupt/collapse chromatin structure by displacing chromatin-associated proteins and/or by recruiting proteases and nucleases to form DNA-degrading complexes or degradosomes [102, 110].

Another important signaling pathway affecting the regulation of apoptosis is the nuclear factor of κB (NF-κB). NF-κB is a sequence-specific transcription factor known to be involved in the inflammatory and innate immune responses. Under normal conditions, NF-κB becomes activated only upon stimulation and subsequently upregulates the transcription of its target genes. NF-κB is activated by many divergent stimuli, including pro-inflammatory cytokines such as TNF-α, TRAIL, interleukin-1β (IL-1β), epidermal growth factor (EGF), T- and B-cell mitogens, bacteria and lipopolysaccharides (LPS), viral proteins, double-stranded RNA, drugs, and a variety of physical and chemical stresses [111]. However, in tumor cells, molecular alterations result in impaired regulation of NF-κB and

become constitutively activated in such cases, leading to deregulated expression of NF-κB-controlled genes [112]. Some genes targeted by NF-κB include cytokines/chemokines and their modulators, immunoreceptors, transcription factors, and regulators of apoptosis such as Bcl-X_L, Fas, FasL, and IAPs [111].

NF-κB is also known to play a pro-apoptotic role, in addition to its more common anti-apoptotic role. Examples of its pro-apoptotic effects in cells include those found in B-cells [113], T-cells [114, 115], and neuronal cells [116, 117]. The anti-apoptotic effects of NF-κB appeared to be cell-type specific and/or dependent on the inducing signal. Normally, NF-κB is transcriptionally inactive in the cytoplasm of most cells as it is bound to its cytoplasmic inhibitor IκBα. Upon stimulation with pro-inflammatory cytokines, such as TNF-α or IL-1, IκBα protein is phosphorylated, ubiquitinated, and subsequently degraded by the proteasome (the role of proteasome is further discussed under proteasome inhibitors). This process exposes the previously masked nuclear localization signal of NF-κB, allowing it to translocate into the nucleus upon IκBα proteolysis and subsequently activate the expression of important target genes involved in cell growth, survival, and adhesion [118, 119]. Activated NF-κB leads to the activation of A1/Bfl-1, a member of the Bcl-2 family, which suppresses Cyt *c* release from the mitochondria [120]. NF-κB activation blocks caspase-8 cleavage and Cyt *c* release, indicating that NF-κB suppresses the earliest signaling components of the caspase cascade. The IAP family genes (*cIAP-1* and *cIAP-2*) and TRAF family genes (*TRAF1* and *TRAF2*) are positively regulated by NF-κB with rapid kinetics following TNF addition [121, 122]. Another member of the IAP family, X-IAP, has been shown to be activated by NF-κB in endothelial cells [123, 124]. Thus, NF-κB activation functions to suppress apoptosis at multiple levels.

The NCCD initially defines "intrinsic apoptosis" as cell death mediated by MOMP and associated with generalized and irreversible ΔΨm dissipation, release of IMS proteins, and respiratory chain inhibition [16]. However, in their latest recommendation, it was proposed that intrinsic apoptosis to be defined as a form of regulated cell death initiated by perturbations of the intracellular or extracellular microenvironment, demarcated by MOMP, and precipitated by executioner caspases, mainly caspase-3 [17]. Figure 17.2 illustrates the intrinsic apoptosis pathway.

17.3 Apoptosis and Cancer

Apoptosis is an essential developmental process to maintain tissue homeostasis, and defects in apoptosis regulation play an important role in cancer development. Deregulation in the apoptosis pathway is one of the reasons why neoplastic cells gain extended life span, develop genetic mutations capable of growth under stress conditions, and undergo angiogenesis [9]. Several key pathways controlling apoptosis are commonly altered in cancer [125]. Tumor resistance to apoptotic cell death is often a hallmark of cancer and contributes to chemoresistance [9]. Alteration of many proteins involved in both intrinsic and extrinsic signaling pathways has been described. For example, overexpression of certain anti-apoptotic proteins, such as Bcl-2, Bcl-X_L, Akt, NF-κB, and IAP protein family, is found in various human tumors [126].

The apoptotic pathway of Fas, one of the TNF receptor family members, is frequently blocked by several mechanisms in cancer, one of which is *Fas* gene mutation [127–129]. *Fas* mutations have been detected in several types of human cancers with frequent allelic losses of chromosome 10q24, where the gene resides [128–130]. Both *TRAIL-R1* and *TRAIL-R2* genes are mapped on chromosome 8p21-22, and allelic losses of the chromosome 8p21-22 have been reported as a frequent event in several cancers, including non-Hodgkin's lymphoma (NHL), lung cancer, breast cancer, colon cancer, prostate cancer, hepatocellular carcinoma, and head and neck cancer [131–137]. Mutations

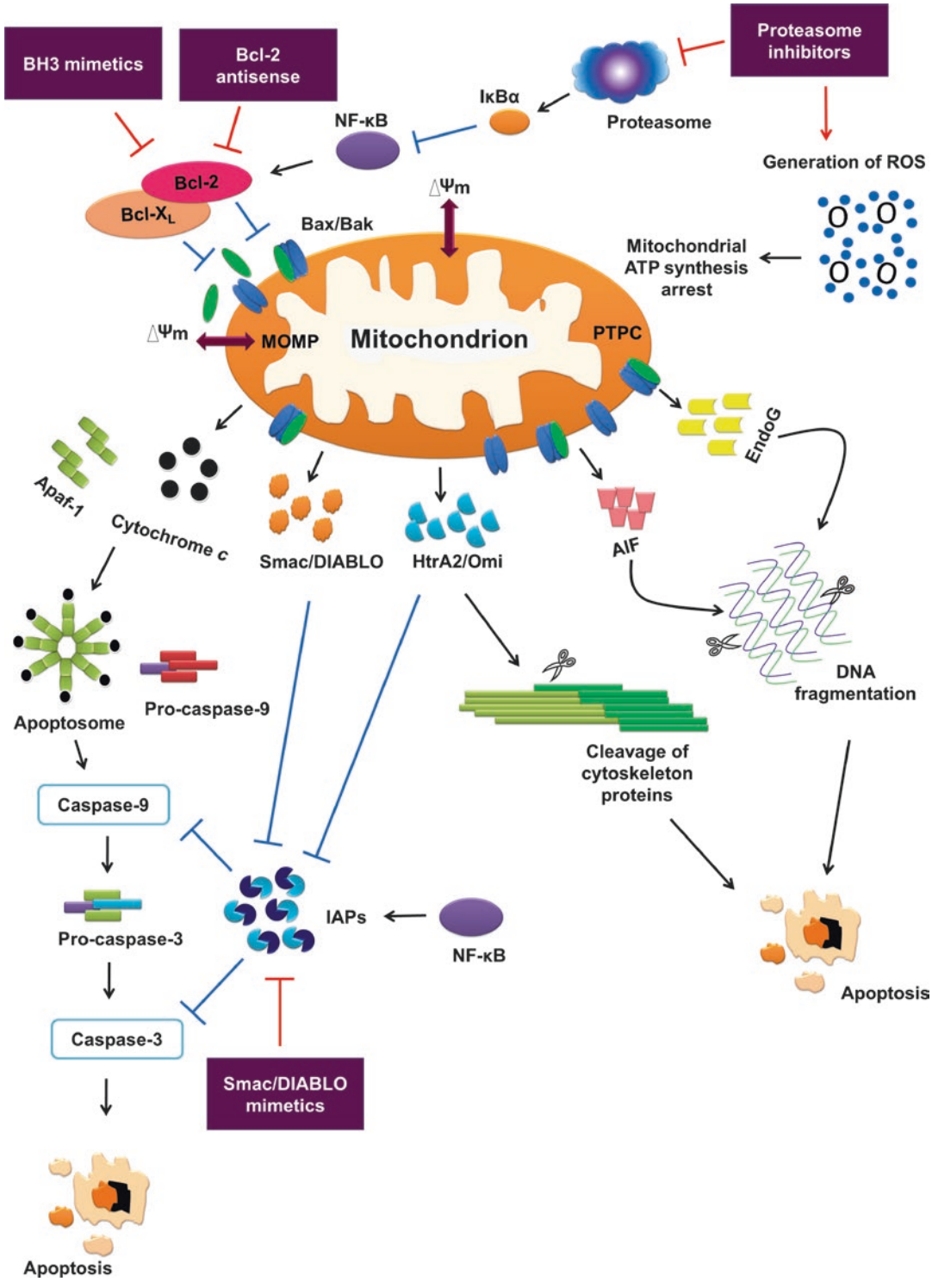

Fig. 17.2 Intrinsic apoptosis signaling pathway and therapeutic targets

of *TRAIL-R2* gene have been reported in head and neck cancer [138] and non-small cell lung cancer (NSCLC) [139]. In addition, somatic mutations of *TRAIL-R1* and *TRAIL-R2* genes are found in NHL [140] and breast cancer [141]. The number of pancreatic tumor tissues with positive membrane staining for TRAIL-R1 and TRAIL-R2 is lower than non-tumor tissues [142]. Loss of TRAIL-R2 expression is associated with poorer prognosis in patients [142]. A significant association is also observed between lower expression of *TNF* genes and poor prognosis in childhood adrenocortical tumors [143].

On the other hand, PP2A inactivation in cancer occurs frequently through the upregulation of CIP2A, a PR65 interactor and PP2A inhibitor [144]. PR65β, a scaffold protein which interacts with the catalytic subunit of PP2A, appears to play a key regulatory role in cancer. This scaffold protein is decreased or mutated in a large fraction of human cancers and has been recently linked to cancer development [145]. On the other hand, Ptc is a tumor suppressor, and mutations of Ptc are associated with neoplasia, especially in basal cell carcinoma and medulloblastoma [38, 39]. DCC expression is shown to be markedly reduced in more than 50% of colorectal tumors. The loss of DCC is not restricted to colon carcinoma but has been observed in other tumor types, including carcinoma of the stomach, pancreas, esophagus, prostate, bladder, breast, male germ tumors, neuroblastomas, gliomas, and some leukemias [35, 146, 147].

Members of the Bcl-2 family of proteins as prominent regulators of apoptosis signaling are often deregulated in many cancers, including lung carcinoma, lymphoma, and glioblastoma [148–152]. Aberrant expression of Bcl-2 is common in chronic lymphocytic leukemia (CLL) and is associated with poor response to chemotherapy and decreased overall survival [153]. *Bcl-2* gene amplification is reported in diffuse large B-cell lymphomas (DLBCL), and overexpression of Bcl-2 protein has been associated with poor prognosis in some forms of NHL [154–156]. Myc/Bcl-2 co-expression in DLBCL is associated with aggressiveness, is more common in the unfavorable activated B-cell (ABC)-like subtypes, and contributes to the overall inferior prognosis of patients with ABC-DLBCL [157]. Single-nucleotide polymorphisms of Bcl-2 are found to have an association with survival in advanced-stage NSCLC patients who received chemotherapy [158]. Furthermore, mutations that inactivate the pro-apoptotic *Bax* gene have been observed in solid tumors and hematological malignancies [159, 160]. Higher Bcl-2 to Bax ratios have been associated with progression of CLL, shorter remission duration, and shorter survival [161, 162]. Therefore, cancer therapeutics that specifically inhibits the anti-apoptotic proteins or activates the pro-apoptotic members of the Bcl-2 family proteins is an attractive strategy to reverse the intrinsic or acquired resistance of cancer cells to apoptosis [163].

Studies have reported that polymorphic variants of the *caspase-8* gene are associated with risk of multiple cancers [164–168]. For example, a six-nucleotide insertion-deletion variant polymorphism (6 N ins/del) of *caspase-8* promoter is linked to a significant decreased risk of bladder and lung cancer in Chinese populations [167, 168]. Since cancer cells are highly dependent on these genetic changes in the apoptotic pathways for survival, designing novel anticancer drugs that selectively kill cancer cells while sparing normal cells seems appealing [169]. Survivin, a member of the IAP family, is undetectable in terminally differentiated adult tissues but abundantly expressed in human cancers such as lung, colon, pancreas, prostate, and breast [163]. Increased survivin mRNA is associated with decreased overall survival in colon cancer patients [170]. Furthermore, increased levels of cIAPs in malignant cells are associated with a shorter relapse-free survival in patients with prostate cancer [171]. Livin or ML-IAP, another member of the IAP family of proteins, is found to be expressed in tumor cells [172, 173]. Thus, the possibility of IAP inactivation through therapeutic intervention is attractive and has gained much interest over the years.

Another important pathway linked to the apoptosis cell death is the p53 pathway, which is often inactivated and deregulated in human cancers [174, 175]. The p53 protein is a transcription

factor with tumor suppressor activities. Its role in tumor suppression relies partly on its ability to regulate the transcription of genes important in cell cycle arrest and in apoptosis. The p53 protein upregulates the expression of a number of genes in response to genotoxic stress, including the pro-apoptotic Bax [176]. It is also found to inhibit the expression of the *Bcl-2* gene [177]. Studies have also shown that Bid is a p53-responsive chemosensitivity gene, which may enhance the cell death response to chemotherapy [178]. The fact that a majority of human cancers harbor mutations in the *p53* gene suggests that such mutations would have contributed to the apoptosis resistance environment. However, the p53 network and the mechanism by which p53 determines the fate of cells remain to be explored.

17.4 Apoptosis Signaling Pathways and Therapeutic Targets in Cancer

17.4.1 TRAIL (TRAIL ligands, Monoclonal Antibodies Against TRAIL-R1 and TRAIL-R2)

TRAIL (Apo2 ligand) induces cell death via the extrinsic pathway by recruiting and activating caspase-8 and caspase-10 to its R1 and R2 receptors [179]. It activates the intrinsic pathway via the TRAIL-caspase-8-tBid-Bax cascade, through the cleavage of Bid, which promotes Bax and Bak oligomerization, leading to Cyt *c* release and activation of caspase-9 [180]. These processes collectively amplify the activities of the related executioner caspases. TRAIL is a promising cancer therapeutic agent, known to induce apoptosis in a wide variety of tumor cells while sparing normal cells [181, 182]. TRAIL activity is also known to be independent of the p53 status, making it potentially effective against chemotherapy-resistant tumors [183]. Many clinical trials have been initiated in cancer patients, using soluble recombinant TRAIL (rhApo2L, codeveloped by Genentech and Amgen; circularly permuted TRAIL (CPT) developed by Beijing Sunbio Biotech) [184–187], monoclonal antibodies (mAbs) (agonists) targeting TRAIL-R1, such as mapatumumab [HGS-ERT1 is developed by Human Genome Sciences (HGS)], and anti-TRAIL-R2 agents such as lexatumumab (HGS-ETR2 is developed by HGS), conatumumab (developed by Amgen), and apomab (developed by Genentech) [188].

Early Phase I/Ib trials of rhApo2L (dulanermin) in advanced cancer [189], advanced NSCLC [190], and NHL [191] reported that this drug was well tolerated by patients and no anti-rhApo2L Abs were detected. A Phase Ib study of dulanermin in combination with modified FOLFOX6 plus bevacizumab in patients with metastatic colorectal cancer was also well tolerated with similar adverse reactions that would be expected from FOLFOX plus bevacizumab [192]. However, only partial response was observed in less than half of the patients, and the median progression-free survival (PFS) was 9.9 months. Dulanermin in combination with rituximab in an open-label Phase Ib/II randomized study revealed that the addition of dulanermin to rituximab in patients with indolent B-cell non-Hodgkin lymphoma was tolerable but did not lead to increased objective responses [193]. Similarly, a recent trial to evaluate the efficacy and safety of dulanermin combined with vinorelbine and cisplatin (NP) as the first-line treatment for patients with advanced non-small cell lung cancer (NSCLC) produced unremarkable results. The median PFS was just 6.4 months in the dulanermin arm versus 3.5 months in the placebo arm. Objective response rate (ORR) was 46.78%, and median OS was 14.6 months in the dulanermin arm versus 13.9 months in the placebo arm [194]. Although these TRAIL-R agonists have been shown to be safe and well tolerated in patients, their respective anticancer activities have been largely disappointing [189, 195–197]. The lack of response may be due to the fact that most primary tumor cells are intrinsically resistant to TRAIL or may acquire resistance during the course of treatment [197].

Circularly permuted TRAIL (CPT) is a recombinant human mutant of Apo2L/TRAIL and is currently undergoing clinical development for the

treatment of multiple myeloma (MM) and other hematologic malignancies [186, 187, 198]. The primary molecular structure difference between CPT and the wild-type Apo2L/TRAIL is that the N-terminus of amino acid 121–135 sequence of Apo2L/TRAIL is connected to the C-terminus of the amino acids 135–281 sequence of Apo2L/TRAIL by a flexible linker. This structure forms stable homotrimers and has potent apoptosis-inducing activity via interaction with DR4/DR5 [198]. CPT apparently has better stability and displays better antitumor activity without significant toxicity against normal cells [199]. Results from a multicenter, open-label, Phase II clinical trial designed to determine the safety, efficacy, and the optimal dose of CPT in combination with thalidomide in patients with relapsed and thalidomide-refractory multiple myeloma (RRMM) were rather unremarkable. Although the addition of thalidomide to CPT was well tolerated with no occurrence of dose-limiting toxicities, the overall response rate (ORR) of 41 efficacy-evaluable patients was just 22.0% (2 complete response; 3 near complete response; 4 partial response) [198]. On the other hand, CPT as single-agent therapy for patients with RRMM produced an overall response rate of just 33.3% with one near-complete response (nCR) and eight partial responses (PRs) [187]. In another Phase 2 study where the safety and efficacy of CPT in combination with thalidomide and dexamethasone (CPT + TD) were evaluated in patients with pretreated RRMM, the median PFS time was 6.7 months for the CPT + TD group versus 3.1 months for the TD group. Serious adverse reactions were reported in 19.7% of the patients [186].

Mapatumumab, a fully human agonistic mAb targeting TRAIL-R1, either used alone or in combination with other chemotherapy drugs in Phase I or Phase II trial has not produced impressive trial outcomes, as in most cases, few patients ended with partial response or stable disease [200–203]. Despite its favorable safety profile, mapatumumab demonstrated limited or no clinical activity in Phase I and II trials in advanced solid malignancies [204, 205], NHL [206], NSCLC [207, 208], refractory colorectal cancer [209], and advanced hepatocellular carcinoma [210]. In a recent randomized, double-blind, placebo-controlled, Phase II study to evaluate the efficacy and safety of mapatumumab in combination with sorafenib in patients with advanced hepatocellular carcinoma (HCC), the results were clearly disappointing [211]. In this large study, a total of 101 patients were recruited, where treatment with mapatumumab and sorafenib was compared with placebo and sorafenib in patients with advanced HCC and was conducted at 29 sites in 6 countries. Both primary endpoint and secondary efficacy endpoints did not demonstrate a mapatumumab-related benefit beyond that achieved with sorafenib alone. These results demonstrated that patients with HCC are unlikely to benefit from adding mapatumumab to their sorafenib-based therapy [211].

Lexatumumab, apomab, and conatumumab are agonistic human mAbs against TRAIL-R2. Generally, the percentage of patients developed partial response or stable disease in several early Phase I trials involving these novel drugs is low, despite being well tolerated. In a Phase I trial and pharmacokinetic study of lexatumumab in pediatric patients with solid tumors, where 24 patients received a total of 56 cycles of lexatumumab over all four planned dose levels, none of the patients experienced complete or partial response [212]. On the other hand, objective activity of apomab was also not demonstrated in a Phase II study among patients with NHL [213], despite some evidence of activity in a Phase I study in patients with advanced malignancies [214]. In a Phase II trial, the addition of apomab to paclitaxel/carboplatin/bevacizumab combination did not improve overall efficacy while increasing the rate of some adverse effects in patients with NSCLC [196, 215]. Unsurprisingly, there are no recent published trial results and ongoing active trials involving both lexatumumab and apomab.

As for conatumumab, a Phase I study in advanced solid tumors showed that this drug is generally well tolerated [216, 217]. Conatumumab in combination with gemcitabine shows evidence of an improved 6-month survival rate and tolerable toxicity in Phase Ib and II metastatic pancreatic cancer trials [218, 219]. In metastatic

colorectal cancer, conatumumab improves progression-free survival (PFS) when combined with FOLFIRI [220], but limited activity when combined with modified FOLFOX6 and bevacizumab [221, 222], and no activity when combined with panitumumab [223]. The effect of conatumumab in NSCLC is similar as compared with rhApo2L [190, 196], of which combination of this drug with paclitaxel and carboplatin did not produce promising results [224, 225]. Combination of conatumumab with other chemotherapy drugs also produced no evidence of activity in soft tissue sarcomas [226]. Ganitumab, an agent targeting the insulin-like growth factor receptor type 1 (IGF1R), and conatumumab were used in a recent Phase Ib/II trial in patients with advanced solid tumors. The study was conducted in six cohorts of advanced non-small cell lung cancer (squamous or non-squamous histology), colorectal cancer, sarcoma, pancreatic cancer, or ovarian cancer patients [227]. Although no dose-limiting toxicities were observed and drugs were well-tolerated, there were no objective responses in all the population tested.

Generally, these trials lacked data on the correlation between patient's TRAIL status and response to therapy. Preferential TRAIL sensitivity and presence of TRAIL-R1 and TRAIL-R2 expression in certain cancers may be crucial factors in patient's response. As such, rhApo2L and agonistic anti-TRAIL-R therapies may be limited to patients with TRAIL-sensitive tumors. The efficacy of TRAIL targeting therapies may be improved if diagnostic methods determining TRAIL sensitivity of clinically detectable human cancers are available [188]. Although there are no more active trials involving most of the drugs in this group, studies involving the combination of conatumumab and other chemotherapy drugs are still ongoing.

17.4.2 Bcl-2 Family Proteins (BH3 Mimetics and Bcl-2 Antisense)

Bcl-2 family proteins can regulate apoptosis both positively and negatively. The Bcl-2 family members consist of anti-apoptotic (Bcl-2, Bcl-X_L, Bcl-W, Bag-1, Mcl-1, and A1/Bfl-1) as well as pro-apoptotic (Bad, Bax, Bak, Bcl-xs, Bid, Bik, and Hrk) molecules [228, 229]. The balance and interaction between Bcl-2 gene family members and posttranslational modifications of Bcl-2-related proteins have been demonstrated to play important roles in regulating cell survival and death. The Bcl-2 family is characterized by specific regions of homology termed Bcl-2 homology (BH1, BH2, BH3, and BH4) domains. Anti-apoptotic proteins have BH1–BH4 domains (e.g., Bcl-2 and Bcl-X_L). On the other hand, pro-apoptotic proteins have either BH1–BH3 domains (e.g., Bax and Bak) or BH3-only domains (e.g., Bid, Bim, Puma, Bad, Noxa, Hrk, Bik) [75, 230, 231]. These domains are critical to the function of these proteins, especially their impact on cell survival and cell death and their ability to interact with other family members and regulatory proteins. The molecular surface of the multidomain anti-apoptotic Bcl-2 protein contains a BH3-binding groove, which accommodates BH3 domain from pro-apoptotic Bcl-2 protein family members. The BH3-only proteins are known to function as antagonists of anti-apoptotic Bcl-2 family proteins and act as tumor suppressors [75]. This forms the basis or platform for subsequent drug discovery strategies based on mimicking BH3 peptides with chemical compounds that bind to the same groove [232].

The earlier observation that apoptosis deregulation in cancer cells primarily affects the upstream of the signaling pathways of Bax/Bak and mitochondria, leaving the downstream core of the apoptotic machinery mostly intact, has led to a therapeutic strategy of which manipulation of the equilibrium between the pro- and anti-apoptotic Bcl-2 family members could possibly restore apoptosis [126, 169]. Since pro-apoptotic BH3 domains directly bind to the hydrophobic grooves of pro-survival proteins with high affinity, and are necessary and sufficient for initiation of apoptosis, agents mimicking the BH3 domains may provide some degree of selectivity against cancer cells. This is mainly because cancer cells are postulated to be more sensitive to inhibition of pro-survival proteins compared with their normal counterparts [9]. Cancer cells often express

high levels of Bcl-2-like anti-apoptotic proteins to evade the apoptotic fate imposed by aberrant cell proliferation, activation of oncogenes, or DNA damage [233]. Therefore, it is possible to design BH3 mimetics to target specific anti-apoptotic proteins that are overexpressed in a particular type of cancer for improved specificity [169]. Several chemicals mimicking BH3 peptides exclusively targeting the Bcl-2 anti-apoptotic proteins have since been described [232, 234, 235]. Another antitumor strategy is direct inhibition of Bcl-2 mRNA, in the form of antisense.

One of the earliest small-molecule BH3 mimetics or more accurately Bcl-2 and Bcl-X_L inhibitor that went through several Phase I/II clinical trials is gossypol, an orally available compound derived from cottonseed extracts [236]. It binds to the BH3-binding grooves of Bcl-2, Bcl-X_L, and Mcl-1 [237]. However, several past clinical trials have not indicated this compound as an effective anticancer agent. Either used alone or in combination, patients treated with gossypol failed to show evidence of tumor regression or any therapeutic responses in several clinical trials [238–240]. A semisynthetic analog of gossypol with improved pharmacologic properties, such as apogossypolone (ApoG2), is found to inhibit the growth of diffuse large cell lymphoma cells in vitro and in vivo [241]. However, this compound has yet to proceed to clinical trials.

A derivative of R-(-)-gossypol (AT-101) is found to be well tolerated in a Phase I trial involving CLL patients [242]. Disappointingly, later studies showed that AT-101 is either not active in patients or the response rates are too low that it did not meet the criteria for additional enrollment in further trials for small cell lung cancer (SCLC) [243, 244]. In NSCLC, patients did not meet the primary endpoint of improved PFS when given a combination of AT-101 and docetaxel [245]. However, AT-101 added to cisplatin and etoposide in another small cohort of patients with SCLC was reported to be encouraging [246]. The slight efficacy gains from AT-101 were probably a result of its role in delaying and/or overcoming chemotherapy resistance, and, as such, it would yield optimal results in the setting of combination cytotoxic therapy [246]. As first-line therapy for metastatic castration-resistant prostate cancer, AT-101 was tolerable but did not extend OS when combined with docetaxel and prednisone in these patients [247]. In another Phase II multicenter study, where men with castration-sensitive metastatic prostate cancer were treated with AT-101 and androgen deprivation therapy, the combination did not meet the pre-specified level of activity [248]. Similarly, the addition of AT-101 to docetaxel in patients with recurrent or distantly metastatic head and neck squamous cell carcinoma did not appear to demonstrate evidence of efficacy [249]. Surprisingly, based on its lack of good efficacy in recent studies, several clinical trials involving combination with lenalidomide and other chemotherapy drugs are currently recruiting patients with multiple myeloma, CLL, and advanced laryngeal cancer. Biological therapies, such as lenalidomide, may stimulate the immune system, and addition to AT-101 may be an effective treatment for relapsed or refractory B-CLL [250].

Obatoclax mesylate (GX15-070) is an indole derivative and a broad-spectrum inhibitor of pro-survival Bcl-2 family proteins, and it has been extensively evaluated in clinical trials. Early Phase I clinical trial of obatoclax mesylate in patients with refractory leukemia and myelodysplasia has demonstrated that the drug is well tolerated up to the highest dose. However, only a single patient with acute myeloid leukemia (AML) with mixed lineage leukemia t(9;11) rearrangement achieved complete remission [251]. In another Phase I trial, where obatoclax was administered to patients with advanced CLL, even though activation of Bax and Bak was demonstrated, it had modest single-agent activity in heavily pretreated patients [252]. In advanced solid tumor and lymphoma, of 35 patients given obatoclax infusions, only 1 patient with relapsed NHL achieved partial response [253]. In both Phase I and II studies in patients with relapsed SCLC, obatoclax added to topotecan produced no difference in response rates as compared to topotecan alone, even though the drug was generally well tolerated [254, 255]. In patients with

extensive-stage small cell lung cancer (ES-SCLC), obatoclax failed to significantly improve ORR, PFS, or OS [256]. In addition, response to this drug in combination with docetaxel is also reported to be minimal in patients with NSCLC [257]. Obatoclax has also showed limited clinical activity in heavily pretreated patients with classic Hodgkin's lymphoma (HL) [258]. Single-agent obatoclax is also not associated with an objective response in patients with acute myeloid leukemia (AML) [259] and has limited first-line activity in patients with myelodysplastic syndromes [260]. Obatoclax appears to have limited efficacy as a single agent or even in combination with some of the more common anticancer drugs.

Another BH3 mimetic, navitoclax (ABT-263) is a selective, potent, and orally bioavailable small-molecule Bcl-2 inhibitor. It has high affinity for the anti-apoptotic Bcl-2 family proteins and kills cancer cells in a Bax/Bak-dependent manner [261]. In a Phase II clinical study, navitoclax exhibited limited single-agent activity against advanced and recurrent SCLC [262]. Navitoclax in combination with either carboplatin and paclitaxel or paclitaxel alone produced significant hematological and non-hematological toxicity and had limited efficacy in the treatment of patients with solid tumors [263]. Similarly, there is lack of objective responses in patients given a regimen of navitoclax combined with gemcitabine or irinotecan with solid tumors [264, 265]. However, when combined with rituximab, moderate response rates are observed in patients with follicular lymphoma and CLL. The combination demonstrated higher response rates for low-grade lymphoid cancers than observed for either agent alone in previous Phase 1 trials [266]. In another similar study, navitoclax in combination with rituximab yielded higher response rates than rituximab alone and resulted in prolonged progression-free survival with treatment beyond 12 weeks [267]. Clinical trials of navitoclax as a single agent or as combination therapy with signaling pathway inhibitors in a variety of cancers such as leukemia, non-small cell lung cancer, melanoma, and other solid tumors are currently ongoing.

Venetoclax (ABT-199) is the first FDA-approved treatment that targets the BCL-2 protein for use in patients with 17p-deleted CLL. Venetoclax is an oral medication, taken daily with food. As a single agent, it is most effective in patients with relapsed CLL and mantle cell lymphoma, where response rates of 80% and complete remission rates of 20% are observed [268]. However, single-agent response rates are modest in follicular lymphoma, diffuse large B-cell lymphoma, multiple myeloma, and AML. Venetoclax is highly selective and known to be more potent than navitoclax [269]. Prior to its approval in 2016, several major studies were carried out for venetoclax in patients with relapsed or refractory CLL or SLL or with non-Hodgkin's lymphoma [270, 271]. These trials showed the potential of BCL-2 antagonism effects of venetoclax across a range of doses and its ability in producing major reductions in tumor burden in all tissue compartments. Side effects were generally limited to low-grade nausea and diarrhea, and the most important toxic effect noted was the tumor lysis syndrome, which the risk can be reduced by a slow-dose ramp-up, careful monitoring, and adequate prophylaxis [272]. Venetoclax has also demonstrated promising clinical activity and favorable tolerability in cohorts of patients with CLL, whose disease progressed during or after B-cell receptor pathway inhibitors (BCRi) (idelalisib or ibrutinib) therapy [273, 274]. In addition to CLL, it has acceptable safety profile and has shown evidence of single-agent antimyeloma activity in patients with relapsed/refractory MM, predominantly in patients with t(11;14) abnormality and those with a favorable BCL-2 family profile [275]. Venetoclax with bortezomib and dexamethasone has an acceptable safety profile and promising efficacy in patients with relapsed/refractory MM [276]. Venetoclax in combination with hypomethylating agents such as decitabine or azacitidine in elderly patients with previously untreated AML is also well tolerated and has shown promising results with the number of patients who achieved remission or complete remission [277].

Randomized trials of combination therapy and Phase III trials in both CLL and AML are underway in all these diseases.

Oblimersen sodium is an 18-mer nuclease-resistant phosphorothioate antisense oligonucleotide designed to bind to the first six codons of the human Bcl-2 mRNA [278]. The use of oblimersen in combination with chemotherapy in a variety of cancers has shown diverse response rates with good tolerability. In the Oblimersen Melanoma Study Group, the addition of oblimersen to dacarbazine improved the multiple clinical outcomes in patients with advanced melanoma and increased overall patient's survival [279]. However, in a later prospective double-blind, placebo-controlled study, this combination did not significantly improve overall survival or progression-free survival in patients with advanced melanoma [280]. In another Phase III trial, the addition of oblimersen to fludarabine and cyclophosphamide significantly increased the complete response/nodular partial response rate in patients with relapsed or refractory CLL [281]. In the same study, a significant 5-year survival benefit was observed with oblimersen in combination with fludarabine and cyclophosphamide. Among patients with fludarabine-sensitive disease who had previously demonstrated maximum benefit with the same treatment, a 50% reduction in the risk of death was observed [282]. However, not all combination therapies produce desirable outcomes. In the Cancer and Leukemia Group B study 10107 (CALGB), although the combination of oblimersen and imatinib was safe and feasible, no clinical benefits were observed in imatinib-resistant chronic myeloid leukemia (CML) patients [283]. In a randomized Phase II study of carboplatin and etoposide with or without oblimersen for extensive-stage SCLC (CALGB 30103), the addition of oblimersen to a standard regimen did not improve any clinical outcome measure [284]. A randomized study of dexamethasone with or without oblimersen sodium in patients with advanced multiple myeloma (MM) demonstrated no significant differences in time to tumor progression or objective response rate [285].

Interestingly, in another Phase I study, the combination of oblimersen, temozolomide, and albumin-bound paclitaxel was well tolerated and demonstrated encouraging activity in patients with advanced melanoma, with objective response rate and disease control rate at 40.6% and 75%, respectively [286]. Some of the common adverse effects associated with oblimersen sodium administration include fatigue, transaminase elevation, and hematological disorders [287, 288]. So far, the FDA has not approved this drug as there was lack of evidence that oblimersen inhibits Bcl-2 in CLL patients or that altering Bcl-2 is beneficial to them. In addition, adding oblimersen to standard chemotherapy has not provided significant improvement in time to progression, overall survival, or secondary endpoints although toxicity was increased [289]. Currently, there are no more new trials involving oblimersen listed in the NIH ClinicalTrials.gov website.

17.4.3 Proteasome Inhibitors

The proteasome is a multicatalytic enzyme complex that degrades intracellular proteins by a targeted and controlled mechanism. The 26S proteasome, a large protein complex, composes approximately 50 subunits that function as a highly specific molecular shredder by hydrolyzing ubiquitinated proteins into small peptides [290]. The 26S proteasome can be further divided into two sub-complexes, a central 20S proteolytic core particle (CP) that is capped at either end by one or two 19S regulatory particles (19S RP). The 20S CP is the degradation unit and contains the active sites required to hydrolyze proteins into peptides [290]. On the other hand, the 19S RP controls the degradation of ubiquitin-tagged substrates by acting as a receptor for poly-ubiquitinated proteins and facilitating their ATP-dependent translocation into the catalytic chamber of the 20S CP [290].

The ubiquitin-proteasome pathway (UPP) is responsible for proteolytic degradation of the majority of damaged and misfolded proteins within the eukaryotic cell. The UPP is essentially

important for controlled degradation of key regulatory proteins involved in a wide variety of cellular functions such as apoptosis [291], cell cycle control, proliferation [292], and transcriptional regulation [293]. However, overactivity of the UPP results in an accelerated turnover of proteins that regulate the cell cycle, leading to a deregulated mitosis, thereby supporting cancer growth [294]. A defect in the proteasome function is associated with the development of different diseases such as neurodegenerative disorders, cardiovascular and rheumatoid diseases, and cachexia, but not cancer, suggesting that cancer cells use the proteasome for their survival [295]. In humans, three deubiquitinases (DUBs) are associated with the 19S RP. Two of these (UCHL5/Uch37 and USP14/Ubp6) are cysteine proteases and members of the ubiquitin C-terminal hydrolases (UCH) and ubiquitin-specific proteases (USP) families, respectively. The expression of the cysteine DUBs UCHL5 and USP14 is also deregulated in cancer. Activities of UCHL5 (along with several other DUBs) are found to be enhanced in tumor biopsies of cervical carcinoma when compared to adjacent normal tissues [296].

The transcription factor NF-κB is inactive in the cytoplasm under normal conditions and is activated when its binding partner, IκBα, is degraded by the proteasome. Constitutive NF-κB activity has been observed in a variety of tumors including MM; sustained activity of NF-κB may lead to aberrant expression of target genes promoting tumor cell proliferation and survival [297]. Bcl-2 is identified as a key target of NF-κB in B-cell lymphoma [298]. NF-κB, a centrally important transcription factor involved in immune and inflammatory cellular responses affecting both cell growth and survival, appears to be pivotally involved in the pathogenesis of aggressive lymphoid malignancies [299]. As a result, the inhibition of proteasome function serves as an important mechanism in anticancer therapy. Proteasome inhibitors have recently emerged as an interesting and potentially new group of chemotherapeutic agents for various human cancers, including breast, prostate, and lung carcinomas, which function in part by stabilizing the IκBα protein and, finally, inhibiting NF-κB activation [119, 300]. Preclinical studies have shown that the proteasome inhibitor, bortezomib, decreases proliferation, induces apoptosis, enhances the activity of chemotherapy and radiation, and reverses chemoresistance in a variety of hematologic and solid malignancy models in vitro and in vivo [301]. Bortezomib is a novel synthetic dipeptide boronic acid that reversibly inhibits the chymotryptic-like activity, and to a lesser extent, the caspase-like activity of the β5- and β1-subunits, of the 20S CP [302].

However, the role of NF-κB as a key determinant of bortezomib-induced cytotoxicity is rather controversial, as several studies have shown that direct inhibition of NF-κB signaling is insufficient to induce apoptosis in bortezomib-sensitive cells [303–305]. Recent studies also found that bortezomib exerts no inhibition of constitutive NF-κB activity in MM or mantle cell lymphoma cells [306, 307]. Results of the genome-wide siRNA screen performed by Chen and coworkers showed that bortezomib induces cell death by interfering with ribosome function and DNA damage pathways and through deregulation of Myc signaling [308]. A separate screen by Zhu and coworkers demonstrates that knockdown of cyclin-dependent kinase 5 (CDK5), as well as a number of other genes, potentiated bortezomib-induced cytotoxicity in MM cells [309]. In addition, proteasome inhibitors are also potent inducers of endoplasmic reticulum (ER) stress [304, 310]. Acute ER stress response caused by proteasome inhibition results in apoptosis [310]. In addition to ER stress, several reports indicate that proteasome inhibitors induce the rapid production of ROS, known to be involved in apoptotic signaling [304, 311, 312].

Bortezomib (Velcade®; Millennium Pharmaceuticals, Inc., Cambridge, MA, and Johnson & Johnson Pharmaceutical Research and Development, L.L.C.) is the first proteasome inhibitor approved by the U.S. Food and Drug Administration (FDA) in 2005 for the treatment of progressive MM in patients who have received at least one prior therapy [313].

The drug was later approved for the treatment of mantle cell lymphoma, a lymphoid malignancy derived from mature B-cells [314–316]. The regulatory approval of bortezomib was based on its efficacy and safety in a large, international, multicenter Phase III prospective study [313]. This randomized, open-label trial compared single-agent bortezomib with single agent, high-dose dexamethasone in patients with progressive MM after at least one prior therapy [317]. Bortezomib manifested significant efficacy and safety, supported by an improved response rate, including achieving near complete responses [313, 317]. Subsequently, further Phase I and II trial results produced encouraging prospects. In a retrospective study [based on data from Phase II (SUMMIT or CREST) or Phase III (APEX) registration studies] to clarify the utility of bortezomib as a repeat therapy, bortezomib retreatment was safe and effective in patients with relapsed MM [318]. In a separate Phase I/II trial, weekly bortezomib plus oral cyclophosphamide and prednisone produced more than 50% complete response rate and an encouraging 1-year survival in relapsed/refractory patients with MM [319]. A regimen consisting of bortezomib, cyclophosphamide, and dexamethasone as induction therapy in previously untreated MM patients is also proven to be effective and tolerable, suggesting that this drug combination induces high response rates independently of cytogenetic risk status [320]. The survival benefit with bortezomib induction/maintenance compared with classical cytotoxic agents in MM is also demonstrated in the Dutch-Belgian Cooperative Trial Group for Hematology Oncology Group-65/German-speaking Myeloma Multicenter Group-HD4 (HOVON-65/GMMG-HD4) Phase III trial [321].

Updated results of a multicenter Phase II PINNACLE study of bortezomib in patients with relapsed or refractory mantle cell lymphoma indicate that single-agent bortezomib is associated with lengthy responses and notable survival in these patients [315]. On the other hand, bortezomib in combination with rituximab, cyclophosphamide, bortezomib, and dexamethasone is also proven to be an effective regimen in relapsed low-grade and mantle cell lymphoma [322]. In addition, combination with cladribine and rituximab was demonstrated to be effective in both advanced relapsed or refractory mantle cell lymphoma and indolent non-Hodgkin lymphoma [323]. However, clinical trials using bortezomib in combination with other chemotherapy drugs in cancers such as HL [324], advanced solid tumors such as breast, ovarian and prostate [325], and metastatic gastroesophageal cancer [326] lacked favorable outcomes. In a recent trial, although daily subcutaneous dose-dense daily regimen of bortezomib showed a dose-dependent plasma exposure with evidence of target inhibition and preliminary signs of clinical activity in solid tumors, the study outcome was limited by cumulative neurological toxicity such as asthenia, anorexia, or ataxia [327]. In another study to evaluate the impact of the addition of bortezomib to rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) on previously untreated patients with non-germinal center B-cell-like (non-GCB) diffuse large B-cell lymphoma (DLBCL), clinical outcomes were not significantly improved by adding bortezomib [328]. Interestingly, a Phase II trial which evaluated the efficacy of bendamustine, bortezomib, and rituximab in patients with previously untreated low-grade lymphoma revealed that the treatment regimen was well tolerated and produced high response rates [329].

It is clear that although bortezomib has potent anti-multiple myeloma activity, not all patients responded to bortezomib, and most responders ultimately relapsed [330, 331]. To date, however, no marker(s) has been identified and validated in a manner that would allow clinical use and distinguish patients likely to respond to bortezomib treatment from those who would not [330]. The most common adverse events are gastrointestinal symptoms, fatigue, thrombocytopenia, and sensory neuropathy, which comprised a major reason of treatment discontinuation [332]. Despite the clinical success of bortezomib in MM and

mantle cell lymphoma, resistance to this drug remains a clinically significant problem. For example, in studies of bortezomib in relapsed refractory patients [331, 332], almost all responding patients ultimately experienced disease progression. Even when bortezomib was used as a single agent in newly diagnosed patients, 52% did not achieve a partial response or a better outcome [333]. Furthermore, the clinical responses to bortezomib in other hematologic malignancies and solid tumors remain low [331, 334]. Currently there are over a hundred clinical trials involving bortezomib either as a single agent or in combination with other chemotherapy drugs in the stage of recruiting, and about a quarter of those trials are Phase III trials.

There are accumulating evidences which support the potential of proteasome inhibitors as immunosuppressants. Proteasome inhibitors are found to interfere with antigen processing and presentation, as well as with the signaling cascades involved in immune cell function and survival, and these agents can be used to reduce antibody production and thus prevent antibody-induced tissue damage. Although several clinical studies have explored the potential of bortezomib for treating immune disorders, such as antibody-mediated organ rejection and graft-versus-host disease (GVHD), and systemic lupus erythematosus, study outcomes were often limited by either bortezomib toxicity or lack of improvement [335–338].

Carfilzomib (previously known as PR-171) is a tetrapeptide epoxyketone-based irreversible proteasome inhibitor, more potent and selective and produces more sustained inhibition of the proteasome [339, 340]. It has been approved by the FDA in 2012 for the treatment of MM. This inhibitor differs structurally and mechanistically from bortezomib. Carfilzomib functions by irreversibly inhibiting chymotrypsin-like activity of the proteasome, whereas bortezomib, a boronic acid dipeptide, inhibits the chymotrypsin-like activity of the 26S proteasome in a reversible manner [341]. The pivotal efficacy study was PX-171-009 (ASPIRE), a randomized, multicenter, Phase III study to compare the efficacy and safety of carfilzomib with lenalidomide and dexamethasone versus lenalidomide and dexamethasone alone in patients with relapsed MM [342]. The ASPIRE study found that the addition of carfilzomib to lenalidomide and dexamethasone (carfilzomib group) significantly improved progression-free survival (PFS) and has a favorable benefit-risk profile as compared with lenalidomide and dexamethasone alone (control group) in patients with relapsed MM [343]. Similar efficacy was reported in Japanese patients with MM [344]. The addition of carfilzomib to the reference regimen has shown efficacy, clinically meaningful and statistically significant improvement in the primary endpoint of PFS [341]. Carfilzomib and dexamethasone regimen is also reported to be more cost-effective as compared with bortezomib and dexamethasone for relapsed/refractory multiple myeloma [345]. A head-to-head comparison of these two proteasome inhibitors in a Phase 3, open-label, randomized controlled trial in patients with relapsed or refractory multiple myeloma (ENDEAVOR trial) indicated that carfilzomib produces clinically meaningful reduction in the risk of death as compared with bortezomib [346]. The carfilzomib-dexamethasone regimen is also found to be superior to bortezomib-dexamethasone regardless of cytogenetic risk [347]. Combination of carfilzomib with alkylating agent such as cyclophosphamide is found to be effective in patients with transplant-ineligible myeloma as well as newly diagnosed MM [348, 349]. Currently various combinations of carfilzomib with other chemotherapy agents such as irinotecan in irinotecan-sensitive malignancies and isatuximab in MM in different phases of trials are ongoing. Other clinical studies are currently exploring the potential benefit of this drug in patients with relapsed AML or acute lymphoblastic leukemia (ALL).

The development of carfilzomib, a second-generation protease inhibitor, represented significant progress toward a less neurotoxic and potentially more efficacious treatment as compared with bortezomib. While the incidence of grade 3 peripheral neuropathy is markedly dimin-

ished with carfilzomib, other aspects of the bortezomib toxicity, including thrombocytopenia, lymphopenia, and fatigue, persisted [350]. However, the dosing frequency and the need for parenteral delivery undoubtedly increase the degree of inconvenience when incorporating these two agents into treatment plans [350]. Ixazomib is the first orally bioavailable option for the treatment of refractory/relapsed MM. The results of the TOUMALINE-MM1 trial paved the way for the approval of ixazomib by the US FDA in November 2015 for the treatment of RRMM in combination with lenalidomide and dexamethasone [351]. However, there is limited direct evidence that ixazomib retains the improved outcomes bortezomib has produced in high-risk disease associated with del(17p) and t(4;14) mutations [350]. The exact role for ixazomib remains to be established, although there are indications that it may be the ideal choice for maintenance regimens.

17.4.4 Inhibitor of Apoptosis Protein (IAP) Antagonists

During apoptosis, natural IAP antagonists such as Smac/DIABLO and HtrA2/Omi translocate from the mitochondria and inactivate IAPs to facilitate caspase activation and cell death. Smac/DIABLO and HtrA2/Omi promote apoptosis by antagonizing the IAPs, such as X-IAP, cIAP-1, and cIAP-2, which are often upregulated in many cancer cells [352]. X-IAP is a potent direct inhibitor of caspase-3, caspase-7, and caspase-9 [353]. Smac/DIABLO contains an IAP-binding motif which forms the basis for the design of the novel class of anticancer drugs named Smac mimetics [354]. Peptides that mimic Smac/DIABLO functions are capable of inducing death or increasing the apoptotic effect of chemotherapeutic agents [62, 352]. In a preclinical study, the synthesized Smac/DIABLO-N7 peptides are found to increase the apoptosis-inducing potential of chemotherapeutic drugs (paclitaxel, doxorubicin, and tamoxifen) and irradiation; in addition, they sensitize TRAIL-resistant cells to undergo apoptosis [355].

Currently, there are several Smac mimetics undergoing evaluation in early clinical trials as cancer therapeutics, including both monovalent compounds, which contain one Smac-mimicking moiety (i.e., LCL161, GDC-0917/CUDC-427, and AT-406/Debio1143) and bivalent agents that are composed of Smac-mimicking elements connected via a chemical linker (i.e., TL32711/birinapant and HGS1029). Monovalent and bivalent Smac mimetics differ in their pharmacologic properties; bivalent compounds are administered intravenously, whereas monovalent compounds are orally bioavailable [356]. AEG35156, an X-IAP antisense oligonucleotide, is the first IAP antagonist that has advanced to human clinical trial. In a randomized Phase II trial of patients with primary refractory AML, the addition of AEG35156 to idarubicin and cytarabine did not improve the rate of remission as compared with the control arm consisting of cytarabine and idarubicin alone [357, 358]. The mRNA level of X-IAP was not determined in this study; therefore, whether efficient knockdown of X-IAP mRNA was achieved in this Phase II trial remains unknown [357]. Later studies on metastatic pancreatic ductal adenocarcinoma also did not produce significant clinical activity [359]. In combination with sorafenib in patients with advanced hepatocellular carcinoma, benefit on PFS was moderate [360]. Some trials were terminated due to toxicity and failure to reach endpoints.

A Phase I report of another novel IAP antagonist, LCL161, indicated that this orally bioavailable agent was well tolerated in patients with advanced cancer. However, no objective responses were observed, despite the fact that LCL161 treatment resulted in target inhibition, as shown by cIAP-1 degradation and cytokine induction [361]. Cytokine release syndrome, including increased levels of TNF-α in the circulation of patients that were treated with LCL161, was identified in a recent Phase I study as a dose-limiting toxicity [362]. Several Phase I/II studies of LCL161 in combination with paclitaxel or topotecan in advanced solid tumors have been completed but are yet to be published. New studies involving combination therapies with other

chemotherapeutic drugs in MM and solid tumors are currently recruiting patients. Two other small-molecule IAP antagonists, HGS1029 and TL32711, were also reported to be well tolerated in Phase I studies and have produced some evidence of antitumor activity as well as suppression of cIAP-1 level [363, 364]. However, dose-limiting toxicity after administration of HGS1029 was observed in about one-third of patients, including elevations of aspartate transaminase, amylase, or lipase, and fatigue [363]. TL32711 or birinapant in two published trials was reported to be well tolerated and exhibited some evidence of antitumor activity [365].

Survivin is a dual functional protein acting as a critical inhibitor of apoptosis (IAP) and key regulator of cell cycle progression [366]. Survivin is overexpressed in many human tumors and has been recognized as a biomarker. Increased survivin usually correlates with poor clinic outcome, tumor recurrence, and therapeutic resistance [367–370]. Survivin is an unconventional drug target, and several indirect approaches have been explored to manipulate its function and the phenotype of survivin-expressing cells. Interference with the expression of the survivin gene, the utilization of its messenger RNA, its intracellular localization, its interaction with binding partners, its protein stability, and the induction of survivin-specific immune responses are some of the strategies. Some of the therapeutics under investigation to target survivin are the low molecular weight molecules, antisense oligonucleotides, siRNA, ribozymes, and immunotherapy [371]. Examples include the use of the low molecular weight molecule inhibitor sepantronium bromide (YM155), the antisense oligonucleotide LY2181308, and survivin-directed autologous cytotoxic T lymphocytes (CTL). The optimum use of survivin inhibitors in the treatment of cancer is thought to be likely in combination with conventional cancer therapies for different cancers [372].

Sepantronium bromide (YM-155) is a novel small molecule which suppresses transactivation of survivin through direct binding to its promoter and selectively suppresses expression of survivin and induces apoptosis [373]. This drug has demonstrated to be safe and to possess antitumor activity in Phase 1 studies [374, 375]. However, Phase II trials reported modest and limited single-agent activity of sepantronium in NSCLC and refractory diffuse large B-cell lymphoma, respectively [376, 377]. In patients with stage III or IV melanoma, pre-specified primary endpoint was not achieved in a Phase II trial [378]. Unfortunately, combination of sepantronium with other chemotherapeutic drugs such as paclitaxel and carboplatin or docetaxel in various cancers also did not produce clinically significant results [379–382].

Gataparsen sodium (LY2181308), a novel 2'-O-methoxymethyl modified antisense oligonucleotide (2-MOE-ASO), is a specific inhibitor of survivin mRNA and is being investigated for efficacy in clinical trials in various groups of cancer patients [383]. It has been reported to be safe in the first-in-human Phase I study, although further studies would be needed to assess its activities [384]. In the most recent studies, adding this drug to standard therapy in patients with NSCLC or prostate cancer failed to elicit a clinically significant efficacy [385, 386]. Table 17.1 summarizes the various drugs targeting the apoptosis pathways and the most recent clinical trial stages based on published reports as well as ongoing trials listed in the NIH ClinicalTrials.gov website.

The crosstalk between apoptosis, ER stress, and autophagy signaling pathway and future directions of cancer therapeutics will be discussed in Chap. 18.

17.5 Concluding Remarks

Apoptosis-targeted therapy has been a critical and important approach in treating and managing cancer. Development of drugs that act either by harnessing the TRAIL pathway, by blocking the action of anti-apoptotic proteins, such as IAPs, small-molecule inhibitors (antisense oligonucleotides), or small interfering RNA and BH3 mimetic, or by targeting the proteasomes are robust strategies for use in cancer therapy. Although some of these drugs have not shown favorable trial outcomes, newer drug candidates such as navitoclax and CPT are potentially useful

Table 17.1 Current therapeutic target in the apoptosis signaling pathway and selected recent clinical trial stages

Pathway	Therapeutic targets	Current drugs	Clinical trial stages (published reports)/type of cancer	Combined with	References
TRAIL	TRAIL-R1 and TRAIL-R2	rhApo2L (Dulanermin)	Phase Ib: Metastatic colorectal cancer	FOLFOX6 and bevacizumab	[192]
			Phase Ib/II: Relapsed indolent B-cell lymphoma	Rituximab	[193]
			Phase III: Advanced non-small-cell lung cancer (NSCLC)	Vinorelbine and cisplatin	[194]
			None active or recruiting		https://clinicaltrials.gov/ct2/home
		Circulatory permuted TRAIL (CPT)	Phase II: Relapsed and refractory multiple myeloma	Thalidomide	[198]
			Phase II: Relapsed and refractory multiple myeloma	–	[187]
			Phase Ib: Relapsed and refractory multiple myeloma	–	[387]
			Phase II: Relapsed and refractory multiple myeloma	Thalidomide and dexamethasone	[186]
			None registered		https://clinicaltrials.gov/ct2/home
	TRAIL-R1	Mapatumumab	Phase II: Advanced non-small cell lung cancer	Paclitaxel and carboplatin	[208]
			Phase II: Advanced hepatocellular carcinoma	Sorafenib	[211]
			None active or recruiting		https://clinicaltrials.gov/ct2/home
	TRAIL-R2	Lexatumumab	Phase I: Solid tumor	–	[212]
			None active or recruiting		https://clinicaltrials.gov/ct2/home
		Apomab (Drozitumab; PRO95780)	Phase I: Advanced cancer	–	[214]
			Phase II: NSCLC	Paclitaxel, carboplatin, and bevacizumab	[215]
			Phase II: NHL	Rituximab	[213]
			None active or recruiting		https://clinicaltrials.gov/ct2/home
		Conatumumab (AMG655)	Phase II: Pancreatic cancer	Gemcitabine	[218]
			Phase Ib/II: Metastatic colorectal cancer	Modified FOLFOX6 and bevacizumab	[222]

			Phase II: Colorectal cancer	FOLFIRI	[220]
			Phase II: Advanced NSCLC	Paclitaxel and carboplatin	[225]
			Phase Ib/II: Advanced solid tumors	Ganitumab	[227]
			Phase Ib: Relapsed ovarian cancer (completed/unpublished)	Birinapant	https://clinicaltrials.gov/ct2/show/NCT01940172
			Phase II: Advanced solid tumors (active, not recruiting)	FOLFOX6, ganitumab, and bevacizumab	https://clinicaltrials.gov/ct2/show/NCT01327612
Bcl-2 family proteins	Anti-apoptotic Bcl-2 family members	AT-101	Phase II: Castration-resistant prostate cancer	Docetaxel and prednisone	[247]
			Phase I: Advanced solid tumors and extensive-stage small cell lung cancer	Cisplatin and etoposide	[246]
			Phase II: Castration-sensitive metastatic prostate cancer	Androgen deprivation therapy	[248]
			Phase II: Recurrent, locally advanced, or metastatic head and neck cancer	Docetaxel	[249]
			Phase I: Solid tumors (completed/unpublished)	Paclitaxel and carboplatin	https://clinicaltrials.gov/ct2/show/NCT00891072
			Phase II: CLL (completed, unpublished)	Rituximab	https://clinicaltrials.gov/ct2/show/NCT00286780
			Phase II: Recurrent glioblastoma multiforme (completed/unpublished)	–	https://clinicaltrials.gov/ct2/show/NCT00540722
			Phase III: Advanced non-small cell lung cancer (completed/unpublished)	Docetaxel and cisplatin	https://clinicaltrials.gov/ct2/show/NCT01977209
			Phase I/II: Relapsed symptomatic multiple myeloma (recruiting)	Lenalidomide and dexamethasone	https://clinicaltrials.gov/ct2/show/NCT02697344

(continued)

Table 17.1 (continued)

Pathway	Therapeutic targets	Current drugs	Clinical trial stages (published reports)/type of cancer	Combined with	References
			Phase I/II: Relapsed B-cell CLL (B-CLL) (recruiting)	Lenalidomide	https://clinicaltrials.gov/ct2/show/NCT01003769
			Phase II: Advanced laryngeal cancer (recruiting)	Docetaxel, cisplatin, and carboplatin	https://clinicaltrials.gov/ct2/show/NCT01633541
		Obatoclax mesylate (GX15-070)	Phase I: Extensive-stage small cell lung cancer	Carboplatin and etoposide	[388]
			Phase I/II: Relapsed or refractory mantle cell lymphoma	Bortezomib	[389]
			Phase I/II: Acute myeloid leukemia	–	[259]
			Phase II: Untreated myelodysplastic syndromes	–	[260]
			Phase II: Extensive-stage small cell lung cancer	Carboplatin and etoposide	[256]
			Phase I: Relapsed CLL	Fludarabine and rituximab	[390]
			None active or recruiting		https://clinicaltrials.gov/ct2/home
		Navitoclax (ABT-263)	Phase I: Solid tumors	Carboplatin and paclitaxel	[263]
			Phase I: Solid tumors	Gemcitabine	[264]
			Phase I: Relapsed or refractory lymphoid malignancies	Rituximab	[266]
			Phase I: Advanced solid tumors	Irinotecan	[265]
			Phase II: B-cell CLL	Rituximab	[267]
			Phase I: Relapsed acute lymphoblastic leukemia (recruiting)	Chemotherapy	https://clinicaltrials.gov/ct2/show/NCT03181126
			Phase I: Relapsed or refractory solid tumors (recruiting)	Sorafenib	https://clinicaltrials.gov/ct2/show/NCT02143401
			Phase I: Advanced or metastatic non-small cell lung cancer (recruiting)	Osimertinib	https://clinicaltrials.gov/ct2/show/NCT02520778
			Phase I/II: Relapsed or refractory lymphoid malignancies (completed/unpublished)	–	https://clinicaltrials.gov/ct2/show/NCT0040680

			Phase I/II: Relapsed small cell lung cancer and other solid tumors (recruiting)	Vistusertib	https://clinicaltrials.gov/ct2/show/NCT03366103
			Phase I/II: Advanced or metastatic solid tumors	Trametinib	https://clinicaltrials.gov/ct2/show/NCT02079740
			Phase I/II: BRAF mutant melanoma or solid tumors	Dabrafenib and trametinib	https://clinicaltrials.gov/ct2/show/NCT01989585
			Phase II: Myelofibrosis (recruiting)	Ruxolitinib	https://clinicaltrials.gov/ct2/show/NCT03222609
		Venetoclax (ABT-199)	Approved by FDA for CLL (2016)		[391]
			Phase I: Relapsed or refractory CLL or small lymphocytic lymphoma (SLL)	–	[270]
			Phase II: Relapsed or refractory CLL with 17p deletion	–	[271]
			Phase I: Relapsed/refractory t(11;14) multiple myeloma	–	[275]
			Phase Ib: Relapsed/refractory multiple myeloma	Bortezomib and dexamethasone	[276]
			Phase II: CLL who progressed during or after idelalisib therapy	Idelalisib	[273]
			Phase II: CLL progressing after ibrutinib	Ibrutinib	[274]
			Phase II: Relapsed/refractory acute myeloid leukemia (AML) and relapsed high-risk myelodysplastic syndrome (recruiting)	Decitabine	https://clinicaltrials.gov/ct2/show/NCT03404193
			Phase II: Relapsed or refractory CLL in the presence of 17p deletion (recruiting)	–	https://clinicaltrials.gov/ct2/show/NCT02966756
			Phase II: CLL (recruiting)	Ibrutinib	https://clinicaltrials.gov/ct2/show/NCT02756897
			Phase III: CLL whose cancer has come back or who had no response to previous cancer treatments including subjects missing part of their chromosome 17 or TP53 gene mutation or who received prior treatment with a B-cell receptor inhibitor (recruiting)	–	https://clinicaltrials.gov/ct2/show/NCT02980731

(continued)

Table 17.1 (continued)

Pathway	Therapeutic targets	Current drugs	Clinical trial stages (published reports)/type of cancer	Combined with	References
			Phase III: AML who are ineligible for standard induction therapy (recruiting)	Azacitidine	https://clinicaltrials.gov/ct2/show/NCT02993523
			Phase III: Mantle cell lymphoma (recruiting)	Ibrutinib	https://clinicaltrials.gov/ct2/show/NCT03112174
			Phase III: AML (recruiting)	Cytarabine	https://clinicaltrials.gov/ct2/show/NCT03069352
			Phase III: CLL (recruiting)	Fludarabine, cyclophosphamide, rituximab, bendamustine, obinutuzumab, and ibrutinib	https://clinicaltrials.gov/ct2/show/NCT02950051
	Bcl-2 mRNA	Oblimersen sodium	Phase III: CLL	Fludarabine and cyclophosphamide	[282]
			Phase III: MM	Dexamethasone	[285]
			Phase I: Advanced melanoma	Temozolomide and albumin-bound paclitaxel	[286]
			Phase II: Chemotherapy-naive patients with advanced melanoma	Dacarbazine	[280]
			None active or recruiting		https://clinicaltrials.gov/ct2/home)
Proteasome		Bortezomib	Approved by FDA for multiple myeloma (MM) (2005)	–	[313]
			Approved by FDA for mantle cell lymphoma (2006)	–	[316]
			Phase I: Advanced solid tumors	–	[327]
			Phase II: Induction therapy in multiple myeloma	Cyclophosphamide and dexamethasone	[320]
			Phase II: Non-germinal center B-cell-like diffuse large B-cell lymphoma	Rituximab, cyclophosphamide, doxorubicin, and vincristine	[328]
			Phase III:MM	Classical cytotoxic agents prior and thalidomide	[321]
			Phase I: Relapsed/refractory non-Hodgkin lymphoma	Everolimus	[392]
			Phase II: Relapsed low grade and mantle cell lymphoma	Rituximab, cyclophosphamide and dexamethasone	[322]
			Phase II: Previously untreated, low-grade lymphoma	Bendamustine and rituximab	[329]

			Phase II: Advanced, newly diagnosed, and relapsed/refractory mantle cell and indolent lymphoma	Cladribine and rituximab	[323]
			Phase III: Cholangiocellular carcinoma (recruiting)	–	https://clinicaltrials.gov/ct2/show/NCT03345303
			Phase III: MM (recruiting)	Selinexor and dexamethasone	https://clinicaltrials.gov/ct2/show/NCT03110562
			Phase III: MM (recruiting)	Dexamethasone	https://clinicaltrials.gov/ct2/show/NCT02811978
			Phase III: Previously untreated MM (recruiting)	Melphalan, prednisone, and daratumumab	https://clinicaltrials.gov/ct2/show/NCT03217812
			Phase III: Relapsed or refractory multiple myeloma (recruiting)	Daratumumab and dexamethasone	https://clinicaltrials.gov/ct2/show/NCT0323497
			Phase III: MM (up to 65 years) (recruiting)	Lenalidomide, dexamethasone, and autologous stem cell transplant	https://clinicaltrials.gov/ct2/show/NCT01208662
		Carfilzomib (PR-171)	Approved by FDA for multiple myeloma (MM) (2012)		[341]
			Phase I: Heavily pretreated MM	Lenalidomide and dexamethasone	[344]
			Phase Ib: Newly diagnosed MM	Cyclophosphamide and dexamethasone	[348]
			Phase Ib: Lung cancer and other irinotecan-sensitive malignancies	Irinotecan	[393]
			Phase I/II: Newly diagnosed transplant-ineligible MM	Weekly carfilzomib, cyclophosphamide, and dexamethasone	[349]
			Phase III: Relapsed or refractory MM	Bortezomib	[346]
			Phase III: Relapsed MM	Lenalidomide and dexamethasone	[343]
			Phase III: Relapsed or refractory MM	Bortezomib and dexamethasone	[347]

(continued)

Table 17.1 (continued)

Pathway	Therapeutic targets	Current drugs	Clinical trial stages (published reports)/type of cancer	Combined with	References
			Phase III: Relapsed and refractory MM (recruiting)	Dexamethasone	https://clinicaltrials.gov/ct2/show/NCT03029234
			Phase III: Plasma cell myeloma (recruiting)	Isatuximab and dexamethasone	https://clinicaltrials.gov/ct2/show/NCT03275285
			Phase III: MM (recruiting)	Lenalidomide and dexamethasone	https://clinicaltrials.gov/ct2/show/NCT02659293
			Phase III: Relapsed or refractory MM (recruiting)	Daratumumab and dexamethasone	https://clinicaltrials.gov/ct2/show/NCT03158688
			Phase III: Plasma cell myeloma (recruiting)	Bortezomib, dexamethasone, lenalidomide, and other quality of life assessment	https://clinicaltrials.gov/ct2/show/NCT01863550
		Ixazomib	Approved by FDA for multiple myeloma (MM) (2015)		[350]
			Phase I: Advanced solid tumors	–	[394]
			Phase I: Relapsed/refractory MM	Lenalidomide-dexamethasone	[395]
			Phase I/II: Relapsed/refractory MM	Pomalidomide and dexamethasone	[396]
			Phase II: Relapsed/refractory cutaneous or peripheral T-cell lymphomas	–	[397]
			Phase III: Relapsed/refractory MM	Lenalidomide and dexamethasone	[398]
			Phase II: Indolent B-cell non-Hodgkin lymphoma (recruiting)	Rituximab	https://clinicaltrials.gov/ct2/show/NCT02339922
			Phase II: MM (recruiting)	Maintenance ixazomib or lenalidomide	https://clinicaltrials.gov/ct2/show/NCT02253316
			Phase III: Newly diagnosed MM not treated with stem cell transplantation (recruiting)	Maintenance	https://clinicaltrials.gov/ct2/show/NCT02312258
			Phase III: Solitary plasmacytoma of bone (recruiting)	Lenalidomide, dexamethasone, and zoledronic acid	https://clinicaltrials.gov/ct2/show/NCT02516423

			Phase III: Relapsed/refractory MM (recruiting)	Pomalidomide and dexamethasone	https://clinicaltrials.gov/ct2/show/NCT03170882
IAP	X-IAP mRNA	AEG35156	Phase I: Advanced cancer	–	[399]
			Phase I/II: AML	Idarubicin and cytarabine	[358]
			Phase II: AML	Idarubicin and cytarabine	[357]
			Phase I: Metastatic pancreatic ductal adenocarcinoma	Gemcitabine	[359]
			Phase II: Advanced hepatocellular carcinoma	Sorafenib	[360]
			None active or recruiting		https://clinicaltrials.gov/ct2/home)
	Pan-IAP	LCL161	Phase I: Advanced cancer	–	[361]
			Phase I: Advanced solid tumor	Paclitaxel	[400]
			Phase I: Advanced solid tumor	–	[362]
			Phase I: Advanced solid tumor (completed/unpublished)	–	https://clinicaltrials.gov/ct2/show/NCT01968915
			Phase I: Advanced solid tumor (completed/unpublished)	Paclitaxel	https://clinicaltrials.gov/ct2/show/NCT01240655
			Phase I: Triple negative breast cancer (completed/unpublished)	Paclitaxel	https://clinicaltrials.gov/ct2/show/NCT01617668
			Phase I/II: Relapsed/refractory small cell lung cancer and selected gynecologic malignancies (recruiting)	Topotecan	https://clinicaltrials.gov/ct2/show/NCT02649673
			Phase II: Relapsed/refractory MM (recruiting)	Cyclophosphamide	https://clinicaltrials.gov/ct2/show/NCT01955434
			Phase I: MM (recruiting)	PDR001 and CJM112	https://clinicaltrials.gov/ct2/show/NCT03111992
			Phase I: Colorectal cancer, non-small cell lung carcinoma, and triple negative breast cancer (recruiting)	PDR001, everolimus, and panobinostat	https://clinicaltrials.gov/ct2/show/NCT02890069
		HGS1029	Phase I: Advanced solid tumor	–	[363]

(continued)

Table 17.1 (continued)

Pathway	Therapeutic targets	Current drugs	Clinical trial stages (published reports)/type of cancer	Combined with	References
			Phase I: Advanced solid tumor (completed/unpublished)	–	https://clinicaltrials.gov/ct2/show/NCT00708006
			None active or recruiting		https://clinicaltrials.gov/ct2/home)
		Birinapant (TL32711)	Phase I: Advanced solid tumor and lymphoma	–	[364]
			Phase I: Refractory solid tumors or lymphoma	–	[401]
			Phase II: Relapsed platinum-resistant or refractory epithelial ovarian cancer	–	[365]
			Phase I: Relapsed ovarian cancer (completed/unpublished)	Conatumumab	https://clinicaltrials.gov/ct2/show/NCT0194017
			Phase I/II: Advanced or metastatic solid tumors (completed/unpublished)	Chemotherapy	https://clinicaltrials.gov/ct2/show/NCT01188499
			Phase I/II: Myelodysplastic syndrome (completed/unpublished)	5-Azacitidine	https://clinicaltrials.gov/ct2/show/NCT01828346
			Phase I/II: Acute myelogenous leukemia, myelodysplastic syndrome, and acute lymphoblastic leukemia (active)	–	https://clinicaltrials.gov/ct2/show/NCT01486784
			Phase I/II: Solid tumors (recruiting)	Pembrolizumab	https://clinicaltrials.gov/ct2/show/NCT02587962
	Survivin	Sepantronium bromide (YM155)	Phase I: Advanced solid tumor or lymphoma	–	[374]
			Phase I: Advanced solid tumor	–	[375]
			Phase II: NSCLC	–	[376]
			Phase II: Melanoma	–	[378]
			Phase II: Refractory diffuse large B-cell lymphoma	–	[377]
			Phase I/II: Advanced non-small cell lung cancer	Paclitaxel and carboplatin	[379]
			Phase II: Stage III (unresectable) or stage IV melanoma	Docetaxel	[380]
			Phase II: HER2-negative metastatic breast cancer	Docetaxel	[381]
			Phase II: Relapsed aggressive B-cell non-Hodgkin lymphoma	Rituximab	[382]

			None active or recruiting		https://clinicaltrials.gov/ct2/home)
		Gataparsen sodium (LY2181308)	Phase I: Advanced solid tumors	–	[402]
			Phase I: Relapsed AML	Idarubicin and cytarabine	[403]
			Phase II: Castration-resistant prostate cancer	Docetaxel and prednisone	[386]
			Phase II: NSCLC	Docetaxel	[385]
			None active or recruiting		https://clinicaltrials.gov/ct2/home)

and need to be explored further. Further understanding the underlying molecular events regulating not just apoptosis but concurrently with autophagy and ER stress may uncover novel targeted interference of these cell death pathways.

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18 Endoplasmic Reticulum Stress and Autophagy in Cancer

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18.1 Introduction

The endoplasmic reticulum (ER) is an important organelle responsible for protein folding and modification and disturbances in the ER environment will lead to ER stress and subsequently causes accumulation of unfolded or misfolded proteins. Although ER stress activates the unfolded protein response (UPR) mechanism to reestablish ER homeostasis, unresolved ER stress can lead to cellular processes such as apoptosis or autophagy. In cancer, tumor cells are dependent on these processes to combat and neutralize the chronic stress and harsh conditions of the tumor microenvironment, leading to tumor survival and tumor expansion; hence, the ER stress response is thought to be cytoprotective. It is now known that ER stress, apoptosis, and autophagy share overlapping molecular pathways and can occur in parallel under similar conditions. Fundamental knowledge in these processes has also generated a great deal of insight into the pathophysiological aspects of cancer, and has provided important considerations in strategizing cancer pharmacotherapy. A number of drugs targeting these processes have been developed and were proven to be promising in both preclinical and clinical studies.

18.2 Endoplasmic Reticulum Stress (ER Stress)

The ER is an intracellular organelle that provides crucial biosynthetic, stress-sensing, and signaling functions in eukaryotic cells [1, 2]. It is the main subcellular compartment for the synthesis, folding, modification, and transport of proteins which are destined to be secreted or embedded in the plasma membrane [3, 4]. The ER is also the major site for the biosynthesis of steroid, cholesterol, and lipid. It is the major intracellular calcium (Ca^{2+}) storage organelle in the cell, and thus plays an important role in calcium homeostasis and calcium-mediated signaling pathways [5]. Nascent proteins are folded and modified correctly in the ER before being transported via the Golgi apparatus to the cell surface or other destination. It is an orchestrated process involving folding, assembly, modification, quality control, and recycling of proteins in a highly oxidizing and calcium-rich ER environment. Proteins translocated into the ER lumen are folded into their proper three-dimensional shapes and modified and assisted by ER-resident enzymes, such as chaperones, glycosylating enzymes, and oxidoreductases [6–8]. Incomplete or misfolded forms are eliminated by quality control systems, including the ER-associated degradation (ERAD) pathway and autophagy [7, 9, 10].

Physiological and pathological conditions such as hypoxia, nutrient fluctuations, altered ER-calcium levels, oxidative injury, inflammation, and viral infections may disrupt the protein folding environment in the ER, causing the accumulation of unfolded or misfolded proteins in the ER lumen [3]. This cellular condition is known as ER stress. ER stress leads to a complex intracellular signal transduction pathway, known as unfolded protein response (UPR), an adaptive mechanism to reestablish ER homeostasis [5, 11]. The UPR primarily aims at reestablishing ER homeostasis by coordinating temporal shut down in protein translation, upregulating ER chaperone genes to increase protein-folding capacity in the ER, and promoting ERAD pathway to remove misfolded proteins [4, 5]. However, when the initial cellular responses fail to restore ER homeostasis, persistent ER stress will elicit an alternative response called the "terminal UPR," which actively promotes cell death to eliminate the damaged cells [7, 12, 13]. Activation of the UPR represents the defining criterion of ER stress, although the terms UPR and ER stress are often used interchangeably [8].

18.3 Unfolded Protein Response (UPR)

The UPR in mammalian cells is governed by three transmembrane ER stress sensors, namely PERK (protein kinase RNA-like ER kinase), IRE1α (inositol-requiring enzyme 1α), and ATF6α (activating transcription factor 6α) [3]. In the absence of ER stress, the ER luminal domains of PERK,

IRE1α and ATF6α are associated with immunoglobulin heavy chain binding protein known as BIP (also known as GRP78), where this interaction maintains all three transmembrane proteins in their inactive state. BIP, a 78-kDa glucose-regulated protein, is well established as an ER chaperone that participates in protein folding and assembly and has been widely used as a marker for ER stress [14]. During ER stress, the accumulating misfolded or unfolded proteins cause BIP to dissociate from the three transmembrane ER stress sensors, and subsequently bind to these misfolded or unfolded proteins. This is due to higher natural affinity of BIP to unfolded proteins compared with the ER stress sensor luminal domains [4]. The release of BIP causes the homodimerization, trans-auto-phosphorylation, and activation of both IRE1α and PERK and translocation of ATF6α to the Golgi apparatus and subsequent activation [8, 12, 15].

Activated PERK phosphorylates eukaryotic translation initiator factor 2α (eIF2α) and attenuates general protein translation, thereby relieving the protein burden on the stressed ER by reducing new protein synthesis and preventing further accumulation of unfolded proteins. Phosphorylation of eIF2α also regulates translation via inhibition of rRNA synthesis [5, 8]. Paradoxically, eIF2α phosphorylation allows selective translation of activating transcription factor 4 (ATF4), a transcription factor that controls the expression of genes encoding ER chaperones (e.g., BIP and GRP94), autophagy, and apoptosis [16, 17]. ATF4 favors the expression of antioxidant response, amino acid biosynthesis, and transport genes to sustain cell survival [4]. Depending on the severity and duration of stress, PERK activation can lead to either survival or cell death [18, 19]. Figure 18.1 illustrates the UPR pathway upon exposure to moderate ER stress.

During prolonged ER stress, ATF4 stimulates the transcription of DNA-damage-inducible transcript 3 (DDIT3; also known as CHOP [CCAAT/enhancer binding protein homologous transcription factor] or GADD153 [growth arrest and DNA damage-inducible gene 153]), a transcription factor that is activated by all three arms of the UPR [5]. DDIT3 itself is a transcription factor that is critical in supporting the ER stress-induced apoptotic program [20]. In addition to its prodeath functions, DDIT3 participates in relieving the general block on translation via induction of

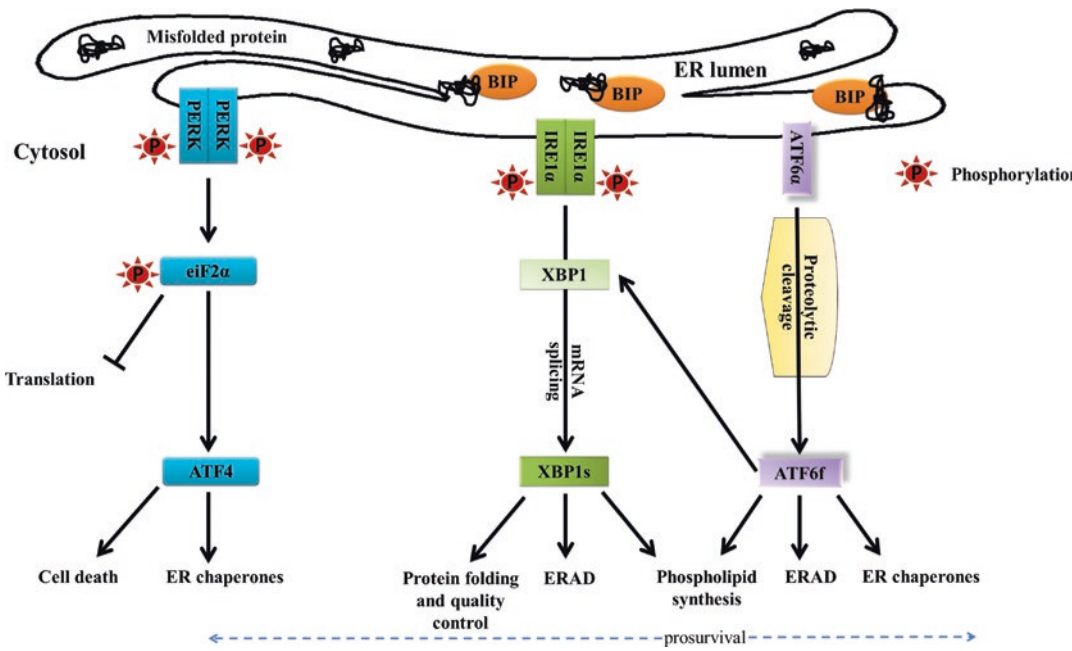

Fig. 18.1 The UPR pathway upon exposure to moderate ER stress

growth arrest and DNA damage-inducible protein 34 (GADD34). GADD34 activates protein phosphatase 1 alpha (PP1A) to dephosphorylate eIF2α and dephosphorylated eIF2α resumes its function in general translation. If the protein folding capacity of the ER has not been reestablished, a premature restoration of protein synthesis will increase protein load in the stressed ER, thus amplifying the damage [5, 8]. Although eIF2α phosphorylation is downregulated during prolonged ER stress, PERK signaling is sustained, possibly to sensitize cells to cell death via DDIT3 induction [17]. Figure 18.2 illustrates the UPR pathway during severe and prolonged ER stress.

Similar to PERK, the release of BIP allows IRE1α to undergo dimerization and autophosphorylation. IRE1α is a bifunctional molecule with serine/threonine protein kinase and endoribonuclease (RNase) activity in its cytosolic domain [8]. Hence, this process leads to the activation of its cytosolic RNase domain, which removes a 26-nucleotide intron from the mRNA encoding the transcription factor X box-binding protein 1 (XBP1), producing mature spliced XBP1 mRNA. The spliced XBP1 mRNA is subsequently translated into an active and stable transcription factor, termed spliced XBP1 (XBP1s). XBP1s regulates the transcription of

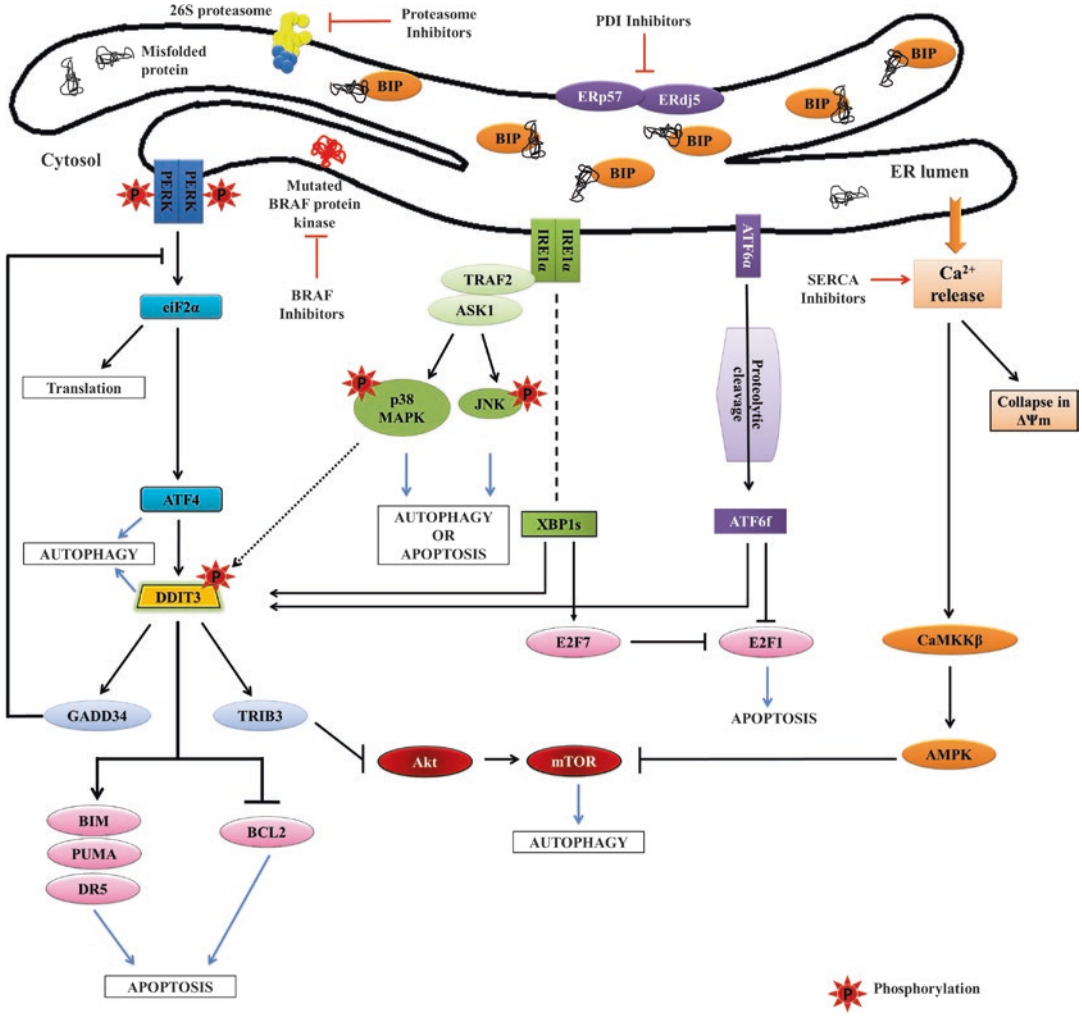

Fig. 18.2 The UPR pathway and ER stress-Ca^{2+} signaling during severe and prolonged ER stress and antitumor targets

several genes involved in protein folding and quality control, ERAD, and phospholipid synthesis [21, 22]. ERAD is a process where misfolded proteins are retro-translocated from ER to the cytosol to be degraded by the 26S proteasome. Meanwhile, phospholipid synthesis is required for ER membrane expansion during ER stress [5, 11]. Through a process known as regulated IRE1-dependent decay (RIDD) of mRNA, IRE1 RNase domain degrades a subset of mRNAs encoding certain proteins of the secretory pathways and proteins located in the ER [11, 16].

Upon severe ER stress, XBP1s upregulates the expression of DDIT3 [5]. On the other hand, prolonged activation of IRE1α recruits the adaptor molecule TNF receptor-associated factor 2 (TRAF2), which further recruits apoptosis signal regulating kinase 1 (ASK1). This leads to a mitogen-activated protein (MAP) kinase activation cascade that activates c-jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK) which further activates BIM and cause the inactivation of BCL-2 [3, 5, 23]. However, IRE1α is turned off upon prolonged ER stress, leading to ablation of the prosurvival XBP1s expression. Attenuation of IRE1α signaling is one possible mechanism to explain the transition from the adaptive UPR to prodeath events [11, 17] (Fig. 18.2).

ATF6α, a type II transmembrane protein, translocates to the Golgi apparatus once released from BIP, where it is proteolytically cleaved to generate a transcriptionally active fragment, termed ATF6f transcription factor. ATF6f mediates the adaptive response to ER protein misfolding by increasing the transcription of genes that increase ER capacity and the expression of *Xbp1* [24, 25]. The transcription target of ATF6f includes genes involved in ERAD, phospholipid synthesis, and ER chaperones, thereby enhancing cellular folding and degradation capacity [8, 16, 17] (Fig. 18.1). ATF6f also contributes to upregulation of DDIT3 during prolonged ER stress [5] (Fig. 18.2).

Taken together, the three UPR transcription factors, ATF4, XBP1s and ATF6f, regulate a large set of partially overlapping UPR target genes during ER stress which modulates adaptation to stress or the induction of cell death under severe conditions [11]. The mechanisms underlying the switch from adaptive phase to prodeath events are still unclear, although it could be possibly through programs that sense the duration of the ER stress condition [17]. If the UPR is successful to increase the protein folding capacity and reduce the amount of misfolded proteins in the ER, BIP reassociates with PERK, IRE1α, and ATF6α, thereby inactivating these signaling modules. However, in case of excessive or prolonged ER stress, signaling pathways leading to cell death, either as apoptosis or autophagy, would be initiated [5, 8]. In certain situations, UPR may upregulate the autophagy machinery to eliminate damaged ER and abnormal protein aggregates [11]. In this context, autophagy is activated as an adaptive mechanism to reestablish ER homeostasis. However, if autophagy reaches a point of no return, cell death will be triggered. Therefore, just like in the case of UPR, persistent ER stress switches the cytoprotective functions of autophagy to cell death-promoting mechanisms [5, 26].

18.4 ER Stress and Cell Death

Several signaling pathways leading to apoptosis and autophagy would be initiated if ER stress is too severe to be relieved [27]. DDIT3 plays an important role in ER stress-induced cellular death, as this factor is a target gene common to all three apical ER stress sensors/executioners [1]. Duration and/or strength of PERK signaling may determine whether prosurvival or prodeath outcome predominates. Transient PERK signaling protects cells by temporarily reducing protein synthesis and thus reducing misfolded protein levels in the ER, but may be insufficient to induce DDIT3 to threshold level, given DDIT3's inherent mRNA and protein instability. Since DDIT3 mRNA and protein have short half-lives, a strong and chronic activation of PERK is necessary to increase steady-state level of DDIT3 to promote cell death [28]. Persistent PERK signaling during prolonged ER stress is known to impair cell proliferation and promotes apoptosis via DDIT3 [29]. DDIT3 represses BCL-2 expression, upregulates BCL-2-interacting

mediator of cell death (BIM) transcription, and promotes translocation of BAX to mitochondria [30–32]. It is also known to bind and induce the promoters of p53 upregulated modulator of apoptosis (*PUMA*), lipocalin 2 (*LCN2*), tribbles homologue 3 (*TRIB3*), and death receptor 5 (*DR5*) [33–37].

As a mediator of the mitochondrial apoptotic pathway, PUMA is known to result in the displacement and activation of BAX/BAK through its binding to antiapoptotic BCL-2 proteins, leading to mitochondrial dysfunction and caspase activation, hence initiating apoptosis [38, 39]. LCN2 is known to exacerbate hypoxia-induced cytochrome c release from mitochondria and caspase-3 activation [40]. Meanwhile, TRIB3 induces both apoptosis and autophagy. It indirectly activates unc-51-like autophagy-activating kinase1 (ULK1) which augments autophagosome formation and reduces autophagy flux. TRIB3 levels inhibit the activity of the kinase Akt by interacting with it and activating forkhead box O1 (FoxO1), a transcription factor that is negatively regulated by Akt, where it is translocated to the nucleus, and induces the proapoptotic gene, *BIM* [41]. It is also noted that DDIT3-mediated DR5 induction is responsible for ER stress apoptosis via caspase 8 [42]. PERK-dependent activation of ATF4 and DDIT3 has been demonstrated to upregulate the transcription of a set of autophagy genes, which are implicated in the formation, elongation, and function of the autophagosome [43].

In addition, IRE1α promotes cell death by recruiting a TRAF2-ASK1 complex, leading to the activation of JNK and p38 MAPK cascades upon prolonged ER stress. JNK promotes apoptosis through the phosphorylation-mediated regulation of Bcl-2 family members [5, 31, 44]. JNK exerts its proapoptotic effect by activating proapoptotic BH3-only protein BIM and by suppressing the antiapoptotic BCL-2 [5]. The p38 MAPK also phosphorylates and suppresses the antiapoptotic BCL-2 protein [45]. BCL-2 not only functions as an antiapoptotic protein, but also acts as an antiautophagy protein via its inhibitory interaction with BECN1. Both JNK and p38 MAPK have been proposed to induce autophagy by promoting dissociation of BECN1 from BCL-2. BECN1 is an essential autophagy regulator that participates in autophagosome formation [5, 45, 46]. In addition, p38 MAPK is known to phosphorylate DDIT3 and enhances DDIT3's ability to function as a transcriptional activator [5, 47] (Fig. 18.2). The apoptosis-inducing activity of the third arm of UPR, ATF6α, has not been widely recognized. This is at least partly due to the fact that ATF6α does not induce apoptosis in cell lines commonly used in research. However, it has been shown that ATF6f mediates apoptosis via suppression of antiapoptotic protein, myeloid cell leukemia sequence 1 (Mcl-1) [48].

The mechanisms underlying the switch from adaptive phase to prodeath events remain elusive, although several hypotheses were suggested. The expression of the transcription factor DDIT3 is thought to be a decisive effector of the switch between adaptive UPR to cell death and the duration and amount of elevated DDIT3 level were hypothesized to be the decisive factor in determining the cell's fate [26]. Upon severe ER stress, ATF4, XBP1s, and ATF6f transcription factors induce the transcription of DDIT3. On the other hand, PERK/eiF2α/ATF4 branch is essential to upregulate DDIT3 protein expression. The transcriptional activity of DDIT3 is then enhanced through the phosphorylation by p38 MAPK [5, 31]. Prolonged high level of DDIT3 protein expression is considered an indicator of the switch to proapoptotic module [8]. DDIT3 alters the balance between prosurvival and proapoptotic Bcl-2 family members and thus promotes apoptosis through the mitochondrial pathway. In addition, a molecular switch to cell death events could also involve TRIB3, a downstream transcriptional target of DDIT3. TRIB3 binds directly to prosurvival Akt kinase, thereby preventing its phosphorylation and reducing its kinase activity. During severe or persistent ER stress, induction of TRIB3 would be more robust, leading to autophagy and apoptosis through TRIB3-mediated inhibition of Akt/mTOR axis [5, 31, 49, 50] (Fig. 18.2).

In fact, IRE1α activities, namely (1) XBP1 mRNA splicing, (2) regulated IRE1-dependent

decay of mRNAs, and (3) JNK/p38 MAPK activation, are also thought to be responsible for the life/death switch under prolonged ER stress conditions [51, 52]. Recently, the role of E2F1 has been described as a potential mechanistic survival/death switch under ER stress conditions [4, 53]. E2F1 is a member of the E2F family of transcription factors involved in several cellular functions such as proliferation, differentiation, and cell death [54, 55]. Upon ER stress induction, E2F7 as one of XBP1 target gene has been demonstrated to be positively regulated and the combined activity of E2F7 and activated ATF6 results in a specific but timely downregulation of E2F1 expression. This results in the removal of E2F1-dependent basal inhibition of both PUMA and NOXA that will induce the apoptotic program [4]. Timely and coordinated expression levels of E2F1 are crucial for determining the survival/death cell fate under ER stress conditions [4].

In addition to the three UPR branches, ER stress-Ca^{2+} signaling also leads to cell death during severe and prolonged ER stress. As ER is the major intracellular calcium storage organelle in the cell, ER stress activation is frequently accompanied by calcium release into the cytosol, causing an increase in cytosolic free calcium ions. Increases in cytosolic calcium concentration upon treatment with different ER stress inducers lead to calcium/calmodulin-dependent kinase kinase-β (CaMKKβ)-dependent activation of AMPK, that ultimately leads to inhibition of mTOR and stimulation of autophagy [5, 56]. In addition, mitochondrial intake of calcium ions following its release into the cytosol from the ER causes a collapse in the inner mitochondrial transmembrane potential (ΔΨm). A long-lasting or permanent ΔΨm dissipation is often associated with cell death [57, 58].

18.5 ER Stress in Cancer and Therapeutic Strategies

Tumor cells are often present within a hostile microenvironment and are confronted with chronic metabolic stress conditions. Following initiation of malignancy, poor vascularization of the tumor mass leads to stressful conditions in the tumor microenvironment, including low oxygen supply, nutrient deprivation, and pH changes. Therefore, many tumor types are thought to be dependent on an adaptive UPR to combat and neutralize the chronic stress and harsh conditions of the tumor microenvironment [5, 26, 44]. On the other hand, most normal cells are not subjected to stress and their UPR pathways are in an inactive state [44].

Both UPR activation and upregulation of BIP represent hallmark of several human cancers. UPR activation enables cancer cells to survive, adapts to adverse environmental conditions, and leads to growth arrest driving dormancy, which promotes resistance to conventional chemotherapy [59–62]. In addition, there are emerging evidences that linked mutations in three sensor genes such as ATF6α, IRE1α, and PERK in tumorigenesis [63–66]. The presence of missense, nonsense, and silent mutations in these genes seems to have tumor- or tissue-specific significance.

While BIP is generally too low to be detected in normal cells, many tumor cell lines display permanently elevated levels of BIP, which reflects the cancer cells' ongoing effort to neutralize the chronic stress within the cells [26]. Elevated BIP is among the critical prosurvival mechanisms of tumor cells to withstand and thrive under detrimental microenvironmental conditions [8]. Similar to BIP, IRE1α/XBP1 signaling pathway is important for tumor growth and survival under stress conditions. An increase in XBP1 expression and splicing has been demonstrated in various human cancers, including breast cancer. Moreover, sustained IRE1α signaling was shown to enhance cell survival and proliferation [44, 67]. PERK/eif2α/ATF4 pathway also plays a role in cancer progression during stress condition. Hypoxia induces activation of the PERK pathway in tumor cells as an adaptive response to promote survival under hypoxic conditions. ATF4 is overexpressed in many solid tumors and is involved in promoting proliferation and survival during nutrient deprivation and severe hypoxia [44, 67].

In addition, several ER stress-associated markers are specifically upregulated in both neuroblastoma and melanoma cells under ER stress

conditions [68]. DDIT3 and four other genes associated with ER stress were induced greater than twofold, namely ERdj5 (PDIA19; an ER-resident protein containing DnaJ and thioredoxin domains), ERp57 (GRP58; PDIA3; an ER-resident protein disulfide isomerase), calreticulin, and calnexin (both ER-resident chaperones) [68]. Protein disulfide isomerase (PDI) family members such as ERdj5 and ERp57 are consistently upregulated in neuroectodermal tumors and a generalized inhibition of PDI activity revealed a significant sensitization of tumor cells to ER-stress apoptosis. PDIs are endoplasmic reticulum chaperone proteins, catalyze disulfide bond breakage, formation, and rearrangement, and are required for protein folding in the endoplasmic reticulum (ER). The observation that knockdown of ERdj5 or ERp57 enhanced the extent of cell death induced by chemotherapeutic drugs suggests that downregulating ER stress responses may be therapeutically valuable; the ER resident proteins ERdj5 and ERp57 may thus be anticancer targets and PDI inhibition in general appears to be a novel therapeutic strategy [68–70]. Recently, there are a few synthetic small molecule PDI inhibitors such as PACMA31, 16F16, and CCF642 which have proven efficacy in cancer models, but have yet to progress to clinical studies [69, 71–73].

Since tumor cells engage adaptive UPR, only a small margin is left for the tumor cells to accommodate additional ER stress. Drugs that aggravate the preexisting ER stress condition in tumor cells may cause a shift from adaptive UPR to severe ER stress, leading to cell death. At the same time, exposure to ER stress-inducing agents causes activation of adaptive UPR in normal cells. Thus, moderate intensity ER stress inducers would be required to sufficiently aggravate ER stress in tumor cells, but at the same time, only modestly trigger ER stress in normal cells, in order to produce tumor-selective cytotoxic outcome. It was hypothesized that exceptionally potent pharmacologic triggers of ER stress might not be ideal in this situation [26].

A variety of distinct pharmacologic agents have been identified to trigger ER stress by different mechanisms. These agents include proteasome inhibitors and sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) inhibitors, among others [26]. Although these compounds affect the UPR pathway, UPR may not be the primary mechanism of action of these drugs [44]. In the context of cancer research, thapsigargin (an inhibitor of SERCA), tunicamycin (an inhibitor of protein glycosylation), and brefeldin A (an inhibitor of protein transport from ER to Golgi) are frequently used in experiments as ER stress inducers to investigate the details of ER stress response [8].

The degradation of the majority of misfolded proteins is mediated by the 26S proteasome through the ERAD pathway [44]. Inactivation of the proteasome by proteasome inhibitors causes accumulation of misfolded proteins bound for the ERAD pathway, thereby triggering the UPR [26]. Bortezomib is a proteasome inhibitor and was approved by the US FDA in 2003 to treat multiple myeloma and mantle cell lymphoma [8]. Treatment of multiple myeloma cells with bortezomib causes rapid upregulation of the components in the UPR, including PERK, ATF4, and DDIT3, resulting in cell death. On the other hand, bortezomib sensitized pancreatic cancer cells to ER stress-induced apoptosis by induction of DDIT3, GADD34 and JNK, while PERK activation and eIF2α phosphorylation were not detected [44]. Several mechanisms have been proposed to explain the cytotoxicity of bortezomib, including effects on NF-kB, cell cycle proteins, apoptosis-regulatory proteins and caspases, as well as ER stress. Although ER stress represents only one of several processes associated with bortezomib-induced cell death, it is conceivable that it might indeed represent the key component, whereas other observed events might be orchestrated secondary to the aggravation of ER stress [8]. Bortezomib is further discussed in Chap. 17.

Inhibitors of human immunodeficiency virus (HIV) protease are known to inhibit the proteasome [26]. Two widely prescribed HIV protease inhibitors, namely nelfinavir and atazanavir, cause the accumulation of polyubiquitinated proteins, aggresome formation, and an increase in BIP and DDIT3 expression [74, 75]. In addition,

nelfinavir has been shown to induce ER stress, autophagy, and apoptosis in vitro and in vivo in nonsmall-cell lung carcinoma [76]. Nelfinavir is currently in clinical trials for repositioning as an anticancer agent [26]. A Phase II trial of nelfinavir in combination with chemoradiation for locally advanced inoperable pancreatic cancer (LAPC) revealed that nelfinavir showed acceptable toxicity and promising survival in pancreatic cancer [77]. The study reports the clinical outcome in 23 patients with LAPC treated with chemoradiotherapy plus nelfinavir which shows moderate median and 1-year overall survival at 17.4 months and 73.4%, respectively [77].

In another Phase II trial of nelfinavir in combination with the proteasome inhibitor bortezomib in 12 patients with advanced hematologic malignancies, promising activity in advanced, bortezomib-refractory multiple myeloma was noted [78]. Nelfinavir alone significantly upregulated the expression of proteins related to UPR in peripheral blood mononuclear cells and inhibited proteasome activity. Of ten evaluable patients in the dose escalation cohort, three achieved a partial response, four stable disease for two cycles or more, while three had progressive disease as best response [78]. Of nine patients given oral nelfinavir before and during radiation therapy for advanced rectal cancer, five patients exhibited good tumor regression on MRI assessed by tumor regression grade (mrTRG) [79]. Unfortunately, nelfinavir monotherapy does not result in a meaningful improvement in clinical outcomes among patients with recurrent adenoid cystic carcinoma [80]. Nelfinavir is currently in clinical trials for various cancers such as cervical intraepithelial neoplasia and advanced renal cancers (Table 18.1). However, atazanavir is not on any clinical trials involving cancer at this moment.

The SERCA is a transmembrane protein that actively imports calcium ions from the cytosol into the ER lumen, thereby establishing a steep calcium gradient between the ER lumen and cytosol. Inhibition of SERCA results in massive leakage of calcium ions from ER to the cytosol and thus efficiently triggers ER stress. Thapsigargin, a naturally occurring sesquiterpene lactone, is an exceptionally potent inhibitor of SERCA. However, its clinical usage is fraught with several challenges; it is quite toxic and not well tolerated by experimental animals. A prodrug of thapsigargin, also known as mipsagargin or G202, has been synthesized and was found to produce substantial tumor regression against a panel of human cancer xenografts in vivo at doses that were minimally toxic to the host [148]. Interestingly, mipsagargin demonstrated an acceptable tolerability and favorable pharmacokinetic profile in a phase I clinical trial in patients with refractory, advanced, or metastatic solid tumors [81].

Certain diaryl-substituted pyrazoles, for example, celecoxib, are another class of compound that has emerged as SERCA inhibitors [26]. Nevertheless, celecoxib might not attain sufficient level of ER stress in tumor tissues because it was initially developed as COX-2 inhibitor. However, celecoxib analogues with minimized COX-2 inhibitory function, but significantly increased ER stress-inducing ability have been developed [8]. AR-12/OSU-03012 is an antitumor celecoxib-derivative that has progressed to Phase I clinical trial as an anticancer agent and has activity against a number of infectious agents including fungi, bacteria, and viruses [149]. It has been shown to suppress tumor cell viability through multiple mechanisms including activation of endoplasmic reticulum stress, inhibition of PDK-1/Akt signaling and the induction of autophagy [150–152]. Although a Phase I clinical trial of AR-12 in adult patients with advanced or recurrent solid tumors or lymphoma has been completed, its overall outcome remain unpublished.

In both oncogenic BRAF melanoma cell lines and in patients who failed clinical treatment for skin melanomas, the presence of oncogenic BRAF was responsible for ER stress induction and cell survival [153, 154]. In particular, human skin melanoma is characterized by oncogenic BRAF mutations, such as $BRAF^{V600E}$. In addition, approximately 8–14% of colorectal cancers (CRC) in early and advanced stages exhibit the $BRAF^{V600E}$ mutation [155–158]. The BRAF serine/threonine protein kinase is a downstream signaling protein in the epidermal growth factor receptor-mediated

Table 18.1 Current therapeutic target in the ER stress and autophagy signaling pathways and clinical trial stages

Pathway	Therapeutic targets	Current drugs	Clinical trial stages/type of cancer	Combined with/versus	References
UPR pathway	SERCA	Mipsagargin	Phase I: Advanced metastatic solid tumors		[81]
			Phase II: Hepatocellular carcinoma (completed/unpublished)	Sorafenib	https://clinicaltrials.gov/ct2/show/NCT01777594
			Phase II: Recurrent or progressive glioblastoma (completed/unpublished)		https://clinicaltrials.gov/ct2/show/NCT02067156
		AR-12	Phase I: Advanced or recurrent solid tumors or lymphomas (completed/unpublished)		https://clinicaltrials.gov/ct2/show/NCT00978523
ERAD	26S proteasome	Bortezomib	Approved by FDA for multiple myeloma (2005) Approved by FDA for mantle cell lymphoma (2006)		The U. S. Food and Drug Administration[a]
			Phase III: Multiple myeloma (recruiting)	Lenalidomide; dexamethasone	https://clinicaltrials.gov/ct2/show/NCT01863550
			Phase III: Stem cell transplant in multiple myeloma (recruiting)	Lenalidomide; dexamethasone	https://clinicaltrials.gov/ct2/show/record/NCT01208662
			Phase III: T-cell acute lymphoblastic leukemia or stage II–IV T-cell lymphoblastic lymphoma (recruiting)	Combination chemotherapy	https://clinicaltrials.gov/ct2/show/NCT02112916
			Phase III: Multiple myeloma (recruiting)	Selinexor; dexamethasone	https://clinicaltrials.gov/ct2/show/NCT03110562
			Phase III: Untreated multiple myeloma (recruiting)	Daratumumab	https://clinicaltrials.gov/ct2/show/NCT02541383
			Phase III: Relapsed and refractory multiple myeloma (recruiting)	Dexamethasone	https://clinicaltrials.gov/ct2/show/NCT02811978
			Phase III: Newly diagnosed myeloma (recruiting)	Elotuzumab	https://clinicaltrials.gov/ct2/show/NCT02495922
		Nelfinavir	Approved by FDA for HIV (1997)		The U. S. Food and drug Administration[a]
			Phase II: Pancreatic cancer	Chemoradiation	[77]
			Phase II: Multiple myeloma	Bortezomib	[78]
			Phase II: Advanced rectal cancer		[79]
			Phase II: Advanced melanoma, lung, or kidney cancer (recruiting)	Nivolumab, radiation therapy	https://clinicaltrials.gov/ct2/show/NCT03050060
			Phase II: Cervical intraepithelial neoplasia (recruiting)		https://clinicaltrials.gov/ct2/show/NCT01925378

			Phase II: Advanced pancreatic cancer (recruiting)	Oregovomab	https://clinicaltrials.gov/ct2/show/NCT01959672
			Phase II: Localized pancreatic cancer (recruiting)		https://clinicaltrials.gov/ct2/show/NCT02024009
			Phase II: Kaposi sarcoma (recruiting)		https://clinicaltrials.gov/ct2/show/NCT0307745
			Phase II: Squamous cell carcinoma larynx (recruiting)	Chemoradiotherapy	https://clinicaltrials.gov/ct2/show/NCT02207439
	BRAF	Vemurafenib	Approved by FDA for unresectable or metastatic melanoma with the $BRAF^{V600E}$ mutation (2011)		The U. S. Food and Drug Administration[a]
			Phase II: Colorectal cancer		[82]
			Phase III: Metastatic melanoma		[83]
			Phase II: Relapsed or refractory advanced solid tumors, non-Hodgkin lymphoma (recruiting)		https://clinicaltrials.gov/ct2/show/NCT03220035
			Phase II: Tumors harboring BRAF genomic alterations (recruiting)		https://clinicaltrials.gov/ct2/show/NCT02304809
			Phase II: Metastatic melanoma (recruiting)	Cobimetinib	https://clinicaltrials.gov/ct2/show/NCT02414750
			Phase II: Metastatic melanoma (recruiting)	Adoptive T-cell therapy with tumor infiltrating lymphocytes	https://clinicaltrials.gov/ct2/show/NCT02354690
			Phase II: $BRAF^{V600E}$ mutation positive craniopharyngioma (recruiting)	Cobimetinib	https://clinicaltrials.gov/ct2/show/NCT03224767
			Phase II: BRAF mutated melanoma with brain metastasis (recruiting)	Cobimetinib	https://clinicaltrials.gov/ct2/show/NCT02537600
			Phase III: Metastatic or unresectable locally advanced melanoma (recruiting)	Atezolizumab; cobimetinib	https://clinicaltrials.gov/ct2/show/NCT02908672
			Phase IV: $BRAF^{V600E}$ mutation-positive malignancies (recruiting)		https://clinicaltrials.gov/ct2/show/NCT01739764
		Dabrafenib	Approved by FDA for unresectable or metastatic melanoma with the $BRAF^{V600E}$ mutation (2014)		The U. S. Food and Drug Administration[a]
			Phase II: Melanoma, untreated brain metastases		[84]
			Phase III: BRAF-mutated metastatic melanoma		[85]
			Phase II: $BRAF^{V600E}$-mutant metastatic NSCLC	Trametinib	[86]
			Phase II: $BRAF^{V600E}$-mutant melanoma brain metastases	Trametinib	[87]

(continued)

Table 18.1 (continued)

Pathway	Therapeutic targets	Current drugs	Clinical trial stages/type of cancer	Combined with/versus	References
			Phase III: Metastatic $BRAF^{V600E/K}$-mutant melanoma	Trametinib; monotherapy	[88]
			Phase III: Metastatic $BRAF^{V600E/K}$-mutant melanoma	Trametinib; monotherapy	[89]
			Phase II: $BRAF^{V600E}$-mutated rare cancers (recruiting)	Trametinib	https://clinicaltrials.gov/ct2/show/NCT02034110
			Phase II: BRAF-mutant melanoma or solid tumors (recruiting)	Trametinib; navitoclax	https://clinicaltrials.gov/ct2/show/NCT01989585
			Phase II: BRAF-mutated ameloblastoma (recruiting)		https://clinicaltrials.gov/ct2/show/NCT02367859
			Phase II: Stage III melanoma (recruiting)	Trametinib	https://clinicaltrials.gov/ct2/show/NCT02231775
			Phase III: Stage III-IV $BRAF^{V600E}$ melanoma (recruiting)	Trametinib: Ipilimumab; nivolumab	https://clinicaltrials.gov/ct2/show/NCT02224781
		Encorafenib	Phase I: Metastatic BRAF-mutant melanoma		[90]
			Phase I: Metastatic BRAF-mutant colorectal cancer	Cetuximab; alpelisib	[91]
			Phase II: Relapsed/refractory multiple myeloma	Binimetinib	https://clinicaltrials.gov/ct2/show/NCT02834364
			Phase III: BRAF V600E-mutant metastatic colorectal cancer (recruiting)	Chemotherapy	https://clinicaltrials.gov/ct2/show/NCT02928224
mTOR signaling pathway	mTOR	Rapamycin (Sirolimus)	Phase I: Relapsed and refractory solid tumors	Cyclophosphamide; topotecan	[92]
			Phase I: Refractory solid malignancies	Sunitinib	[93]
			Phase I: Invasive bladder cancer	Intravesical BCG	https://clinicaltrials.gov/ct2/show/NCT02753309
			Phase II: Metastatic, RAI-refractory, differentiated thyroid cancer (recruiting)	Cyclophosphamide	https://clinicaltrials.gov/ct2/show/NCT03099356
			Phase II: Metastatic hormone-resistant prostate cancer (recruiting)	Docetaxel; carboplatin	https://clinicaltrials.gov/ct2/show/NCT02565901
			Phase II: Recurrent/refractory germ cell tumors (recruiting)	Erlotinib	https://clinicaltrials.gov/ct2/show/NCT01962896
			Phase II: Advanced, radioactive iodine refractory Hurthle cell thyroid cancer (recruiting)	Sorafenib	https://clinicaltrials.gov/ct2/show/NCT02143726

			Phase II: Recurrent/refractory solid and CNS tumors (recruiting)	Metronomic chemotherapy	https://clinicaltrials.gov/ct2/show/NCT02574728
		Temsirolimus (CCI-779)	Approved by FDA for advanced RCC (2007)	–	The U. S. Food and Drug Administration[a]
			Phase I: Advanced solid tumors	Cetuximab	[94]
			Phase I: Recurrent pediatric solid tumors	Perifosine	[95]
			Phase I: Advanced NSCLC	Pemetrexed	[96]
			Phase II: Advanced endometrial cancer	Temsirolimus and alternating megestrol acetate; tamoxifen	[97]
			Phase II: Relapsed/refractory primary CNS lymphoma		[98]
			Phase II: Resistant ovarian cancer/advanced/recurrent endometrial carcinoma		[99]
			Phase I: Advanced cancers (recruiting)	Metformin	https://clinicaltrials.gov/ct2/show/NCT01529593
			Phase I: Advanced cancers (recruiting)	Bevacizumab; valproic acid; cetuximab	https://clinicaltrials.gov/ct2/show/NCT01552434
			Phase I: Advanced or metastatic solid tumors (recruiting)	Cetuximab	https://clinicaltrials.gov/ct2/show/NCT02215720
			Phase I: Relapsed acute lymphoblastic leukemia and non-Hodgkin's lymphoma (recruiting)	Etoposide; cyclophosphamide	https://clinicaltrials.gov/ct2/show/NCT01614197
		Everolimus (RAD001)	Approved by FDA for advanced RCC (2009), advanced pancreatic neuroendocrine tumor (2011), renal angiomyolipoma (2012) and hormone receptor-positive, HER2-negative breast cancer (2012)		The U. S. Food and Drug Administration[a]
			Phase I: Prostate cancer	Radiation	[100]
			Phase I: Triple-negative breast cancer	Doxorubicin; bevacizumab	[101]
			Phase I: Diffuse large B-cell lymphoma	R-CHOP-21	[102]
			Phase Ib: Metastatic gastroesophageal adenocarcinoma	mFOLFOX6	[103]
			Phase II: Elapsed estrogen receptor-positive high-grade ovarian cancers	Letrozole	[104]
			Phase II: Hormone receptor-positive, HER2-negative metastatic breast cancer	Dexamethasone	[105]

(continued)

Table 18.1 (continued)

Pathway	Therapeutic targets	Current drugs	Clinical trial stages/type of cancer	Combined with/versus	References
			Phase II: Relapsed or refractory indolent lymphoma		[106]
			Phase II: Metastatic clear cell renal cell cancer	Pazopanib	[107]
			Phase II: Advanced follicular-derived thyroid cancer		[108]
			Phase II: Castration-resistant prostate cancer	Bicalutamide	[109]
			Phase II: Peripheral T-cell lymphoma	CHOP	[110]
			Phase II: Metastatic non-clear cell renal cell carcinoma	Sunitinib	[111]
			Phase III: Estrogen receptor-positive metastatic breast		[112]
			Phase III: Hormone receptor positive metastatic breast cancer (recruiting)	Aromatase inhibitors	https://clinicaltrials.gov/ct2/show/NCT02511639
			Phase III: High risk of relapse, ER+ and HER2− primary breast cancer (recruiting)	Adjuvant hormone therapy	https://clinicaltrials.gov/ct2/show/NCT01805271
			Phase III: Metastatic breast cancer (recruiting)	Fulvestrant; exemestane	https://clinicaltrials.gov/ct2/show/NCT02404051
			Phase IV: Progressive pancreatic neuroendocrine tumors (recruiting)		https://clinicaltrials.gov/ct2/show/NCT02842749
			Phase IV: Liver cancer (recruiting)		https://clinicaltrials.gov/ct2/show/NCT02081755
		Ridaforolimus (Deforolimus; AP23573)	Phase I: Solid tumor cancers	Paclitaxel; carboplatin	[113]
			Phase I: Advanced solid tumors		[114]
			Phase II: Advanced breast cancer	Dalotuzumab; exemestane	[115]
			Phase II: Estrogen receptor-positive breast cancer	Dalotuzumab; exemestane	[116]
	PI3K/mTOR	BEZ235	Phase II: Advanced pancreatic neuroendocrine tumors		[117]
			Phase II: Locally advanced or metastatic transitional cell carcinoma		[118]

			Phase I: Advanced solid tumors (completed/unpublished)		https://clinicaltrials.gov/ct2/show/NCT01195376
			Phase I: Advanced solid tumors (completed/unpublished)		https://clinicaltrials.gov/ct2/show/NCT01343498
			Phase Ib: Castration-resistant prostate cancer (completed/unpublished)	BKM120	https://clinicaltrials.gov/ct2/show/NCT01634061
			Phase Ib/II: HER2 negative, locally advanced or metastatic breast cancer (completed/unpublished)	Paclitaxel	https://clinicaltrials.gov/ct2/show/NCT01495247
			Phase I/II: Advanced breast cancer (completed/unpublished)		https://clinicaltrials.gov/ct2/show/NCT00620594
Proautophagics		Temozolomide	Approved by FDA for glioblastoma (2005)	Radiotherapy	[119]
			Phase I: Recurrent malignant gliomas	Metronomic temozolomide	[120]
			Phase I/II: Glioblastoma	Immunotherapy	[121]
			Phase I/II: Recurrent malignant gliomas	Nimustine	[122]
			Phase I/II: Primary CNS lymphoma	Induction chemotherapy; whole-brain radiotherapy	[123]
			Phase II: Glioblastoma	Bevacizumab; Irinotecan	[124]
			Phase II: High-grade glioma	Radiotherapy; lomustine	[125]
			Phase II: NSCLC with brain metastases	Pemetrexed	[126]
			Phase II: Metastatic melanoma	Ipilimumab	[127]
			Phase II: Refractory or relapsed neuroblastoma	Irinotecan-temozolomide with temsirolimus or dinutuximab	[128]
			Phase II: Metastatic colorectal cancer		[129]
			Phase II: Refractory or relapsed neuroblastoma	Bevacizumab, irinotecan	[130]

(continued)

Table 18.1 (continued)

Pathway	Therapeutic targets	Current drugs	Clinical trial stages/type of cancer	Combined with/versus	References
			Phase II: Glioblastoma	Neoadjuvant temozolomide; hypofractionated accelerated radiation therapy	[131]
			Phase III: High-risk low-grade glioma	Radiotherapy	[132]
			Phase III: Glioblastoma (recruiting)	Radiotherapy; nivolumab	https://clinicaltrials.gov/ct2/show/NCT02667587
			Phase III: Glioblastoma (recruiting)	Nivolumab	https://clinicaltrials.gov/ct2/show/NCT02617589
			Phase III: Extensive stage small-cell lung cancer (recruiting)		https://clinicaltrials.gov/ct2/show/NCT02772107
			Phase III: Recurrent glioblastoma (recruiting)	Bevacizumab	https://clinicaltrials.gov/ct2/show/NCT03149575
			Phase III: High-grade gliomas (recruiting)	Interferon-alpha	https://clinicaltrials.gov/ct2/show/NCT01765088
		Arsenic trioxide	Approved by FDA for acute promyelocytic leukemia (2000)		The U. S. Food and drug Administration1
			Phase II: Relapsed or refractory malignant lymphoma		[133]
			Phase II: Hepatocellular carcinoma	Locoregional therapy	[134]
			Phase II: Recurrent or refractory stage 4 neuroblastoma or metastatic paraganglioma/pheochromocytoma	(131)I-MIBG	[135]
			Phase II: Acute promyelocytic leukemia	All-trans retinoic acid; chemotherapy	[136]
			Phase II: Relapsed small-cell lung cancer		[137]
			Phase III: Acute promyelocytic leukemia	All-trans retinoic acid	[138]
			Phase III: Non-high-risk acute promyelocytic leukemia	All-trans retinoic acid	[139]
			Phase III: Acute promyelocytic leukemia	Anthracyclines	[140]
			Phase III: Untreated acute promyelocytic leukemia (recruiting)	Tretinoin; chemotherapy	https://clinicaltrials.gov/ct2/show/NCT02339740
			Phase III: High-risk acute promyelocytic leukemia (recruiting)	Tretinoin; chemotherapy	https://clinicaltrials.gov/ct2/show/NCT02688140

Autophagy inhibitors		Chloroquine	Phase I: Metastatic or unresectable pancreatic cancer		[141]
			Phase I: Stage IV small-cell lung cancer (completed/unpublished)	Gemcitabine	https://clinicaltrials.gov/ct2/show/NCT00969306
			Phase I/II: Breast ductal carcinoma in situ (completed/unpublished)		https://clinicaltrials.gov/ct2/show/NCT01023477
			Phase I: Advanced solid tumors (recruiting)	Carboplatin; gemcitabine	https://clinicaltrials.gov/ct2/show/NCT0207153
			Phase I/II: IDH1/2-mutated solid tumors (recruiting)	Metformin	https://clinicaltrials.gov/ct2/show/NCT02496741
			Phase II: Breast cancer (recruiting)		https://clinicaltrials.gov/ct2/show/NCT02333890
			Phase II: Advanced or metastatic breast cancer (recruiting)	Taxane chemotherapy	https://clinicaltrials.gov/ct2/show/NCT01446016
		Hydroxychloroquine	Phase I: Advanced solid tumors and melanoma	Temozolomide	[142]
			Phase I: Advanced solid tumors	Vorinostat	[143]
			Phase I: Relapsed/refractory myeloma	Bortezomib	[144]
			Phase I/II: Glioblastoma	Radiation; temozolomide	[145]
			Phase I/II: Pancreatic adenocarcinoma	Gemcitabine	[146]
			Phase II: Metastatic pancreatic adenocarcinoma		[147]
			Phase II: Colorectal cancer (recruiting)	Vorinostat; Regorafenib	https://clinicaltrials.gov/ct2/show/NCT02316340
			Phase II: Recurrent breast cancer (recruiting)	Everolimus	https://clinicaltrials.gov/ct2/show/NCT03032406
			Phase I/II: Pancreatic cancer	Gemcitabine; abraxane	https://clinicaltrials.gov/ct2/show/NCT01506973
			Phase I/II: Advanced BRAF mutant melanoma	Dabrafenib; trametinib	https://clinicaltrials.gov/ct2/show/NCT02257424
			Phase I/II: Renal cell carcinoma	Aldesleukin	https://clinicaltrials.gov/ct2/show/NCT01550367

[a]https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm

MAP kinase pathway, which activates MEK through its phosphorylation. $BRAF^{V600E}$ mutation leads to constitutive BRAF kinase activity, which sustains the MAP kinase signaling pathway. $BRAF^{V600E}$-mediated p38 MAPK activation stimulates both the IRE1α/ASK1/JNK and TRIB3 pathways. BCL-XL/BCL-2 phosphorylation by active JNK releases BECN1, whereas TRIB3 inhibits the Akt/mTOR axes, resulting in an increase in basal autophagy [154].

Vemurafenib and dabrafenib are BRAF inhibitors which have been approved by the USA FDA and EMA for the treatment of BRAF-mutated metastatic melanoma. In an open-label, multicenter 2-year follow-up of vemurafenib in 3219 patients with $BRAF^{V600}$ mutation-positive metastatic melanoma, data suggest that long-term vemurafenib treatment is effective and tolerable [83]. Although vemurafenib and dabrafenib demonstrated impressive antitumor activity in advanced melanoma with objective response rates around 50% [85, 159], disappointing results were seen for patients with $BRAF^{V600E}$-mutated colorectal cancer. In the Phase II study evaluating vemurafenib in patients with metastatic $BRAF^{V600E}$-mutated colorectal cancer, of 21 patients, only one patient had confirmed partial response (5%) and the median progression-free survival (PFS) was 2.1 months [82]. Dabrafenib monotherapy did not show meaningful clinical activity with only one confirmed partial response among the 11 patients with $BRAF^{V600E}$-mutated colorectal cancer included in the Phase I trial [84]. Encorafenib, another potent and selective oral BRAF inhibitor, showed signs of efficacy in patients with BRAF-mutant advanced melanoma but lack of objective response in patients with colorectal cancer [160]. All three drugs are currently in several clinical trials for other tumors (Table 18.1).

Treatment of tumor cells with drugs that trigger further ER stress might result in two desirable anticancer outcomes. First, the drugs by themselves might result in increased antitumor effects. Second, the overload and subsequent breakdown of the UPR adaptive system might increase the tumor cells' sensitivity toward conventional chemotherapeutic agents [26]. Targeting of alternative pathways is an attractive strategy to improve antitumor therapy in apoptosis-resistant cancer. In view of the fact that ER stress is basally activated in many cancers, aggravation of the preexisting ER stress condition and the subsequent activation of autophagy represent an alternative therapeutic target to improve cancer therapy [27].

18.6 Autophagy

The ubiquitin-proteasome system (UPS) and lysosomes are two primary intracellular protein degradation pathways recognized in eukaryotic cells. Differences between these two major protein degradation systems depend on their functional significance and the type of substrates taken in for degradation [161]. The UPS catalyzes the rapid degradation of abnormal proteins and short-lived regulatory proteins, leading to a control of a diversity of essential cellular processes [162]. In the lysosomal protein degradation pathway, degradation of extracellular materials is mediated by endocytosis, whereas degradation of intracellular long-lived cytoplasmic proteins and damaged organelles is mediated by three types of autophagy, macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA), which are classified based on their transport of cytoplasmic materials into the lysosome for degradation [163, 164].

Autophagy literally means self-digestion in Greek [165]. Macroautophagy, usually refers to autophagy, is responsible for the turnover of unnecessary or dysfunctional organelles and proteins, such as damaged mitochondria [166]. These processes are important to maintain a well-controlled balance between anabolism and catabolism to facilitate normal cell growth and development. It is also a survival pathway, required during starvation or growth factor deprivation, as it provides an alternative energy source [167, 168]. Autophagy process provides catabolic intermediates for intracellular production of ATP when energy supplies are limited. It plays an essential role during starvation, cellular differentiation, cell death, cell survival, aging, and tumor prevention [164, 166, 169].

Autophagy pathway is a multistep process characterized by induction, vesicle nucleation, extension, and completion of an isolation membrane to form an organelle called autophagosome [170]. Briefly, the autophagy process begins with the formation of a preautophagosomal structure known as isolation membrane or phagophore [171]. The isolation membrane engulfs and elongates to form the autophagosome, surrounding the components destined to be recycled. The autophagosome, which is a double membrane-bounded structure, undergoes maturation, and fuses with both endosomal and lysosomal vesicles to form autolysosome [171–173]. The sequestered contents are subsequently degraded by lysosomal hydrolases and are recycled. Based on morphological features, the term "autophagic cell death" has been described in instances of cell death that are accompanied by massive cytoplasmic vacuolization.

The core autophagy machinery is composed of four major functional groups: (1) the unc-51-like kinases (ULKs) (ATG1-ATG13-ATG17 kinase complex), (2) the Class III phosphatidylinositol-3-kinase catalytic subunit type 3 (PI3KC3) complexes, including Class III PI3K (the mammalian orthologue of vascular protein sorting 34; VPS34), p150/VPS15 (the mammalian orthologue of Vps15), BECN-1 (the mammalian orthologue of ATG6/Vps30) and ATG14L (ATG14), (3) two ubiquitin-like conjugation systems: ATG12 and ATG8, and (4) ATG9 and its cycling system [174]. The ULKs (the mammalian orthologues of ATG1, which exist in a large complex with mammalian ATG13), focal adhesion kinase family interacting protein of 200 kDa (FIP200; the mammalian homologue of ATG17), and the recently identified ATG101 play a crucial role in autophagy induction [175–179]. ULK1 is part of a family of kinases in humans (ULK1–4). Isoform ULK1 is the most important component in autophagy and in some cells lines, blocking both ULK1 and ULK2 is necessary to completely shut down autophagy [180].

The ULK1 kinase regulates proautophagic signals by phosphorylating many substrate proteins [181]. The numerous substrates of ULK1 include itself and other subunits of the ULK1 complex; other elements of the core autophagy machinery, including PI3KC3–C1 subunits such as BECN1 and ATG9; and other autophagy-related proteins such as AMBRA1 [180, 181]. Autophosphorylation of the kinase domain's activation loop at Thr180 of ULK1 is essential for activation upon autophagy induction [182, 183]. Subsequently, phosphorylation of these downstream molecules by ULK1 is an important step in the initiation of autophagy.

The early stages of the phagophore membrane nucleation are dependent on the Class III PI3KC3 complex which consists of the Class III PI3KC3 protein, its regulatory protein kinase p150/VPS15, and BECN1 [184]. BECN1 is a 60-kDa tumor suppressor protein and is identified from a yeast two-hybrid screen as a BCL-2 interacting protein [185]. Several studies have demonstrated that several binding molecules positively regulate BECN1 activity and autophagosome formation and maturation. For example, ultraviolet radiation resistance-associated gene (UVRAG), ATG14L, and activated molecule in BECN1 regulated autophagy protein (AMBRA1) associate with BECN1 to activate autophagy [186–190].

The Class III PI3KC3 phosphorylates phosphatidylinositol to generate PI(3)P which is an essential early event in autophagy initiation, downstream of ULK1 [187, 191, 192]. PI3KC3 forms two distinct complexes, known as complexes I and II (PI3KC3–C1 and PI3KC3–C2) which contain the catalytic subunit VPS34/Vps34, the putative protein kinase VPS15/Vps15 and BECN1/ATG6 [187, 192]. PI3KC3–C1 contains ATG14L/ATG14, which directs the complex to phagophore initiation sites [186, 187, 193–196]. PI3KC3–C1 facilitates elongation meanwhile PI3KC3–C2, which contains UVRAG, directs endosome and autophagosome maturation [180].

The next stage of phagophore membrane elongation (expansion and closure of the autophagosome) requires two ubiquitin-like systems [197]. The ubiquitin-like protein ATG12 conjugates with ATG5 in an ATG7- and ATG10-dependent manner [161]. The ATG5–ATG12 complex interacts with ATG16 to form a stable and large multimeric complex called the ATG16L complex,

which localizes on the outer surface of the extending autophagosomal membrane [170]. This complex is important in the stimulation and localization of the microtubule-associated protein 1 light chain 3 (LC3) conjugation reactions. LC3 is first cleaved by ATG4 to expose a C-terminal glycine residue required for subsequent activation and conjugation reactions [198]. It is then conjugated to the lipid phosphatidylethanolamine (PE), also via ATG7 and E2-like ATG3, and is subsequently recruited to both outer and inner surfaces of the autophagosomal membrane [197, 199]. Actually, two forms of LC3 are produced posttranslationally in various cells; the unconjugated form (LC3-I) is in the cytosol, while the conjugated form (LC3-II) targets the autophagosomal membrane with the assistance of the ATG16L complex [199, 200]. ATG16L complex is a ubiquitin-protein ligase (E3)-like enzyme that functions as a scaffold for LC3-II lipidation by localizing to the source membranes during autophagosome formation [200, 201]. The association of LC3-II to the autophagosome is crucial for membrane elongation of the autophagosome and the final limitation of the membrane to form the vacuoles [161]. The ATG5–ATG12–ATG16 complex is recycled, while the LC3 complex stays on the membrane until it is degraded by the lysosome [161]. In mammalian autophagy, LC3-II protein is used as an index of autophagosome formation or as an autophagosomal marker [202]. These conjugation systems are considered to be uniquely important to the autophagosome formation and have been identified as possible drug targets in cancer [203].

ATG9 system is required for phagophore expansion. It is the only transmembrane protein in the autophagy core machinery and has been proposed to play a key role in directing membrane from donor organelles for autophagosome formation [204]. ATG9 trafficking from the plasma membrane and trans-Golgi network involves two conserved sorting signals for proper function in autophagy, namely ATG9 interaction with the AP1/2 clathrin adaptor complex and phosphorylation of ATG9 at Tyr8 by SRC kinase and at Ser14 by ULK1. SRC kinase directly phosphorylates Tyr8 of ATG9 and promotes the interaction of ATG9 with the AP1/2 complex and leads to the movement of ATG9 away from the juxtanuclear region [205]. As with Tyr8, phosphorylation at Ser14 enhances the binding of ATG9 with the AP2 complex and promotes ATG9-AP1 interaction. Zhou and co-workers showed that phosphorylation of ATG9 at both the Tyr8 and Ser14 sites is required for maintaining proper autophagy under both basal conditions and in response to starvation-induced stress [205]. Finally, ATG9 binds the small Rab GTPases (RABGAP) protein TBC1D5, and both TBC1D5 and the AP2 complex contribute to the correct sorting of ATG9-containing vesicles during the initiation of autophagy [206].

The completed autophagosome membrane subsequently fuses with lysosome via the actions of the lysosomal proteins including the lysosomal-associated membrane protein 1 (LAMP1), LAMP2, member of RAS oncogene family (Rab7), and UVRAG [207]. The eventual autolysosome is a single membrane-bound acidic vesicle where the contents are digested and recycled by lysosomal hydrolases such as cathepsins (CTS), and its nutrient and energy are recycled [208]. These single membrane autolysosomes filled with degraded cytoplasmic materials can be easily observed using transmission electron microscopy (TEM) [170]. In addition, the adapter protein sequestosome 1 (SQSTM1/p62), which targets specific substrates to autophagosomes and LC3II are degraded along with other cargo proteins and are used as a measure of autophagy flux [209]. The autophagy cargo receptor p62/SQSTM1 binds ubiquitin on cargo to deliver cargo proteins to autophagosomes by docking onto LC3 on autophagosomes. P62 itself is an autophagy substrate that accumulates when autophagy is inhibited [210].

The Nomenclature Committee on Cell Death (NCCD) recommends that the term “autophagic cell death” be used based on some biochemical and functional considerations, before indicating that a cell death is mediated by autophagy. Some of the considerations include making sure that the investigated cell death can be suppressed by the inhibition of the autophagic pathway using

chemicals and/or genetic means (e.g., gene knock-out or RNAi silencing of essential autophagy modulators such as AMBRA1, ATG5, ATG12, or BECN1) [211].

One of the most-studied and important pathways involved in autophagy regulation is the PI3K-Akt-mTOR signaling pathway. The mammalian target of rapamycin, commonly known as mTOR, is a serine/threonine kinase which belongs to the family of phosphatidylinositol 3-kinase-related kinases. It regulates translation and cell growth by its ability to phosphorylate both binding protein of eukaryotic translation inhibition factor eIF4E (4E-BP1) and p70 ribosomal S6 kinase (p70S6k). Upon stimulation by a variety of signals including cytokines, growth factors, cellular stress such as heat shock, hypoxia, and oxidative stress, PI3K is recruited to the inner cell membrane via phosphorylated receptor tyrosine kinases and catalyzes the phosphorylation of phosphatidylinositol-3,4-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). The recruitment of inactive Akt from the cytosol to the plasma membrane requires that the pleckstrin homology (PH) domain of Akt binds to PIP3 synthesized at the plasma membrane by PI3K. Akt is then phosphorylated at Thr308 by phosphatidylinositol-dependent kinase 1 (PDK1) [212, 213]. PTEN phosphatase antagonizes PI3K-Akt signaling by converting PIP3 back to PIP2 [212]. (Fig. 18.3).

Upstream PI3K and Akt activation by growth factors leads to the activation of mTOR and sub-

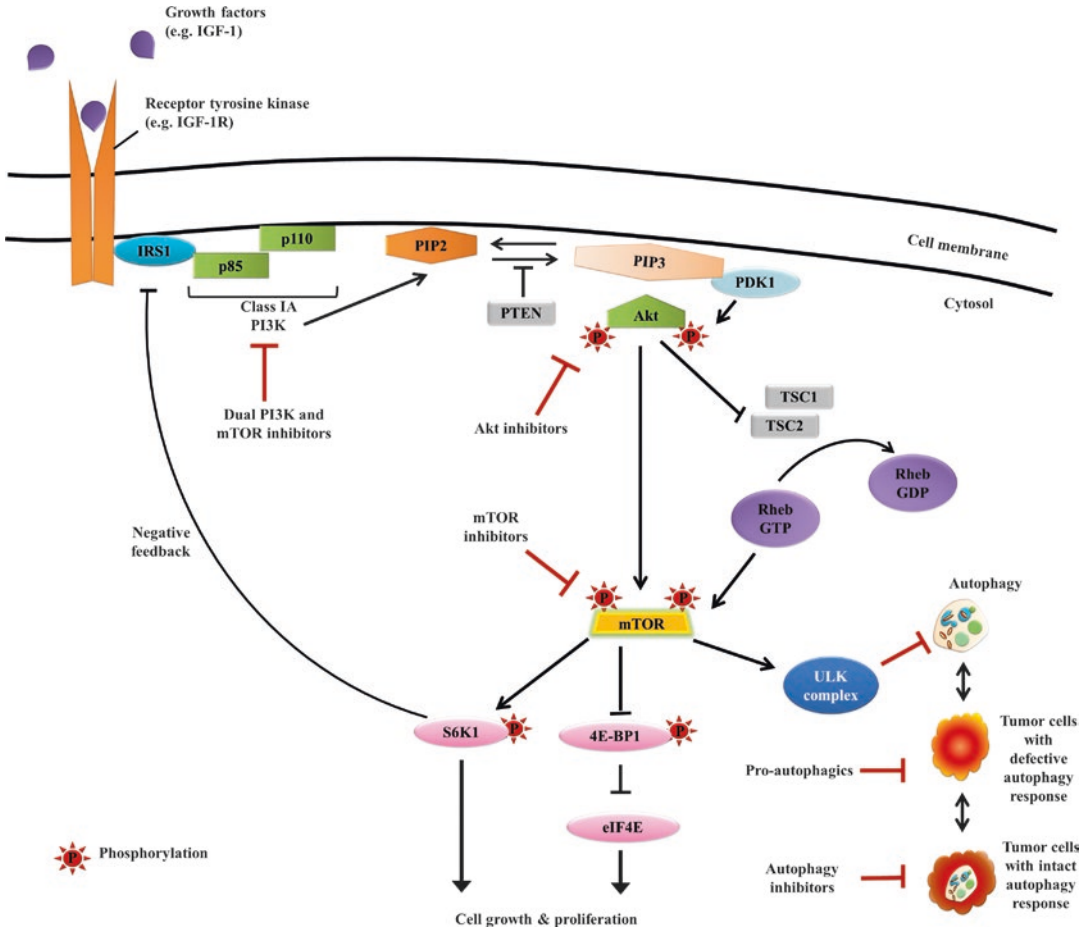

Fig. 18.3 Autophagy signaling pathway and antitumor targets

sequently phosphorylation of downstream substrates. Phosphorylation of p70S6k promotes ribosome biogenesis, and increases the capacity of the translational machinery for protein synthesis [214]. Phosphorylation of 4E-BP1 initiates the transcription of a subset of mRNAs important for cell growth and proliferation [214–216]. The mTOR kinase is a key regulatory component that controls the induction of autophagy [217]. Inhibition of mTOR (by nutrient depletion, starvation, or rapamycin) leads to cell cycle arrest, inhibition of cell proliferation, immunosuppression, and induction of autophagy. Increased levels of the mTOR kinase are found to inhibit the autophagy process, resulting in an increased in cell growth and tumor development [173]. Rapamycin, a specific mTOR inhibitor, complexes with the cytosolic receptor FK506-binding protein (FKBP12), and subsequently binds to a distinct region of mTOR upstream of the catalytic domain [218]. It induces autophagy and inhibits the proliferation of a variety of cells [219].

In eukaryotic cells, mTOR exists in two different complexes: mTORC1; a rapamycin-sensitive complex defined by its interaction with the supplementary protein Raptor (regulatory-associated protein of mTOR) and mTORC2; a rapamycin-insensitive complex defined by its interaction with Rictor (rapamycin-insensitive companion of mTOR) [220–222]. mTORC1 and mTORC2 accessorial complexes consist of mTOR, mammalian lethal with SEC13 protein 8 (mLST8) (also known as GßL) and DEP domain-containing mTOR-interacting protein (Deptor) [223]. mLST8 binds to the kinase domain of mTOR, and stabilizes the interaction of Raptor with mTOR in a rapamycin-sensitive pathway [224]. Raptor is the first protein shown to bind directly to mTOR that is required to mediate mTOR regulation of p70S6k and 4E-BP1 activities [221, 225]. On the other hand, PRAS40 and Deptor play roles as distinct negative regulators of mTORC1 [226, 227].

In a rapamycin-sensitive mTOR signaling pathway, much of the knowledge about mTORC1 function comes from the use of rapamycin, a bacterial macrolide antibiotic [228]. Upon entering the cell, rapamycin binds FK506-binding protein (FKBP12), its intracellular receptor, which subsequently binds to the FKBP12-rapamycin binding domain (FRB) of mTOR, thus inhibiting the mTORC1 functions [229, 230]. Rapamycin weakens the interaction between mTOR and Raptor [231]. However, the exact mechanism of how rapamycin and several rapamycin derivatives bind to FKBP12 to inhibit mTORC1 signaling is not completely understood [232]. Various conditions including starvation or lack of nutrients such as amino acids and/or glucose mimic rapamycin treatment, hence inhibit mTOR function in cultured cells, as indicated by rapid inactivation of p70S6k and hypophosphorylation of the 4E-BP1 [233].

Studies have shown that mTORC1 controls autophagy through the regulation of a protein complex consisting of ULK1, mAtg13, and FIP200 [176, 178, 234]. ULK complex is directly controlled by mTOR, leading to maintenance of the mAtg13 hyperphosphorylation state and suppression of autophagy induction [235]. A study has demonstrated that inhibition of mTOR by rapamycin leads to dephosphorylation of ULK1, ULK2, and mATG13, and activates ULKs to phosphorylate FIP200. These results suggested that the ULK-ATG13-FIP200 complexes are direct targets of mTOR and important regulators of autophagy in response to mTOR signaling [178]. One of the most important proteins involved in the regulation of mTORC1 activity is the tuberous sclerosis complex (TSC), which is a heterodimer of two proteins, TSC1 (also known as hamartin) and TSC2 (also known as tuberin) [230]. TSC1 and TSC2 function as a GAP (GTPase-activating protein) that negatively regulates a small GTPase called Rheb (Ras homologue-enriched in brain). TSC1 and TSC2 inhibit mTORC1 signaling by transforming Rheb into its inactive GDP-bound state [236, 237].

On the other hand, mTORC2 consists of mTOR, mLST8, Rictor, Deptor, mammalian stress-activated map kinase-interacting protein 1 (mSIN1; also known as MAPKAP1), and the recently identified protein observed with Rictor (PROTOR) [223, 238]. Rictor is defined as a novel mTOR-interacting protein defining a sec-

ond raptor-independent mTOR complex [220, 239]. Unlike mTOR-Raptor, the mTOR-Rictor complex does not bind to FKBP12-rapamycin, and is insensitive to rapamycin treatment [220, 222]. Therefore, rapamycin treatment does not represent a complete inhibition of mTOR function [240]. mTORC2 stimulates cell signaling through activation and phosphorylation of the proproliferative and prosurvival kinase Akt [241]. Akt regulates cellular processes such as metabolism, survival, apoptosis, growth, and proliferation by phosphorylating various effectors. mTORC2 activates Akt directly by phosphorylation at Ser473, which is a site needed for its maximal activation [242, 243].

In addition, mTORC2 controls various members of the AGC subfamily of kinases which includes serum and glucocorticoid-induced protein kinase 1 (SGK1) and several members of PKC family including PKCα [220], PKCε [244], PKCδ [245], and PKCζ [246]. mTORC2 is also known to phosphorylate mammalian Ste20-like kinases 1 (MST1) which is a core component kinase in the Hippo signaling pathway [247]. The Hippo pathway is composed of a group of evolutionarily conserved protein kinases that inhibit cellular growth and promote apoptosis [248, 249]. MST1 phosphorylates and activates large tumor suppressor (LATS) kinases, which in turn phosphorylate and inhibit Yes-associated protein 1 (YAP1), a co-transcription factor that promotes proliferation and survival [250]. mTORC2 is reported to be involved in the regulation of cytoskeletal organization through Rho GTPases and PKCα [220, 239]. Inhibitors of mTOR kinase domain have been developed to suppress the activity of both mTOR complexes (mTORC1 and mTORC2) [251, 252]. Figure 18.3 illustrates the simplified autophagy signaling pathways.

18.7 Autophagy and Cancer

The role of autophagy in cancer is rather perplexing. It is widely known that the autophagic pathway is deregulated in tumor cells. Several proteins and pathways related to autophagy signaling are deregulated during cancer development [189, 253]. Cell lines derived from hepatic, pancreatic, and breast carcinoma exhibit low autophagic activity, as compared with normal cells from the same origin [189, 254]. Autophagic capacity is known to increase during premalignant stages of pancreatic carcinogenesis, and then decreases during the transition of pancreatic adenoma into adenocarcinoma, suggesting that a decreased autophagic activity possibly contributes to the malignancy of pancreatic cancer [255, 256]. A decrease in autophagic capacity is also observed during animal experimental carcinogenesis, where cells from preneoplastic liver nodules or primary hepatocellular carcinomas induced by chemical carcinogens showed a decreased autophagic capacity as compared to normal liver cells [256, 257]. In addition, BECN1 is found to be mono-allelically deleted in a high percentage of ovarian, breast, and prostate cancers (based on the 17q21 and gene mapping studies). However, BECN1 is adjacent to the known tumor suppressor gene breast cancer 1 (BRCA1) on chromosome 17. Genomic analysis of BECN1 in The Cancer Genome Atlas (TCGA) demonstrated that allelic loss of BECN1 does not occur independently of codeletion with BRCA1, suggesting instead that BRCA1 loss is the driver mutation in hereditary and sporadic breast cancer [258–260].

There is a direct link between tumorigenesis and the disruption of the autophagy signaling pathways. PTEN deletions as well as the amplifications of both Class III PI3K and Akt are found in several cancers [261, 262]. The mTOR signaling pathway is constitutively activated in many tumor types. For example, the mTOR pathway is frequently found to be hyperactive in cancers such as breast cancer, suggesting that mTOR is an attractive target for cancer drug development and therapy [263–265]. The mTOR signaling network contains a number of tumor suppressor genes which includes *PTEN*, *LKB1* (liver kinase B1), *TSC1/2*, and a number of proto-oncogenes such as *PI3K*, *Akt,* and *eIF4E* genes [266]. Several alterations in genes such as *KRAS*, *EGFR*, *LKB1*, *PTEN*, *PIK3CA* (encoding the p110 catalytic subunit of PI3K), as well as *Akt1* mutations, *EGFR* and *PIK3CA* amplification, and *PTEN*

deletion have been described in NSCLC, which lead to uncontrolled mTOR pathway signaling [267]. In addition, dysregulation of the mTOR pathway appears to be more common in squamous lung carcinoma than adenocarcinoma [267, 268].

Cancer-related changes in pathways at the downstream of mTOR such as p70S6k and eIF4E are reported in breast carcinoma [269, 270]. In addition, malignant cell types undergo massive autophagosomes and eventually cell death when responding to anticancer agents and traditional herbs indicate the potential utility of autophagic cell death induction in cancer therapy [173, 271, 272]. Autophagic cell death characterized by an increase in the number of autophagic vacuoles in the cytoplasm, followed by cell demise has been observed in various diseases such as Alzheimer's disease [273], Huntington's disease [274–277], and Parkinson's disease [278]. Thus, manipulation of autophagy is considered an attractive strategy to increase the efficacy of cancer treatments, prevent cancer development, and limit tumor progression.

However, autophagy is divergent in nature in both tumor suppression and tumor progression [279]. Although the argument supports that if cells cannot activate autophagy, protein synthesis will predominate over protein degradation and cellular growth continues (typical characteristic of tumor cells), that was not the case for most. For example, a study in human epidermoid lung carcinoma cells revealed that the autophagic pathway in response to nutrient deprivation is not downregulated when compared to their normal counterparts [280]. Human colon cancer cells which are able to survive for long period of time in the absence of nutrients have a high rate of autophagic activity [281]. Studies in colorectal cancer cells revealed that these cancerous cells harbor functional autophagic machinery to prolong cell survival during shortages of nutrients [282]. A study by Fuji and coworkers has also shown that strong LC3 expression in the peripheral area of pancreatic cancer tissue is correlated with poor outcome and short disease-free period [283]. Activated autophagy observed in pancreatic cancer cells is thought to be a response to factors in the cancer microenvironment, such as hypoxia and poor nutrient supply. In addition, autophagy was found to be upregulated in RAS-transformed cancer cells to promote cancer cells growth, survival, tumorigenesis, invasion, and metastases [284–286]. Upregulation of autophagy in cancer cells is caused by direct activation of the transcription factors of the microphthalmia-associated transcription factor (MiTF)/TFE family that control autophagy and lysosomal biogenesis or by removal of a repressive phosphorylation on the autophagy initiation machinery [286–288].

In lung cancer, deletion of *Atg7* dramatically alters tumor pathology from carcinomas to that of benign oncocytomas [289, 290]. ATG7-deficient tumors accumulate dysfunctional mitochondria and prematurely induce p53 and proliferative arrest. As defective mitochondria is a major autophagy substrate, this indicates that benign human tumors manifest a phenotype of defective autophagy, perhaps explaining their benign status [286]. Autophagy has been identified as the key mechanism of cell survival in estrogen receptor-positive (ER+) breast cancer cells undergoing treatment with 4-hydroxytamoxifen (4-OHT) [291]. Antiestrogen therapy is the standard treatment for ER+ breast cancers which improves overall survival and provides chemoprevention [292, 293]. Unfortunately, approximately half of the women treated with antiestrogen therapy either do not respond or their breast cancer ultimately acquires resistance during treatment [294, 295]. Studies have shown that autophagic activity reduces the efficacy of chemotherapy and tamoxifen therapy in ER+ breast cancer cells [291, 296, 297], supporting the thesis that blocking autophagy signaling pathways may provide a new mechanism of anticancer therapy for resistant tumors.

In another example, electron microscopy examination of autophagic vesicles in melanoma tumors from 12 patients enrolled in a Phase II clinical trial of temozolomide and sorafenib therapy revealed that autophagic index (mean number of autophagic vacuoles per cell) is significantly higher in patients who derived little or no clinical

benefit from the combination of temozolomide and sorafenib treatment. Patients who had stable disease or responded to therapy had low levels of autophagy in their tumors. These findings further validate the preclinical evidence that autophagy plays a critical role in resistance to chemotherapy. Results of this study indicate that pretreatment levels of autophagy can predict resistance to therapy. Patients with aggressive melanoma are more likely to have higher levels of autophagy in their tumor and therefore may respond to autophagy inhibition as a therapeutic strategy [298]. Hence, the divergent nature of autophagy has resulted in strategies for using proautophagics or autophagy inhibitors depending on the inherent nature of the cancer involved.

18.8 Autophagy Signaling Pathways and Therapeutic Strategies in Cancer

18.8.1 mTOR Signaling Pathway Inhibitors

Rapamycin (Sirolimus) as the first prototype of an mTOR inhibitor has poor aqueous solubility and strong immunosuppressive properties. Therefore, its utilization at doses capable of exerting anticancer effects is rather limited [299]. Nevertheless, trials utilizing rapamycin as a single agent or combination therapy are still being carried out. In a Phase I study of rapamycin and sunitinib in patients with advanced NSCLC, combination of rapamycin and sunitinib is reported to be well tolerated and has warranted further investigation in Phase II trials [300]. However, the same was not observed in another recent study. Combination of sunitinib and rapamycin was observed to be quite toxic in all cohorts of patients with refractory solid malignancies [93]. The addition of rapamycin was thought to be able to decrease the sunitinib-induced VEGF production, but on the contrary, VEGF levels went further up along with sunitinib and rapamycin administration; it only came down during the sunitinib-off weeks [93]. However, in another recent Phase I trial, combination of oral rapamycin, topotecan, and cyclophosphamide was well tolerated in patients with relapsed/refractory solid tumors. Biomarker studies demonstrated modulation of angiogenic pathways with reduction of thrombospondin-1 and soluble vascular endothelial growth factor receptor-2 levels, respectively [92]. Several Phase II trials with rapamycin in combination therapy are currently recruiting patients with bladder, thyroid, prostate, and central nervous system (CNS) tumors (Table 18.1).

Various rapamycin analogues have since been developed. Temsirolimus (CCI-779) is the first mTOR inhibitor approved by the US FDA for cancer treatment, and is considered a first-line treatment for patients with advanced renal cell carcinoma (RCC) with poor prognostic features [301]. A number of clinical trials were carried out for this drug, mainly as combination therapy with other chemotherapy drugs. Moderate clinical activity was observed in patients with bone and soft-tissue sarcoma given a combination of temsirolimus and cixutumumab in a Phase II trial [302] and in patients with metastatic adrenocortical carcinoma, the same combination therapy resulted in 40% of patients achieving prolonged stable disease [303]. Similarly, in a recent Phase I study of temsirolimus in combination with cetuximab in patients with advanced solid tumours, both the median PFS and overall survival (OS) were <1 year and less than half of the patients had stable disease at the end of the trial, indicating modest clinical activity [94].

In another recent Phase I study combining perifosine (an Akt inhibitor) and temsirolimus, although stable disease was seen in 9 of 11 subjects with high-grade gliomas, no partial or complete responses were achieved [95]. However, the combination of these Akt and mTOR inhibitors was considered safe and feasible in patients with recurrent/refractory pediatric solid tumors [95]. When temsirolimus was tested as a single therapy in patients with relapsed or refractory primary CNS lymphoma in a Phase II trial, complete response was seen in five patients (13.5%), partial response in 12 patients (32.4%), and an overall response rate of just 54% [98]. In platinum-refractory/resistant ovarian cancer or

advanced/recurrent endometrial carcinoma, although temsirolimus treatment was well tolerated, it did not meet the predefined efficacy criteria [99]. Phase I and Phase II clinical trials with temsirolimus and sorafenib carried out in patients with metastatic melanoma did not produce sufficient activity to justify further use [304, 305]. Similarly, in a Phase II trial for metastatic colorectal cancer, temsirolimus had limited efficacy in chemotherapy-resistant KRAS mutant disease [306].

Everolimus is another rapamycin analogue which was already approved as an anticancer agent. Everolimus (RAD001; rapamycin derivative 001) is a hydroxyethyl ether derivative of rapamycin that has been developed for oral administration [307]. This drug was approved by FDA for use in a variety of cancers, including advanced renal cell carcinoma, advanced pancreatic neuroendocrine tumors, renal angiomyolipoma, and HER2-negative breast cancer. Everolimus is structurally similar to temsirolimus, binds to an intracellular protein, FKBP12, forming a complex that inhibits the mTOR kinase. In a recent Phase I trial to assess safety and efficacy of everolimus in combination with liposomal doxorubicin and bevacizumab in patients with advanced metaplastic triple negative breast cancer, only patients with the presence of PI3K pathway aberration were associated with a significant improvement in objective response rate, but not the clinical benefit rate [101]. A randomized Phase II study indicated that combination therapy of everolimus with tamoxifen increased the clinical benefit rate (defined as the percentage of all patients with complete or partial response or stable disease at 6 months), time to progression (TTP), and overall survival compared with tamoxifen alone in postmenopausal women with aromatase inhibitor-resistant metastatic breast cancer [308]. Further Phase III trials in combination therapy with aromatase inhibitors and adjuvant hormone therapy in hormone receptor positive metastatic cancer are currently underway.

Everolimus given for 14 days in combination with R-CHOP-21 (rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone delivered in a 21-day cycle) in patients with diffuse large B-cell lymphoma was proven to be safe. A total 23 of 24 patients achieved an overall response, and all 23 attained a complete metabolic response by PET, suggesting that drugs that target the PI3K-mTORC pathway added benefit when combined with standard R-CHOP [102]. The combination of everolimus plus CHOP was also effective in patients who are newly diagnosed with peripheral T-cell lymphomas, with objective response rate up to 90% [110]. The combination of mFOLFOX6 and everolimus in patients with metastatic gastroesophageal adenocarcinoma was also considered to be an active regimen with 83% of the patients experiencing a partial response [103]. Everolimus as a single therapy has demonstrated clinically relevant antitumor activity in patients with advanced differentiated thyroid cancer; median PFS and OS were 9 and 18 months, respectively [108].

Ridaforolimus (deforolimus or AP23573) has been tested in Phase I and Phase II clinical trials, and has shown promising results in several tumor types including sarcoma [299, 309]. Ridaforolimus received fast track and orphan drug status from the US FDA, as well as orphan status from the European Medicines Agency. Latest Phase I trials indicate that ridaforolimus as single therapy or in combination with other chemotherapy drugs was safe and well-tolerated [113, 114]. However, in a previous Phase II trial study on the efficacy and safety of single-agent ridaforolimus in patients with relapsed or refractory hematologic malignancies, results were unremarkable. Of the 52 patients evaluated, partial responses were noted in five subjects, while hematologic improvement and stable disease were observed in less than half of the patients [310]. In addition, the combination of ridaforolimus and dalotuzumab was no more effective than exemestane in patients with advanced ER-positive breast cancer, and the incidence of adverse events was higher [116]. Thus, the combination was not further pursued.

PI3K/Akt/mTOR pathway is often constitutively activated in human tumor cells and thus has been considered as a promising drug target. BEZ235 is a potent imidazo (4,5-c) quinoline

derivative that inhibits PI3K and mTOR kinase activities by binding to the ATP-binding cleft of these enzymes, and induces G1 arrest [311]. Preclinical studies have suggested that BEZ235 is a potent dual PI3K/mTOR modulator with favorable pharmaceutical properties. For example, it inhibits VEGF-induced HUVEC cell proliferation and survival in vitro and VEGF-induced angiogenesis in vivo [312]. The compound also inhibits microvessel permeability in BN472 mammary carcinoma grown orthotopically in syngeneic rats, suggesting that this compound is potentially antiangiogenic [312]. Deregulated angiogenesis and high tumor vasculature permeability are known VEGF-mediated characteristics of human tumors. In addition, BEZ235 is found to produce significant tumor growth inhibition in xenograft models of pancreatic cancers and breast cancer cells [313, 314]. However, in a Phase II trial of the BEZ235 in patients with everolimus-resistant pancreatic neuroendocrine tumours, BEZ235 was poorly tolerated by patients. Although evidence of disease stability was observed, the study did not proceed to stage two [117]. Similarly, BEZ235 showed modest clinical activity and an unfavorable toxicity profile in patients with advanced and pretreated transitional cell carcinoma, with just a minority of patients experienced a clinical benefit [118]. Several Phase I/II clinical trials of BEZ235 in patients with advanced solid malignancies such as prostate and breast cancer were completed, but reports on the safety and efficacy of this drug have yet to be published.

18.8.2 Proautophagics

Temozolomide is the first proautophagic cytotoxic drug used to overcome apoptosis resistance in cancer cells, and was approved for use in glioblastoma multiforme (GBM) [119]. It has demonstrated therapeutic benefits in patients with glioblastoma, and has been evaluated for several types of apoptosis-resistant cancers [315]. Temozolomide is a prodrug, a monofunctional alkylating agent, and is chemically related to dacarbazine. It is the 3-methyl derivative of the experimental anticancer drug, mitozolomide. The ability of temozolomide in inducing autophagic cell death was reported in various preclinical studies [316–319]. In addition, temozolomide has demonstrated proapoptotic activities in malignant melanoma cells [320]. In a systematic assessment of three randomized controlled trials addressing whether temozolomide holds any advantage over conventional therapy for high-grade gliomas, it was shown that temozolomide is an effective therapy for GBM. The drug prolongs survival, delays disease progression, and has a low incidence of early adverse events [321]. Similar outcomes were observed in a Phase II study involving erlotinib in combination with radiation therapy and temozolomide to treat GBM and gliosarcoma. Patients treated with the combination of erlotinib and temozolomide during and following radiotherapy had better survival than historical controls [322].

In a later Phase II trial, patients with unresectable or multifocal glioblastoma, an upfront regimen of temozolomide and bevacizumab was well tolerated, and provided a significant level of disease stabilization [323]. In patients with recurrent glioblastoma, either used as a single agent in a dose-intense schedule or in combination with other chemotherapeutic agents, temozolomide was proven to be well tolerated and safe [324–326]. In pediatric patients with recurrent solid tumors or brain tumors, low-dose temozolomide improved tolerability and was convenient as outpatient therapy [327]. However, in a recent Phase II trial, bevacizumab plus irinotecan combination resulted in a superior PFS-6 rate and median PFS compared with temozolomide in patients with glioblastoma that harbors a nonmethylated O(6)-methylguanine DNA methyltransferase promoter [124]. Patients with an O(6)-methylguanine–DNA methyltransferase (MGMT) nonmethylated (nmMGMT) glioblastoma (GBM) have a particularly short median survival of 12.6 months and do not substantially benefit from temozolomide chemotherapy [124, 328].

The combination of adjuvant temozolomide and lomustine, an alkylating agent, was associated with a significant improvement in OS and event-free survival (EFS) compared with adju-

vant temozolomide alone in the Children's Oncology Group ACNS0126 study [125]. This effect was most apparent in patients whose tumors had MGMT overexpression, as well as those who did not undergo gross-total resection and in those with glioblastomas. In a current Phase II study, neoadjuvant temozolomide was associated with an encouraging favorable long-term survival with acceptable toxicity in patients with glioblastoma [131]. Temozolomide in combination with vorinostat was also well tolerated in children with recurrent CNS malignancies with myelosuppression [329]. Vorinostat is a broad inhibitor of histone deacetylase (HDAC) activity which induces apoptosis, inhibits angiogenesis, and downregulates immunosuppressive interleukins. Several Phase III trials using temozolomide in combination with targeted monoclonal antibodies or interferon-alpha in glioblastomas and high-grade gliomas are currently recruiting patients (Table 18.1).

However, poor therapeutic effects were observed in patients with NSCLC. In a current efficacy and safety study of temozolomide in a total of 31 pretreated patients with NSCLC, only two patients achieved partial response and three had stable disease [330]. Moreover, the researchers pointed out that prolonged low daily doses of temozolomide produce minimal activity in patients with advanced NSCLC. In a recent Phase II study, combination therapy of pemetrexed and temozolomide group achieved the same efficacy in PFS and OS as the pemetrexed and cisplatin group, but with less toxicity. High-dose pemetrexed plus temozolomide may be a better regimen for treating NSCLC with brain metastasis due to its better safety profile [126]. A further Phase III study in patients with extensive small-cell lung cancer is currently underway.

Arsenic trioxide (ATO) has recently been introduced as part of a regimen in the therapy and management of acute promyelocytic leukemia (APL) [331]. It is now considered to be "the most biologically active single drug in APL" by a panel of International Leukemia Experts for the European Leukemia Net. The North American Intergroup Study Cancer and Leukemia Group B (CALGB) 9710 demonstrated that adults with APL receiving two cycles of ATO consolidation had significantly improved OS and decreased relapse risk (RR) [332]. It also achieves great success as a single agent and in combination with all-trans retinoic acid (ATRA) in the treatment of APL.

Arsenic trioxide (ATO) is known to induce both autophagy and apoptosis depending on cell types; therefore, its role as an autophagy inducer remains largely uncertain. In some preclinical trials, ATO induces the autophagy pathway in ovarian carcinoma cells, and synergizes with everolimus to induce the cytotoxicity of ovarian cancer cells. The enhanced cytotoxicity is accompanied by the upregulation of ATG5-ATG12 conjugate and LC3-II, a hallmark of autophagy [333]. In another recent study, ATO induces the autophagic degradation of the BCR-ABL1 oncoprotein, known to cause chronic myeloid leukemia (CML) and Ph+ acute lymphoblastic leukemia (ALL) [334]. However in other studies, in the presence or absence of ionizing radiation and in specific low concentrations, ATO induces apoptosis in MTLn3 cells, known to be highly malignant and resistant to both radio- and chemotherapy [335]. Interestingly, in human glioma cells, ATO induces both autophagy and apoptosis in vitro and in vivo, mediated by the inhibition of PI3K/Akt and activation of MAPK signaling pathway [336].

In a Phase I clinical study, ATO given concomitantly with radiation therapy in children with newly diagnosed anaplastic astrocytoma, glioblastoma, or diffuse intrinsic pontine glioma, was safe and well tolerated by patients throughout the entire dose escalation [337]. ATO was also reported to be well tolerated when used in combination with temozolomide and radiotherapy in malignant gliomas [338], or when used in combination with bortezomib, high-dose melphalan, and ascorbic acid in multiple myeloma (MM) patients [339]. A Phase II study to evaluate the efficacy and feasibility of a sequential treatment consisting of induction and consolidation with ATO followed by autologous hematopoietic cell transplantation for relapsed APL revealed that ATO demonstrates outstanding efficacy. Of the 23 patients who underwent autologous hema-

topoietic cell transplantation with PML-RARα-negative PBSC graft, posttransplant relapse occurred only in three patients, and there was no transplant-related mortality. The 5-year event-free and overall survival rates were 65% and 77%, respectively [340].

A recent study showed that the combination of ATO and ATRA exerts at least equal and probably superior antileukemic efficacy compared with ATRA and standard chemotherapy in low- and intermediate-risk APL [136]. In a Phase III study in which a chemotherapy-free ATRA and ATO treatment regimen was compared with the standard chemotherapy-based regimen (ATRA and idarubicin) in both high-risk and low-risk patients with APL, ATRA and ATO have a high cure rate and less relapse and a lower incidence of liver toxicity [138]. Similarly, a recent Phase III trial showed that ATRA-ATO had an edge over ATRA-chemotherapy over time and that there was significantly greater and more sustained antileukemic efficacy in low- and intermediate-risk APL [139]. ATO consolidation cycles are well tolerated in pediatric patients with APL and allow significant reduction in cumulative anthracycline doses while maintaining excellent survival and a low relapse risk for both standard and high-risk patients with APL [140]. Other Phase III clinical trials using ATO as combination therapy with other chemotherapy drugs and/or tretinoin are currently ongoing for APL.

18.8.3 Autophagy Inhibitors

The knowledge that autophagy plays a role as a cell survival pathway in response to therapeutic and cellular stresses in the tumor microenvironment (which is highly acidic and hypoxic) implies that autophagy may work in favor of cancer cells. Therefore, inhibition of protective autophagy may break the resistance mechanism for survival of the harsh tumor microenvironment and lead to cell death [341]. Since autophagy activities are known to differ according to stages of cancer, modulation of autophagy is postulated to enhance efficacy of anticancer therapy. In a preclinical study, effects of imatinib, with or without different types of autophagy inhibitors, on human malignant glioma cells were investigated [342]. It was demonstrated that suppression of imatinib-induced autophagy by 3-methyladenine (3-MA) or siRNA against ATG5 (which inhibits autophagy at an early stage) attenuates the imatinib-induced cytotoxicity. On the other hand, inhibition of autophagy at a late stage by bafilomycin A1 or RTA 203 enhances imatinib-induced cytotoxicity through the induction of apoptosis [342]. The therapeutic efficiency of imatinib may be augmented by inhibition of autophagy at a late stage, which could help sensitize the glioma cells to anticancer therapy [342].

The current autophagy inhibitors used in trials for human cancer are chloroquine (CQ) and hydroxychloroquine (HCQ). Both drugs are widely used as antimalarial agents and have gained much attention as potential chemosensitizers in treating tumors when used in combination with cytotoxic chemotherapeutic agents [343–345]. CQ inhibits lysosomal acidification and prevents autophagy by blocking autophagosome fusion and degradation [344, 346, 347]. CQ also sensitizes cancer cells to chemotherapeutic agents through autophagy-independent mechanisms and has other anticancer effects that are independent of its effects on autophagy [348].

A number of clinical trials have revealed the promising role of CQ, an autophagy inhibitor, as a novel antitumor drug. In an early glioblastoma study, where patients were treated with CQ in conjunction with radiation and temozolomide, the results showed a significantly prolonged median survival compared with controls [349]. Addition of CQ to conventional treatment for GBM also improves mid-term survival of patients [350]. Gemcitabine–CQ combination as a first- or late-line treatment in patients with metastatic or unresectable pancreatic cancer is well tolerated and shows promising effects on the clinical response [141]. A number of Phase I/II trials in solid tumors such as breast cancer are currently recruiting patients.

Although initial glioblastoma studies that used CQ in combination with chemotherapy and radiation therapy revealed median survival greater than control, there was no significant

improvement in survival of patients with glioblastoma treated with HCQ [145]. A Phase II trial of HCQ as a single agent in patients with previously treated metastatic pancreatic cancer demonstrated no clinical benefit and provided inconsistent evidence of autophagy inhibition [147]. Since this study was carried out in patients with advance disease, thus, there was a limitation for HCQ to improve end-stage disease outcome [348]. The results appear to be similar to an earlier Phase I study involving patients with advanced NSCLC. Although HCQ, with or without erlotinib, was found to be safe and well tolerated, the overall response rate was as low as 5% [351]. In other Phase I/II trials, HCQ in combination therapy with other drugs such as temozolomide, vorinostat, bortezomib, and gemcitabine are proven to be safe and tolerable among patients with advanced solid tumors and myeloma [142–144, 146]. So far, clinical trials of CQ and HCQ as autophagy inhibitors have demonstrated the safety of targeting autophagy for cancer therapy. More potent and autophagy-specific inhibitors such as Lys05 and drugs that target ULK1, VPS34, and ATG4B are in development and early preclinical stage [348]. Table 18.1 summarizes the various drugs targeting the autophagy pathways and clinical trial stages based on published reports as well as other trials listed in the NIH ClinicalTrials.gov website.

18.9 Crosstalk in ER Stress, Autophagy, and Apoptosis

Many cellular processes including apoptosis, autophagy, translation, and energy metabolism are controlled by the ER stress and mTOR signaling pathway. However, the crosstalk among these three signaling pathways has been identified only recently. It has been shown that Akt inactivation mediates ER stress-induced cell death. Long-term exposure to ER stress dephosphorylates Akt, induces DDIT3 expression, and causes cell death. Treatment with PI3K inhibitor alone also decreases phosphorylation of Akt, upregulates DDIT3 expression, and causes cell death, suggesting that PI3K/Akt inhibition specifically induces DDIT3 expression. Thus, Akt inactivation is important in ER stress-induced DDIT3 expression and cell death [352]. In addition, ER stress-induced apoptosis has been reported to be partly mediated by reduced insulin signaling through reduced Akt phosphorylation and increased glycogen synthase kinase 3β (GSK3β) activity. GSK3β is a proapoptotic Akt substrate whose activity is inhibited by Akt phosphorylation [353]. Prolonged ER stress has been shown to inhibit Akt/TSC/mTOR pathway, induce DDIT3 expression, and trigger apoptosis cell death [354]. On the other hand, ER stress negatively regulates Akt/TSC/mTOR pathway to enhance autophagy-mediated cell death [355].

It has been suggested that ER stress promotes autophagy and/or apoptosis via TRIB3-dependent inhibition of Akt/mTOR pathway [49, 50]. It was also proposed that ATF4 negatively regulates mTOR via DNA damage inducible transcript 4 (DDIT4, also known as Redd1) expression in response to ER stress. DDIT4 is a cellular stress responsive gene that has been shown to inhibit mTOR activity [356, 357]. ER stress also leads to CaMKKβ-dependent activation of AMPK, which ultimately leads to inhibition of mTOR and stimulation of autophagy [358]. In addition, it has been demonstrated that ER stress induces BIP expression and promotes an interaction between BIP and Akt. The physical interaction between BIP and Akt at the plasma membrane of cells following induction of ER stress prevents Akt phosphorylation [359]. To sum up, these observations suggest that ER stress may negatively regulate Akt and/or mTOR activity via various pathways, and ultimately leads to cell death.

It is also widely accepted that reactive oxygen species (ROS) generation precedes downstream cellular cascades, including those that determine cell fate either survival (autophagy) or death (apoptosis). Excessive ROS production disrupts the electron transport chain and produces reactive oxygen molecules, leading to depolarization of the mitochondrial membrane and initiation of mitochondria-induced apoptosis. However, ROS generation has also been shown to occur downstream after apoptotic stimulation (TRAIL-induced), or autophagy inhibition [360–362]. However, cell

fate outcomes are largely dependent on the amount of ROS generated and the cell's antioxidant response. During starvation, reactive oxygen molecules are produced as a result of Class III PI3K activation that stimulates autophagy through oxidation of ATG4, ultimately increasing the formation of lipidated LC3-rich autophagosomes [363]. Both $O2^-$ and $H2O2^-$ induce autophagy through AMPK activation and subsequent mTOR inhibition, and by transcriptional regulation of autophagy genes such as SQSTM1 (p62) and BECN1 [364–366]. A number of studies have similarly demonstrated that exogenously applied ROS leads to autophagy induction or apoptosis.

Functional relationships between apoptosis and autophagy are gaining much interest, as both cell deaths are not mutually exclusive. Perturbations in the apoptotic machinery, such as caspase inhibition, have been reported to induce both autophagic cell death and necroptosis [367, 368]. Inhibition of autophagy in cancer cells results in an accelerated cell death that manifests the hallmarks of apoptosis including chromatin condensation, MOMP, and activation of caspases [369]. In some cases, mixed phenotypes of both autophagy and apoptosis are detected in response to common stimuli [346, 369]. Studies in a variety of experimental systems indicate that autophagic cell death is likely to be context- and cell type-dependent. Autophagy can delay the onset of apoptosis following starvation, DNA damage, and hemodynamic stress [173]. For example, 1-day fasting causes liver autophagy in rats, but when starvation is prolonged for a few days, hepatocytes succumb to apoptosis [370]. Similarly, hematopoietic cell lines withdrawn from growth factor first activate autophagy, and eventually apoptosis [167]. Studies have also demonstrated that certain compounds have the ability to trigger both apoptosis and autophagic cell deaths simultaneously in cancer cells [371, 372]. Blocking of one pathway will trigger the activation of another [373]. Researchers have also hypothesized that there are factors (either external or internal) that may affect the preferential shunting into either biochemical cascades that will ultimately result in either apoptosis or autophagic cell death [374].

Crosstalks between autophagy and apoptosis exist at multiple levels because both pathways share mediators and pathway regulators. Several signals and pathways involved in autophagy are in common with apoptosis. Starvation and oxidative stress can trigger both apoptosis and autophagy. BCL-2 proteins function to inhibit both apoptosis and autophagy, providing another clue to the interplay between both processes. BECN1, the essential autophagy protein and haploinsufficient tumor suppressor, interacts with several cofactors such as AMBRA1, BIF-1, and UVRAG to activate the lipid kinase Class III PI3K, and induce autophagy [375]. In normal conditions, BECN1 is bound to and inhibited by BCL-2 or the BCL-2 homologue BCL-X_L, well-characterized apoptosis regulators, which involve an interaction between the BH3 domain in BECN1 and the BH3 binding groove of BCL-2/BCL-X_L. BH3-only proteins can competitively disrupt the interaction between BECN1 and BCL-2/BCL-X_L to induce autophagy. Nutrient starvation can stimulate the dissociation of BECN1 from its inhibitors, either by activating BH3-only proteins (such as BAD) or by post-translational modifications of BCL-2 (such as phosphorylation) that may reduce its affinity for BECN1 and BH3-only proteins [375]. Antiapoptotic BCL-2 family members participate in the inhibition of autophagy, whereas the proapoptotic BH3-only proteins participate in the induction of autophagy.

A recent finding suggests a link between autophagy and the extrinsic apoptotic pathway mediated by p62/SQSTM1. Autophagy is recently known to be responsible in selective degradation of polyubiquitinated proteins via SQSTM1, which encodes for p62 protein. P62 interacts with LC3 via its LC3 interacting region (LIR). Recent studies indicate that p62 is recruited to damaged mitochondria via binding to ubiquitinated outer mitochondrial membrane proteins, suggesting that p62 may serve as an autophagy receptor for ubiquitinated proteins and damaged mitochondria [376–378]. In addition to its role in autophagy, p62 mediates a cell's decision to undergo apoptosis or survival through its organization of signaling complexes in the cyto-

plasm [377, 379, 380]. Upon cytokine stimulation, p62 activates the NF-κB pathway, which subsequently induces the prosurvival genes, such as antiapoptotic and cell proliferation genes, and induces the expression of inflammatory genes such as cytokines, chemokines, and adhesion molecules [380–383]. However, p62 is also found to activate caspase-8 in the extrinsic apoptosis pathway, resulting in the initiation of apoptosis and cell death [379].

The expression of Patched (Ptc) induces apoptosis, but this activity is suppressed by its ligand, sonic hedgehog (SHH). Interestingly, hedgehog inhibition is found to induce autophagy through upregulation of BNIP3, and is also found to increase apoptosis in hepatocellular carcinoma cells at the same time [384]. In a recent study, apoptosis suppressed by the knocking down of PP2A can be reversed by the administration of 3-MA, a known autophagy inhibitor. The elevated accumulation of LC3-II and the decline of the autophagy substrate p62 are also observed in PP2Ac-small interfering RNA transfected cells. However, overexpression of PP2Ac suppresses the accumulation of LC3-II and restores p62 [385]. Interestingly, 3-MA increases cell death induced by diamindichloridoplatin (DDP), which suggests the protective function of autophagy in DDP-induced cell death [385].

18.10 Future Directions

There are increasing evidences that three major processes, i.e., apoptosis, ER stress, and autophagy, share overlapping molecular pathways and can occur in parallel under similar conditions. Fundamental knowledge in apoptosis, ER stress, and autophagy has also generated a great deal of insight into the pathogenesis of cancer, and has provided important considerations in strategizing cancer pharmacotherapy. Much effort and investment have been devoted to experimental drugs modulating apoptosis, ER stress, and autophagy. A number of drugs have proven to be promising during preclinical and clinical studies, but these drugs appear to be effective in one type of cancer and not in other. The percentage of patients who totally responded or partially responded to these treatments, either as single agent or in combination therapies, is relatively low, even though the outcome of these trials suggests some potential. These unforeseen effects are probably due to the specific-targeted nature of the therapy, in addition to the interconnected relationships between these cell death pathways. The contradictory role of autophagy and the status of autophagy in the human tumors concerned remain speculative, and further complicate the response to conventional anticancer treatment.

Currently, modulating apoptosis, ER stress, and autophagy by various means may be an important strategy to fight against the disease. Cancers which are resistant to the apoptotic effects of certain chemotherapy drugs may be sensitive to drugs that evoke ER stress or autophagic cell deaths. An intact autophagy pathway has a role in promoting carcinogenesis as well as in suppressing it. It also has a role in the development of resistance to treatment. Therefore, if autophagy response and activity are normal in tumors, combining standard chemotherapy drugs with autophagy inhibitors may sensitize tumor cells to anticancer agents. Cancer cells which present defects in the autophagy pathway may be managed by replacement of autophagy-inducing signals, e.g., proautophagics, or by inhibiting mTOR kinase. In some other cases, utilizing both autophagy and apoptosis inducers may present a deadly strategy against highly resistant tumors. Thus, devising personalized pharmacotherapeutic strategy based on the autophagy status of the tumors has become an attractive option and offers significant potential to be translated into the clinic.

Combination of anticancer drugs of many different classes with autophagy inhibitors and inducers is underway but with little rationale for deciding or selecting patients who are most likely to benefit from these therapies. So far, targeted drugs like oblimersen, bortezomib, and mTOR inhibitors such as everolimus and ridaforolimus have shown to be useful in some clinical trials. These novel classes of drugs appear to work synergistically in combination with other chemotherapeutics, and have also shown specific

activities against certain cancers. Clinical trials of CQ or HCQ as autophagy inhibitors have also demonstrated the safety of targeting autophagy for cancer therapy. Since these drugs are specifically targeted against certain molecules or receptors in the pathway, further unveiling of the tumor's characteristics such as receptor or protein status may be critical in assessing patient's response and clinical trial success. Furthermore, a number of known genes that play a role in these cell death pathways are either activated or inactivated in several cancers. This will certainly affect not only the promotion and progression of cancer, but also their response to treatment. Therefore, to optimize and personalize treatment strategies, the genetic profile of the tumors is important.

For example, RAS- and BRAF-mutant tumours are often associated with high levels of autophagy and exhibit autophagy dependency. These would be good markers to select patients in which autophagy can be inhibited therapeutically [285, 348, 386–388]. Other markers include signal transducer and activator of transcription 3 (STAT3) and IL6 in breast cancer cells [389], JNK1 in colon cancer [390], and EGFR-mutated or amplified tumors [391]. Some clinical trials have already used these markers to evaluate efficacy and for validation. This may provide information on the optimal point in the pathway to be targeted, and can also be identified as prognostic markers. At the same time, the development of both robust tissue markers and relevant techniques that can be used in the clinical context needs to occur along with novel treatments, which will be another challenge.

Although recent studies have incorporated some predictive biomarkers by examining tumor status, the utility of such practice remains non-conclusive. For example, the expression of peptidyl O-glycosyltransferase GaLNT14 has been proposed to be a potential marker of dulanermin or Apo2L/TRAIL activity in NSCLC as high GaLNT14 mRNA and protein expression in tumor cell lines are associated with Apo2L/TRAIL sensitivity [392]. An increase in PFS and OS was observed in GaLNT14-positive patients with advanced NSCLC in the dulanermin arm, indicating the potential predictive response biomarker for Apo2L/TRAIL-based cancer therapy [393]. On the other hand, in a Phase Ib/II trial on mapatumumab, a humanized mAb against TRAIL-R1, strong expression of TRAIL-R1 (indicated by immunohistochemical staining) did not appear to be a prerequisite for the effectiveness of mapatumumab in patients with relapsed or refractory follicular lymphoma [394]. Noteworthy, in the two patients who experienced a partial or complete response, the TRAIL-R1 staining was either undetected or weak [394]. However, this could be an isolated case, and trials with bigger sample size should be carried out. Tumor profiling would remain as a good strategy to identify patients who may respond to the relevant treatment.

Fundamental knowledge of cell death pathways remains an area of major interest among scientists in the field of cancer. More studies to characterize these pathways and identify potential targets, and further evaluation of the efficacy of the current drugs in various cancers are certainly warranted.

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Prognostic Value of Innate and Adaptive Immunity in Cancers

19

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Contents

19.1 Introduction

Human carcinogenesis is a dynamic process that depends on a large number of variables and is regulated at multiple *spatial* and *temporal* scales [1–4]. According to the theory of multistep carcinogenesis, cancer cells accumulate a number of molecular changes to eventually become fully malignant. The "reductionist" view of cancer expressed in myriads of molecular biology-based investigations stated that all the information necessary for a cell to transform itself into a neoplastic cell can be attributed to changes at the genomic level [5]. This is mainly based on the fact that the genome carries all of the information related to any cell process, and that any cellular transformation is due to a specific genomic change [6]. Cancer is recognized as a highly heterogeneous disease: more than 200 distinct types of human cancer have been described, and various tumor subtypes can be found within specific organs. In addition, tumors have somatic mutations and epigenetic changes, many of which are specific to

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the individual neoplasm [7]. This genetic and phenotypical variability primarily determines the self-progressive growth, invasiveness and metastatic potential of neoplastic disease, and its response or resistance to therapy, and it seems that the multilevel complexity of cancer explains the clinical diversity of histologically similar neoplasia [8–11]. Carcinogenesis might be depicted as a *nonlinear* process, the behavior of which does not follow clearly predictable and repeatable pathways. The behavior of a linear system changes progressively in response to an environmental factor. In contrast, the behavior of nonlinear complex systems may be perceived as surprising and unpredictable. Periods of inactivity may be punctuated by sudden change, apparent patterns of behavior may disappear, and new patterns may unexpectedly emerge [2, 12]. Moreover, nonlinear systems do not react proportionally to the magnitude of their inputs and depend on their initial conditions, i.e., small changes in the initial conditions may generate very different end points [13]. These characteristics are commonly highlighted by the frequency with which differences in progression or therapeutic response are seen in the same tumor type, and by the fact that cancer morphology does not always reveal a similar underlying biology [14]. Gliomas and glioblastoma, the most aggressive and most common of all primary malignant brain tumors, are genetically heterogeneous, are relatively less antigenic, and are less responsive to immunotherapy than other cancers [15]. It is now accepted that tumors grow in a complex network of epithelial and mesenchymal cells, vascular and lymphatic vessels, and inflammatory and immune cells [16–18]. The interactions of the neoplastic cells with their microenvironment are tortious, taking advantage of energy and nutriments carried by the blood vessels and growth factors produced by inflammatory and stromal cells and fighting for space to expand and escape the immune attack [19, 20]. Hypoxic conditions also affect the stromal compartment, where stromal cells are in close contact with the cancer cells [10]. When tumor cells metastasize in distant organs, the crosstalk starts again and the overall aggressiveness of a cancer, and therefore, the clinical outcome of the patient will greatly depend on these complex interactions.

19.2 Immune Infiltration as a Major Player of the Tumor Microenvironment

Among the various factors, which influence tumor establishment, growth, local invasion, and metastasis, the impact of immunity has been debated for a long time [21, 22]. While inflammation is now recognized as an enabling characteristic of human cancer [23, 24], the immune system is programmed to recognize tumors from their inception. Immune surveillance against the tumor is stimulated by the presence of tumor-associated antigens (TAA) and by stress-induced molecules [24]. It is known that tumor-associated antigen targets in solid tumors exhibit heterogeneity with regard to intensity and distribution, posing a challenge for chimeric antigen receptor (CAR) T-cell therapy. Novel CAR designs, such as dual TAA-targeted CARs, tandem CARs, and switchable CARs, in conjunction with inhibitory CARs, are being investigated as means to overcome antigen heterogeneity [24]. Only in the past decade, however, studies in murine models led to the understanding of the role of the immune system in cancer progression, a process termed cancer immunoediting [25]. Immunoediting is a process composed of three phases: first, the elimination of tumor cells by immune surveillance; then an equilibrium phase, during which the tumor is subjected to immune-mediated latency and the immune system is in balance with the tumor; and the last phase, during which tumor cells escape immune restraints and co-opt the immune system to promote malignancy. Tumor cells mediate a complex and dynamic immunoediting procedure that results in increased vascular efflux into the draining lymphatics, an immunosuppressive microenvironment rich in regulatory T lymphocytes, dysfunctional antigen presentation, and downregulation of normal effector lymphocyte responses [26]. Tumor cells employ diverse mechanisms to escape from

immune surveillance and manipulate the immune system and the microenvironment to facilitate the development of a malignant phenotype. These include mechanisms that promote escape, such as the downregulation of TAA and the decrease in expression/secretion of pro-inflammatory cytokines, as well as mechanisms that induce immune suppression, such as the production of immune suppressive cytokines, metabolites, and immune checkpoint molecules. Immunoediting enables tumor cells to evade immune system detection, disseminate from the initial niche, survive in the circulation, and settle at new metastatic sites.

Histopathological analyses of solid tumors reveal that they are infiltrated by cells of the innate and adaptive immunity (Fig. 19.1) [27–29]. Macrophages are heterogeneous, multifunctional, myeloid-derived leukocytes that are part of the innate immune system, playing wide-ranging critical roles in basic biological activities, including maintenance of tissue homeostasis involving clearance of microbial pathogens [30]. Macrophages represent a significant portion of the tumor mass, where they are commonly termed tumor-associated macrophages (TAMs) [31]. These cells are generated from blood monocytes

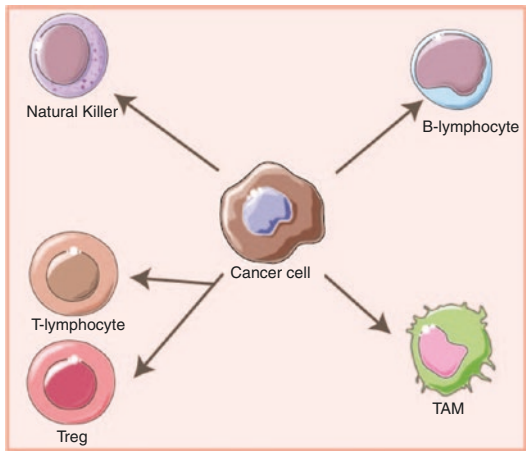

Fig. 19.1 Histopathological analyses of solid tumors reveal that they are infiltrated by cells of the innate and adaptive immunity. Cancer cells and unmodified genetic cells might be depicted as a "microunit" whose behavior is determined by complex relationships among the microenvironment components

[32], which differentiate into two distinct macrophage types, identified as M1 (or classically activated) and M2 (or alternatively activated). M1- and M2-polarized macrophages are endowed with opposite functional roles in terms of tumor suppression and immune stimulation. Several transcription factors, such as peroxisome proliferator-activated receptors, signal transducers and activators of transcription, CCAAT-enhancer-binding proteins, interferon regulatory factors, Kruppel-like factors, GATA-binding protein 3, nuclear transcription factor-κB, and c-MYC, were found to promote the expression of specific genes, which dictate the functional polarization of macrophages [33, 34]. Indeed, whereas M1 cells, by virtue of their ability to elicit Toll-like receptor (TLR) pathway, enhance immune responses and restrain tumor progression, M2 macrophages switch off the immune system and promote tumor development. Mast cells, myeloid-derived suppressor cells (i.e., the most abundant type of hematopoietic cells in the immune system) [35, 36], and neutrophils [37, 38] have also been reported to invade the intratumoral space. Dendritic cells (DCs) are found in different locations within a tumor, most immature Langerhans cell-type DCs home in the tumor nests and are tightly linked to malignant cells, whereas both immature interstitial DCs and plasmacytoid DCs are located in the stroma [39]. The ability to mount an effective antitumor immune response requires coordinate control of $CD4^+$T-cell and $CD8^+$T-cell function by antigen presenting cells, most importantly DCs. In some cases, tumors create an immunosuppressive microenvironment that helps protect tumor cells from immune recognition. In many cases, this defect can be traced back to a failure of DCs to recognize, process, and present tumor antigens to T-cells [40, 41]. Mature DCs concentrate in lymphoid islets adjacent to the tumor nests and some draining lymph nodes. NK cells are usually found in the stroma of most tumors [42, 43] but can be also found in close contact with tumor cells in renal cell carcinoma. The distribution of lymphocytes may be differently orchestrated depending on the tumoral spatial organization [44]. T lymphocytes are mainly located in the core, often referred as the center of

the tumor, its invasive margin [45], and in adjacent lymphoid islets. Among T lymphocytes, most of them have a memory phenotype, naïve cells being found mostly in adjacent lymphoid islets [46]. Some $CD8^+$ T lymphocytes contact malignant cells, whereas others are dispersed in the stromal compartment. Forkhead/winged helix transcription factor $(FoxP3)^+$ T lymphocytes, T-lymphocyte helper 17 (Th17), T-follicular cells help (TFH), and B lymphocytes concentrate in the stromal tissue and in lymphoid islets. A similar organization is found in metastatic sites, as in the primary tumors, although diversity is observed within tumors and between patients. Correlations between the levels of immune cell infiltration of tumors and clinical outcome have been investigated in several cancers of unrelated histological origin [47–50]. A strong lymphocytic infiltration has been reported to be associated with good clinical outcome in different tumor types and subtypes, including melanoma, head and neck, breast, bladder, ovarian, colorectal, renal, prostatic, and lung cancer [47, 48, 50–55]. The analysis of other T lymphocytes has also yielded apparently contradictory results. Th17 cells have been reported to be associated with poor prognosis in colorectal, lung, and hepatocellular carcinoma or have been reported to predict better survival in some esophageal and gastric cancers [56]. The effect of intratumoral B lymphocytes in cancer remains far from clear; B-cells have recently been appreciated as paracrine mediators of solid tumor development [57], although their capability to enhance T-cell activation might have a positive impact in the organization of the antitumor immune response [58]. Here, we discuss the role played by innate and adaptive immune system in the local progression and metastasis of human cancer of unrelated histological origin and the prognostic information that we can currently understand and exploit.

19.3 Cellular Players of the Innate Immunity in Cancer

Rudolf Virchow (1821–1902) first observed infiltrating leukocytes in tumors and proposed the inflammation as a primary site of cancer occurrence [59]. Later, epidemiological and experimental studies have associated chronic infections to about 15–20% of tumors [60, 61] and linked inflammation to tumorigenesis, by modulation of a variety of complex processes, including the increased cell proliferation, rate of mutagenesis, angiogenesis [62], and inhibition of apoptosis. For these reasons, inflammation has been acknowledged as a critical element in cancer occurrence and has been included as a new "hallmark of cancer" [23]. The inflammation is the protective response of the body against various harmful stimuli; however, the aberrant and inappropriate activation tends to become harmful [63].

19.3.1 Tumor-Associated Macrophages (TAM)

A number of studies appraised tumor-associated macrophages (TAM) as crucial mediators of the connection between inflammation and cancer occurrence [64–68]. TAMs secrete a plethora of cytokines and chemokine, which are the soluble mediators of inflammation and are mainly responsible to mediate such processes [69]. It is widely accepted that in the majority of cancers, TAMs have a pro-tumoral effect [70]. However, these cells are intrinsically "plastic" in their functions, and in the complexity of tumor microenvironment, they were shown to acquire antagonistic properties ranging from immune suppressive to immune-stimulatory properties. While the antitumor role of TAM has been previously linked to the orchestration of T-lymphocyte antitumor immune response, recent findings have shown that tumor immune surveillance can be firmly directed by TAMs when "educated" by specific treatments in a T-cell independent fashion [71]. The functional plasticity of macrophages is regulated by environmental stimuli; thus, their immune profile results in the identification of two distinct polarized functions, schematically simplified as M1/M2 classification. Macrophages are recruited at peripheral sites by locally secreted chemotactic factors and cytokines, including inflammatory chemokines and growth factors [i.e., vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and macrophage colony-stimulating factor (M-CSF)]

[72]. These cytokines can also promote macrophage survival and polarization. Although mobilization of the circulating pool of monocytes is the main mechanism of macrophage recruitment, local proliferation can contribute to macrophage accumulation at the tumor site [73]. In the tumor context, both tumor and stromal cells secrete a variety of chemo-attractants for blood circulating monocytes, including CCL-2, originally discovered as a tumor-derived chemotactic factor [74]. Molecular profiling analyses of both human and murine TAMs have evidenced a profile closer to that of M2 macrophages [75, 76], whose remodeling, immunosuppressive activities, and production of trophic factors for tumor and stromal cells functionally correlate to important pro-tumor activities [77], including proteolytic activity [78], remodeling of the extracellular matrix [79], and induction of angiogenesis [80]. Liu et al. have shown that M2-polarized TAMs increased fibroblastic morphology, upregulated mesenchymal markers (i.e., Vimentin and Snail) at the mRNA and protein levels, and increased proliferation, migration, and metalloproteinase MMP2 and MMP9 proteolytic activity in pancreatic cancer cells [81]. In addition, it has been shown that the inhibitor of MMP-9 has been associated with decreased survival in breast cancer [82]. Leifler et al. identified MMP-9 as a potent player in modulating the innate immune response into antitumor activities [82]. Notably, TAMs exert their pro-tumor functions both directly acting on tumor cells and indirectly, by orchestrating the suppression of the adaptive immune response. Macrophages, whether adequately activated, have the capability to both directly kill tumor cells [83, 84], a property mediated by contact dependent [85] as well as independent mechanisms [71], and orchestrate an antitumor adaptive immune response, through the activation of cytotoxic lymphocytes.

19.3.2 Tumor-Associated Neutrophils (TAN)

Although TAMs are the most prevalent innate cellular components of the tumor microenvironment, the role of tumor-associated neutrophils (TANs) on tumor progression has been reconsidered [86, 87]. Accordingly, TANs have been shown as a source of cytokines and chemokine and in different settings also anti-inflammatory mediators, thus likely to mediate a dual effect on tumor progression depending on their polarization state, i.e., N1 and N2 [88, 89]. TAMs and TANs functional polarization and prognostic value reflect the intrinsically plasticity as it varies along the tumor type, location in the tumor tissue (i.e., necrotic and hypoxic areas), and the tumor stage. Studies have demonstrated specific examples of tumor-mediated signals (such as transforming growth factor-β, TGF-β) that induce the formation of a pro-tumorigenic N2 phenotype capable of supporting tumor growth and suppressing the antitumor immune response. However, there are also studies showing that TAN can also have an antitumorigenic N1 phenotype [90]. Many patients with advanced cancer show high levels of neutrophilia, tumor neutrophils are connected to dismal prognosis, and the neutrophil-to-lymphocyte ratio has been introduced as a significant prognostic factor for survival in many types of cancer. Neutrophils constitute an important portion of the infiltrating immune cells in the tumor microenvironment, but controversy has long surrounded the function of these cells in the context of cancer. In particular, it remains unclear whether these different populations represent bona fide subsets or simply activation/polarization states in response to local cues [91, 92].

19.4 Cellular Players of the Adaptive Immunity in Cancer

It has been accepted that immune cells infiltrate the tumor stroma, and they are essential players of the tumor microenvironment. The cells of the adaptive immune system are mainly represented by $CD8^+$ cytotoxic T lymphocytes (CTLs) and $CD4^+$ T-helper lymphocytes. The main function of $CD4^+$ T lymphocytes is to sustain activation of other cells, including macrophages, B-cells, and CTLs, by release of several cytokines, such as interleukn-2 (IL-2), tumor necrosis factor alpha (TNFα), and interferon gamma (INFγ). Identification and specific elimination of tumor

cells are mediated by CTLs CD8+ T-cells [93, 94], which produce perforin and granzyme B [95]. The recognition by lymphocytes of antigens after first encounter is kept at a higher activation level compared to the baseline. Activated T lymphocytes have long life, are more reactive to stimulation than naïve T lymphocytes, and are detectable by specific surface molecules, suggesting that their presence in the context of solid tumors has important implications. Accordingly, antigen-experienced CTLs phenotypically switch CD45 isoform from CD45RA to CD45RO when they are activated [96].

T-lymphocyte activation is also modulated by a subpopulation of T lymphocytes indicated as T-regs, which suppress immune responses [97]. The transcription factor *FOXP3* is a specific T-reg cell marker [97, 98]. T-regulatory cells were discovered more than 20 years ago and have remained a topic of intense investigation by immunologists [99]. T-reg lymphocytes include different subpopulations, although the most investigated are CD4+ CD25+ [97, 100]. However, these markers are not completely specific for T-regs because CD25 and *FOXP3* might also be expressed by activated CTLs [96]. Accordingly, the specificity of tumor-infiltrating T-reg cell antigen has yet to be determined in humans. T-regs may exert different functions according to the tumor contexture, i.e., they might block anti-tumor immunity or decrease chronic pro-tumor inflammation [96].

In the clinical setting of some human cancers, the lymphocytic reaction can comprise different components beside dispersed tumor-infiltrating lymphocytes (TILs) and include discrete "lymphoid aggregates," resembling lymph-node-like structures. These aggregates are similar to those observed in chronic inflammatory conditions, where tissues harboring target antigens are infiltrated by cellular effectors of the adaptive immune system, which organize anatomically and functionally as in secondary lymphoid organs, with recruitment of B-cells and T-cells, follicular dendritic cells with germinal centers, and specialized vessels suited to mediate traffic of immune cells [101, 102]. Those structures are named tertiary lymphoid tissue (TLT) and might be involved in the organization of the immune response. Few reports exist that TLTs are present also in cancer [103, 104]. Moreover, the concept of ectopic lymphoid structures within solid tumors has only recently become appreciated, and it is still unclear whether these structures retain functional immune activities to mediate recruitment and activation of TILs.

19.5 Prognostic Value of Innate and Adaptive Cells of the Immune System in Cancer

The stromal compartment of solid tumors is infiltrated by immune and inflammatory cells expressing a wide array of specific markers and exerting critical effects on tumor outcome depending on their specific subset, density, spatial location [105] and the staging of tumor at diagnosis [106–108]. It is widely accepted that in preclinical studies, cellular mediators of the innate immunity favor tumor progression [23, 77, 109]. Accordingly, the quantification of the number of CD68+ TAMs was linked to a poor prognosis in pancreatic cancer and Hodgkin's lymphoma [110, 111]. Recently, it has been shown that tumor and stroma DC, NK cells, M1-polarized TAMs, CD8+ T-cells, and B-cells were associated with improved prognosis and tumor PD-L1, and stromal M2 TAMs and T-reg cells had poorer prognosis in non-small-cell lung cancer [112]. In the case of pancreatic cancer, expression of M1 markers of macrophage polarization was associated with better prognosis, while M2 markers were linked to worst prognosis [111]. In lung cancer, IL10+–CD68+ TAMs were associated with worst prognosis in patients with late-stage disease at diagnosis [113], while in a subsequent study, a high ratio of M1/M2 macrophages was a feature of patients with good outcome [114]. Thus, according to the simplified view of macrophage polarization provided by Mantovani et al., in clinical studies, macrophages infiltrate tumor nest as a heterogeneous population, which seem to retain different functional and molecular properties that may vary according to the instructions

provided by the tumor milieu. On the contrary, in colorectal cancer, it was shown a correlation of high number of TAMs with a better prognosis [85, 115], and in a later study, this correlation held true regardless of TAM polarization [116]. Discrepancies among clinical studies on prognostic abilities of innate immune cells underline the importance of the tumor type when trying to determine TAM's influence on tumor progression. Wu et al. found that TAMs identified only with CD68 have no significant correlation with the prognosis and clinicopathological parameters of bladder cancer patients. However, TAMs detected with CD163 could serve as a prognostic marker for bladder cancer patients. These findings invite further research on the role of TAM subsets in bladder cancer patients [117]. Beside the parenchymal cells, the liver also contains resident and infiltrating myeloid cells involved in immune responses to pathogens and much less is known about their interplay with hepatitis B virus (HBV). While it is still unclear if liver macrophages play a role in the establishment and persistence of HBV infection, several studies disclosed data suggesting that HBV would favor liver macrophage anti-inflammatory phenotypes and thereby increase liver tolerance. In addition, alternatively activated liver macrophages might also play in the long term a key role in hepatitis B-associated pathogenesis, especially through the activation of hepatic stellate cells [118]. Further clinical data are warranted to study whether TAM effect might differ along tumor progression and, in a clinical relevant scenario, with regard to chemotherapy treatments. Several retrospective clinical studies on colorectal, melanoma, ovarian, breast, and non-small-cell lung tumors generally underlined the adaptive immune cell tumor infiltration as a prognostic indicator of good patient's prognosis [103, 105, 119–123]. Variability with respect to prognostic potential of the markers employed relies on the specific population of T lymphocytes and the type of tumor settings investigated. In this view, colorectal cancer represents a paradigm since its *milieu* is highly permeated by adaptive immune cells with potential antitumor abilities. A seminal paper by Galon et al. claimed that concomitant local infiltration of $CD3^+$ lymphocytes at the tumor invasive margin and in the intratumoral location was a better predictor of survival than the tumor-node-metastasis (TNM) staging system [105]. However, TNM is still the gold standard to predict CRC patient prognosis, while TILs are not employed to date in clinical practice. A subsequent study from Laghi et al. raised doubts on previous claim and showed that while $CD3^+$ T-infiltrating lymphocytes (TILs) were not independent by TNM staging in predicting patient's prognosis, TILs were a strong prognostic factor only among lymph-node negative but not among lymph-node-positive CRCs [106]. Later, Mlecnik et al. showed that an immune score was reproposed, although represented by partly overlapping subpopulations of TILs (i.e., $CD8^+$ and $CD45RO^+$), which had to be concomitantly located at the tumor invasive margin and intratumoral in each CRC specimen [124]. By these means, these immune features identified a benchmarking population with a dismal prognosis and devoid of TILs, representing only 6.5% of the CRCs (stages I–III) [124]. This strategy fostered statistical analysis but might not provide proper clinical prognostic relevance when addressing surveillance strategies and allocation to chemotherapy in the overall population of CRC. The biological relevance of tumor lymph-node infiltration in the context of TIL prognostic abilities was previously shown in ovarian cancer in a study suggesting a negative interaction of nodal status with antitumor immunity [107]. In CRC, the density of activated $CD8^+$TILs decreased in patients with metastatic lymph nodes and advanced tumor staging, suggesting that immune escape might occur along CRC disease progression [125]. Accordingly, in a different study, the expression of *eomesodermin,* a transcription factor critically involved in the production of perforin, is inversely associated with tumor lymph-nodal involvement [126]. In melanoma, these observations were supported by the fact that a primary tumor devoid of TILs was shown to predict sentinel lymph node metastasis. These studies underline that the plasticity of TILs with regard to their recruitment and antitumor activity seems to differ along the clinical progression of

different solid cancers [108]. Therefore, future design of clinical trials aimed to employ TILs as diagnostic tools or novel immunotherapy strategies should take into account these considerations. Recruitment of T-reg cells to the tumor milieu is another mechanism of tumor immune evasion. In ovary cancer, recruitment of T regs decreased specific antitumor TILs and associated with a worst prognosis [127]. In hepatocellular, renal cell, and breast carcinomas, the number of $CD4^+CD25^+Foxp3^+$ cells was associated with worst patient's outcome [128–130], although not independently by other histopathological features in the case of breast cancer. Counterintuitively, different CRC studies showed that a high density of $Foxp3^+$ cells was independently associated with better prognosis [131–133]. This discrepancy might be explained by hypothesizing that $Foxp3^+$ cells instead of inhibiting antitumor immunity seem to decrease chronic pro-tumor inflammation. However, the biological basis explaining the differing roles of T-reg cells in tumor progression with respect to the tumor type is still unknown. New experimental models properly simulating tumor development will be helpful in better understanding T-reg activity on tumor. It is indubitable that the estimation of risk of recurrence for patients with CRC must be improved. A robust immune score quantification is needed to introduce immune parameters into cancer classification. Recently, an International Panel assessed the prognostic value of total tumor-infiltrating T-cell counts and cytotoxic tumor-infiltrating T-cell counts with the consensus Immunoscore assay in patients with stage I–III CRCs. The Immunoscore provides a reliable estimate of the risk of recurrence in patients with colon cancer. These results support the implementation of the consensus Immunoscore as a new component of a TNM-Immune classification of cancer [45].

19.6 Concluding Remarks

Solid tumors contain a heterogeneous mixture of malignant and nonmalignant cells within an extracellular matrix supported by an irregular vascular network [134, 135]. The cancer microenvironment makes up the stroma of the neoplasm and is the tissue that determines tumor growth, progression, and ability to initiate metastases. Because of the role that the cancer microenvironment plays in each stage of tumor development, better knowledge about the interactions of the tumor with its microenvironment would seem to be of the utmost importance for developing new treatment strategies [136, 137]. It has been ascertained that cancerous stroma coevolves alongside tumor progression, thereby promoting the malignant conversion of epithelial carcinoma cells [138]. However, tumor stroma is infiltrated by a variety of immune cells with the ability to influence tumor development and with a relevant impact on prognosis. The understanding that the immune system plays a dual role in cancer progression has led to the recent development of targeted immunotherapies [139]. Immune surveillance against the tumor is stimulated by the presence of TAA and by stress-induced molecules. Only in the past decade, however, have studies in murine models led to the understanding of immune system roles in cancer progression, a process termed cancer immunoediting [25].

It is indubitable that the analysis of the type, quantity, location, and the functions of the immune infiltrate becomes a primary step in understanding the history of cancer in a clinical relevant perspective. A comprehensive analysis of all components of the lymphocytic infiltrates in the context of their localization, organization, and impact at various steps of tumor progression remains largely, if not entirely, to be addressed in prospective studies [140, 141]. As recently reported by Hamada et al., further studies examining tumor molecular alterations and additional factors in the tumor microenvironment may inform development of immunoprevention and immunotherapy strategies [142]. In parallel, understanding the mechanisms of efficient immune reactions, the place where they are initiated, the cellular and molecular mediators involved, and their impact at different stages of the disease should provide new tools and goals for more effective and less toxic-targeted therapies.

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Immunogenetics of Cancer

20

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20.1 Introduction

The influence of genes in the development of cancers can be very high, very well depicted in numerous hereditary cancers or very low in some cancers. Although the roles played by genes in the pathophysiology and prognosis of the malignant transformation are highly variable in different cancers, their role cannot be ignored. For sure, polymorphisms in immune-related genes, known as immune polymorphisms, have an undeniable role in shaping undeniable but complex interactions between the immune system and malignancies which can significantly influence the face of malignancy with respect to predisposition, nature, prognosis, and response to treatment in each individual.

20.2 Cancers: Why Are There Different Faces?

It has long been observed that individuals are different with respect to predisposition nature, prognosis, and response to treatment in cancer [1, 2]. Since the first observations, scientific minds have been preoccupied with the question that, what is the reason for this high interindividual variation. Nowadays, it is obvious that behind the ugly scene of cancers, there is a complex interplay between genes and environment and this question can be answered straightforwardly by the high variability of genetic and environmental factors for each individual [1]. Although it is estimated that less than 0.1% of the genome is different between any two individuals, this variability is equal to at least several million nucleotide differences per individual [3, 4]. Genetic effect in the development of cancers is investigated by analyzing the rate of heritability in twin studies with shared and unshared environment [5]. The influence of genes can be very high in melanoma, leukemia, and prostate cancer as well as numerous hereditary cancers like familial adenomatous polyposis, or it can be very low in some cancers like cancer of the cervix or head and neck [5, 6] (Fig. 20.1). Although the roles played by genes in the pathophysiology and prognosis of the malignant transformation are highly variable in different cancers, their role cannot be ignored [7, 8]. Malignant transformation is not just a result of a cell-autonomous process and is shaped by intrin-

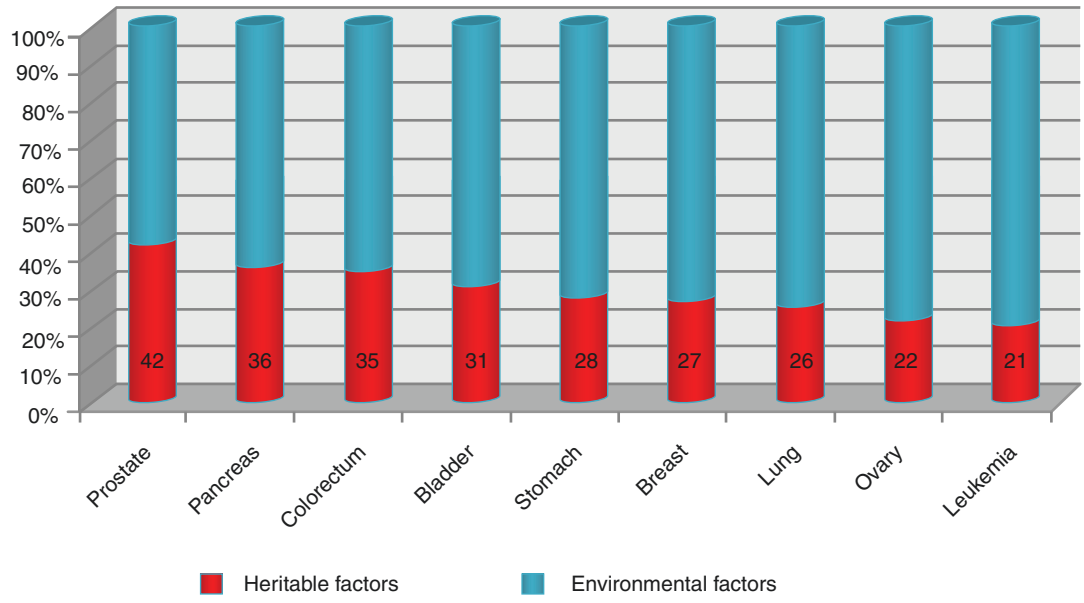


Fig. 20.1 Heritability of cancers in different sites based on the information available from twin studies

sic properties, but also its cross talk with microenvironment governed by the immune and endocrine systems, stroma, vascular system, and other systems [7]. Therefore, this heritability results from additive effects of low-penetrance genetic factors, each one contributing a small amount of risk [7].

20.3 Immune Polymorphism

The role of immune system in defense against malignancies was proposed in the early 1990s by Paul Ehrlich [9]. So far this book, page by page, has tried to show the undeniable but complex interactions between the immune system and malignancies. This complex interaction mostly results from the manipulation of the immune system by cancer cells evoluting to prevent self-destruction [9]. Four phenomena contribute to the escape of malignant cells from the immunosurveillance:

1. *Immunoedition*: Natural selection of malignant cells which are most successful in deceiving the immune system occurs by the pressure of the immune system itself. This pressure combined with the genetic instability of the cancers leads a somatic evolution toward variants proficient in immune escape in primary tumor lesions [10–12]. This struggle between cancer and immune cells is bidirectional wherein immune response is also programmed according to cancer antigen presentation.
2. *Downregulation of the local immune system:* Several tumors can manipulate the local defense by producing inhibitory molecules such as indoleamine 2,3-dioxygenase (IDO) and different cytokines or expression of apoptose-inducing ligands such as Fas-ligand [10, 13].
3. *Tolerance induction and losing immunogenicity*: The absence of costimulatory molecules, localization in natural environment of healthy cells and therefore absence of danger signals, losing human leukocyte antigen (HLA) class I molecules, and aberrant expression of immunomodulatory nonclassical HLA class I antigen (Ag) can all induce tolerance in the immune system [10, 13, 14].
4. *Host immunodeficiency*: Any deficiency in the immune status of individuals can predispose them to various malignancies.

In addition, once the immune escape occurred, the immune system can profoundly influence the

prognosis, natural history, and response to different therapies either by direct effects on malignant cells or indirect effects on angiogenesis and inflammation [10, 13–15].

The immune system of each individual is subject to variability due to different environments, different diets and nutritional status, and different antigenic exposures and most importantly due to an uncountable number of polymorphisms in genes governing the immune system elements and cells [16, 17].

Genetic polymorphisms are defined as variations in human genome present in at least 1% of the population [18]. These polymorphisms were beneficiary either in their cross talk with certain environmental factors alone or in combination with their associated polymorphisms, or they were at least neutral enough not to compromise the life of the individual bearing them; therefore, they were not erased by the evolutionary pressure [16, 18, 19]. Immune response-associated genes are not an exception, and they have an uncountable number of polymorphisms [16]. For example, HLA region includes the most polymorphic genes in the human genome [16]. This high variety in immune-associated genes is a product of a long interaction with an environment consisting of numerous ever-evolving pathogens [16]. In this context, the majority of polymorphisms had the chance to be beneficiary in defense against some pathogens [17, 20, 21].

Single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTRs) (a repeat unit includes 15–100 nucleotides), and microsatellites are three important types of polymorphisms [22].

SNP is defined as a difference in a single nucleotide in the DNA sequence and is estimated to account for 90% of the human genome variations. Microsatellites, scattered through the genome with an average density of one in every 2000 pb, are variable tandem repeats of 2–8 bp, most commonly CA dinucleotide, and their alleles are differentiated by the number of repeats (Fig. 20.2) [22, 23].

Polymorphisms are able to change the immune function at several levels from expression patterns to posttranslational modifications:

1. Some polymorphisms might change DNA methylation and consequently chromatin structure and expression patterns [24, 25].
2. Some polymorphisms may disrupt transcription factor binding sites (TFBSs) and consequently influence the expression [22, 26, 27].
3. mRNAs splicing patterns can be modified by polymorphisms as a result of deletion of a splice site, creation of a new splice site, or modification of exon-splicing enhancers and silencers [26].
4. MicroRNAs (miRNAs) are important elements in gene regulation with various actions. Their binding sites might be disrupted as a result of polymorphisms [26].
5. Some polymorphisms can cause mRNA instability and its early destruction [22, 28].
6. Polymorphisms may create premature termination codons [26].
7. Exonic polymorphisms can substitute an amino acid in protein sequence, change protein structure, and consequently alter protein function [22, 27, 28].

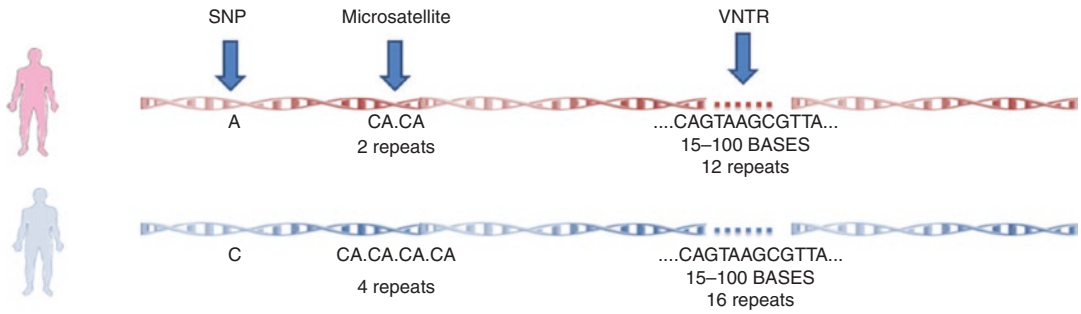


Fig. 20.2 Different types of polymorphisms in the human genome

8. Some polymorphisms may change posttranslational modification (PTM) site and consequently influence posttranslational modifications [26].

Therefore, it seems that this high genetic variability in immune response-associated genes known as immune polymorphism contributes to the observed interindividual differences [16, 21]. The aforementioned differences aside from cancer development contribute in various cancer manifestations, behavior, prognosis, and response to different treatment strategies [29–31].

20.4 Immunogenetics

20.4.1 Background

Immunogenetics, as the meeting point of two exciting fields of immunology and genetics, is a new but rapidly expanding field of science studying this immune polymorphism in order to understand the governance of genetics on the immune system [16, 32, 33].

Although the term "immunogenetics" was used earlier [34], the first milestone in the history of immunogenetics was coincident with the failed study of blood transfusion in 1952 [35]. This failure resulted in the discovery of HLA system [16, 36], which attracted the attention of biomedical researchers to interindividual differences in the immune system. From that point on, for decades, investigators tried to associate different complex diseases with various HLA types using serological methods [37, 38]. However, modern immunogenetics required more than one century of biomedical advances remarked by Mendel's laws of heredity in 1865 [18, 39], discovery of chromosomes as the cellular basis of heredity in 1902, discovery of DNA double helix as the molecular basis of heredity in 1953 [40], decoding the genetic codes, and last but not least the completion of Human Genome Project in April 2003 [18, 41, 42]. Human Genome Project not only contributed to the discovery of genetic polymorphisms but also provided an infrastructure for other large-scale projects like International HapMap Project and "1,000 Genomes Project" [43]. Discovery of approximately 25–35% of estimated nine to ten million SNPs is just one of the uncountable achievements of such projects [16, 42–44]. Genetic polymorphisms in the immune system contribute to a large part of the interindividual variation in immune response, and today, immunogenetic studies have provided a vast knowledge of the effects of immune polymorphism on the host defense. However, just the estimation that there is one SNP per every 290 bp shows that there is much more to be brought to light [43, 44].

20.4.2 Immunogenetic Tools

Along with the concert of conceptual advancements, tools employed in this field have changed in order to gather immunogenetic information more accurately, in less time and less cost [16]. Twin studies recruit twins in order to remark the importance of genetic component in susceptibility to traits and diseases [18, 45]. The result of such studies provides a rough estimation of genetic contribution to interindividual differences in immune system by comparison of concordance rates of immune traits between monozygotic and dizygotic twins [18, 38, 45]. The higher the concordance difference is, the greater the heritability [8, 18].

Upon introduction of immune polymorphism, several association studies tried to show the contribution of specific genes using the candidate gene approach or hypothesis-driven approach [18, 46]. This approach includes looking into the differences between patients and controls in allele frequencies of SNPs in genes selected based on the known pathophysiologic pathways of the disease. These studies at first employed restriction enzymes to identify specific SNPs called restriction fragment length polymorphisms (RFLPs) in the restriction site of the enzyme [47]. This approach is also known as a reductionist approach, since studies employing this approach investigate only a few genes and polymorphisms at a time [18, 46, 48].

In the early 1990s, discovery of hundreds of informative microsatellites provided the possibility of a dramatic change in the approach of

immunogenetic studies from a hypothesis-driven approach to positional approach [4, 18, 49]. The information from this project provided the immunogenetic scientists with the most suitable SNPs for genotyping in order to indirectly gather as much as information about the genome variation of an individual [18, 50]. These SNPs, which are representative of a block of SNPs, are known as tagSNPs. The extent of LD in a region determines the number of tagSNPs required to cover a region. The lower the LD is in a region, the higher number of tagSNPs is needed and therefore the higher the cost of genotyping the region is [51]. Nowadays, availability of high-throughput gene technologies such as gene chips or microarrays has enabled investigators to genotype cost-effectively, rapidly, and almost effortless hundreds of thousands to millions of SNPs at the same time [4, 38, 46, 49]; therefore, this approach is also known as "nonreductionist" approach [4]. These technological advancements were employed in community-based and large-scale GWASs in order to identify trait-associated regions with higher resolution. The results of such studies are trait-associated SNP (TAS) as a representative of the true casual variant which might be each of the known and unknown variants in whole TAS block. The TAS block is defined as all known and unknown polymorphisms in strong LD with the tagSNP [4, 18, 52]. Therefore, LD along with technological advances turned SNPs, the most common and more importantly the most stable genetic variations in human DNA, into application [53].

However, there are major limitations in GWASs to be overcome.

1. Generally, the genetic component of complex diseases originates from several major susceptibility loci and a component of as many as a dozen minor susceptibility loci known as polygenes (Fig. 20.3). These polygenes individually have small to medium impact on the overall genetic component; therefore, GWASs require a large study sample with homogenous ethnicity and phenotype to have enough high power to identify these polygenes [4, 23, 52, 54]. This is a major problem in immunogenetic studies of cancers as patients with cancers present with highly variable phenotypes. As a result, the odds ratio for each allele is typically below 1.5, and the *P* value should be less than 10^{-6} to show a significant association [7, 55].

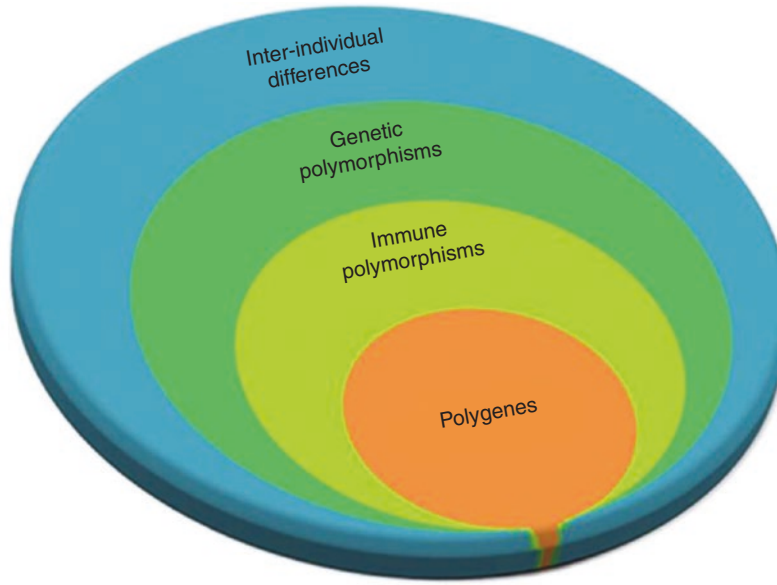


Fig. 20.3 Immune polymorphism component in interindividual differences

2. The genetic component and therefore effect of any risk allele decreases by increased exposure of populations to environmental risk factors which is the reason why some results could not be replicated in different populations [7]. For example, increased prevalence of acquired immune deficiency syndrome (AIDS) in some African populations predisposes population to different cancers disregarding their genetic background [56, 57]. This is also the case in regard to some extreme dietary patterns, smoking habits, and other environmental factors [58, 59].
3. Some cancer susceptibility variants have non-additive interactions with other genetic and environmental factors. It is possible that the effect of one variant depends on the presence of one or several specific alleles in another locus or even certain environmental risk factors. Therefore, such susceptibility variants can be detected only in GWASs with samples of patients with particular genetic and environmental background [7].
4. At least 10% of SNPs within a range of 1 kpb of hotspots are untaggable which means they do not have any LD with tagSNPs [51]. The presence of these numerous untaggable SNPs always limits the power of GWASs in finding all possible genetic associations [44]. Therefore, GWASs should employ additional sequencing within known recombination hotspots [44].
5. GWASs are less effective in some old population like African countries, since LD is generally lower in these populations due to the longer duration being affected by genetic recombination [4, 18, 52, 53].
6. The different LD, hotspots, and haplotype patterns in different populations might complicate replication studies in different populations [53]. For example, in some population, the causal variant may be separated from the associated TAS block by a hotspot.
7. Sometimes the associated TAS block does not include a causative allele but an allele beneficiary for the affected individuals with the disease, and therefore the natural selection has selected them instead of those affected individuals without the allele [16].
8. Population stratification is another source of bias in such studies as the association of the trait and TAS block may be due to an ancient branching of the population bearing both causal trait alleles and the TAS block; however, this bias can be minimized by the careful selection of the control group or by assessing population structure and correcting for it [18, 53, 60].
9. If certain alleles are associated with a more aggressive disease and lower survival, they are less presented in patients and may not be detected as a susceptibility allele [61].

After identification of associated TAS blocks by GWASs, the actual functional variant in the associated TAS block can be found by further genetic association studies employing more accurate low-throughput technologies and other SNP markers in order to finely map the associated genes and alleles in the associated TAS block [49]. In these studies, allele frequencies of polymorphisms are compared in groups of cases and controls. However, results of such association studies are often contradictory due to the heterogeneous nature of the cancers, numerous gene–gene and gene–environment interactions [62, 63]. In addition, another source of discrepancy between these studies is the limitation in study design. For example, using hospital-based controls can result in a serious selection bias since polymorphisms under investigation might have association with the diseases that hospital-based controls may have [64, 65]. Moreover, some association studies failed to consider other genetic and environmental risk factors such as socioeconomic status, nutritional statues, smoking patterns, etc. [60]. Lacking such information may cause serious confounding bias [66]. Therefore, in order to get the most benefit from results of genetic association studies and to systematize their findings, employing meta-analyses as a powerful statistical method is essential [28, 67]. Meta-analysis by pooling the results of old studies allows us to see the whole picture of the effect of a certain polymorphism [28].

Regardless of interspecies differences, there are similarities in cancer development between humans and rodents, and therefore mouse studies are a complementary tool for genetic association studies within human population [7, 68, 69]. Numerous genetically engineered mouse (GEM) models provide a simplified model of various cancers with controllable genetic and environmental background in which the effects of a unique polymorphism on the malignancy can be studied [7, 70].

Exact mechanism of action of polymorphisms can be identified using different bioinformatic tools and in vitro studies [26]. Numerous bioinformatic online and offline tools are available which can predict the effect of polymorphisms by considering amino acid biophysical properties, active site residues, metal and lipid binding sites of gene product, TFBSs, splice sites and its regulatory motifs, miRNA binding sites, and PTM sites (Table 20.1) [26]. However, bioinformatics is limited by the extent of our knowledge [24, 26].

Different in vitro methods are developed to identify functional polymorphisms. The most important ones are reporter gene assay and electrophoretic mobility shift assay (EMSA) (Figs. 20.4 and 20.5) [24]. The reporter gene

Table 20.1 A small example of different bioinformatic tools

Title	Address	Description
dbSNP	http://www.ncbi.nlm.nih.gov/SNP/	A database for SNP information
Ensembl	http://www.ensembl.org/	A database for genome information, comparative genomics, variation, and regulatory data
HapMap Consortium	http://www.hapmap.org/	A database for haplotype blocks
SNPper	http://snpper.chip.org/	Online tool available for SNP analysis
SNP3D	http://www.snps3d.org/	Online tool available for functional analysis of SNPs based on structure and sequence analysis
SNPeffect	http://snpeffect.vib.be/index.php	A database for phenotyping human SNPs and for finding information regarding SNPs effect on structure stability functional sites, structural features, and PTM sites
MutDB	http://www.mutdb.org/	Online database for human variation data with protein structural information and other functionally relevant information

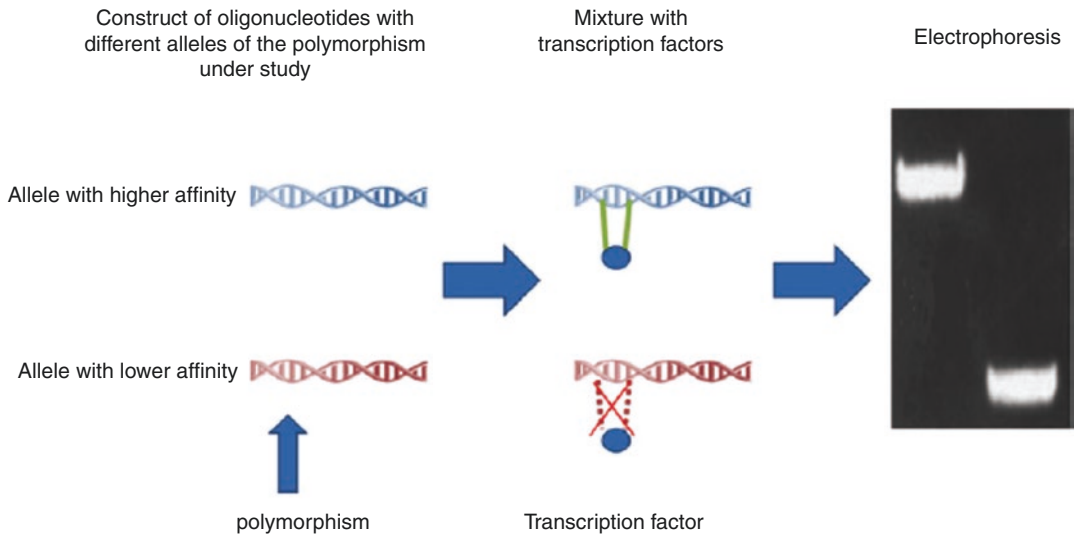


Fig. 20.4 EMSA, an in vitro experiment to measure binding affinities of different TFBS for transcription factors

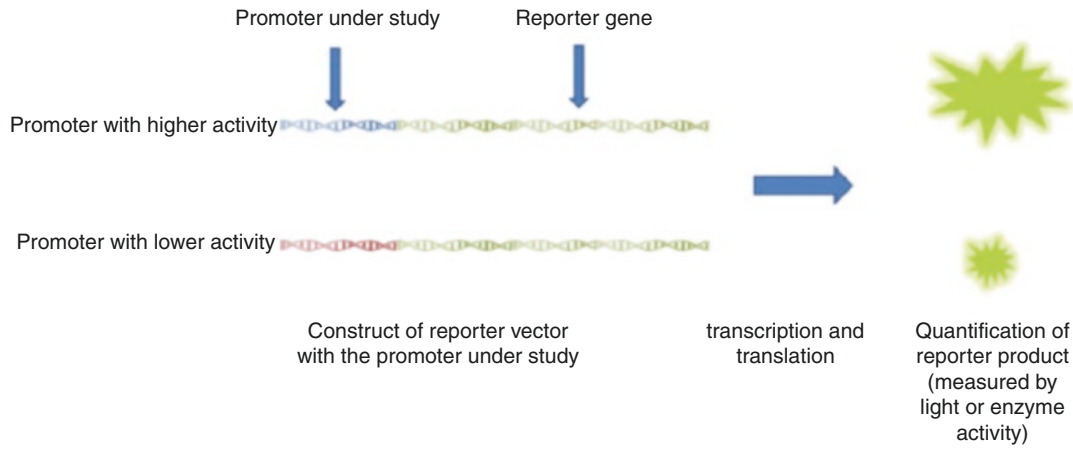


Fig. 20.5 Reporter gene assay, an in vitro tool to measure strength of different promoters

assay employs a reporter gene with a quantifiable product and clones the promoter of interest in its upstream [24, 71, 72]. Therefore, quantification of reporter gene product can provide information about the promoter strength [24, 71, 72]. On the other hand, EMSA can measure the effect of different polymorphisms on the affinity of TFBS sequence for different transcription factors. In these studies, double-stranded oligonucleotide containing the polymorphism of interest is mixed with nuclear extract with various transcription factors [24, 73, 74]. Higher affinity for these factors results in the formation of more protein–DNA complex resulting in retardation of mobility in electrophoresis [24, 73, 74].

The results from immunogenetic studies should always be interpreted with consideration of information from immunogenomics and immunoproteomics [38]. It should be noted that information from each type of study, i.e., GWASs, genetic association studies, in vitro and mouse studies, and bioinformatics, are just pieces of the complex puzzle of immunogenetics and cancer. No individual method is precise enough to see the final picture (Fig. 20.6).

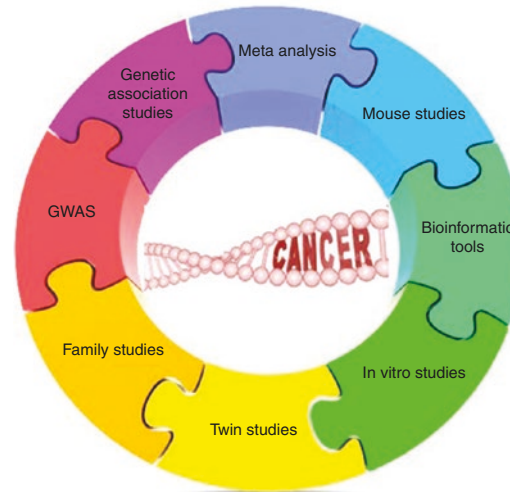


Fig. 20.6 Different methods in immunogenetic studies are pieces of a complex puzzle

20.5 Immunogenetics: A Champion in Fighting the Losing Battle Against Cancer

The application of immunogenetics in cancer is more than promising. Some variations in immune polymorphism reduce the immune capacity in

clearing either malignant transformations or cancer-inducing infectious agents and predispose bearing individuals to various cancers as exaggerated in case of most primary immunodeficiency diseases [4, 18, 21, 38]. Although each individual variant has a little informative potential for clinical application, understanding their interactions and therefore their cumulative effect is of high clinical importance [7].

Immunogenetic studies not only can help clinicians in risk assessment of individuals for susceptibility to certain cancers in order to employ preventive strategies but also may open new windows for treatment [4, 18, 21, 38, 52, 75–77]. GWASs might result in the identification of unexpected genes which in turn result in identification of new pathways in pathophysiology of cancers [52]. These new pathways not only provide a broader insight into how and why of the cancers but also may suggest new molecular targets for prevention and immunopharmacology and immunotherapy [4, 18, 38, 47, 52]. Keeping in mind that immune system provides the only antineoplastic reaction completely specific to cancer cells, it is vital to completely understand the genetic factors governing the immune system–cancer interactions and employ this knowledge in eliminating the cancers [4, 78]. In addition, this knowledge might begin a post-genomic era in individualized medicine [4, 38]. The presence of some variants in immune-associated genes might affect the success or failure in applying a particular therapy and immunogenetic information provides a way to predict toxicity and clinical effectiveness of different immune-based therapies [4, 16, 22, 38]. Therefore, employing the knowledge from immune polymorphism in prediction of treatment outcome may justify the application of an expensive partly effective treatment option [4, 16, 38, 79].

20.6 Human Leukocyte Antigen

20.6.1 Background

Human leukocyte antigens are specialized elements of the immune system in recognition of self from non-self. HLA is responsible for presenting Ags to T-cells and therefore serves as a door to the specific immune system. HLA class I Ags are on the surface of almost all nucleated cells and generally present processed endogenous antigens to $CD8^+$ cells [15, 80]. Presentation of abnormal Ags derived from intracellular pathogens or malignant transformations potentially initiates a cytotoxic T lymphocyte (CTL) response and consequently targets cell lysis [81]. By their interaction with killer cell immunoglobulin-like receptors (KIRs) on the surface of natural killer (NK) cells, HLA class I antigens regulate lytic activity of NK cells. Therefore, any change in either expression or structure of HLA class I profoundly influences T and NK cell mediated immunity [11].

On the other hand, HLA class II Ags are exclusively expressed on the surface of professional antigen-presenting cells (APC) and present processed exogenous Ags to T helper (Th) cells. Following presentation of unfamiliar Ags and in the presence of appropriate costimulatory molecules, Th cells activate effector elements of the immune system [15, 81].

Both classes of Ags comprise an intracellular, transmembrane, and an extracellular part which includes highly polymorphic antigen binding groove. From the evolutionary view, this high variety favors the chance of heterozygosity and consequently Ag presenting potential for each individual along with a significant increase in the general repertoire of the whole species for Ag presentation [16, 81].

20.6.2 Genes Behind HLA

HLA loci, located in 6p21.3 region, occupy only a small part of major histocompatibility complex (MHC) genetic system which is home to at least 220 genes [82, 83] (Fig. 20.7). MHC is divided into three classes of genes distributed from centromere to telomere. Class II with 0.9 mb is the nearest one to the centromere; class I with 1·9 Mb is near telomere, and class III with 0·7 Mb lies in between [84]. The first two classes encode for HLA class I and II and the third class consists of

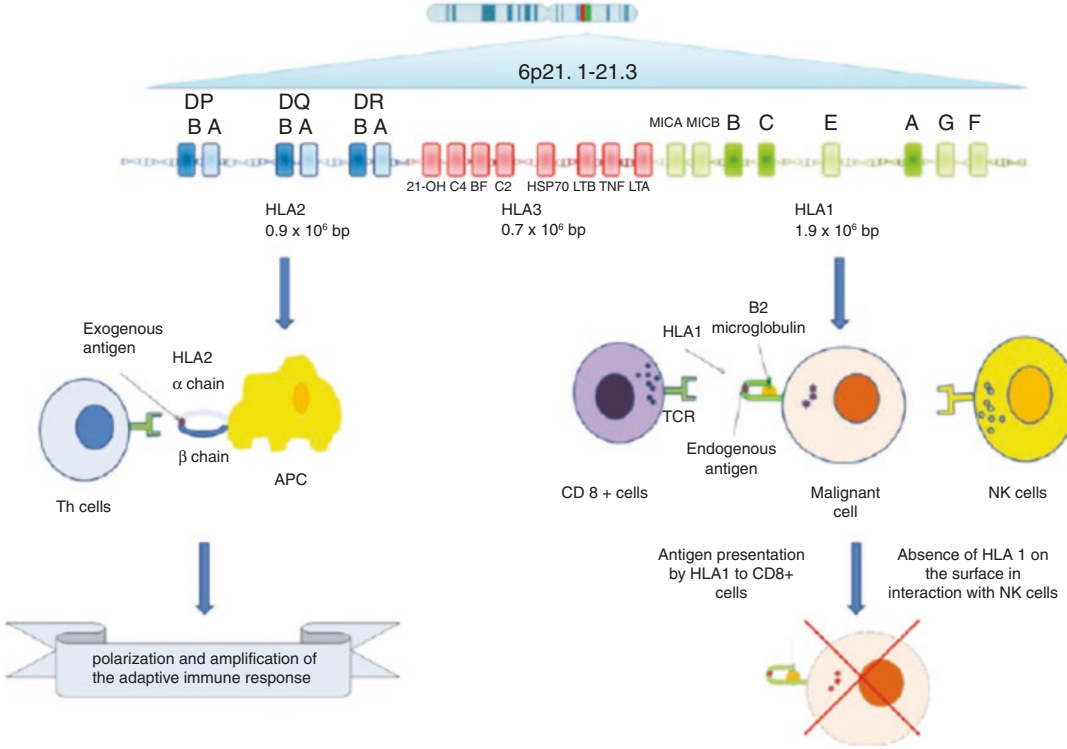


Fig. 20.7 HLA as the gate of adaptive immunity from genes to function

a group of genes encoding some members of the complement system, some cytokines like tumor necrosis factor alpha (TNF-α), heat shock proteins (HSP), and an enzyme called 21-OH hydroxylase [36, 84].

In class I, there are three highly polymorphic classic genes known as *HLA-A*, *HLA-B*, and *HLA-C*, while there are a number of nonclassical genes known as *HLA-E*, *HLA-F*, and *HLA-G* [85, 86]. Class I genes encode the highly polymorphic heavy chain of HLA class I (45 kDa) which later joins the non-polymorphic B2 microglobulin encoded by chromosome 15 [85, 86]. Classic genes consist of eight exons, but the most important exons are exons 2 and 3 encoding for peptide binding groove. Other exons encode for transmembrane region and cytoplasmic tail [36, 87]. There are two HLA haplotypes based on HLA-B leader peptide dimorphism, one that supplies the CD94/NKG2 ligands and the other that supplies KIR ligands. Genotypes harboring the first variant have more diverse and potent NK cells [88]. Beside these highly polymorphic classic HLA class I genes, there are three other HLA genes in class I known as *HLA-E*, *HLA-F*, and *HLA-G* which are more conserved. Most probably, they are not involved in Ag presentation but in interaction with more conserved parts of the immune system. For example, *HLA-E*, which is minimally polymorphic, regulates cytotoxic activity of NK cells by interacting with CD94/NKG2 lectin-like receptors. The conservation within this gene guarantees that there is a constant protection for healthy cells in most people and provides a minimum safeguard for autoimmunity [37, 89, 90]. Some of them like HLA-G are expressed on trophoblastic cells and placental chorionic endothelium and induce immune tolerance during pregnancy [85, 91–95].

Class II consists of classic genes called *DP*, *DQ*, and *DR* and nonclassic genes known as *DM* and *DO*. Classic genes encode for one highly polymorphic beta chain (26–28 kDa) and a less polymorphic alpha chain (33–35 kDa) [84].

Therefore, there are six classic *D* genes in this region. Genes for alpha chain consist of five exons, while beta chains are encoded by six exons. The exons 2 and 3 in both set of genes are responsible for encoding peptide binding domains [36].

HLA class I and *II* genes are the most polymorphic genes in the human genome with 2365, 3005, and 1848 alleles for *HLA-A*, *HLA-B*, and *HLA-C*, respectively, and 2156 alleles for class II genes (based on IMGT/HLA database, release 3.13 on July 2013) [96]. This high polymorphism is mostly clustered in several hypervariable blocks in exons 2 and 3 which are responsible for encoding antigen binding groove. Therefore, a unique combination of sequence motifs in these hypervariable regions determines each allele [15]. This genetic structure is accompanied by high LD not only between *HLA* genes but also non-HLA genes constituting extended haplotypes [97]. The majority of polymorphisms in hypervariable regions result in amino acid substitutions in peptide binding grooves, which in turn dramatically changes Ag binding affinity of the final product [15]; on the other hand, variants in noncoding regions influence transcription, translation, and splicing and thereby expression levels [81].

Nowadays, with a few exceptions, HLA alleles are named by six or even eight digits. The first two digits are representative of the serological family the allele belongs to, while the third and fourth digits distinguish between different sequences affecting amino acid sequences. The next two digits are identifiers of synonymous polymorphisms, and seventh and eighth digits are used to distinguish intronic polymorphisms or ones located into untranslated regions [98].

20.6.3 From Polymorphisms to Clinic

HLAs are involved in cancer immunity and therefore in susceptibility and prognosis mainly by presenting certain Ags known as tumor-associated antigens (TAAs). TAAs are the first contact of malignant cells with adaptive immunity. Since introduction of the first TAA in melanoma patients in 1991, a broad heterogeneous group of Ags was discovered and associated with different malignancies. This heterogeneous group can be divided into four classes of Ags [9, 99]:

1. Cancer–testis Ags are a result of epigenetic alterations leading to reactivation of silence genes. One of the famous examples is Ags from MAGE family. These Ags are not exclusive to just one type of cancer. The reason for this naming is that they are normally expressed in MHC-negative testicular germ cells and placental trophoblasts.
2. Differentiation Ags are normally expressed in the tissue of origin of the tumor, like melan-A, and tyrosinase in melanomas.
3. Unique tumor Ags are products of mutated tumor suppressor genes and oncogenes like abnormal product of RAS or p53. Fusion proteins as a result of chromosomal aberrations are also included in this group.
4. Infectious tumor Ags are expressed by oncogenic viruses associated with some malignancies. The examples are latent membrane proteins 1 and 2 (LMP-1 and LMP-2) in Epstein–Barr virus (EBV)-associated Hodgkin's lymphoma (HL) and E6 and E7 associated with human papillomavirus (HPV)-associated cervical cancer.

Nowadays, hundreds of HLA association studies prove that HLA alleles are important elements in predisposition to cancer. Seven mechanisms are suggested for complex relationship of HLA genotypes and susceptibility, prognosis, recurrence, and clinical response to immunotherapy and tumor vaccines:

1. *Efficiency in TAA presentation*: One of the major factors in Ag presenting ability of different HLA is the affinity of their Ag binding grooves to different epitopes. This affinity is highly dependent on the amino acid sequence in the hypervariable regions. Even one change in this sequence due to polymorphisms profoundly influences binding affinities to TAAs and Ags used in tumor vaccines and therefore susceptibility prognosis and response to tumor vaccines [37, 86, 100–102]. For instance, *HLA-*

A∗0207 is associated with susceptibility to EBV-associated lymphoma in East Asian population, while *HLA-A∗0201* is a protective factor; however, this huge difference at the clinical level is a result of a single amino acid change (Y99 to C) at the protein level [103, 104].

2. *Interaction with T-cells and NK cells*: Change in variable regions and constant regions involved in interaction with T-cells and NK cells can change HLA potential for inducing an effective immune response [101, 105]. This includes changes in cytokine profile, the other mainstay of immunity against cancers [106].
3. *Efficiency in inducing immune response to infectious agents*: Antigen binding abilities of different HLA alleles influence immune reaction to infectious agents associated with malignant transformation. For example, EBV is frequently emphasized as an important environmental factor in the pathogenesis of HL and nasopharyngeal carcinoma (NPC) [107]. Latent membrane protein-1 (LMP-1) and Epstein–Barr virus nuclear antigen (EBNA-4 and EBNA-6) proteins produced during latent infection by EBV are efficiently presented by A∗0201 and A∗1101, respectively [87]. Therefore, these alleles can induce a strong immune response which consequently results in resolving the infection and lower chance of malignant transformation. Another example is the protective effect of DQB1∗0301 allele on hepatitis C virus (HCV) infection, HCV-associated liver cirrhosis, and HCV-associated hepatocellular carcinoma (HCC). This allele can efficiently present majority of immunodominant epitopes of HCV [108]. Another supportive finding is that increased level of HLA-A expression is associated with uncontrolled HIV activity through blocking the NK cell mediated immunity [109]. Virus-induced cancers specifically HPV-associated cancers further induce somatic mutations in HLA genes and related gene families [110].
4. *Change in HLA expression patterns*: In some malignancies like melanoma, Burkitt's lymphoma, and carcinoma of the cervix and lung, HLA expression and Ag processing machinery are disturbed in order to prevent TAA presentation and consequently immune recognition of malignant cells. This mechanism is one of the major pathways for the immune escape of tumoral cells [11, 111]. Some polymorphisms within the noncoding regions can influence expression levels [37]. In addition, some HLA alleles are specifically lost during malignant transformation [112]. Loss of HLA-A2 in colorectal cancers, breast cancer, and cervical cancer or lower expression levels of HLA-DR4 and HLA-DR6 in melanoma is a good example for these phenomena [113, 114]. On the contrary, some alleles like HLA-B∗4405 are not dependent on some elements of the regular Ag processing machinery like transporter associated with Ag presentation (TAP) and therefore can present antigens without susceptibility to viral-induced diminished TAP function [115]. Moreover, the polymorphic nature of HLA is lost during the evolution of cancer cells. The loss of heterozygosity in HLA (LOHHLA) is a method to evaluate the prevalence of HLA loss in cancer clonal and subclonal microenvironments [116]. The increased HLA homozygosity leads to the loss of neoantigen identification and subsequent subclonal propagation of cancer cells.
5. *Increased susceptibility to chronic infections or autoimmunity*: Some HLA haplotypes and alleles are associated with various chronic inflammatory diseases which in turn predispose individuals to various cancers [79, 117]. Excess growth factors and prolonged proliferation in the background of chronic destruction increase the risk of malignant transformation [117]. In addition, chronic immune stimulation of B-cells and prolonged and repeated DNA double-strand breaks associated with somatic hypermutation (SHM) and class switch recombination (CSR) significantly increase the chance of malignant transformation, and therefore, autoimmunity and chronic infection are important risk factors for some hematological malignancies like non-Hodgkin's lymphoma (NHL) [117]. In these cases, HLA alleles can affect the extent of immune reaction and stimulation of B-cells [117]. For instance, *HLA-*

DRB1∗0301, *HLA-B∗0801 HLA-DRB1∗0101*, and *HLA-DRB1∗0401*, the susceptibility alleles of NHL are associated with autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome, and celiac disease [102, 108, 118]. The more prominent example is the paradoxical relationship of DQB1∗0301 with HCV infection and HCV-related B-cell lymphoma. While DQB1∗0301 is associated with a better immunologic control of HCV and a self-limiting infection, it is a susceptibility factor for HCV-related NHL. In this case, efficient presentation of viral antigens by DQB1∗0301 in the context of persistent HCV infection results in $CD4^+$-dependent chronic stimulation of B-cells [108].

6. *Sensitivity to mutation*: It is suggested that some HLA alleles are more susceptible to mutations like rearrangements of the DNA material and crossover. Such dramatic alterations might influence the function of oncogenes or tumor suppressors in the proximity of HLA genes. An example of such an oncogene is Waf1/p21 gene, located in 6p21.1 [105].
7. *Linkage disequilibrium*: LD with non-HLA genes of class III or even nonclassical HLA in the form of extended haplotypes can justify some of the founded associations. LD with non-HLA genes like *TNF-α*, in context with extended haplotype, can influence the relationship between toxicity of immunotherapy and HLA alleles. For example, high TNF-α increases the IL-2 toxicity in patients with melanoma [119, 120].
8. *HLA subtypes naturally suppressing the immunity against tumors*: HLA-G and HLA-E are both involved in cancer development through inhibiting NK cell mediated cytotoxicity by immunoglobulin-like transcript 2 (ILT2) [121]. Thus, polymorphisms resulting in higher expression of these HLAs are associated with poor prognosis and high recurrence, as studied about HLA-G+3027 in HL [122]. Hypoxia-induced factor 1 (HIF-1) which is released in hypoxic conditions of cancer hypermetabolic states is a regulator of HLA-G expression [123].

20.6.4 HLA Typing and HLA Association Studies: Lessons from the Past

HLA has a history as long as immunogenetics itself. An observation of transfusion failures in 1952 paved the road to the discovery of the first HLA allele by Dausset [124]. Since 1958, there was a continuous international effort in order to share experimental data and HLA typing technologies, identify new HLA alleles and serotypes, and uncover the role of HLA system in pathogenesis of numerous diseases [36]. The result of such effort was the identification of over 9500 alleles for HLA class I and II over a short period of four decades [36]. Along with the discovery of new alleles, the first nomenclature committee was held in 1987 followed by several nomenclature committees to unify the nomenclature and classification [36].

Early studies employed low-resolution serological methods which detected HLA on T-cells or B-cells [125]. Although these serological methods were subject to huge development in detection methods from complement-dependent cytotoxicity test to ELISA method, flow cytometry, and Luminex technique, the real breakthrough in HLA association studies was the introduction of PCR and high resolution DNA-based typing methods [36]. This technology allowed not only detection of high HLA polymorphisms with higher sensitivity and specificity but also the detection of new alleles with more flexibility by simply adding new probes to the old panels [126]. Nowadays, the old DNA-based method employing PCR-RFLP has been replaced by more rapid tests [126]. Generally, they either identify PCR products containing hypervariable regions by hybridization with sequence-specific probes (SSO) or employ sequence-specific primers (SSP) to identify variants as part of PCR process itself [15, 36, 127]. The latter was extensively used back in mid-1990s [15, 36, 127]. Even though aberrant typing as a sign of new allele can be followed by direct DNA sequencing, both methods are ineffective in case there is a new allele [15]. Later this limitation was overcome by polymerase chain reaction-sequence-based

typing which can directly detect the sequence of alleles. In this method which is based on dye terminator chemistry, dye bounded 2,3 dideoxynucleotides are used as substrates for PCR process. Randomly addition of labeled dideoxynucleotides, and consequently, a stop in elongation of DNA chain result in the development of numerous DNA fragments with different sizes. These DNA fragments can easily be separated by capillary electrophoresis, and the ending dideoxynucleotides can be identified by specific fluorescence emitted from the related dye.

In parallel, huge efforts were made to understand the role of these alleles in etiology and natural history of several diseases. In oncology, the first association was found in HL in 1967 [37]. This finding triggered a series of HLA association studies on different cancers worldwide. The fruit of this global movement was finding association between HLA alleles and susceptibility to several hematological malignancy including HL, NHL, childhood acute lymphoblastic leukemia, Kaposi's sarcoma, chronic myeloid leukemia (CML), and also non-hematological malignancies including nasopharyngeal carcinoma, thyroid cancer, renal cell carcinoma (RCC), cervical cancer, and both melanoma and non-melanoma skin cancers [15, 128]. Moreover, investigations on natural history of cancers showed relationship of several alleles from both classes with mortality in ovarian cancer, non-small cell lung carcinoma, head and neck squamous carcinoma, and local recurrence in melanoma [77, 101, 105]. Several studies showed importance of HLA context in the outcome of immunotherapy and tumor vaccines in melanoma, RCC, cervical carcinoma, and CML [77, 100, 120, 129].

Although the result of such studies was inconsistent in some cases, most studies pointed to the undeniable role of HLA polymorphism in susceptibility, prognosis, natural history, and response to immunotherapy in different cancers [37].

These past experiences emphasize that a prestigious HLA association study is a complex art rather than a simple case–control study and several factors should be considered in interpreting their results. In this regard, results of meta-analysis of these association studies are more reliable (Table 20.2).

Table 20.2 Significant results from published meta-analysis of HLA associations with cancers

Alleles	Cancer site	Number of cases	Number of controls	OR ± 95% CI	Population included	References
DQB1∗03	Hepatocellular carcinoma	398	593	0.65 (0.48–0.89)	China, Italy, Spain, Egypt	Xin et al. [130]
	Cervical cancer	163	410	0.85 (0.74–0.97)	USA, England, Senegal, Sweden, Uppsala, Japan, Venezuela, China, Brazil, India, Mexico, Tunisia	Zhang et al. [131]
DQB1∗0301	Cervical cancer	917	2742	1.14 (1.06–1.23)	USA, England, Senegal, Sweden, Uppsala, Japan, Venezuela, China, Brazil, India, Mexico, Tunisia	Zhang et al. [131]
DQB1∗02	Hepatocellular carcinoma	398	593	1.78 (1.05–3.03)	China, Italy, Spain, Egypt	Xin et al. [130]
	Cervical cancer	441	1361	0.91 (0.82–0.99)	USA, England, Senegal, Sweden, Uppsala, Japan, Venezuela, China, Brazil, India, Mexico, Tunisia	Zhang et al. [131]
DQB1∗0402	Cervical cancer	142	1731	1.31 (1.04–1.64)	USA, England, Senegal, Sweden, Uppsala, Japan, Venezuela, China, Brazil, India, Mexico, Tunisia	Zhang et al. [131]
DQB1∗05	Cervical cancer	213	823	1.18 (1.01–1.38)	USA, England, Senegal, Sweden, Uppsala, Japan, Venezuela, China, Brazil, India, Mexico, Tunisia	Zhang et al. [131]

(continued)

Table 20.2 (continued)

Alleles	Cancer site	Number of cases	Number of controls	OR ± 95% CI	Population included	References
DQB1∗0502	Hepatocellular carcinoma	257	349	1.82 (1.14–2.92)	China, Spain	Xin et al. [130]
DQB1∗0602	Hepatocellular carcinoma	173	226	0.58 (0.36–0.95)	China, Spain	Xin et al. [130]
DQB1∗0603	Cervical cancer	236	3608	0.62 (0.53–0.72)	USA, England, Senegal, Sweden, Uppsala, Japan, Venezuela, China, Brazil, India, Mexico, Tunisia	Zhang et al. [131]
DRB1∗01	Hepatocellular carcinoma	2030	2817	0.53 (0.29–0.96)		Liu et al. [132]
	Nasopharyngeal carcinoma	786	1282	0.55 (0.39–0.78)	USA, China, Greece, Tunisia, Singapore	Yao et al. [133]
DRB1∗03	Nasopharyngeal carcinoma	1152	1600	1.55 (1.30–1.86)	USA, China, Greece, Tunisia, Singapore	Yao et al. [133]
	Cervical cancer	272	1352	0.74 (0.59–0.91)	China	Wei et al. [134]
DRB1∗07	Hepatocellular carcinoma	281	466	1.65 (1.08–2.51)	China, Italy, Spain, Egypt	Lin et al. [135]
		156	224	2.1 (1.06–4.14)	China	Lin et al. [135]
		125	242	1.41 (0.83–2.42)	Italy, Spain, Egypt	Lin et al. [135]
DRB1∗08	Nasopharyngeal carcinoma	786	1282	1.44 (1.08–1.92)	USA, China, Greece, Tunisia, Singapore	Yao et al. [133]
	Cervical cancer	87	967	0.68 (0.52–0.90)	China	Wei et al. [134]
DRB1∗09	Nasopharyngeal carcinoma	786	1282	1.33 (1.06–1.67)	USA, China, Greece, Tunisia, Singapore	Yao et al. [133]
DRB1∗10	Nasopharyngeal carcinoma	686	1123	1.82 (1.02–3.26)	USA, China, Greece, Tunisia	Yao et al. [133]
DRB1∗11	Hepatocellular carcinoma	2030	2817	0.58 (0.38–0.88)		Liu et al. [132]
	Nasopharyngeal carcinoma	786	1282	0.62 (0.42–0.91)	USA, China, Greece, Tunisia, Singapore	Yao et al. [133]
DRB1∗12	Hepatocellular carcinoma	281	516	1.59 (1.09–2.32)	China, Italy, Spain, Thailand	Lin et al. [135]
		206	324	1.73 (1.17–2.57)	China, Taiwan	Lin et al. [135]
		75	192	0.3 (0.04–2.47)	Spain, Italy	Lin et al. [135]
	Nasopharyngeal carcinoma	786	1282	0.62 (0.47–0.81)	USA, China, Greece, Tunisia, Singapore	Yao et al. [133]
		2030	2817	1.49 (1.08–2.07)		Liu et al. [132]
DRB1∗14	Hepatocellular carcinoma	2030	2817	1.89 (1.27–2.82)		Liu et al. [132]
DRB1∗15	Hepatocellular carcinoma	281	466	1.7 (0.8–3.59)	China, Italy, Spain, Egypt	Lin et al. [135]
		156	224	3.22 (1.63–6.37)	China	Lin et al. [135]
		125	242	0.8 (0.34–1.89)	Spain, Egypt, Italy	Lin et al. [135]
	Cervical cancer	362	1307	1.62 (1.36–1.93)	China	Wei et al. [134]

Table 20.2 (continued)

Alleles	Cancer site	Number of cases	Number of controls	OR ± 95% CI	Population included	References
DRB1∗0701	Cervical squamous cell carcinoma	1445	2206	1.59 (1.09–2.35)	Iran, USA, England, Sweden, France, Brazil	Yang et al. [136]
		1083	1248	1.29 (1.02–1.63)	Caucasians	Yang et al. [136]
DRB1∗1301	Cervical squamous cell carcinoma	2743	3904	0.63 (0.52–0.78)	Iran, USA, England, Sweden, France, Brazil	Yang et al. [136]
		2013	2360	0.61 (0.48–0.77)	Caucasians	Yang et al. [136]
DRB1∗1302	Cervical squamous cell carcinoma	1877	2966	0.49 (0.36–0.68)	Iran, USA, England, Sweden, France, Brazil	Yang et al. [136]
		2013	2360	0.75 (0.57–0.98)	Caucasians	Yang et al. [136]
DRB1∗1501	Cervical squamous cell carcinoma	1915	2628	1.42 (1.23–1.65)	Iran, USA, England, Sweden, France, Brazil	Yang et al. [136]
		2191	2628	1.22 (1.01–1.47)		Yang et al. [136]
DRB1∗1502	Cervical squamous cell carcinoma	1424	2184	1.87 (1.08–3.26)	Iran, USA, England, Sweden, France, Brazil	Yang et al. [136]
DRB1∗1503	Cervical squamous cell carcinoma	432	894	3.4 (1.69–6.87)	Iran, USA, England, Sweden, France, Brazil	Yang et al. [136]
DRB1∗1602	Cervical squamous cell carcinoma	1314	2234	0.61 (0.38–0.98)	Iran, USA, England, Sweden, France, Brazil	Yang et al. [136]
DRB1∗0403	Cervical squamous cell carcinoma	1796	2050	2.05 (1.02–4.12)	Caucasians	Yang et al. [136]
DRB1∗0405	Cervical squamous cell carcinoma	1496	1700	6.13 (1.03–36.33)	Caucasians	Yang et al. [136]
DRB1∗0407	Cervical squamous cell carcinoma	1796	2050	2.71 (1.11–6.61)	Caucasians	Yang et al. [136]
DRB1∗0901	Cervical squamous cell carcinoma	1796	2050	0.58 (0.34–0.99)	Caucasians	Yang et al. [136]

20.6.5 Typing Methods

Indeed, immunogenetic studies are deeply influenced by technological advances. Low-resolution serologic HLA typing was one of the major limitations in early studies [87]. Serologic typing is only enabled to identify the family of alleles. This family often comprises a heterogeneous group of alleles with different affinities and different potential for Ag presentation. Since distribution of alleles belonging to the same serotype is different in various populations, such studies often obtained conflicting results in different populations. One of the best historical examples is HLA association studies in nasopharyngeal carcinoma (NPC).

NPC, as an epithelial carcinoma of the head and neck origin, was one of the main focuses of early HLA association studies. Early serological studies showed an association between HLA-A2 and NPC in Chinese population, while studies in Caucasians found HLA-A2 as a protective allele for both NPC and EBV-associated HL [115, 137–141]. Later, higher-resolution studies showed

HLA-A∗02:07, a common allele in Chinese population but rare among Caucasians, as the main risk factor, while HLA-A∗02:01, a common allele in Caucasians, was shown to be the actual protective factor in this population [142, 143]. Further associations among DRB1 and DQB1 variants with cervical squamous cell and hepatocellular cancers were identified, which are depicted in Table 20.2. It is possible that future studies employing higher-resolution methods reveal even new causal variants within the current associations.

20.6.6 Environmental Factors

Various environmental and genetic factors play roles behind scenario of cancer, and malignant transformation is the result of a complex interaction between these factors. It is often the case that certain genetic factors need certain environmental factors to play their role in pathogenesis of cancer. The role of environmental factors in HLA association studies is more prominent in virus-associated malignancies like HL, NPC, and cervical cancer. Each virus has different strains with different Ags and the prevalence of these strains is not the same in different populations. Each strain is best presented by certain HLA alleles. Therefore, one HLA allele efficient for presenting Ags of one population's prevalent strain may not present Ags of another population's prevalent strain efficiently [87]. Such a phenomenon might be extended to other environmental factors like virus prevalence, viral load, diet, cigarette smoking, and socioeconomic status, all of which are highly dependent on the population under study [78, 144]. For instance, pathogenesis of cervical cancer is dependent on persistent infection with high-risk human papillomavirus (HPV) and this risk factor itself is highly related to socioeconomic status, sexual relationship, and prevalence of high-risk variants in the region [144, 145].

20.6.7 Linkage Disequilibrium

MHC region is home to more than 200 genes beside classic HLA genes. Due to the low recombination rates, these genes are often in strong linkage disequilibrium together [82]. This strong LD can complicate finding the actual causal allele. The problem gets worse when the causal allele is an unknown allele in strong LD with the associated allele. This limitation can be overcome by whole genome sequencing (WGS) of the region in close proximity of the associated allele [107]. One example is the association of NPC with HLA-A∗0207 and HLA-B∗4601 which are in strong LD. In this case, either allele, both of them, or even a third allele in LD with both of them might influence the pathogenesis of NPC [143].

Some studies reported extraordinary LD in MHC region between alleles from one class and alleles of other classes and even non-HLA genes. This extraordinary haplotypes are known as extended haplotypes [87]. Thus, in interpreting results of HLA association studies or design of one, non-HLA genes such as the *transporter associated with Ag processing (TAP) MHC class I chain-related A (MIC-A), heat shock proteins (HSP)*, and *TNF-α* which are located nearby or within the classic HLA genes should be considered [82, 87]. These extended haplotypes are especially of importance in immunogenetic studies of cancers, since numerous elements of the immune system are in the front line of defense against cancer.

For instance, the ancestral haplotype 8.1 (AH 8.1: HLA-A∗01-B∗08-Cw∗07-DRB1∗03-TNF-G308A), in which HLA alleles are in LD with TNF-α, is the most frequent extended MHC haplotype in Caucasian populations [119]. Primarily, this extended haplotype was associated with clinical course of NHL [79, 119]; however, later studies showed that polymorphism in *TNF-α* gene has a more prominent effect in this association compared to Cw∗07 and DRB1∗03 alleles [9, 79]. In this case, polymorphisms in TNF-α promoter influence TNF-α expression levels. TNF-α level consequently affects the extent of immune activation upon tumor challenge. In addition, increased TNF-α impairs Ag presentation potential of APCs and by its effect on cytokine profile results in a bias toward Th2 immune responses [79]. All these factors can contribute to the exacerbation of systemic symptoms, anemia, hypoalbuminemia, and poor outcome [9].

Another example is the association of HLA-A∗03 and chronic myeloid leukemia (CML) [82]. A translocation between *t*(9,22)(q34;q11) creating a truncated chromosome 22 known as Philadelphia chromosome is present in majority of patients with CML [146]. Depending on the precise location of the fusion, different fusion proteins are encoded. Keeping this in mind and the absence of costimulatory molecules on CML cells, it is improbable that the association of HLA-A∗03 is due to its efficiency in presenting fusion proteins and its ability to induce an effective immune response [82]. However, this allele is in LD with the C282Y mutation of the *hemochromatosis gene*, a susceptibility marker for CML [82].

In some cases, an optimal immune response is dependent on optimal Ag presentation by both HLA classes and the presence of certain alleles in non-HLA genes. An absence of one of these optimal alleles may result in anergy and immune escape. In some populations, these alleles might be in LD in form of an unknown extended haplotype, while in other populations this haplotype might be absent [61]. One of such associations has been reported between cervical squamous cell carcinoma and multi-locus haplotype of B∗4402-Cw∗0501-DRB1∗0401-DQB1∗0301 [61].

20.6.8 HLA and Cancer Diagnosis, Prognosis, and Treatment

HLA typing and studying their association with cancer development would be beneficial if utilized in improving the early diagnosis, defining the prognosis of cancers and their response to treatment strategies. Quantifying HLA-G expression level regardless of the HLA subtype may be a predictive tool in distinguishing benign and malignant lesions and determining the prognosis of cancerous lesions [147, 148]. Moreover, targeting HLA-G by siRNA and specific Abs has resulted in improved NK cell mediated cytolysis and Th2 related cytokine expression, preventing tumor progression [149, 150].

Not only considering certain HLA types which influence the response of cancer cells to different immunotherapy methods helps in predicting the efficacy of an immunotherapy method [151], but also this is used to reinforce development of specific cancer vaccines through determining HLA hotspots of neoantigen recognition [152].

T-cell mediated immunotherapy methods like anti-CTLA-4 and PD-1 inhibitors and checkpoint blockade therapies are strongly affected by HLA expression patterns [111]. Thus, restoring HLA expression patterns is one pivotal solution for improving the response to these treatments requiring T-cell activation. On the other hand, abnormal expression of certain biomolecules like SHP2 in prostate cancer is the main cause of HLA suppression [153]. Targeting these negative regulators readily increases T-cell mediated immunity.

20.7 The Cytokine Network

20.7.1 Background

Cytokines are a group of soluble regulatory factors by which the immune system controls and modulates different activities of its cells. Each cytokine triggers certain cascade of events in their target cells by binding to their receptors and activating intracellular signal transduction pathway [16, 22]. Cytokine network is responsible for coordination of effector actions of different elements of the immune system, as well as the differentiation and proliferation of different immune cells. In addition, secretion of antibodies and inflammation is tightly regulated by complex interaction between these cytokines [15, 25, 28].

Chronic inflammation, by inducing chronic tissue damage and compensatory cell proliferation, is considered a major promoter of malignant transformations. As an example, nitric oxide, produced during inflammation, might damage DNA structure in different tumor suppressor genes and oncogenes [154]. Therefore, any dysregulation in cytokine network can result in excessive production of tumor-inducing factors, DNA damage, angiogenesis, and dysplasia and consequent development of various inflammatory diseases including different cancers [28, 155]. Cytokine network is a determinant factor in the development of metastasis and natural history

of cancers [28]. In some cancers, malignant cells can manipulate cytokine network in order to escape immunosurveillance or promote their own proliferation [154, 156]. In addition, cytokine network can influence the outcome and toxicity of different immunotherapy methods [15, 22, 157]. Several cancers including hepatocellular carcinoma (HCC), oral squamous cell carcinoma, melanoma, the gastric, pancreatic, and prostate cancer were associated with high levels of certain proinflammatory or antiinflammatory cytokines [28].

Cytokine levels are not the same in all individuals. Interindividual differences in cytokine levels in both baseline and stimulated phases are a result of both genetic and environmental factors [157]. Since there is not an intracellular storage for cytokines, their secretion is dependent on the transcriptional and translational rates of their genes [16, 28]. Not surprisingly, genes responsible for encoding cytokines and their receptors are relatively polymorphic [15, 22, 25]. Several polymorphisms in their gene can affect their expression, structure, and activity [22, 25, 28, 154, 158]. Most of these polymorphisms are in noncoding regions including promoter or intronic sequences and exonic regions are usually highly conserved [15, 16]. So far, numerous genetic association studies have been suggested as associations of these SNPs with various cancers in different populations. However, results of such studies were often inconsistent, and the reported associations varied not only in different populations but also in different cancers and even in their different subtypes [155]. Therefore, a meta-analysis of these studies can show some more conclusive evidence of these associations.

In addition to polymorphisms of cytokine genes, there are other polymorphic elements such as various transcription factors and cytokine-specific receptors which are involved in actions of cytokine network [22, 28]. For instance, polymorphisms in the nuclear factor-kappa B (*NF-κB*) gene, one of the most important transcription factors, can result in extensive changes in the cytokine network by altering transcription of TNF-a, IL-1, IL-6, and IL-8 [22]. Although the exact roles of these polymorphisms in tumor immunology are less clear, the relevance of this role is becoming more and more apparent in recent years [22].

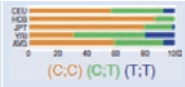
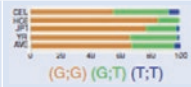
20.7.2 Interleukin-1 Superfamily

IL-1α and IL-1β and their antagonist IL-1Ra are members of this superfamily with pleiotropic effects on inflammation, immunity, and hemopoietic system. High levels of IL-1 are found in tumor sites; however, IL-1 family plays an ambivalent role in tumor immunity. IL-1 induces cytokine secretion from T-cells to potentiate the differentiation and function of immunosurveillance cells. On the other hand, IL-1 induces the expression of adhesion molecules, matrix metalloproteinases, growth factors, and angiogenic factors and promotes invasiveness and metastasis of malignant cells [159, 160].

20.7.2.1 Interleukin-1 α

IL-1α is encoded by seven exons of a gene located in 2q14. Variant −889C>T (rs1800587) is one of the common promoter variants of IL-1α gene (Table 20.3). Although the promoter containing T

Table 20.3 Genotype details for SNPs of IL-1

SNP	GMAF[a] [161]	Population diversity[b] [162]	Change at DNA level	Change at protein level	Effect on cytokine level
rs1800587	$T = 0.253$		−889C>T	NA[c]	T allele: ↑
rs17561	$T = 0.203$		+4845G>T	Ala114Ser	T allele: ↑

[a]GMAF: the minor allele frequency in 1094 worldwide individuals provided from 1000 genome phase 1 genotype data
[b]*CEU* European, *CHB* Han Chinese, *JPT* Japanese Tokyo, *YRI* Yoruba African, *AVG* Mathematical average of all samples
[c]*NA* not applicable

allele has been shown to result in a marginally higher level of expression, at the protein level, T allele was associated with significantly increased IL-1α levels which could not be justified by only different expression patterns. Further studies showed that this SNP has high LD with an exonic SNP in +4845G>T (rs17561) resulting in substitution of alanine with serine at the position of 114 which results in more efficient process of pre-IL-1α compared to Ala114 and consequently higher release of IL-1α [25].

20.7.2.2 Interleukin-1 β

High levels of IL-1β have been shown to be associated with increased risk of most human cancers and also poor prognosis in cancer patients [154, 156, 163]. IL-1β is encoded by a 7.5 kb gene with seven exons located on 2q14. Its expression is regulated by two distal and proximal promoter elements [164, 165]. So far, several polymorphisms have been identified in this gene. −511C>T (rs16944) and −31C>T (rs1143627) are two common variants in the promoter region, and +3954C>T (rs1143634) is a common synonymous polymorphism in coding region of IL-1β gene (Table 20.4) [28].

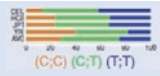
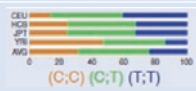
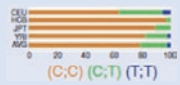
In northern and western European ancestry (CEU), −511C>T (rs16944) and −31C>T (rs1143627) had strong LD ($r^2 = 0.94$) [28, 156]. In vivo, −511T/−31T haplotype has been associated with higher IL-1β levels in the lungs and gastric mucosa. It is suggested that −31C>T (rs1143627) is the causal variant of this haplotype [25, 165]. In the same line, in vitro studies like luciferase reporter assay showed higher expression of luciferase gene with promoter containing T allele in −31C>T (rs1143627) [25]. Results of EMSA studies suggested that this higher expression is a result of higher affinity for several transcription factors as a result of a change in a TATA-box motif [25].

T allele in rs1143634 was associated with increased IL-1β secretion and several inflammatory diseases [156]. However, no evidence on the functionality of +3954C>T (rs1143634) is available, and it seems that +3954C>T (rs1143634) is just a marker for a functional polymorphism such as −31T>C (rs1143627) [25, 28].

A meta-analysis of 81 case–control studies with 19,547 patients with HCC, gastric, lung, blood, cervical, esophageal, prostate, breast, and skin cancers and 23,935 controls showed that, overall, −511C>T (rs16944) has no significant association with cancers [156], while another meta-analysis of 26 studies with 8083 patients with cancer and 9183 controls showed a significant association of +3954C>T (rs1143634) with increased risk of cancers in a dominant model which is in accordance with the results of another meta-analysis of 33 studies (Table 20.5) [156, 175].

Meta-analysis of the association between −511C>T and gastric cancer development among Chinese population reported a significant association under all genetic models [167]. Another meta-analysis of studies on associations between *IL-1β* gene polymorphisms and gastric cancer published from January 2000 to December 2009 (including 18 studies with 4111 controls and 3295 cases for −511C>T (rs16944), 21 studies with 5883 controls and 3786 cases for −31T>C

Table 20.4 Genotype details for SNPs of IL-1β

SNP	GMAF [161]	Population diversity [162]	Change at DNA level	Change at protein level	Effect on cytokine level
rs16944	*T* = 0.465		−511C>T	NA	T allele: ↑
rs1143627	*C* = 0.4808		−31C>T	NA	T allele: ↑
rs1143634	*T* = 0.146		+3954C>T	NA	UA[a]

[a]*UA* unavailable

Table 20.5 Significant results from published meta-analysis of associations of IL-1β polymorphisms with cancers

Alleles	Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
rs16944	Gastric cancer	2041	2441	TT + CT vs. CC	1.23 (1.09–1.37)	Italy, Japan, China, Korea, Portugal, UK, mixed Asian	Vincenzi et al. [166]
		5136	5332	T vs. C	1.21 (1.07–1.37)	China	Chen et al. [167]
				TT vs. CC	1.41 (1.11–1.80)		
				CT vs. CC	1.26 (1.05–1.50)		
				TT + CT vs. CC	1.31 (1.08–1.58)		
				TT vs. CC + CT	1.24 (1.05–1.47)		
		9066	11,192	TT vs. CC + CT	1.15 (1.03–1.29)	Polish/Scotland, Portugal, Germany, China, USA, Taiwan, Brazil, Korea, Costa Rica, Italy, Japan, Honduras, Finland, Spain, Sweden, India, Romania	Park et al. [168]
	Cervical cancer	836	980	TT vs. CC	1.74 (1.28–2.36)	Egypt, Korea, India, China	Xu et al. [156]
				CT vs. CC	1.71 (1.32–2.23)		
				TT + CT vs. CC	1.74 (1.35–2.23)		
		1210	1388	TT vs. CC	1.56 (1.22–1.99)	Egypt, Korea, India, China	Wu et al. [169]
				CT vs. CC	1.61 (1.31–1.99)		
				TT + CT vs. CC	1.60 (1.31–1.95)		
		836	980	T vs. C	1.38 (1.05–1.82)	Korea, India, China, Egypt	Lee and Song [170]
				TT + CT vs. CC	1.72 (1.34–2.21)		
				TT vs. CC	1.74 (1.28–2.37)		
	Hepatocellular carcinoma	890	821	CT vs. CC	0.75 (0.60–0.94)	Japan, Taiwan, Thailand	Xu et al. [156]
				TT + CT vs. CC	0.68 (0.47–0.99)		
	Blood cancers	3839	3762	CC + CT vs. TT	1.19 (1.04–1.37)	Italy, Spain, Germany, USA, Canada, Greece	Xu et al. [156]
	Prostate cancer	1425	1563	T vs. C	0.86 (0.77–0.96)	Mixed population	Xu et al. [171]
				TT vs. CC	0.74 (0.58–0.94)		
				TT vs. CC+CT	0.79 (0.63–0.98)		

Table 20.5 (continued)

Alleles	Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
rs1143627	Lung cancer	3435	4719	TT + TC vs. CC	1.23 (1.06–1.43)	China, Italy, mixed European, Denmark	Li and Wang [172], Peng et al. [173]
	Gastric cancer	1535	2585	TT + TC vs. CC	1.16 (1.01–1.33)	Korea, Mexico, China, Brazil, Italy, USA	Vincenzi et al. [166]
	Hepatocellular carcinoma	1039	1588	CC + CT vs. TT	1.31 (1.09–1.57)	Japan, Taiwan, Morocco	Jin et al. [174]
	Prostate cancer	787	771	CT vs. CC	1.35 (1.00–1.80)	Mixed population	Xu et al. [171]
rs1143634	Malignancy	8083	9183	TT + CT vs. CC	1.15 (1.01–1.30)	Sweden, Poland, China, UK, Germany, Tunisia, Costa Rica, Oman, USA, Greece, Netherlands, Norway, Japan	Xu et al. [156]
	Gastric cancer	2359	3613	CT vs. CC	1.16 (1.03–1.32)	USA, China, UK, Germany, Italy, Japan, India, Sweden, Oman	Zhang et al. [175]
	Oral cancer	346	417	CT vs. CC	0.65 (0.45–0.94)	Greece, China	Zhang et al. [175]
				TT + CT vs. CC	0.69 (0.49–0.98)		
	Lung cancer	8907	9760	CC + CT vs. TT	0.92 (0.86–0.99)	Norway, Japan, France, USA	Li and Wang [172]

(rs1143627) polymorphism, 10 studies with 3610 controls and 1559 cases for +3954C>T (rs1143634)) showed significantly increased risk of cancer in individuals with IL-1β −511T allele. In stratified analysis for different ethnicities, such an association was present in Caucasians but not in Asians or in Hispanics. This study also showed such an association for intestinal-subtype and noncardia gastric cancer [176, 177]. However, this study did not show any significant association between gastric cancer risk and −31T>C (rs1143627) and +3954C>T (rs1143634) [176]. Older studies conducted on 2005 and 2007 more or less showed such pattern for this SNP [166, 177]. However, a meta-analysis of five studies published up to September 2008 showed association of +3954C>T (rs1143634) and gastric cancer risk in Chinese and Japanese population [178]. Interestingly, a meta-analysis conducted on 6108 gastric cancer patients reported that although there was no significant association between −31T>C and gastric cancer, this polymorphism significantly increased gastric cancer risk in the presence of H. pylori infection [179]. In line with that, another study on 20,000 cases and controls found a significant association of −511C>T with gastric cancer, enhanced by H. pylori infection and Asian ethnicity [168].

Studies on cervical cancer patients have also reported significant association of −511C>T with cervical cancer development under allelic, homozygote, heterozygote, and dominant genetic models [169, 170]. Another systematic review evaluating associations of HCC with polymorphisms of IL-1 gene (reported up to September 2010) and a meta-analysis of 1279 patients with lung cancer and 2248 controls failed to support any significant increase in risk for −511C>T (rs16944) and −31C>T (rs1143627) [173, 180]. However, a meta-analysis of the association

between −31C>T, +3954C>T, and lung cancer resulted in significant association for both polymorphisms [172].

A comprehensive study on prostate cancer and different polymorphisms of IL-1β showed that there is a significant association between −511C>T and prostate cancer in allelic, homozygote, and recessive models and also between −31C>T and prostate cancer only in heterozygote model [171]. No significant association was detected between +3954C>T and prostate cancer.

20.7.2.3 Interleukin-1Ra (IL-1Ra)

IL-1RA has antiinflammatory properties by competing with IL-1 cytokines in binding to their receptors. This cytokine is encoded by *IL-1RN* gene located on 2q14.2. Its transcript may contain six, five, or four exons [25, 154]. There is an 86-bp variable number tandem repeat (VNTR) in intron 2 of this gene [25]. The short alleles of this VNTR contain only two repeats (IL-1RN∗2), while long alleles may have three to six repeats (IL-1RN L) [62, 176]. The more prevalent allele containing four repeats is named IL1RN∗1 [181]. In vitro and in vivo studies have shown extensive associations of this variant with the members of IL-1 superfamily. IL-1RN∗2 was associated with not only higher IL-1RA levels but also enhanced IL-1β production and decreased IL-1α production [182]. However, the final result of IL-1RN∗2 was decreased IL-1RA/IL-1β ratio, followed by prolonged proinflammatory immune response [25]. Although intronic VNTR contains potential binding sites for an interferon-α silencer, an interferon-β silencer, and an acute-phase response element, all leading to its functional importance, these associations are suggested to be a result of LD with other variants [164, 183]. Some authors suggested that the enhancing effect of IL-1RN∗2 on IL-1RA levels is dependent on the presence of −511T allele or the absence of +3954T in IL-1β [25].

A meta-analysis of 71 case–control studies (including 37 studies on gastric cancer, 6 studies on HCC, 4 on cervical cancer, 4 on breast cancer, 4 on lung cancer, and 16 studies on other cancers) with 14,854 cases and 19,337 controls showed that overall carriers of IL-1RN∗2 are significantly more susceptible to cancer (Table 20.6) [175].

A systematic review on 3322 prostate cancer patients and 2147 controls revealed no significant association between *IL-1RN* polymorphism and this cancer [171]. However, two studies on cervical cancer patients reported significant association between IL-1RN∗2 and cervical cancer [169, 170].

Table 20.6 Significant results from published meta-analysis of associations of IL-1RN VNTR with cancers

Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
Malignancy	14,854	19,337	22 vs. LL	1.37 (1.07–1.75)	40 studies of Asian descendents, 29 of Caucasian descendents, and 2 with mixed ethnicity	Zhang et al. [175]
			2L vs. LL	1.19 (1.07–1.32)		
			22 + 2L vs. LL	1.25 (1.12–1.41)		
			2 vs. L	1.23 (1.10–1.38)		
Breast cancer	1145	1102	2L vs. LL	0.74 (0.58–0.93)	Japan, Germany, Korea, India	Zhang et al. [175]
			22 + 2L vs. LL	0.78 (0.62–0.97)		
Cervical cancer	782	762	2 vs. L	1.41 (0.98–2.03)	Portugal, China, India	Lee and Song [170]
	1663	1374	22 vs. LL	2.64 (1.29–5.40)	Portugal, China, India	Wu et al. [169]
			22 vs. 2L + LL	2.15 (1.06–4.38)		
			22 + 2L vs. LL	1.60 (1.07–2.38)		
Gastric cancer	3209	4856	2L vs. LL	1.22 (1.05–1.41)	Portugal, China, Germany, Brazil, Taiwan, Thailand, UK, Italy	Zhang et al. [175]
			22 + 2L vs. LL	1.25 (1.09–1.43)		
			2 vs. L	1.20 (1.05–1.38)		
	3418	5789	22 + 2L vs. LL	1.26 (1.06–1.51)	Arab, Brazil, Netherland, Korea, USA, China, Italy, Mexico, South Korea, Germany, Taiwan, Portugal, Poland	Xue et al. [176]
			22 vs. LL	2.64 (1.29–5.40)		
			22 vs. 2L + LL	2.15 (1.06–4.38)		

20.7.3 Interleukin-4

Interleukin-4 (IL-4) is a pleiotropic cytokine with major roles in regulation of humoral immunity by its various effects on production of several other cytokines and dedifferentiation of B-cells and promoting expression of class II MHC Ags [28, 154]. It also has potent antitumor activity against various tumors by its inhibitory effect on the growth of tumor cells and its growth stimulatory effect on lymphocytes [184, 185].

IL-4 gene is located on the long arm of chromosome 5 (5q31.1), and through recent years, many variants identified on this gene. Among these variants, two important polymorphisms are −589C>T (rs2243250) and −33C>T (rs2070874) which are both promoter SNPs of which T alleles are associated with increased production of IL-4 in in vivo studies [28, 186]. The other variant of this gene is a 70-bp VNTR at intron 3 (Table 20.7) [186].

A meta-analysis of 8715 patients with various cancers and 9532 controls presented in 23 case–control studies found no significant association between −589C>T (rs2243250) and overall cancer susceptibility. This study also did not find any significant relationship in stratified analysis for ethnicity or different cancer types [187]. In line with that, another study on 1317 colorectal cancer patients and 1659 controls did not report significant association of this SNP with colorectal cancers neither globally nor race-dependently [188]. Similarly, a systematic review on the association of IL-4 SNPs with risk of glioma did not result in a significant relation [189]. However, another meta-analysis of 14 studies involving 3562 cancer cases found that T allele in rs2243250 was significantly associated with decreased oral cancer risk and increased risk of RCC [190]. Another meta-analysis proposed an association between the same SNP with decreased risk of gastric cancer only in Caucasians [191]. Gastrointestinal cancers' association with IL-4 SNPs has been investigated in a meta-analysis regarding rs2243250 and rs2070874. The study stated that despite rs2243250, T allele carriers in rs2070874 are associated with increased risk of gastrointestinal cancers, especially gastric cancer and studies conducted outside Asia [192].

A recent meta-analysis on 10,873 cancer cases and 14,328 controls reported significant association of −589C>T (rs2243250) with all-type cancers with considerable heterogeneity among studies [193]. When analyzed stratifically, the association remained significant in gastric, breast, lung, prostate cancer, and leukemia. Also, in population-based subgroups, the risk of cancer development in Caucasians and Asians was in sig-

Table 20.7 Genotype details for SNPs of IL-4

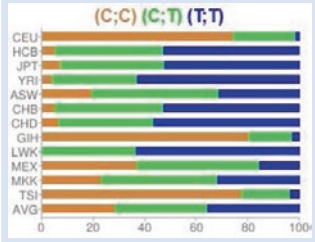
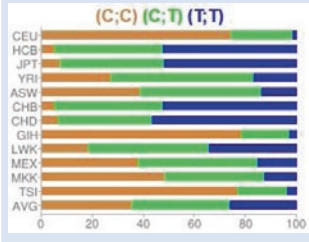
SNP	GMAF [161]	Population diversity [162]	Change at DNA level	Change at protein level	Effect on cytokine level
rs2243250	$T = 0.484$		−589C>T	NA	T allele: ↑
rs2070874	$T = 0.428$		−33C>T	NA	T allele: ↑

Table 20.8 Significant results from published meta-analysis of associations of −589C>T (rs2243250) in IL-4 gene with cancers

Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
All-type	3334	4803	CT vs. TT	0.82 (0.68–0.98)	Caucasian	Jia et al. [193]
			CT vs. TT + CC	0.79 (0.66–0.96)		
All-type	5350	6731	CT vs. CC + TT	0.89 (0.82–0.97)	Asian	Jia et al. [193]
Oral cancer	270	225	TT vs. CC	0.40 (0.19–0.84)	China, India	Zhenzhen et al. [190]
			TT + CT vs. CC	0.45 (0.22–0.94)		
RCC	467	518	TT vs. CC	1.98 (1.06–3.69)	China, Spain	Zhenzhen et al. [190]
			TT vs. CC + CT	1.43 (1.05–1.95)		
Gastric cancer	1477	2412	CT vs. TT	0.75 (0.61–0.91)	Netherland, Scotland, Spain, Italy, China, Taiwan	Jia et al. [193]
			CT vs. TT + CC	0.77 (0.66–0.91)		
			C vs. T	1.15 (1.01–1.32)		
	1700	892	TT + CT vs. CC	0.80 (0.66–0.97)	Caucasian	Sun et al. [191]
			T vs. C	0.83 (0.70–0.98)		
Breast cancer	1001	1298	CC vs. CT	1.21 (1.00–1.46)	India, USA	Jia et al. [193]
			TT vs. CC	0.56 (0.33–0.97)		
			CC vs. CT + TT	1.25 (1.04–1.51)		
			C vs. T	1.25 (1.06–1.47)		
Lung cancer	1930	2342	CT vs. CC + TT	0.84 (0.75–0.97)	Taiwan, China	Jia et al. [193]
Prostate cancer	588	652	CT vs. TT	1.48 (1.14–1.92)	USA, China	Jia et al. [193]
			TT vs. CC	0.48 (0.31–0.74)		
			CT vs. CC + TT	1.33 (1.05–1.69)		
			TT vs. CC + CT	0.64 (0.50–0.82)		

nificant association with this SNP. Moreover, this study has evaluated the association of two other IL-4 polymorphisms. The rs2070874 was significantly associated with oral cancer and leukemia development and susceptibility of cancer in Asian population [193]. The association between rs79071878 polymorphism and cancer risk was also significant generally in all-type cancers and with a small sample size in bladder cancer and breast cancer (Tables 20.8, 20.9, and 20.10) [193].

20.7.4 Interleukin-6 (IL-6)

IL-6, a 23.7 kD proinflammatory cytokine, is involved in inducing acute-phase response, differentiation of monocytes to macrophages, proliferation of T-cells, and Th2 cytokine production [194]. It has been previously shown to be of importance in susceptibility, natural history, and prognosis of several malignancies including prostate cancer, colorectal carcinoma, and breast cancer [25, 28]. This cytokine is encoded by a gene on chromosome 7p21 with five exons [195]. Two common promoter variants of IL-6, −174G>C (rs1800795) and −572G>C (rs1800796), were extensively studied in different inflammatory diseases (Table 20.11). −174G>C (rs1800795) is the first identified common promoter variant of IL-6 [25]. C allele in both of these variants was associated with lower IL-6 levels in several studies [158, 162, 196–201]. However, such an effect on IL-6 levels was not confirmed by some studies on −174G>C

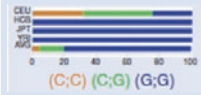
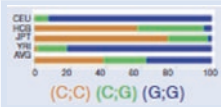
Table 20.9 Significant results from published meta-analysis of associations of −33C>T (rs2070874) in IL-4 gene with cancers

Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
All-type	1535	1962	CT vs. CC + TT	0.85 (0.73–0.98)	Asian	Jia et al. [193]
Leukemia	30	40	CC vs. CT	3.27 (1.02–10.45)	Iran	Jia et al. [193]
			CT vs. TT	0.03 (0.00–0.57)		
			CT vs. CC + TT	0.24 (0.08–0.77)		
Oral cancer	140	120	CT vs. TT	1.93 (1.13–3.29)	India	Jia et al. [193]
			CT vs. CC + TT	1.67 (1.00–2.77)		
			TT vs. CC + CT	0.50 (0.31–0.82)		
			C vs. T	1.69 (1.16–2.48)		
Gastrointestinal cancer	2101	3318	T vs. C	1.11 (1.00–1.24)	China, Korea, Spain, European countries	Cho and Kim [192]
	1576	1889	T vs. C	1.28 (1.03–1.58)	Asian	Cho and Kim [192]
Gastric cancer	1367	2583	T vs. C	1.17 (1.03–1.34)	China, Korea, European countries	Cho and Kim [192]

Table 20.10 Significant results from published meta-analysis of associations of IL-4 VNTR (rs79071878) with cancers

Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	Reference
All-type	1896	2526	23 vs. 22	1.40 (1.09–1.79)	Taiwan, Turkey, India, China	Jia et al. [193]
			33 vs. 22	0.62 (0.44–0.87)		
			33 vs. 22 + 23	0.69 (0.55–0.88)		
			22 vs. 33	1.26 (1.00–1.58)		

Table 20.11 Genotype details for SNPs of IL-6

SNP	GMAF [161]	Population diversity [162]	Change at DNA level	Change at protein level	Effect on cytokine level
rs1800795	*C* = 0.185		−174G>C	NA	C allele: ↑
rs1800796	*C* = 0.290		−572G>C	NA	C allele: ↑

(rs1800795) [158, 196–200]; therefore, this inconsistency might be the result of partial LD between this SNP and an actual functional SNP [25]. EMSA studies showed that −572G>C (rs1800796) is not in a TFBS; therefore, its influence on IL-6 serum levels probably results from strong LD with a functional variant such as −6331T>C (rs10499563) [196]. C allele in −572G>C (rs1800796) is highly associated with T allele in −6331T>C (rs10499563) [196]. Interestingly, T allele in this SNP is associated with higher expression of IL-6 gene [196]. −6331T>C (rs10499563) is near the distal promoter of IL-6 located between −5202 and −5307.

EMSA studies showed that T allele in −6331T>C (rs10499563) resulted in more affinity for Oct-1 of which binding changes the chromatin structure and locates the distal promoter to the transcription start site [25].

There is no systematic review reporting the significant association of these polymorphisms with increased risk of hematologic malignancies [202]. A systematic review of 12 case–control studies on breast cancer (published till December 2009) with 10,137 cases and 15,566 controls found no significant association between −174G>C (rs1800795) and susceptibility to breast cancer [158]. Another stratified study revealed a protective effect of the rs1800795 (OR:0.51) for breast cancer in Asians (378 cases and 432 controls), whereas this SNP was oppositely associated (OR:2.51) with genitourinary cancers in the same ethnicity (496 cases and 600 controls) [203]. Similar to a meta-analysis with 6481 patients with colorectal cancer and 7935 controls, another study of 7210 patients and 9467 controls did not show any significant association in any genetic model between −174G>C (rs1800795) and colorectal cancer [204, 205]. However, in stratified analysis in a subgroup of patients with the history of current or habitual use of NSAIDs (3061 cases and 4024 controls), carriers of C allele in −174G>C (rs1800795) had significantly lower risk for colorectal cancer (Table 20.12) [204]. This study did not show any significant association between colorectal cancer and −572G>C (rs1800796) in 2574 cases and 3344 controls [204]. In line with this, two recent meta-analyses on gastric cancer patients did not confirm any effect of these two SNPs on susceptibility to cancer [210, 211]. Whereby according to a stratified random effect meta-analysis −174G>C (rs1800795) SNP was associated with increased risk of colorectal cancers in European population with 4164 cases and 5469 controls [207].

Table 20.12 Significant results from published meta-analysis of associations of −174G>C (rs1800795) in IL-6 gene with cancers

Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
Colorectal cancer	3061	4024	GC + CC vs. GG	0.75 (0.64–0.88)	Individuals from Denmark, USA, and Spain who regularly or currently took NSAIDs	Yu et al. [204]
Skin cancer	1130	1260	GC vs. GG	1.28 (1.06–1.54)	Spain, UK, Czech Republic, Sweden, Bulgaria, Denmark, USA	Wu et al. [206]
			CC + GC vs. GG	1.26 (1.05–1.50)		
Melanoma	530	596	C vs. G	1.19 (1.01–1.41)	USA, UK, Spain, Bulgaria	Wu et al. [206]
Colorectal cancer	4164	5469	C vs. G	1.07 (1.01–1.14)	Europe	Wang and Zhang [207]
Liver cancer	587	850	C vs. G	0.74 (0.61–0.89)	Caucasian, Asian, Mixed	Tian et al. [208]
			CC vs. GC + GG	0.59 (0.36–0.95)		
			CC + CG vs. GG	0.67 (0.52–0.88)		
Breast cancer	378	432	CC + CG vs. GG	0.51 (0.37–0.70)	Ancestral North Indians	Joshi et al. [203]
Genitourinary cancers	496	600	CC vs. CG + GG	2.51 (1.59–3.96)	Ancestral North Indians	Joshi et al. [203]
Hepatocellular carcinoma	1448	3160	CC vs. GG	0.36 (0.16–0.85)	Italy, USA, Japan	Liu et al. [209]
			GG + GC vs. CC	2.82 (1.26–6.28)		

Table 20.13 Significant results from published meta-analysis of associations of −572G>C (rs1800796) in IL-6 gene with cancers

Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
Cancer	9985	13,045	CG + GG vs. CC	1.33 (1.09–1.63)	China, Korea, USA, Singapore, Japan, Sweden	Du et al. [215]
			CG vs. CC	1.32 (1.08–1.62)		
Prostate cancer			GG vs. CG + CC	1.26 (1.02–1.57)	Sweden, USA, China	Du et al. [215]
Prostate cancer	11,613	13,992	C vs. G	0.735 (0.61–0.89)	Asian, African, Caucasian	Magalhães et al. [216]
			CC vs. GC + GG	0.54 (0.34–0.87)		
			CC + CG vs. GG	0.78 (0.63–0.97)		

In addition, two systematic reviews of 2949 and 2801 patients with lung cancer and 3375 and 3234 controls considering −174G>C (rs1800795) SNP and one study with 2691 cases and 3067 controls on −572G>C (rs1800796) did not show any significant association between these SNPs and lung cancer [173, 212, 213]. The −174G>C (rs1800795) polymorphism was also significantly associated with increased risk of skin cancer and specifically melanoma due to a seven-study based meta-analysis (1130 cases and 1260 controls) [206]. In contrast to an insignificant association with cancer risk in a meta-analysis with a sample size of 3387 cases and 4529 controls [214], another meta-analysis on 19 case–control studies (9985 cases and 13,045 controls) demonstrated a significant association between −572G>C (rs1800796) and risk of all-type cancer [215]. When stratified by cancer type and ethnicity, the association remained significant for prostate cancer and Asians, respectively. Moreover, another systematic review supported the significant association of allele C in −572G>C SNP and prostate cancer within 11 vs. 992 controls [216].

Additionally, systematic reviews conducted on the association of IL-6 SNPs and risk of HCC reported a significant negative association between SNP rs1800795 and HCC, but there was no significant association with −572G>C (rs1800796) polymorphism [209]. A rather individual meta-analysis considering the Mendelian randomization analysis reported an association of decreased level of IL-6 due to −174G>C SNP and decreased risk of liver cancer, wherein for 1 pg/Ml reduction in IL-6 level, the risk of liver cancer is reduced by 12% [208] (Table 20.13).

20.7.5 Interleukin-8

IL-8, a member of human α-chemokine subfamily, has a major influence on tumor invasion and metastasis by its stimulatory properties on angiogenesis and inflammation [25, 28, 63, 217, 218]. A gene located on chromosome 4q13–q21 with four exons is responsible for encoding this cytokine [217]. Fifteen functional SNPs have been identified within this gene including –251A>T (rs4073), +396T>G (rs2227307), and +781C>T (rs2227306) (Table 20.14) [28]. –251A>T (rs4073), located in the promoter region, was identified in 2000. Although there was little evidence on the functionality of this SNP in vitro, several in vivo studies showed higher levels of IL-8 in carriers of A allele [25]. On the contrary, one study showed higher transcription for T allele in gastric carcinoma cell line [155, 219]. EMSA studies showed that T allele in +781C>T allele (rs2227306) is associated with higher binding ability for a transcription factor (C/EBPb) [25]. Several studies showed associations of –251A>T (rs4073) with lung, gastric, colorectal, bladder, and prostate cancer in different populations (Table 20.15) [186].

A meta-analysis of 13,189 patients with lung, prostate, breast, colorectal, and nasopharyngeal cancers and 16,828 controls showed that carriers of A allele in –251A>T (rs4073)

Table 20.14 Genotype details for SNPs of IL-8

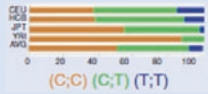
SNP	GMAF [161]	Population diversity [162]	Change at DNA level	Change at protein level	Effect on cytokine level
rs4073	*T* = 0.497	UA	–251A/T	NA	A allele: ↑
rs2227306	*T* = 0.294		+781C/T	NA	T allele: ↑
rs2227307	*G* = 0.422	UA	+396 T/G	NA	UA

Table 20.15 Significant results from published meta-analysis of associations of −251T/A (rs4073) in IL-8 gene with cancers

Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
Malignancy	13,189	16,828	AA vs. TT	1.21 (1.08–1.36)	Tunisia, Iran, Denmark, UK, Croatia, Germany, USA, Greece, China, Japan, Portugal, Spain, Mexico, Finland, France, Norway, Poland, Korea, India, Netherlands	Wang et al. [155]
			AA + TA vs. TT	1.12 (1.03–1.23)		
	5633	8240	AA + TA vs. TT	0.90 (0.83–0.97)	Population-based studies	Gao et al. [219]
	12,917	17,689	A vs. T	1.07 (1.00–1.15)	Taiwan, Thailand, China, Japan, Korea, Tunisia, USA, UK, Iran, Denmark, Greece, Croatia, Spain, Sweden, Finland, Poland, Brazil, Mexico, Portugal	Wang et al. [220]
			AA vs. TT	1.15 (1.01–1.30)		
			AA vs. AT + TT	1.08 (1.02–1.14)		
Gastric cancer	3036	3082	AA vs. TT	1.48 (1.13–1.95)	Asia	Wang et al. [155]
			TA vs. TT	1.20 (1.04–1.40)		
			AA + TA vs. TT	1.27 (1.08–1.48)		
	4274	6498	AA vs. TT	1.28 (1.02–1.62)	Japan, Iran, China, Korea, Finland, Spain, Mexico, Poland	Wang et al. [155]
			AA + TA vs. TT	1.17 (1.01–1.36)		
Nasopharyngeal cancer	440	459	AA vs. TT	2.04 (1.38–2.99)	Tunisia, China	Wang et al. [155]
			TA vs. TT	1.59 (1.19–2.13)		
			AA + TA vs. TT	1.70 (1.30–2.24)		
	545	568	AA + TA vs. TT	1.48 (1.16–1.89)	Tunisia, China	Gao et al. [219]
	1293	652	AT vs. TT	1.33 (1.05–1.67)	Tunisia, China, Romania, Poland, Russia, Slovakia, Czech Republic	Wang et al. [220]
			AA + AT vs. TT	1.41 (1.13–1.75)		
			AA vs. AT + TT	1.40 (1.08–1.81)		
Oral cancer	1324	1879	AA vs. AA + TA	1.23 (1.03–1.46)	China, Taiwan, Thailand, Greece, Japan, France	Wang et al. [221]
			AT vs. TT	1.25 (1.07–1.47)		

Table 20.15 (continued)

Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
Breast cancer	683	880	TA vs. AA	1.44 (1.09–1.91)	Iran, China	Huang et al. [222]
			AA vs. AA + TA	1.435 (1.11–1.86)		
	717	537	TA vs. AA	0.54 (0.40–0.74)	Tunisia	Huang et al. [222]
			AA vs. AA + TA	0.74 (0.57–0.95)		
	1262	1419	TT vs. AA + TA	0.69 (0.57–0.86)	Tunisia, China, UK	Huang et al. [222]
Lung cancer	309	312	A vs. T	1.48 (1.04–2.11)	China, India	Gao et al. [223], Wang et al. [224]
			AA vs. AT + TT	1.35 (1.02–1.92)		

were more susceptible to different cancers [155]. Another study reviewed results of 45 studies including 14,876 cases and 18,465 controls and showed such an association only among hospital-based studies and surprisingly showed significantly decreased risk of cancers for AA genotype among population-based studies [219].

Additionally, a systematic review of 12,917 cancer patients and 17,689 controls reported significant association between −251A>T (rs4073) and cancer in overall population. When analyzed in stratas, it was significantly associated with breast cancer, nasopharyngeal cancer, Asians, and hospital-based studies [220]. It should be noted that hospital-based studies have an increased chance of a selection bias since hospital-based controls might have disease conditions under the influence of the studied polymorphism [217].

Another systematic review of ten papers including 2195 gastric cancer patients and 3505 controls confirmed that AA genotype was a risk factor for gastric cancer in whole population and in Asian population. In stratified analysis for tumor location and histology, this association remained significant only in the cardia gastric cancer and diffused type [63]. A meta-analysis evaluating papers on gastric cancer published from January 2000 to January 2011 (18 papers including 6554 controls and 4163 cases) also found such an association in Asians but not in Caucasians. However, unlike the previous study, when stratifying for pathology types, the association remained significant only in intestinal-type cancer but not in the diffused type [225].

Although a systematic review of 1324 patients with oral cancer and 1879 controls reported in six studies (published till October 2012) showed higher risks of oral cancer in carriers of A allele in –251A>T (rs4073) [221], another systematic review with six studies did not support this association [226]. In subgroup analysis for ethnicity, there were only significant associations among Caucasians but not in Asians [221].

Meta-analyses on six case–control studies have also indicated significant association of −251T>A (rs4073) with lung cancer development in Asian population, but not among all populations [223, 224].

On the contrary, T allele in this SNP was associated with an increased risk of breast cancer in Asian and African populations. However, this study showed no significant associations between this SNP and breast cancer in 1880 breast cancer patients and 2013 controls [222]. There were no any significant associations between this SNP and colorectal cancer in a meta-analysis of nine case–control studies with 3019 cases and 3984 controls [227].

20.7.6 Interleukin-10

IL-10 is a pleiotropic, immunoregulatory cytokine which can affect both the innate and adaptive immune systems [228]. IL-10 has pleiotropic effects on tumor immunology. It plays an antiinflammatory role by inhibiting production of proinflammatory mediators such as IL-1α, IL-1β, IL-6, IL-8, IL-12, TNF-α, and IFN-γ [25, 67]; in addition, IL-10 inhibits presentation of tumor Ags by suppressing the expression of HLA molecules [154, 157]. On the other hand, IL-10 induces proliferation in B-cells and T-cells and regulates angiogenesis in various cancers [28, 229].

Twin studies demonstrated that IL-10 levels are significantly influenced by genetic factors with a heritability of 74% [25, 230]. IL-10 is encoded by five exons of a gene located on 1q31–1q32. At least 40 SNPs have been identified in this gene [66, 67, 231]. Several common variants including −1082 A>G (rs1800896), −819 C>T (rs1800871), and −592 A>C (also called −571 rs1800872) have been identified within the promoter region of this gene (Table 20.16) [229].

In vivo studies showed higher levels of IL-10 in individuals with GCC haplotype of these three SNPs, while ATA haplotype was associated with the lowest levels of IL-10 [25, 157]. It is suggested that −1082A>G (rs1800896) is the most functional SNP of these three variants and G allele in this SNP results in higher IL-10 levels [25]. EMSA studies showed different affinities of alleles of this SNP for a nuclear protein identified as poly ADP-ribose polymerase1 (PARP-1) which acts as a transcription repressor [25, 66]. So far, several studies have evaluated the associations of different IL-10 polymorphisms with various cancers including lung cancer, breast cancer, cervical cancer, gastric cancer, melanoma and nasopharyngeal cancer, and prostate cancer [66, 67]. A systematic review evaluated the association of −1082A>G (rs1800896) with risk of malignancy by reviewing results of 61 articles (published up to September 2010) with a total of 14,499 cancer patients and 16,967 controls. This study found no significant association between alleles of this SNP and overall susceptibility to cancers. However, carriers of G allele in Asian population had significantly more susceptibility to various cancers. In stratified analysis for cancer types, there was increased risk of lung cancer and NHL in carriers of G allele (Table 20.17) [66].

Table 20.16 Genotype details for SNPs of IL-10

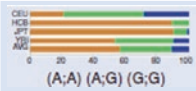
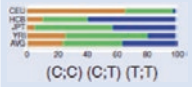
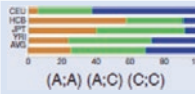
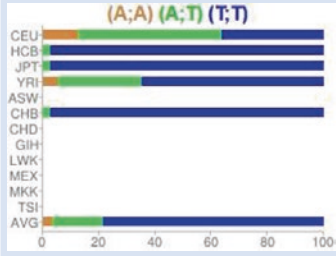
SNP	GMAF [161]	Population diversity [162]	Change at DNA level	Change at protein level	Effect on cytokine level
rs1800896	*G* = 0.303		−1082A>G	NA	G allele: ↑
rs1800871	*T* = 0.409		−819C>T	NA	UA
rs1800872	*C* = 0.409		−592A>C	NA	UA
rs1800890	*A* = 0.2259		−3575T>A	NA	A allele: ↑

Table 20.17 Significant results from published meta-analysis of associations of polymorphisms of IL-10 gene with cancers

Alleles	Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
rs1800872	All-type	16,785	19,713	AA vs. CC	0.90 (0.83–0.98)	UK, Spain, Korea, USA, China, Taiwan, Turkey, France, Costa Rica, Australia, Scotland, Poland, Italy, Netherland, Germany, Finland, Mexico, Japan, Sweden, Denmark, Croatia, Norway	Ding et al. [232]
				AA vs. AC + CC	0.92 (0.86–0.98)		
	HCC	354	1683	CC vs. AA + AC	1.68 (1.25–2.26)	Taiwan, Korea, China, Japan	Wei et al. [67]
				C vs. A	1.29 (1.12–1.49)		
	Cervical cancer	2396	1388	A vs. C	1.16 (1.04–1.31)	Korea, Netherlands, Sweden, India, China	Ni et al. [233]
				AA + AC vs. CC	1.18 (1.01–1.39)		
	Gastric cancer	1526	2538	AA vs. CC + AC	0.81 (0.68–0.97)	(Asians) China, Korea, Japan	Xue et al. [225]
	Lung cancer	601	1008	CC + AC vs. AA	1.8 (1.28–2.54)	Chinese, Denmark, Germany	Xue et al. [225]
				CC vs. AA	2 (1.24–3.23)		
	DLBCL	719	1539	AA vs. CC + AC	0.63 (0.43–0.94)	USA, France	Cao et al. [234]
				AA vs. CC	0.64 (0.43–0.96)		
rs1800871	Gastric cancer	989	2350	TT vs. CT + CC	0.82 (0.7–0.96)	(Asians) China, Korea, Japan	Xue et al. [235]
		1960	3705	T vs. C	0.87 (0.77–0.97)	East Asian, Latinos, Caucasian	Cui et al. [236]
		1459	1770	T vs. C	0.85 (0.73–0.98)	East Asian	Cui et al. [236]
	Lung cancer	311	507	C vs. T	1.27 (1.01–1.58)	China, Denmark, Germany	Peng et al. [173]
				CC vs. TT	2.27 (1.32–3.89)		
	Head and neck cancers	1676	2230	C vs. T	1.15 (1.04–1.21)	Italy, China	Niu et al. [237]
				CC vs. TT	1.28 (1.03–1.59)		

Table 20.17 (continued)

Alleles	Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
rs1800896	Gastric cancer	4289	5965	A vs. G	0.489 (0.335–0.713)	China, USA, Italy, Korea, Costa Rica, Honduras, Finland, Japan, Spain	Pan et al. [229]
		3631	6431	GG + GA vs. AA	1.41 (1.13–1.76)	China, USA, Italy, Korea, Costa Rica, Honduras, Finland, Japan, Spain, mixed European	Ni et al. [238]
	Head and neck cancers	2258	2887	G vs. A	1.56 (1.27–1.92)		Niu et al. [237]
				AG vs. AA	1.64 (1.32–2.05)		
				GG vs. AA	2.24 (1.69–2.97)		
				GG + GA vs. AA	1.7 (1.36–2.14)		
				GG vs. AG + AA	1.89 (1.23–2.9)		
	Nasopharyngeal carcinoma	623	1018	GG + GA vs. AA	1.77 (1.39–2.26)	Italy, China, Tunisia	Yu et al. [239]
				G vs. A	1.53 (1.06–2.20)		
				AG vs. AA	1.75 (1.38–2.21)		
	Oral cancer	1357	1509	G vs. A	1.76 (1.36–2.27)	China, Greece, Germany	Niu et al. [237]
				AG vs. AA	1.71 (1.15–2.54)		
				GG vs. AA	3.13 (2.06–4.77)		
				GG + GA vs. AA	1.83 (1.26–2.66)		
				GG vs. AG + AA	2.69 (1.77–4.09)		
	Lung cancer	315	507	GA vs. AA	3.16 (1.16–8.63)	Taiwan, Germany, Turkey	Peng et al. [173]
				GG vs. AA	2.07 (1.16–3.70)		
				GG + GA vs. AA	3.17 (1.31–7.68)		
	NHL	2338	1999	GA vs. AA	1.18 (1.02–1.36)	Australia, Maryland, Sweden, France, Athens, Germany	Wang et al. [66]
				GG + GA vs. AA	1.17 (1.02–1.35)		
	DLBCL	1191	1610	GG + GA vs. AA	1.30 (1.08–1.57)	Australia, France, Sweden, Germany, USA	Cao et al. [234]
				GA vs. AA	1.32 (1.08–1.61)		
	FL	10,226	12,215	G vs. A	1.12 (1.04–1.21)	Australia, France, Sweden, USA, Germany, Italy, Greece, Asia, Poland, Norway	Zhang et al. [240]
				GG vs. AG + AA	1.20 (1.06–1.37)		
				GG vs. AA	1.24 (1.06–1.45)		
	Malignancy	1733	2003	GA vs. AA	1.80 (1.17–2.76)	(Asian) China, Taiwan, Kentucky, Korea, Japan	Wang et al. [66]
				GG vs. AA	3.32 (1.62–6.82)		
				GG + GA vs. AA	1.67 (1.07–2.60)		
				GG vs. AA + GA	2.93 (1.43–6.03)		

rs1800890	Melanoma	377	371	AA + AT vs. TT	0.70 (0.52–0.95)	Germany, USA	Dai et al. [241]
				TA vs. TT	0.67 (0.49–0.92)		
	NHL	10,703	11,823	AA vs. TA + TT	1.13 (1.04–1.23)	USA, Germany, Norway, Poland, Denmark, Sweden, Asia, Australia	Zhang et al. [240]
	DLBCL	10,703	11,823	AA vs. TT	1.34 (1.19–1.52)	USA, Germany, Norway, Poland, Denmark, Sweden, Asia, Australia	Zhang et al. [240]
				AT vs. TT	1.12 (1.03–1.22)		
				AA vs. TA + TT	1.26 (1.13–1.41)		
				AA + AT vs. TT	1.17 (1.08–1.27)		
				A vs. T	1.15 (1.09–1.22)		
		9048	7773	AA vs. TT	1.29 (1.13–1.47)	USA, Germany, Sweden	Dai et al. [241]
				AA vs. TA + TT	1.21 (1.07–1.36)		
				AA vs. TA + TT	1.16 (1.06–1.28)		
				TA vs. TT	1.13 (1.02–1.24)		
		4020	13,316	A vs. T	1.16 (1.08–1.25)	Canada, Italy, Spain, UK	Cao et al. [234]
				AA + AT vs. TT	1.20 (1.08–1.33)		
				AA vs. TA + TT	1.25 (1.09–1.44)		
				AA vs. TT	1.34 (1.15–1.56)		
				AT vs. TT	1.15 (1.03–1.28)		
	FL	10,703	11,823	AA vs. TT	1.26 (1.08–1.46)	USA, Germany, Norway, Poland, Denmark, Sweden, Asia, Australia	Zhang et al. [240]
				AA vs. TA + TT	1.20 (1.04–1.37)		
				AA vs. TA + TT	1.15 (1.04–1.27)		
				A vs. T	1.13 (1.05–1.21)		

A HuGE review on 15,942 cancer patients and 22,336 controls found no significant relation between −819C>T and cancer development. Within subgroups, only a moderate decreased risk was reported in Asian population [242]. The first systematic review of gastric cancer studies showed significant association between −1082A>G (rs1800896) and gastric cancer not in overall population but only when the analysis was limited to the Asian populations [243]. This finding was supported by another review of studies regarding −1082A>G in digestive cancer patients [244]. However, another systematic review of 22 studies with 4289 patients and 5965 controls evaluated the association of −1082A>G (rs1800896) with susceptibility to gastric cancer. This meta-analysis showed that carriers of G allele have significantly increased the risk for gastric cancer especially in Caucasian populations [229]. Another meta-analysis with 3631 patients and 6431 controls showed similar results; nonetheless, results remained significant in Asian population but not in Caucasians. This study, in stratified analysis, showed that this association is significant in cardiac subtype and intestinal-type but not in noncardia subtype or diffuse-type cancer [238]. Moreover, a meta-analysis on 623 patients and 1018 controls showed that risk of nasopharyngeal carcinoma has a significant association with rs1800896 but not with the other two polymorphisms [239].

A large sample meta-analysis on multiple center studies showed a significant association of −592C>A with decreased risk of cancer development. When analyzed among subgroups, this negative association remained in smoking-related cancers, Asian population, and hospital-based studies [232].

Regarding −819C>T (rs1800871), a systematic review based on 11 studies and 4008 controls and 1490 cases showed significantly increased risk for carriers of C allele among Asians but not Caucasians. Such increased risk was also noted for diffuse-subtype cancer but not for intestinal-subtype [235]. Another meta-analysis supported the protective role of rs1800871 T allele in gastric cancer, especially in adenocarcinoma, Asian population, and population-based studies [236].

A systematic review of studies on −592A>C (rs1800872) found significantly increased risk of gastric cancer in carriers of C allele only in Asian populations but not in Caucasians and Latinos. In stratified analysis for noncardia and cardia subtypes or intestinal, diffuse, or mixed subtypes, no significant association was found [225]. The association of −592C>A with gastric cancer in Asian population was also confirmed in another study [245].

A meta-analysis of seven articles published on association of −1082A>G (rs1800896) and HCC with 1012 HCC cases and 2308 controls showed no association between this SNP and susceptibility to HCC. The same systematic review based on the results of four studies showed carriers of C allele in −592A>C (rs1800872) had an increased risk of HCC. This study also showed no significant association between −819C>T (rs1800871) and HCC based on results of three studies [67].

A meta-analysis reviewed the results of 13 studies with 9692 patients with prostate cancer and 10,488 healthy individuals as controls. However, this review did not show any significant association for the three SNPs which was in accordance with the results of an older review on the basis of ten studies [231, 246]. Another review which analyzed results of eight studies with 1636 breast cancer patients and 1670 controls did not show any altered risk of breast cancer for different alleles of −1082A>G (rs1800896). This review also showed no significant associations between −592A>C (rs1800872) and breast cancer in any genetic model [247].

In addition to its regulating effects on the immune system, IL-10 can induce transcription of one of the promoters of HPV [233]. Therefore, polymorphisms of this cytokine were under focus of researchers in the field of cervical cancer. However, no significant association was found between −1082G>A (rs1800896) and susceptibility to cervical cancer in two meta-analysis studies [248]. Also significantly increased susceptibility to cervical cancer was detected in carriers of A allele in −592A>C (rs1800872) [233].

A comprehensive systematic review on head and neck cancers with 2258 patients and 2887 controls revealed significant association of

−1082A>G with oral, nasopharyngeal, and head and neck cancers in general and in Asian and Caucasian race subgroups [237]. The same study did not find any significant relation with −592A>C but it showed significant association of head and neck cancers with −819C>T polymorphism [237].

Systematic review of 10,703 NHL patients and 11,823 controls indicated a significant association of −3575T>A polymorphism with NHL and likewise with DLBCL, FL, Caucasians, and hospital-based studies [240]. Association of −1082A>G with FL was also reported in this study, regardless of ethnicity [240]. Another meta-analysis on different cancer patients showed significant association of −3575T>A with reduced risk of melanoma and increased risk of DLBCL [241]. In line with that, a meta-analysis reported significant association of −3575T>A, −1082A>G, and −592C>A with the risk of DLBCL, while no association was detected for −819C>T [234].

20.7.7 Interleukin-12

Interleukin-12 (IL-12) is a proinflammatory cytokine with several functions including differentiation of Th1 pathway, the critical pathway involved in protection against malignancy [25]. It can also induce IFN-γ production by T and NK cells and therefore suppress angiogenesis. In addition, IL-12 has a major role in the reactivation and survival of memory $CD4^+$ T-cells which results in repolarization of $CD4^+$ T-cells from dysfunctional antitumor Th2 into Th1 cells [249, 250].

IL-12 is composed of two parts, a p35 unit which is encoded by *IL-12a* on 3q25.33 and a p40 unit encoded by *IL12b* on 5q33.3 [25]. One common variant in IL-12b gene, including +1188A>C (rs3212227) in 3′ UTR, and three common variants of IL-12a including +277G>A (rs568408) in 3′ UTR, IVS2 T>A (798T>A; rs582054), and −564T>G (rs2243115) in 5′UTR have been extensively studied previously (Table 20.18) [251]. In vitro and in vivo studies showed that A allele in +1188A>C (rs3212227) was associated with higher expression and greater mRNA stability [25, 252]. It is suggested that +277G>A (rs568408) may disrupt exon-splicing enhancers and miRNAs binding and therefore results in an unstable IL-12 mRNA and lower IL-12 secretion [253].

One meta-analysis of ten studies involving 2954 cancer patients and 3276 controls showed significant associations between +1188A>C (rs3212227) and susceptibility to cancer (Table 20.19). In addition, by stratified analysis for cancer type, this study showed significantly increased susceptibility to cervical cancer and nasopharyngeal cancer in C allele carriers [252].

A meta-analysis of 18 studies evaluated the associations of polymorphisms of both *IL-12* genes and cancer susceptibility. This study reviewed results of 13 studies on +1188A>C (rs3212227), including nine studies in Asians,

Table 20.18 Genotype details for SNPs of IL-12

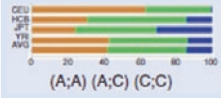
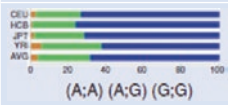
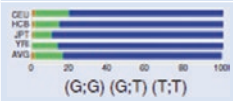
SNP	GMAF [161]	Population diversity [162]	Change at DNA level	Change at protein level	Effect on cytokine level
rs3212227	*C* = 0.338		+1188A>C	NA	A allele: ↑
rs568408	*A* = 0.128		+277G>A	NA	G allele: ↑
rs582054	*A* = 0.489	UA	+798T>A	NA	UA
rs2243115	*G* = 0.107		−564T>G	NA	UA

Table 20.19 Significant results from published meta-analysis of associations of 1188A>C (rs3212227) in IL-12b with cancers

Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
Malignancy	2954	3276	CC + AC vs. AA	1.32 (1.06–1.63)	UK, Bulgaria, China, France	Chen et al. [252]
			AC vs. AA	1.30 (1.07–1.57)		
			CC vs. AA	1.39 (1.05–1.86)		
			CC vs. AC + AA	1.17 (1.02–1.33)		
	10,404	10,861	C vs. A	1.14 (1.02–1.27)	UK, USA, Italy, China, Russia, Korea, Bulgaria, Tunisia	Zhou et al. [251]
			AC + CC vs. AA	1.20 (1.01–1.15)		

three studies in Caucasians, and one in Africans, and showed increased risk of all cancers in C allele carriers. This association remained significant in Asian population but not in Caucasians [251]. This study like the previous one showed increased susceptibility to cervical and nasopharyngeal cancer in carriers of C allele. However, no significant association was found between cancer susceptibility and +277G>A (rs568408). Also, there was no significant association for +564T>G (rs2243115) and IVS2 T>A (rs582054) of IL-12a [251].

20.7.8 Tumor Necrosis Factor-α and Lymphotoxin-α

Tumor necrosis factor-α (TNF-α), by its triggering effect on the cytokine cascade of IL-1, IL-6 and other mediators, is one of the most important proinflammatory cytokines in the maintenance and homeostasis of the immune system, inflammation, and host defense [254]. TNF-α has both procarcinogenic and anticarcinogenic properties, and its importance in cancer is evidenced by previous studies which repeatedly reported high levels of TNF-α in cancer patients [255–257]. Some tumor cells can even produce TNF-α in an autocrine manner [154]. Consistent with its name, high levels of TNF-α result in tumor necrosis, but low levels of this cytokine impair antitumor immune response and induce tumor angiogenesis and therefore is associated with increased tumor growth, progression, invasion, and metastasis of tumor cells [255–258]. In addition, TNF-α levels can influence weight loss cachexia, and anemia in the host and also its response to treatment [259].

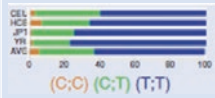
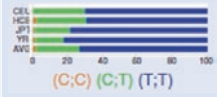
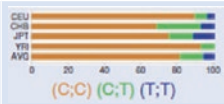
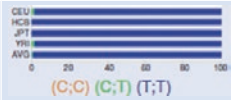
Lymphotoxin-α (LTA), another cytokine of the TNF family, is similar to TNF-α with respect to amino acid sequence, receptors, and biologic activities [255–258].

TNF-α is encoded by a gene located on chromosome 6 (region p21.3) and is a member of HLA class III. −308G>A (rs1800629) and −238G>A (rs361525) are two common promoter variants of *TNF-α* gene [25]. Other variants include −1031C>T (rs1799964), −863C>A (rs1800630), −857C>T (rs1799724), −376G>A (rs1800750), and IVS1 + 123G>A (rs1800610) (Table 20.20) [25]. The *LTA* gene is located in the same region and has an *Nco*I restriction fragment length polymorphism (+252A>G) in its first intron (rs909253).

A allele of −308G>A (rs1800629) is associated with higher levels of TNF-α [260]. While several in vitro studies did not show any functionality for this SNP, some authors suggested that this allele had more affinity for a transcriptional activator and another study showed that A allele disrupts a 10-bp binding region for activator protein-2 (AP-2) (a repressor protein) [25, 259]. Of interest, −308G>A (rs1800629) is in high LD with +252G>A, a functional SNP in *lymphotoxin alpha* gene, and other *HLA* genes within ancestral haplotype, *HLA A1-B8-DR3-DQ2-TNF_308A-LT_252A* [259, 261, 262].

An allele of −238G>A (rs361525) was associated with lower levels of TNF-α in peripheral blood mononuclear cells carrying TNF-α −238A allele [255]. However, several in vitro studies did

Table 20.20 Genotype details for SNPs of TNF-α and lymphotoxin-α

SNP	GMAF [161]	Population diversity [162]	Change at DNA level	Change at protein level	Effect on cytokine level
rs1800629	*A* = 0.096		−308G>A	NA	A allele: ↑
rs361525	*A* = 0.051	UA	−238G>A	NA	G allele: ↑
rs1799964	*C* = 0.200		−1031C>T	NA	C allele: ↑
rs1800630	*A* = 0.145		−863C>A	NA	A allele: ↑
rs1799724	*T* = 0.097		−857C>T	NA	T allele: ↑
rs1800610	*A* = 0.102	UA	IVS1 + 123G>A	NA	UA
rs1800750	*A* = 0.013		−376G>A	NA	A allele: ↑
rs909253	*C* = 0.398		+252 A>G	NA	G allele: ↑

not provide any evidence on the functionality of this SNP [25].

A Japanese in vitro study showed that C allele in rs1799964 is associated with higher production of TNF-α by concanavalin A (Con A)-activated peripheral blood mononuclear cells [263]. Reporter assays showed increased promoter activity for A allele of −376G>A (rs1800750), and EMSA studies showed more affinity of this allele for Oct-1 transcription factor compared to other allele [22, 25]. In vivo studies showed that individuals carrying at least one allele out of three (−1031C, −863A, −857T) had higher TNF-α production and higher transcriptional activity [22, 264]. In the same line, minor alleles of −863C>A (rs1800630) and −857C>T (rs1799724) were associated with higher promoter activity and more affinity for Oct-1 transcription factor [22, 25, 264]. On the contrary, one study showed that −863A allele had less affinity for NF-κB [22, 25, 265].

In vitro studies showed that phytohemagglutinin-activated mononuclear cells having +252G allele (rs909253) produce more LTA and interestingly TNF-α [266, 267].

Previously, several associations have been reported between TNF-α polymorphisms and susceptibility to NHL, gastric carcinoma, breast cancers, prostate, uterine endometrium, lung, cervix, and nasopharynx. However, a meta-analysis reviewed 34 studies (published up to March 2011) including 34,679 cancer patients and 41,186 controls and found no significant association between −238G>A (rs361525) polymorphism and susceptibility to cancer [268]. In line with this, a meta-analysis of 30,000 breast cancer cases and 30,000 controls from 30 studies of the breast cancer association consortium could not find any significant association between −238G>A (rs361525) and susceptibility to breast cancer [269].

Although no significant association was found between −308G>A and breast cancer and its subtypes, −308G>A was reported to be significantly associated with distant metastasis of triple negative breast cancers [270]. Similarly, a

meta-analysis on 12,360 cases and 15,310 controls revealed no significant association of −308G>A with breast cancer [271]. However, in stratified analysis, −308G>A was a protective factor in postmenopausal women while a risk factor in premenopausal women [271]. A review of 18 studies with 11,320 breast cancer patients and 14,112 controls found a significant relationship between −308G>A (rs1800629) polymorphism and breast cancer only in Caucasian population (Table 20.21) [254]. In addition, after excluding hospital-based studies a significant decreased risk in carriers of A allele was found. This study also reviewed 33,112 patients and 35,814 (reported in 35 studies) and found no significant association for −238G>A (rs361525). This study also did not find any significant association between breast cancer and −863C>A (rs1800630) and −857C>T (rs1799724), −1031 C>T (rs1799964) polymorphisms, which may be due to the fact that the overall sample analyzed for these polymorphisms was very small [254]. Consistent with the previous study, a meta-analysis of 11 studies on 10,184 patients with breast cancer and 12,911 controls found that G allele in −308G>A (rs1800629) is associated with significantly increased risk of breast cancer [258]. Another meta-analysis evaluated 10,236 breast cancer cases and 13,143 controls presented in 13 studies [293]. This study could confirm such a decreased breast cancer risk in carriers of −308A allele only in Caucasians [293]. However, no significant association between breast cancer susceptibility and other polymorphisms of TNF-α was found [293]. In accordance with previous studies, a recent systematic review on 37,257 patients and 39,564 controls supported the lack of association between rs361525 and breast cancer development [294]. A meta-analysis of 4625 breast cancer patients and 4373 controls for LTA −252A>G (results from seven studies published up to January 2012) did not find any significant association between genotypes of this polymorphism and breast cancer. However, in stratified analysis for ethnicity, carriers of G allele had significantly increased risk of breast cancer in Asian population [295].

A systematic review of 11 studies with 3094 cervical cancer cases and 3037 controls found that carriers of AA genotype for −308G>A (rs1800629) had 39% increased risk of cervical cancer compared with −308GA/GG genotypes [257]. In addition, in stratified analysis, such an association remained significant in Asian population [257]. This meta-analysis by its review on 1190 cases and 1784 controls showed decreased risk of cervical cancer in carriers of A allele in −238G>A (rs361525) [257]. In a similar way, significant association was detected between allelic model of −308G>A and −238G>A with cervical cancer in overall population and specifically in Caucasians [272]. In a meta-analysis of 13 studies reported up to October 2011 which involved 3294 cervical cancer patients and 3468 controls, no association was found between −308G>A (rs1800629) and cervical cancer [251]. However, in Caucasian and African population, significantly increased risk of cervical cancer was observed in carriers of A allele in this SNP. This study also meta-analyzed results of six studies on −238G>A (rs361525) (2416 cases and 2010 controls) and found that carriers of −238A allele had lower risk of cervical cancer which remained significant in Caucasian populations [251]. A recent meta-analysis reviewed results of 12 case–control studies including 1751 cases with upper aerodigestive tract (UADT) cancer and 3345 controls [280]. Oropharynx cancer was investigated in six of these studies, while five studies investigated esophagus cancer and one investigated larynx cancer. Squamous cell carcinoma and adenocarcinoma were investigated in nine and two studies, respectively, and one study investigated both cancer types. This study overall found a significant increase in risk of UADT cancer in carriers of AA genotype in −308G>A (rs1800629) compared to individuals who had GA or GG genotypes [280]. In addition, significantly increased risks were found in oropharynx cancer but not in esophagus cancer or larynx cancer. In the subgroup analysis for histologic type, this association remained significant only for squamous cell carcinoma, but not for adenocarcinoma [280]. Another meta-analysis with a sample size of 5617 reported no significant association

Table 20.21 Significant results from published meta-analysis of associations of polymorphisms of TNF-α with cancers

Alleles	Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
rs1800629	Cervical cancer	2710	2877	AA vs. GG	2.09 (1.34–3.25)	(Caucasian) India, USA, Portugal, Costa Rica, Sweden	Zhou et al. [251]
				AA vs. GA + GG	2.09 (1.35–3.25)		
		3094	3037	AA vs. GG	1.41 (1.03–1.92)	Sweden, India, Costa Rica, South Africa, Mexico, Portugal, Zimbabwe, USA, South Korea	Liu et al. [257]
				AA vs. GA + GG	1.39 (1.02–1.90)		
		3769	3914	A vs. G	1.19 (1.02, 1.38)	USA, India, Sweden, Costa Rica, Argentina, Portugal, Korea, China, Zimbabwe, South Africa	Jin [272]
				AA + GA vs. GG	1.17 (1.00–1.38)		
	Breast cancer	10,184	12,911	G vs. A	1.08 (1.02–1.14)	Italy, Tunisia, UK, Iran, USA, Poland, Croatia, Russia, Germany	Fang et al. [258]
				AA vs. GA + GG	1.10 (1.04–1.17)		
		10,254	12,926	AA + AG vs. GG	0.91 (0.85–0.97)	(Caucasians) Italy, USA, Poland, UK, Russian, Croatia, Germany	Yang et al. [254]
	HCC	2357	3161	AA vs. GG	1.97 (1.01–3.83)	USA, Turkey, China, Japan, Thailand, Italy	Wei et al. [265]
				AG vs. GG	1.88 (1.23–2.88)		
				AA + AG vs. GG	1.80 (1.19–2.72)		
		1665	2177	AA + AG vs. GG	1.74 (1.12–2.72)	Israel, Turkey, China, Italy, Thailand, USA, Japan	Yang et al. [180]
		1385 (HBV-related)	3079	AA + AG vs. GG	1.38 (1.05–1.82)	Japan, India, China, USA, Tunisia, Thailand, Italy, Korea	Xiao et al. [273]
		2070	2468	AA + AG vs. GG	1.74 (1.20–2.54)	Turkey, USA, China, Thailand, Japan, Italy	Guo et al. [274]
				GA vs. GG	1.58 (1.05–2.39)		
	Gastric cancer	4399	6855	AA vs. GG	1.49 (1.11–1.99)	South Korea, Taiwan, USA, Portugal, Colombia, China, Germany, Japan, Mexico, Brazil, Italy, Honduras, Poland, Finland, Spain	Gorouhi et al. [275]
				GA vs. GG	1.14 (1.02–1.27)		
		7009	12,119	AA + GA vs. GG	1.20 (1.07–1.34)	Korea, Taiwan, USA, Portugal, China, Germany, Mexico, Italy, Brazil, Finland, Honduras, Spain, Poland, Japan, Australia, Romania, Europe	Yang et al. [276]

(continued)

Table 20.21 (continued)

Alleles	Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
				A vs. G	1.18 (1.07–1.30)		
				AA vs. GG	1.29 (1.07–1.56)		
				AA vs. GG + GA	1.20 (1.00–1.45)		
		3335	5286	A vs. G	1.23 (1.11, 1.36)	USA, Spain, Korea, China, Finland, Germany, Mexico, Portugal, Honduras, Italy, Brazil, Japan	Zhang et al. [277]
				AA vs. GG	1.78 (1.28, 2.48)		
				AA vs. GG + GA	1.65 (1.21, 2.25)		
				AA + GA vs. GG	1.21 (1.08, 1.36)		
		2626	4801	A vs. G	1.32 (1.12–1.56)	Portugal, Romania, USA, Poland, Finland, Netherland, Italy, UK, Spain	Zhu et al. [278]
				AA vs. GG	1.76 (1.37–2.26)		
				AA vs. GG + GA	1.62 (1.27–2.07)		
				AA + GA vs. GG	1.35 (1.14–1.60)		
		3898	8885	AA + GA vs. GG	1.23 (1.12–1.34)	Romania, Portugal, Spain, USA, South Korea, China, Mexico, Germany, Honduras, Poland, Finland, Brazil, Colombia, Italy, Japan	Guo et al. [274]
	Colorectal cancer	2837	3601	AA vs. GG	1.46 (1.07–1.97)	Western countries	Min et al. [279]
				GA vs. GG	1.05 (0.93–1.19)		
	UADT cancer	1751	3345	AA vs. GA + GG	1.54 (1.07–2.21)	China, India, USA, Australia	Wang et al. [280]
	Oropharynx	944	1712	AA vs. GA + GG	2.68 (1.34–5.35)	China, India, USA	Wang et al. [280]
				AA vs. GG	2.70 (1.35–5.36)		
				AA vs. GA	2.59 (1.23–5.46)		
	Oral cancer	1280	1508	GG vs. AA	0.19 (0.04–1.00)	Taiwan, India, China, Greece, Germany, Thailand, USA	Chen et al. [281]
				GG + GA vs. AA	0.22 (0.06–0.82)		
	Urogenital cancers	11,613	12,542	A vs. G	1.18 (1.06–1.32)	Asian, Caucasian, African-American	Cai et al. [282]
				GA vs. GG	1.19 (1.04–1.37)		
				AA + GA vs. GG	1.20 (1.07–1.36)		
	Cervical cancer	4332	4337	A vs. G	1.28 (1.08–1.52)	Asian, Caucasian, African-American	Cai et al. [282]
				GA vs. GG	1.27 (1.02–1.60)		
				AA + GA vs. GG	1.29 (1.06–1.58)		

	NHL	9812	11,518	AA vs. GG	1.51 (1.26–1.80)	Tunisia, Germany, France, Sweden, UK, Japan, Austria, Poland, USA, Serbia, Greece, Denmark, Egypt, China, Iran, Poland, Norway, Australia	He et al. [283]
				AA vs. GA + GG	1.47 (1.23–1.75)		
	DLBCL	34,041	42,730	A vs. G	1.35 (1.27–1.44)	China	Yang et al. [284]
	SCC	2836	5235	AA vs. GG	1.62 (1.15–2.29)	China, Croatia, Thailand, USA, India, Serbia, Germany, Denmark, Poland, Australia	Liu et al. [285]
				AA vs. GA + GG	1.56 (1.10–2.20)		
	Lung cancer	2436	2573	A vs. G	1.13 (1.00–1.27)	Asian, Caucasian	Xie et al. [286]
	Prostate cancer	5757	6137	AA + AG vs. GG	1.53 (1.09–2.14)	Portugal, Finland, India, USA, Spain, China, UK	Ma et al. [287]
				AG vs. GG	1.48 (1.05–2.08)		
rs361525	Cervical cancer	2416	2010	A vs. G	0.61 (0.47–0.78)	South Korea, USA, India, Sweden, Costa Rica	Zhou et al. [251]
				GA vs. GG	0.59 (0.45–0.77)		
				GA + AA vs. GG	0.59 (0.46–0.77)		
		1190	1784	GA vs. GG	0.54 (0.40–0.73)	Costa Rica, Mexico, USA, India, Korea	Liu et al. [257]
				GA + AA vs. GG	0.55 (0.41–0.74)		
		2522	2253	A vs. G	0.64 (0.51–0.80)	USA, India, Costa Rica, Argentina, Korea	Jin [272]
				AA + AG vs. GG	0.62 (0.49–0.79)		
	HCC	1572	1875	A vs. G	1.32 (1.04–1.69)	China, Thailand, Italy, Taiwan, South Korea, China	Cheng et al. [288]
				AG vs. GG	1.32 (1.02–1.71)		
				AA + AG vs. GG	1.33 (1.03–1.72)		
		938	1370	AG vs. GG	1.63 (1.17–2.26)	China, Korea, Thailand, Italy, Japan	Wei et al. [265]
				AA + AG vs. GG	1.62 (1.18–2.22)		
		1572	1875	A vs. G	1.32 (1.04–1.69)	Asian, Caucasian	Cheng et al. [288]
				AG vs. GG	1.32 (1.02–1.71)		
				AA + AG vs. GG	1.33 (1.03–1.72)		
		608 (HBV-related)	1967	AA vs. GG	4.776 (1.28–17.819)	China, Tunisia, Thailand, Italy, Korea	Xiao et al. [273]
	GI cancers	4849	8567	AG vs. GG	1.19 (1.00–1.40)	Korea, China, Japan, Italy, Finland, Spain, Thailand, Netherland, USA, Iran, Australia	Hui et al. [289]
				A vs. G	1.19 (1.03–1.39)		
				AA + AG vs. GG	1.20 (1.02–1.41)		
	Gastric cancer	7795 (all)		A vs. G	1.32 (1.02–1.72)	Asian, Caucasian	Yu et al. [290]
				AG vs. GG	1.32 (1.01–1.72)		

(continued)

Table 20.21 (continued)

Alleles	Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
				AA + AG vs. GG	1.34 (1.02–1.76)		
	Oral cancer	598	634	G vs. A	2.75 (1.25–6.04)	Taiwan, India, China, Thailand	Chen et al. [281]
				GG vs. GA + AA	2.23 (1.18–4.23)		
rs1800630	HCC	627	1004	AC vs. CC	1.72 (1.03–2.88)	China, Korea, Thailand, Italy, Japan	Wei et al. [265]
				AA + AC vs. CC	1.65 (1.06–2.57)		
		558 (HBV-related)	1422	AC vs. CC	1.89 (1.23–2.92)	China, Thailand, Korea, Italy	Xiao et al. [273]
rs1799724	Gastric cancer	1118	1591	T vs. C	1.17 (1.01–1.35)	China, Japan	Zhang et al. [277]
		1835	3219	T vs. C	1.19 (1.07–1.33)	Asian, Caucasian	Cen and Wu [291]
				TT vs. CC	1.44 (1.03–2.02)		
				TC vs. CC	1.19 (1.05–1.36)		
				TT/TC vs. CC	1.21 (1.07–1.38)		
		1897	3219	T vs. C	1.12 (1.01–1.25)	Brazil, Korea, Poland, Japan, Italy, China	Wang et al. [292]
				TC vs. CC	1.16 (1.02–1.33)		
				TT/TC vs. CC	1.16 (1.02–1.32)		
	HCC	807	1510	TT vs. CC	1.65 (1.06–2.57)	China, Korea, Japan	Wang et al. [292]
				TT vs. TC/CC	1.61 (1.04–2.49)		

of −308G>A with esophageal SCC and adenocarcinoma [296]. Moreover, another study reported that −308G>A was not significantly associated with head and neck cancers neither overall nor in subgroup analysis [297]. The association of Oral cancers with TNF-α polymorphisms was analyzed in a meta-analysis with 1280 cases for −308G>A and 598 cases for −238G>A [281]. This study revealed a significant association between both SNPs and risk of oral cancers [281].

A meta-analysis on 2436 cases and 2573 controls proposed a significant association between −308G>A and lung cancer. In stratified analysis the association remained significant in small cell lung cancer, non-small cell lung cancer, and in Asian subgroup [286].

A wide-spectrum meta-analysis on digestive system cancers overall revealed a significant association of −238G<A with all-type GI cancers worldwide and also individually in Asian subgroup [289]. The same results were achieved in another study on −308G>A and digestive system cancers [274]. This study also detected significant association in Caucasians, gastric cancer, and HCC subgroups. A meta-analysis on gastric cancer and −308G>A (rs1800629) reviewed 5225 patients and 8473 controls in 26 papers. This study found a significant increase in risk of gastric cancer in carriers of A allele in comparison with G allele [298]. Another meta-analysis on gastric cancer evaluated 4399 cases and 6855 controls presented in 24 studies published up to October 2007 [275]. This study found a significant increase in risk of gastric cancer in carriers of AA genotype in −308G>A (rs1800629) polymorphism. In stratified analysis, AA genotype was significantly associated with an increased risk of noncardia cancers and intestinal type of gastric cancer compared to the GG genotype [275]. Another meta-analysis on gastric cancer and −308G>A (rs1800629) polymorphism included 19 studies with 3335 gastric cancer patients and 5286 controls [277]. In addition, this study included five studies with 1118 gastric cancer patients and 1591 controls for −857C>T (rs1799724). This study also found a significant increase in risk of gastric cancer in carriers of A allele and AA genotype in −308G>A (rs1800629) compared with G allele in the whole population and in Caucasians but not in East Asian [277]. This study also found a weak but significant association between T allele of −857 C>T (rs1799724) and gastric cancer risk compared with the C allele [277]; whereas in another study a significant association of −857 C>T with gastric cancer was observed under four genetic models [291]. Similarly, two reviews on 7009 and 2626 gastric cancer patients reported a significant association of −308G>A with gastric cancer which also remained significant in Caucasians [276, 278]. Moreover, −238G>A has been observed to be significantly associated with gastric cancer, particularly in Asian population [290].

A meta-analysis on TNF-α SNPs in colorectal cancer patients suggested that while −308G>A is contributed to increased risk of colorectal cancers, −238G>A is not significantly associated with them [279].

Several systematic reviews have been published on the associations of TNF-α polymorphisms and susceptibility to HCC. The most recent one evaluated results of 11 case–control studies (reported up to July, 2012) with a total of 1572 HCC cases and 1875 controls revealed an increased risk of HCC in carriers of A allele in −238G>A (rs361525) [288]. In stratified analysis, this association remained significant only in Asian populations [288]. Another meta-analysis included 2357 cases and 3161 controls presented in 17 studies published till November 2010 [265]. This study showed that A allele in both −238G>A (rs361525) and −308G>A (rs1800629) was associated with an increased risk of HCC. In stratified analysis for ethnicity, these associations remained significant in Asians but not in Caucasians [265]. AA and AC genotypes in −863C>A (rs1800630) were also associated with increased HCC risk compared to CC genotype. However, this study did not find any significant association for −857C>T (rs1799724) and −1031C>T (rs1799964) polymorphisms [265]. The pattern for −238G>A (rs361525) and −308G>A (rs1800629) was also repeated in other systematic reviews [180, 299, 300]. The association of −238G>A with HCC was confirmed in another

review with 1.572 patients under allelic, heterozygote, and dominant models [288]. In addition, when a meta-analysis was restricted to HBV-related HCCs, significant association was revealed between −308G>A, −238G>A, −863C>A, and HBV-related HCC either when analyzed with healthy controls or when analyzed with HBV-carriers [273].

A recent meta-analysis exclusively on −857C>T revealed a significant association of this SNP with gastric cancer and hepatocellular cancer, while no significant association was found for colorectal, cervical, and prostate cancer development [292].

Urogenital cancers were also reported to be associated with −308G>A (rs1800629) in a meta-analysis with 11,613 patients and 12,542 controls. When analyzed in subgroups, the association remained significant in cervical cancer, urothelial cancer, and Caucasian population groups [282]. A meta-analysis of seven case–control studies with 1311 bladder cancer cases and 1436 controls found that carriers of A allele in −308G>A (rs1800629) had an increased risk of bladder cancer [301]. A multicenter study investigated associations between six polymorphisms of TNF-α (rs1799964, rs1800630, rs1799724, rs1800629, rs361525, rs1800610) and prostate cancer risk in 2321 cases and 2560 controls from two nested case–control studies within the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trials and the Cancer Prevention Study II Nutrition Cohort for [302]. Overall, this study found no significant association between these polymorphisms and prostate cancer risk. But this study found a significant decreased risk in carriers of T-C-T-G-A haplotype in rs1799964, rs1800630, rs1799724, rs1800629, and rs1800610 compared to the most frequent haplotype (T-C-C-G-G) [302]. In subgroup analysis, T allele in −1036 C>T (rs1799724) in individuals who did not regularly use NSAID was associated with significantly less susceptibility to prostate cancer compared to the CC genotype. In addition, when limiting analysis to non-advanced tumors, carriers of −1036T or A allele in IVS1+123G>A (rs1800610) had a significantly decreased chance for prostate cancer [302]. Another study on two TNF-α polymorphisms (rs1800629 and rs361525) reported a significant positive association of −308G>A (rs1800629) with risk of prostate cancer under dominant and heterozygote models; but no significant association was detected for −238C>T (rs361525) [287].

Another multicenter study evaluated associations of −308G>A (rs1800629) with NHL in 7999 cases and 8452 controls from participating studies from the InterLymph Consortium. Carriers of −308A allele had increased risk for NHL, B-cell NHL, diffuse large B-cell lymphoma (DLBCL), and other marginal zone lymphoma. However, no significant associations were found between −308G>A (rs1800629) and chronic small lymphocytic lymphoma CLL/SLL or T-cell NHL [303]. Although this study also did not find any significant association between LTA +252A>G (rs909253) and NHL, carriers of G allele in this SNP had increased risk to DLBCL and mycosis fungoides [303]. In addition, the dominant model of this SNP was associated with leukemia development in a meta-analysis with 1509 cases and 4075 controls, while no association was detected among TNF-α polymorphisms [304]. A study on Chinese Han population with 34,041 cases and 42,730 controls demonstrated a significant association of −308G>A with DLBCL under allelic genetic model [284]. In accordance with previous studies, another multicenter study supported the significant association of −308G>A with NHL [283]. In stratified analysis, the African and Caucasian population was shown to be associated with increased risk of NHL, while Asian population was associated with reduced risk of lymphoma [283]. This study did not report any significant association for the −238G>A and −857C>T SNPs. Interestingly another meta-analysis suggested that the association of −308G>A with NHL is population dependent, wherein −308G>A is a protective factor in Asians (OR = 0.75, 95% CI: 0.66–0.86, $p < 0.001$), but a risk factor in Caucasians (OR = 1.22, 95% CI: 1.06–1.40, $p = 0.007$) [305].

In a meta-analysis of 33 studies with 14,435 cancer patients and 10,583 healthy controls, statistically significant increase in risk of malignant transformation was found in carriers of G allele in +252A>G (rs909253) which remained signifi-

cant in both Asian population and Caucasians [306]. A meta-analysis on 11 individual case–control studies with 2270 cases and 4404 controls found that G allele of +252A>G (rs909253) is associated with a significant increase in risk of gastric cancer, but this risk was significant only in Asians, but not Caucasians [307]. An older study also showed such a risk only in Asians especially those with *H. pylori* infection [308].

A recent comprehensive systematic review on the association of SCCs and TNF-α SNPs with overall 2836 SCC patients and 5235 controls resulted in significant association of recessive and codominant models of rs1800629 polymorphism with lung and oral SCC and all-type SCC in Asian population [285]. The same study did not report significant association of rs361525 polymorphism with SCCs except for a reduced risk of lung SCC in only two studies with 196 patients [285]. Furthermore, no association was found between rs1800629 and risk of skin cancers [285].

20.7.9 Interferon Gamma (IFN-γ)

Interferon gamma (IFN-γ) is a proinflammatory cytokine of Th1 subset with major roles in antitumor immune response. This cytokine enhances differentiation of lymphocytes and their function and Ag presentation through inducing expression of HLA molecules [154]. In addition, it inhibits angiogenesis in various tumors [65, 309].

IFN-γ gene with four exons and a length of 5.4 kb is located on chromosome 12q24 [309]. Two common SNPs including an intronic SNP +874T>A (rs2430561) and a promoter variant in (−179T>G (rs2069707)) have been previously identified [25, 65, 309]. This promoter variant is adjacent to a HSF-binding motif. In addition, there is a CA repeat microsatellite within the first intron of the gene ranging from 12 to 15 repeats [25, 309]. It was shown that allele 2 of the microsatellite and T allele in +874T>A (rs2430561) are in complete LD [25].

In vitro studies showed that T allele of +874T>A (rs2430561) is associated with higher IFN-γ production. EMSA studies showed that this allele has higher affinity for NF-κB which is in accordance with the location of this SNP in the first intron of the gene, a region related to binding of NF-κB [65, 309] (Table 20.22).

A meta-analysis of 17 studies with 1929 cancer cases and 2830 controls showed a nonsignificant increase in risk of cancer in the presence of AA genotype for +874T>A (rs2430561). However, this study showed significantly increased susceptibility in individuals with AT genotype compared with TT genotype (Table 20.23) [309]. Another meta-analysis with 32 studies and 4524 cases and 5684 controls did not find a significant association either [65]. Interestingly, in stratified meta-analy-

Table 20.22 Genotype details for SNPs of IFN-γ

SNP	GMAF [161]	Population diversity [162]	Change at DNA level	Change at protein level	Effect on cytokine level
rs2430561	*G* = 0.2686	NA	+874T>A	NA	T allele: ↑

Table 20.23 Significant results from published meta-analysis of associations of +874T>A (rs2430561) in IFN-γ gene with cancers

Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
Cervical cancer	661	835	AT vs. TT	1.10 (1.02–1.19)	India, South Africa	Mi et al. [309]
	1116	1290	TA vs. TT	1.47 (1.14–1.90)	Indonesia, India, China, Africa, Brazil	Sun et al. [310]
			TT + TA vs. AA	1.40 (1.10–1.78)		
Breast cancer	527	715	TT vs. AA	1.58 (1.10–2.27)	Iran, Italy, Turkey, China, USA	Liu et al. [65]
			TT vs. AT + AA	1.53 (1.14–2.06)		
HCC	859	1482	UA	1.38 (1.12–1.70)	Japan, China, Korea, India, USA, Tunisia, Brazil	Zhou et al. [311]

sis for ethnicity, carriers of T allele had significantly increased susceptibility to cancer in European and African population but not in Asian population [65]. This study also found that TT genotype significantly contributes to the risk of breast cancer in all ethnicities [65]. Similarly, a meta-analysis on 5630 cancer patients and 6096 controls did not result in any significant association except for the recessive model in African population [312]. Another meta-analysis on 859 HCC patients reported a significant association of rs2430561 with the risk of HCC development, especially with the background of HBV infection [311]. Moreover, a significant association of cervical cancer development with dominant and codominant genetic models of +874T>A polymorphism was found in a meta-analysis with 1116 cases and 1290 controls [310]. In a systematic review of 420 leukemia patients and 767 controls, +874T>A polymorphism was reported to be associated with chronic leukemias, albeit negatively in CLL patients [313]. The three latter studies were all significant when analyzed among Asian population [310, 311].

20.7.10 Transforming Growth Factor-β (TGF-β)

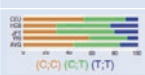
Transforming growth factor-β (TGF-β) is a functional mediator of epithelial and fibroblast cell proliferation and a regulator of immune cell populations [314]. In early stages of tumor progression, it acts as a tumor suppressor; however, in advanced cancers, TGF-β induces many activities that lead to growth, invasion, and metastasis of cancer cells [314–316].

TGF-β family consists of three isoforms with pleiotropic roles in cancer immunity [317–319]. TGF-β1 as the most common isoform of this family has enhancing effects on angiogenesis and its regulatory role in growth, differentiation, and apoptosis of different cells [64, 157, 319]. It also results in escape of malignant cells from immunosurveillance by suppressing expression of HLA molecules [154, 157, 318, 319].

TGF-β1 gene is located in the long arm of chromosome 19 (19q13.1). +869T>C (rs1800470; also called +29T>C, or rs1982037) is a common variant in the first exonic region of *TGF-β1* which results in substitution of leucine to proline at codon 10 in signal sequence [317]. +915G>C (also called +74 or rs1800471) is another exonic variant resulting in an arginine-to-proline substitution at codon 25. −509C>T (rs1800469) and −800G>A are two promoter variants in a proximal negative regulatory region (Table 20.24) [320, 321]. In vivo studies showed that T allele in −509C>T (rs1800469) was associated with higher levels of TGF-β1 in plasma and also higher expression [25, 75]. Despite some contrary results, C allele in +869T>C (rs1800470) was associated with higher secretion of TGF-β1 in in vitro studies [25, 318]. Arginine in +915G>C (rs1800471) was also associated with higher levels of TGF-β1 in in vivo studies [25]. In vitro studies showed that A allele in −1287G>A (rs11466314), another variant of this gene, is associated with higher expression of TGF-β1 [25]. EMSA studies showed that C allele in −387C>T (rs11466315) had greater affinity for Sp1 and Sp3 complexes [25].

Results of 40 case–control studies (including 3 studies with African population, 14 on Asian descendants, and 23 studies with European population) with 16,166 patients with various cancers and 19,126 controls were analyzed in a systematic review. Although this meta-analysis did not

Table 20.24 Genotype details for SNPs of TGF-β

SNP	GMAF [161]	Population diversity [162]	Change at DNA level	Change at protein level	Effect on cytokine level
rs1800470	*G* = 0.444	UA	+29T>C	Pro10Leu	C allele: ↑
rs1800471	*G* = 0.046	UA	+74G>C	Arg25Pro	G allele: ↑
rs1800469	*T* = 0.359		−509C>T	NA	T allele: ↑

find any significant association with overall risk of cancer, its result suggested that individuals with C allele in +869T>C (rs1800470) have significantly greater risk for prostate cancer. This finding was supported by another review with 2604 prostate cancer patients [322]. However, in subgroup analysis this allele was significantly associated with all-type cancers in Asian populations and prostate cancer in Caucasians [319, 322] (Table 20.25).

A meta-analysis of 30 studies including 20,401 patients with breast cancer and 27,416 controls showed increased risk of breast cancer in individuals with C allele in +869T>C (rs1800470). In stratified analysis, this association remained significant in Caucasian population and population-based studies [64, 327]. However, three other meta-analyses, one with a sample size of 24,021 cases and 31,820 controls and the others with almost half of this sample could not find such an association [327–329]. Another recent meta-analysis of 20,022 cases and 24,423 controls could find this increased risk for C allele just in Caucasians [321]. This study also reviewed results of eight studies with 10,633 cases and 13,648 controls for −509C>T (rs1800469) and did not find any significant association between alleles of this polymorphism and risk of breast cancer in accordance with another meta-analysis (including 10,197 patients with breast cancer and 13,382 healthy controls) [321, 330, 331]. Some authors suggested that the effect of TGF-β1 is different according to expression of estrogen receptor and progesterone receptor in breast cancer tumors, in a way that recessive and allelic models of −509C>T polymorphism are associated with ER-positive breast cancers [320, 332].

Meta-analysis of 1315 lung cancer patients and 1448 normal controls reported a significant association of lung cancer with dominant model of 1800470 polymorphism, overall and within

Table 20.25 Significant results from published meta-analysis of associations of SNPs of TGF-β gene with cancers

Alleles	Cancer site	Number of cases	Number of controls	Analysis type	OR ± 95% CI	Population included	References
rs1800470	Malignancy	5183	6524	CC vs. TT	1.26 (1.03–1.53)	(Asian) Korea, China, Japan	Wei et al. [319]
				CT vs. TT	1.20 (1.01–1.43)		
	Prostate cancer	2605	3129	CT vs. TT	1.28 (1.01–1.61)	USA, Germany, Brazil, Japan	Wei et al. [319]
				CC + CT vs. TT	1.24 (1.02–1.52)		
		2604	3129	C vs. T	1.08 (1.00–1.16)	Asian, Caucasian, African	Cai et al. [322]
	Breast cancer	20,401	27,416	CT vs. TT	1.05 (1.00–1.09)	Mixed from Asian, Caucasian, and African	Qiu et al. [64]
				CC + CT vs. TT	1.05 (1.01–1.09)		
	Lung cancer	1315	1448	TT + TC vs. CC	1.23 (1.03–1.47)	Asian, Caucasian	Fan et al. [323]
rs1800469	Gastric cancer	2130	2374	TT vs. CC + CT	1.35 (1.1–1.65)	India, China	Li et al. [75]
		2928	3480	TT vs. CC + CT	1.23 (1.09–1.38)	China, USA, India	Chang et al. [324]
	Colorectal cancer	994	2335	CC vs. TT	1.62 (1.30–2.02)	Iran, Germany, Korea, China	Fang et al. [325]
				TC + CC vs. TT	1.30 (1.08–1.58)		
				CC vs. TC + TT	1.48 (1.26–1.75)		
		4440	6785	CC + CT vs. TT	1.18 (1.06–1.32)	USA, UK, Iran, China, Korea, Germany	Wang et al. [326]
	Colon cancer	1760	2454	CC + CT vs. TT	1.31 (1.05–1.63)	UK, USA, China	Wang et al. [326]

Asian descendants [323]. This association remained significant in NSCLC subgroup. In spite of the previous study, a systematic review on 1167 lung cancer patients revealed no significant association of both 1800470 and 1800469 polymorphism with lung cancer development except for a subgroup analysis of +869T>C (rs1800470) among Caucasians [333].

A systematic review analyzed results of 55 studies with a total number of 21,639 cancer patients and 28,460 controls for associations of −509C>T (rs1800469) and susceptibility to different cancers. Although there was no a significant association between overall risk of cancer and genotypes of this SNP, this study found increased susceptibility of carriers of C allele to colorectal cancer particularly in Caucasians [334]. In addition, a meta-analysis of five studies with 994 colorectal cancer patients and 2335 controls found increased risk of colorectal cancer for C allele of −509C>T (rs1800469) which remained significant only in Asian population but not Caucasians in stratified analysis [325]. On the other hand, a systematic review of seven original articles with a total of 2130 patients with gastric cancer and 2374 controls found significantly increased susceptibility to gastric cancer in carriers of T allele in −509C>T (rs1800469) in a recessive model [75]. This association was supported by an updated review on 2928 cases and 3480 controls, while no association was detected for +869T>C and +915G>C [324]. Another meta-analysis pooled the results of 29 case–control studies with 8664 patients with digestive tract cancers and 12,532 controls. This study did not show any significant association with overall risk of digestive tract cancers. However, this study found that C allele in −509C>T (rs1800469) is significantly contributed to the risk of digestive tract cancers in Caucasians. In addition, carriers of C allele in the whole study sample had increased risk for colorectal cancer [335]. Another systematic review of 12 studies with 4440 colorectal cancer patients and 6785 controls could find such an association only in colon cancer [326].

20.8 Concluding Remarks

In the recent decades, a great scientific effort has uncovered the importance of immune polymorphisms in cancers. However, this uncovered part, although is promising, only reminds us that there is much more to reveal in this field. There comes a day that gathering immunogenetic data becomes one main part of every clinical trial in cancer. This information will help understand more about subgroups of patients, natural history of the cancers, responsiveness of cancer to treatment, or toxicity of treatment, all in relation to immune polymorphism [16]. One day, it might be possible to assess the degree of predisposition to different cancers for each individual and to employ preventive measurement, and in case of suffering from cancers, to efficiently choose between treatment options and predict their clinical effectiveness [28]. Although it seems a vague dream in the far future, it is becoming closer to reality everyday considering the pace of scientific advancements.

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Epigenetics and MicroRNAs in Cancer

21

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Contents

21.1 Introduction

MicroRNAs (miRNAs) are small noncoding RNAs (ncRNAs) which regulate gene expression by directly binding mostly, but not exclusively, to the 3′-untranslated region (3′-UTR) of target mRNAs [1]. In 1993, Victor Ambros first identified a small ncRNA, called lin-4, able to regulate the expression of a gene called lin-14 involved in the development of *C. elegans* [2]. In 2001, Lagos-Quintana et al. [3] showed for the first time that many of these small ncRNAs (in the meantime called microRNAs) are present not only in invertebrates but also in vertebrates. In 2002, Croce's group provided the first evidence of miRNA involvement in cancer by showing that a specific cluster of miRNAs (namely, the miR-15a/16-1 cluster) is located in the frequently deleted chromosomal region 13q14 in chronic lymphocytic leukemia (CLL) [4]. In 2005, Frank Slack supported this molecular evidence of

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miRNA involvement by demonstrating that let-7 directly targets the *RAS* oncogene in lung cancer [5]. In the same year, Cimmino et al. [6] found that the miR-15a/16-1 cluster directly targets the antiapoptotic *BCL2* gene in human CLL. From this time on, we assist at a plethora of studies identifying dysregulation of miRNAs in almost all types of human cancers and unraveling their contribution to human carcinogenesis by identifying which genes are modulated by the dysregulated miRNAs. Overall, these studies clearly state that aberrancies of the miRNome (defined as the full spectrum of miRNAs in a specific genome) contribute to human cancer development and can be therapeutically targeted to restore miRNA expression to normal [7]. Moreover, it has become clearer that miRNA involvement goes beyond cancer, since they are involved in a variety of biological processes, spanning from development, differentiation, apoptosis, and proliferation to senescence and metabolism [8–13].

MiRNAs are genes, like any other *protein coding gene (PCG)*, transcribed by RNA polymerase II into a capped and polyadenylated precursor, called pri-miRNA [14, 15]. A double-stranded RNA-specific ribonuclease called Drosha, in conjunction with its binding partner DGCR8 (DiGeorge syndrome critical region gene 8, or Pasha), cleaves the pri-miRNA into a hairpin-shaped RNA precursor (pre-miRNA), about 70–100 nucleotides (nt) long [16]. Transferred to the cytoplasm by Exportin 5, the pre-miRNA is cleaved into an 18–24 nt duplex by a ribonucleoprotein complex, composed of a ribonuclease III (Dicer), and TRBP (HIV-1 transactivating response RNA binding protein). Finally, the duplex interacts with a large protein complex called RISC (RNA-induced silencing complex), which includes proteins of the Argonaute family (Ago1-4 in humans), which drives one strand of the duplex (the so-called mature miRNA) mainly, but not exclusively, to the 3′-UTR of the target mRNAs. Overall, miRNAs exert its effect by modulating the expression of the target mRNAs either by mRNA cleavage or by translational repression. In 2007, Vasudevan et al. [17] discovered that miRNAs can also increase the expression of target mRNAs. Each miRNA can target several different transcripts. For instance, it has been demonstrated that a cluster of two miRNAs (namely, miR-15a and miR-16) can affect the expression of about 14% of the human genome in a leukemic cell line [18]. In addition, the same mRNA can be targeted by several miRNAs [19].

Epigenetics is defined as all heritable changes in gene expression not associated with concomitant alterations in the DNA sequence. In a traditional sense, gene epigenetic regulation usually includes DNA promoter methylation and chromatin histone modifications which are catalyzed by specific enzymes, overall indicated as effectors of the epigenetic machinery. However, if we consider the above definition, also *miRNA* gene regulation sensu stricto represents a component of epigenetics. Interestingly, it has been discovered that there is a two-way correlation between miRNAs and other epigenetic mechanisms: miRNAs can regulate the expression of effectors of the epigenetic machinery and miRNA genes undergo the same epigenetic regulatory mechanisms of any other *PCG*. These two main aspects of miRNome-epigenome cross-regulation and their implications in human carcinogenesis will be the main focus of this chapter.

21.2 MiRNAs Regulate Effectors of the Epigenetic Machinery

In 2007, Fabbri et al. [20] provided the first evidence that miRNAs can affect the expression of epigenetically regulated *PCG* in cancer by directly targeting key effectors of the epigenetic machinery, such as DNA methyltransferases (DNMTs). The miR-29 family (composed of miR-29a, miR-29b, and miR-29c) can directly silence the expression of de novo *DNMT3A* and *DNMT3B* in non-small cell lung cancer (NSCLC), leading to a global hypomethylation status of cancer cells and re-expression of *tumor suppressor genes (TSGs)* such as *FHIT* and *WWOX*, whose expression is silenced in NSCLC by promoter hypermethylation. As a result of the re-expression of these TSGs, NSCLC cells undergo apoptosis both in vitro and in an in vivo xenograft model [20]. Subsequently, Garzon et al. [21]

showed that in addition to directly targeting de novo *DNMTs*, miR-29b is also capable of targeting the *maintenance DNMT1*, even though in an indirect way: by directly silencing Sp1, a transactivator of *DNMT1*. These combined effects of miR-29s on all three major *DNMTs* highlight their relevance for epigenetic processes and explain the profound effects of their restoration on the global methylation status of cells. MiRNAs such as the miR-29 family, able to directly target effectors of the epigenetic machinery, have been called "epi-miRNAs." In mouse embryonic stem (ES) cells, two independent groups have shown that members of miR-290 cluster directly target RBL2, an inhibitor of *DNMT3* genes [22, 23]. ES Dicer null cells are characterized by no expression of the miR-290 cluster, overexpression *of RBL2*, and disruption of de novo methylation pathway, leading to increased telomere recombination and aberrant telomere elongation. Restoration of the miRNA cluster reverted this phenotype [22, 23]. Interestingly, the regulatory effect of miR-290 cluster on de novo *DNMTs* was not observed in human embryonic kidney 293 cells following Dicer knockdown, suggesting that miR-290 targeting effect on *DNMT3s* might be cell- and/or species-specific [22].

Another important family of epi-miRNAs is the miR-148a/b-152 family. In 2008, Duursma et al. [24] showed that miR-148a and miR-148b can indeed bind to the coding region (not the 3′-UTR) of DNMT3b mRNA, affecting the expression of this gene. This seminal study also concluded that by binding to this unusual site, miR-148 family might be responsible for the several different splice variants of DNMT3b [24]. A role for the miR-148a/b-152 family was further confirmed in cholangiocarcinoma, where it was shown that these miRNAs, in addition to miR-301, can directly target DNMT1, and their expression is silenced by IL-6, which is involved in cholangio-cancerogenesis [25]. This paper provided the first evidence of a correlation between epi-miRNAs, inflammation, and cancer. In 2010, Das et al. [26] showed that all-trans-retinoic acid (ATRA)-treated neuroblastoma cells undergo downregulation of MYCN, hence leading to overexpression of MYCN repressed miRNAs such as miR-152, miR-26a/b, and miR-125a/b. They also showed that these miRNAs are epi-miRNAs in this model, since they downregulate DNMT1 and DNMT3B expression, leading to re-expression of epigenetically silenced NOS1, which promotes neural cell differentiation. Also, the expression of miR-152 was normally downregulated with concurrent increase of *DNMT1* expression in HBV-induced HCCs [27]. More recently, Wang et al. [28] identified miR-342 as another epi-miRNA involved in colon carcinogenesis. They showed that the expression of *miR-342* is inversely correlated to DNMT1 levels in colorectal cancer (CRC) tissues and cell lines, and that this miRNA targets DNMT1, leading to reactivation of epigenetically silenced TSGs such as *ADAM23*, *Hint1*, *RASSF1A*, and *RECKS*. Functionally, restoration of miR-342 resulted in a reduction of *DNMT1* expression, reduced cell proliferation, and invasiveness in CRC cells and inhibition of tumor growth and lung metastasis formation in nude mice [28]. In 2010, viral epi-miRNAs have been shown to control the epigenetic machinery of host cells through DNMTs [29]. MiR-K12-4-5p, a Kaposi sarcoma-associated herpesvirus (KSHV) miRNA, was found to regulate the expression of DNMT1, 3A, and 3B indirectly, by targeting the expression of Rbl2, a known repressor of DNMT1, 3A, and 3B transcription. Ectopic expression of miR-K12-4-5p reduces Rbl2 protein expression and increases DNMT1, 3A, and 3B mRNA levels in 293 cells, thus affecting the overall epigenetic reprogramming of the host cell [29].

Epi-miRNAs are also involved in regulating the expression of histone deacetylases (*HDACs*) and Polycomb Repressive Complex (*PRC*) genes. For instance, HDAC4 is a direct target of both miR-1 and miR-140 [30, 31], while miR-449a binds to the 3′-UTR region of *HDAC1* [32]. *HDAC1* is upregulated in several kind of cancers, and *miR-449a* re-expression in prostate cancer cells induces cell-cycle arrest, apoptosis, and a senescent-like phenotype by reducing the levels of HDAC1 [32]. Recently, Jeon et al. [33] showed that miR-449a, b regulate *HDAC1* expression by directly targeting its 3′UTR transcript, indicating that this might be one of the reasons for the low

miR-449a, b expression and the high expression of *HDAC1* in lung cancer. MiR-140 has also been shown to be involved in chemoresistance mechanisms by targeting *HDAC4* [34]. Inhibition of endogenous miR-140 by locked nucleic acid (LNA)-modified anti-miRNAs partially sensitized resistant colon cancer stemlike cells to 5-FU treatment by increasing HDAC4 levels, leading to a G_1 and G_2 phase arrest [34]. Low expression of miR-9 along with high expression levels of *HDACs* (*HDAC4* and *5*) were discovered in Waldenstrom macroglobulinemia (WM) [35]. Mir-9 targets HDAC4 and HDAC5 in WM cells. Overexpression of miR-9 causes downregulation of HDAC4, 5, leading to an upregulation of acetylated-histone-H3 and acetylated-histone-H4. This provides evidence that the loss of miR-9 might be responsible for upregulation of HDAC4 and HDAC5 in WM cells, contributing to the pathogenesis of WM disease [35].

EZH2 is the catalytic subunit of the Polycomb Repressive Complex 2 (PRC2) and is responsible for heterochromatin formation by trimethylating histone H3 lysine 27 (H3K27me3), leading to the silencing of several TSGs. Varambally et al. showed that in prostate cancer cell lines and primary tumors, the expression of miR-101 decreases during cancer progression, inversely correlating with an increase of EZH2. These findings are suggestive of a role as epi-miRNA for miR-101, a hypothesis which was tested and confirmed by showing that miR-101 directly targets EZH2 both in prostate and in bladder cancer models [36, 37]. Moreover, miR-101-mediated suppression of EZH2 inhibits cancer cell proliferation and colony formation, revealing a TSG role for miR-101, mediated by its modulatory effects on cancer epigenome [37]. The inverse correlation between *miR-101* and *EZH2* was also observed in glioblastoma [38], gastric cancer [39], and NSCLC [40]. In prostate cancer it has been shown that *miR-101* can be inhibited by androgen receptor and HIF-1α/HIF-1β [41]. Ectopic expression of miR-26a targets *EZH2* in Burkitt's lymphoma, leading to reduced cell proliferation, increased percentage of cells in G1-phase, and increased apoptosis in Raji and Namalwa cells [42]. Intriguingly, the authors also found that c-Myc negatively regulates miR-26a, therefore maintaining high *EZH2* expression levels in cells and significantly contributing to c-Myc-induced tumorigenesis [42]. In 2009, Juan et al. [43] analyzed a regulatory double-negative feedback loop between miR-214 and EZH2 in controlling PcG-dependent gene expression during differentiation. PcG proteins suppress the transcription of miR-214 in undifferentiated skeletal muscle cells (SMC). Ectopic expression of miR-214 directly targets *EZH2*, increases myogenin expression, and promotes muscle differentiation [43]. *EZH2* is also highly expressed in nasopharyngeal carcinoma (NPC) patients and correlates with a higher risk of relapse [44]. *MiR-26a*, *miR-98*, and *miR-101*, whose expression is consistently downregulated in human NPC specimens when compared to normal nasopharyngeal epithelial tissue samples, have been shown to directly target *EZH2* [44], suggesting a prognostic role for these three miRNAs in NPC. Recently, there has been an extensive series of studies unraveling the central role of miR-101 in the regulation of *EZH2*, in several types of cancer. In hepatoma tissues, it was shown that miR-101 and miR-29c are downregulated, but their expression can be restored (leading to reduced levels of EZH2, EED, and H3K27me3 proteins) after treatment with TPA (12-O-tetradecanoylphorbol 13-acetate), which is dependent on protein kinase C (PKC) and ERK pathways in HepG2 cells [45]. Also, Smiths et al. [46] have established a pro-angiogenic effect of miRNA-101 working together with EZH2 and VEGF during the process of angiogenesis. The group analyzed the expression of miR-101 in endothelial cells derived from glioma patients and found it to be low. VEGF downregulates the expression of miR-101 resulting in increased protein expression of EZH2 and induces the elongation of endothelial cells leading to a pro-angiogenic response. Transfection with pre-miR-101, or EZH2 siRNA, or treatments with DZNep, a small inhibitor of EZH2 methyltransferase activity, reverses this process in HBMVECs controls, providing a network between VEGF/miR-101/EZH2 proteins toward pro-angiogenic response in endothelial cells

[46]. A summary of the described epi-miRNAs is provided.

Overall, these studies indicate that epi-miRNAs can modulate several key effectors of the epigenetic machinery, which indirectly affects the expression of epigenetically regulated genes. Considering that inactivation of *TSGs* by epigenetic mechanisms represents one of the main strategies adopted by cancer cells to promote their oncogenic phenotype, it is of the utmost importance to completely dissect these mechanisms, since they could provide new molecular targets for anticancer treatments.

21.3 MiRNAs Are Epigenetically Regulated in Several Types of Human Cancers

As previously anticipated, the relationship between miRNome and epigenome is bidirectional. Not only do miRNAs regulate the expression of effectors of the epigenetic machinery, but they also undergo the same epigenetic regulation of any other *PCG*.

By treating bladder cancer cell lines with both a DNA demethylating agent (5-aza-2′-deoxycytidine, 5-AZA) and an HDAC inhibitor (4-phenylbutyric acid), Saito et al. found that about 5% of all human miRNAs increased their expression levels [47]. MiR-127 was the most upregulated after this treatment, and its re-expression led to direct targeting and downregulation of the oncogene *BCL-6*, inducing a tumor suppressor function. MiR-127 is part of a cluster which includes miR-136, miR-431, miR-432, and miR-433 and is embedded in a CpG island region; however, *miR-127* is the only member of the cluster whose expression increases upon treatment with the two epigenetic drugs [47]. Moreover, when each drug was used alone, no variation in *miR-127* expression was observed [47], suggesting that both DNA methylation and histone modifications affect the epigenetic regulation of *miR-127*. This seminal work shows that indeed miRNAs undergo epigenetic regulation, that it is a complex epigenetic regulation (involving both methylation and histone modifications), and that there are differences among miRNAs which even belong to the same cluster. Lujambio et al. created a double knockout (DKO) for DNMT1 and DNMT3B in the CRC cell line HCT-116 and compared miRNA expression profile of DKO and wild-type cells. About 6% analyzed miRNAs were re-expressed in the DKO cells [48]. Among them, miR-124a (embedded in a CpG island heavily methylated in this cell line) was re-expressed, reducing the levels of its direct target gene *CDK6* and impacting on the phosphorylation status of CDK6-downstream effector Rb protein [48]. Prosper's work has identified a signature of 13 miRNAs embedded in CpG islands, with high heterochromatic markers (such as high levels of K9H3me2 and/or low levels of K4H3me3) in acute lymphoblastic leukemia (ALL) patients [49, 50]. Among these, miR-124a was methylated in 59% of ALLs, and its promoter hypermethylation was associated with higher relapse rate and mortality rate vs. non-hypermethylated cases; hence, miR-124a promoter methylation status was an independent prognostic factor for disease-free and overall survival [50]. Finally, supporting Lujambio's results, also in ALL the impact of miR-124a in the CDK6-Rb pathway was confirmed by showing that miR-124a directly silences *CDK6* [50]. Hypermethylation of miR-124a promoter is also involved in the formation of epigenetic field defect which is a gastric cancer predisposing condition characterized by accumulation of abnormal DNA methylation in normal-appearing gastric mucosa, mostly induced by *H. pylori* infection [51]. These findings also suggest that miR-124a promoter hypermethylation is an early event in gastric carcinogenesis. MiR-107, another epigenetically controlled miRNA, targets *CDK6* in pancreatic cancer as well and impacts this oncogenic pathway [52]. In HCT-116 cells, deficient for DNMT1 and DNMT3B, Bruckner et al. showed increased expression of let-7a-3, an miRNA normally silenced by promoter hypermethylation in the wild-type cell line [53]. In lung adenocarcinoma, primary tumors let-7a-3 promoter was found hypomethylated with respect to the normal counterpart [53], whereas hypermethylation of let-7a-3 promoter was described in epithelial ovarian cancer, paralleled the low expression

of insulin-like growth factor-II expression, and was associated with a good prognosis [54]. Therefore, DNA methylation could act as a protective mechanism by silencing miRNA with oncogenic function. Also, the miRNA-200 family participates in the maintenance of an epithelial phenotype, and loss of its expression can result in epithelial to mesenchymal transition (EMT). Furthermore, the loss of expression of miR-200 family members is associated with an aggressive cancer phenotype. Vrba et al. [55] found that hypermethylation of the miR-200c/141 CpG island is closely linked to their inappropriate silencing in cancer cells, and the epigenetic regulation of this cluster appears evolutionarily conserved, since similar results were obtained in mouse. Interestingly, no variation in miRNA expression was observed in lung cancer cells treated with either demethylating agents or HDAC inhibitors or their combination [56]. Another miRNA which is under epigenetic control is miR-1. In hepatocarcinoma, miR-1 is frequently silenced by promoter hypermethylation [57]. However, in DNMT1 null HCT-116 cells (but not in DNMT3B null cells), hypomethylation and re-expression of miR-1-1 were observed [57], revealing a key role for the maintenance DNMT in the regulation of this miRNA. Han et al. [58] observed that neither *5-AZA* nor *DNMT1* deletion alone can recapitulate miRNA expression profile of DKO DNMT1/DNMT3B HCT-116 cells. Also, Lehmann et al. [59] found that in breast cancer cell lines, 5-AZA re-activates miR-9-1 (hypermethylated in up to 86% of primary tumors), but not miR-124a-3, miR-148, miR-152, or miR-663 (hypermethylated as well). Previously, Meng et al. [60] observed that in malignant, but not in normal cholangiocytes, 5-AZA induces re-expression of miR-370. Overall, these results indicate that the epigenetic control of miRNAs is both cancer specific and miRNA specific. More recently, Chang and Sharan [61] reported that *BRCA1* recruits the *HDAC2* complex to the miR-155 promoter, which is consequently silenced epigenetically through the deacetylation of H2A and H3 histones. The study also showed the upregulation of miR-155 in BRCA1-deficient or BRCA1-mutant human tumors. The knockdown of miR-155 in a BRCA1 mutant tumor cell line attenuates in vivo tumor growth. However, a knockdown of BRCA1 results in a two- to threefold increase in miR-155 levels in vitro. In contrast, a 50–150-fold increase in miR-155 in human breast cancer cell lines or tumor samples was observed, suggesting that this increase may not be caused only by *BRCA1* loss; other transcription factors may activate the miR-155 promoter after it is epigenetically activated due to the loss of BRCA1 [61]. Mazar et al. [62] studied which miRNAs were re-expressed upon treatment of a melanoma cell line with demethylating agents. Among the 15 re-expressed miRNAs, miR-375 and miR-34b were also involved in melanoma progression [62]. Liu et al. [63] found that miR-182 was significantly upregulated in human melanoma cells after combined treatment with 5-AZA and trichostatin A. Genome sequence analysis revealed the presence of a prominent CpG island 8–10 kb upstream of miR-182, but methylation analysis showed that this genomic region was exclusively methylated in melanoma cells, not in normal human melanocytes. Since miR-182 has been shown to harbor oncogenic properties, this finding raises a possible concern for melanoma patients treated with epigenetic drugs [63]. MiR-31 maps at 9p21, a genomic region frequently deleted in solid cancers including melanoma. Asangani et al. [64] found recurrent downregulation of miR-31 in melanoma primary tumors and was associated with genomic loss or epigenetic silencing by DNA methylation and EZH2-mediated histone methylation. Moreover, miR-31 overexpression resulted in downregulation of EZH2 and a derepression of its target gene rap-1GAP. The increased expression of EZH2 was associated with melanoma progression and poor overall survival [64].

Nickel (Ni) compounds are well described human carcinogens. Recently an important regulatory double-negative feedback loop has been discovered between *miR-152* and *DNMT1* in nickel sulfide (NiS)-transformed human bronchial epithelial (16HBE) cells [65]. Expression of *miR-152* was specifically downregulated by promoter hypermethylation, whereas ectopic expression of *miR-152* resulted in a remarkable reduction of *DNMT1* expression in transformed cells.

Interestingly, treatment with 5-AZA or knock down of DNMT1 reversed this process. Further, inhibition of *miR-152* expression in 16HBE cells was found to increase *DNMT1* expression and DNA methylation. Moreover, ectopic expression of *miR-152* caused a significant decrease of cell growth, whereas inhibition of *miR-152* reversed this process in 16HBE cells, suggesting the existence of an important functional negative feedback loop between *miR-152* and *DNMT1*, likely to play an important role in NiS-induced lung carcinogenesis [65]. The relationship between miRNA and cognate host gene epigenetic regulation was addressed by Grady et al. by studying miR-342, located in an intron of the *EVL* (*Ena/Vasp-like*) gene [66]. EVL promoter hypermethylation occurs in 86% of colorectal cancers and is already present in 67% of adenomas, suggesting that it is an early event in colon carcinogenesis. The combined treatment with 5-AZA and the HDAC inhibitor trichostatin A restores the synchronized expression of EVL and miR-342. The *EGFL7* gene, frequently downregulated in several cancer cell lines and in primary bladder and prostate tumors, hosts miR-126 in one of its introns. While the mature miR-126 can be encoded by three different transcripts of the cognate host gene, each of them with its own promoter, *miR-126* is concomitantly upregulated with one of *EGFL7* transcripts which has a CpG island promoter, when cancer cell lines are treated with inhibitors of DNA methylation and histone deacetylation, indicating that silencing of intronic miRNAs in cancer may occur by means of epigenetic changes of cognate host genes [67]. In summary, miRNAs are encoded by either *ncRNA* genes with their own promoters or by noncoding sequences in introns of *PCGs*. In the latter case, miRNA expression is usually driven by the same promoters of the corresponding *PCGs*.

The role of miRNA epigenetic modifications in the metastatic process has also been investigated by several groups. Lujambio et al. [68] treated three lymph-node metastatic cell lines with 5-AZA and identified three miRNAs which showed cancer-specific CpG island hypermethylation: miR-148a, miR-34b/c, and miR-9. The reintroduction of miR-148a and miR-34b/c in cancer cells with epigenetic inactivation inhibited cell motility and their metastatic potential in xenograft models and was associated with downregulation of miRNA oncogenic target genes, such as *c-MYC, E2F3, CDK6*, and *TGIF2* [68]. Finally, promoter hypermethylation of these three miRNAs was significantly associated with metastasis formation also in human malignancies [68]. MiR-34b/c cluster is also epigenetically regulated in CRC (promoter hypermethylation in 90% of primary CRC samples vs. normal colon mucosa) [69], whereas epigenetic silencing of miR-9 and miR-148a (together with miR-152, miR-124a, and miR-663) was described also in breast cancer [59].

Finally, Fazi et al. showed that transcription factors can recruit epigenetic effectors at miRNA promoter regions and contribute to the regulation of their expression. The AML1/ETO fusion oncoprotein is the aberrant product of *t*(8, 21) translocation in acute myeloid leukemia (AML) and can bind to the pre-miR-223 region. The oncoprotein recruits epigenetic effectors (i.e., DNMTs, HDAC1, and MeCP2), leading to aberrant hypermethylation of the CpG in close proximity to the AML1/ETO binding site and H3-H4 deacetylation of the same chromatin region [70]. In SkBr3 breast cancer cell line, Scott et al. were able to demonstrate that 27 miRNA expression levels are rapidly modified (5 up- and 22 downregulated) by a treatment with the HDAC inhibitor LAQ824 [71], indicating that some miRNAs are mainly silenced by histone modifications. In A549 lung cancer cell line, the HDAC inhibitor SAHA deregulates 64 miRNA (>twofold change) targeting genes involved in angiogenesis, apoptosis, chromatin modification, cell proliferation, and differentiation [72]. A list of the discussed epigenetically regulated miRNAs is provided.

In summary, these studies convincingly support an epigenetic regulation of miRNAs, and the fact that cancer cells adopt epigenetic mechanisms to silence/re-express key miRNAs modulating relevant PCGs for the development of their oncogenic phenotype. The metastatic process also seems to be driven, at least in part, by the selected epigenetic regulation of miRNAs, in addition to the well-known epigenetic regulation of relevant PCGs.

21.4 Concluding Remarks

The series of studies listed in this chapter should have convinced the readers that a tight connection relates miRNAs and epigenetics, and this relationship harbors significant implications in the development and spreading of malignancies. Aberrancies of the miRNome can effectively be reversed by overexpressing miRNAs that are downregulated in cancer and/or by silencing miRNAs overexpressed by cancer cells. Synthetically generated miRNA-mimic molecules can be effectively delivered to cancer cells. Conversely, miRNAs can be administered as anti-miRNA molecules in case the silencing of a miRNA needs to be achieved. Most commonly, anti-miRNAs can be administered as antagomiRs [73], or LNA anti-miRNAs [74], which are oligonucleotides complementary to the sequence of the targeted mature miRNA, but biochemically modified to reduce the risk of degradation by cellular RNAses, and are conjugated with cholesterol to facilitate their entrance in the cells. By designing mimics and/or anti-miRNAs of epi-miRNAs, a profound modulation of several epigenetically regulated PCGs is anticipated. Similarly, epigenetic drugs such as 5-AZA and histone active drugs will directly affect the expression of several epigenetically regulated miRNAs, as well as indirectly the expression of those mRNAs modulated by these epigenetically regulated miRNAs. The overall effect on cell phenotype is the combination of these modifications in the transcriptome and miRNome. Therefore, a clear and deep understanding of these basic mechanisms is necessary in order to avoid re-expression of oncogenes and/or onco-miRNAs. Despite the complexity suggested by these interactions, an increasing number of excellent works is bringing us on the right track by dissecting the complexity of such mechanisms and supporting a general optimistic view: that in a future not too far to come, we will be able to effectively translate these discoveries into new strategies to fight cancer, resulting in decreased mortality.

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The Role of DNA Methylation in Cancer

22

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22.1 Introduction

It is becoming increasingly clear that along with genetic instability and mutations, as common events in cancer development, epigenetic and its regulatory mechanisms have a crucial role in malignant cellular transformation [1–3]. The term "epigenetics" was first described by Waddington [4] and literally means "above genetics." It refers to heritable modifications which lead to altered gene expression profiles, without making any changes in primary genome sequence [5]. Epigenetic processes, which include CpG island methylations, histone modifications, and gene expression regulation through non-coding RNAs, are of essential parts in normal developmental processes. However, aberrant epigenetic mechanisms contribute to deviated gene function and carcinogenesis [6, 7].

Among the epigenetic mechanisms mentioned above, DNA methylation is one of the most deeply studied epigenetic alterations [8]. In 1982, Gama-Sosa et al. observed the significant variation of 5-methylcytosine (5-mc) content and distribution in DNA samples from different tissues [9]. This finding led to the next study in 1983, which presented the overall and considerable different 5-mc content of DNA samples obtained from normal and tumor tissues [10]. In the same year, Feinberg and Vogelstein [11, 12] reported altered DNA methylation of specific genes in some human cancer cells compared with their normal counterparts. Afterwards, over three decades of research on DNA methylation, it is now revealed that this phenomenon as one of the most important epigenetic modifications is involved in the variety of biological procedures and play a critical role principally through regulating gene activity [13]. It is not surprising, therefore, that aberrant DNA methylation may lead to inappropriate gene expression and consequently uncontrolled cell growth which are the hallmarks of cancer [14–16].

This chapter focuses on the molecular basis of DNA methylation, tumor-related genes and tumor-specific methylation, clinical approaches of using DNA methylation as a biomarker in early diagnosis, prognosis and also as a therapeutic target, and common methods for assessing DNA methylation.

22.2 Molecular Basis of DNA Methylation

DNA methylation usually occurs at the 5′ position of cytosine within CpG dinucleotides and results in gene silencing. The CpG dinucleotides are distributed throughout the human genome, while CpG islands (CGIs) are condensed clusters of CpG dinucleotides of the genome, which are frequently positioned in the 5′-flanking promoter regions of genes. CGIs are predominantly free of methylation in "housekeeping" genes and provide an active transcription, although certain CpG islands, which are involved in imprinting and X inactivation, become normally methylated.

As shown in Fig. 22.1, transferring of a methyl group from S-adenosylmethionine (SAM) as a methyl-donor to the fifth-position of the cytosine residue in a CpG dinucleotide as a methyl-acceptor is catalyzed by DNA methyltransferases (DNMTs) [7, 17, 18].

Although the focus of this chapter is on the role of CpG island promoter methylation in inhibiting gene expression, regulating the role of methylation in non-CpG island promoters such as 5′-CpNpG-3′ or non-symmetrical 5′-CpA-3′ and 5′-CpT-3′ [19] should not be overlooked.

Cytosine is converted to 5-methylcytosine by transferring a methyl group from s-adenosyl methionine to fifth-position of cytosine. This reaction is catalyzed by DNA methyltransferases (DNMTs).

22.3 DNA Methyltransferases (DNMTs)

DNMT1, DNMT3A, DNMT3B are three active members of the highly conserved DNMT family in mammalians. The fourth protein, DNMT3L, is the regulatory subunit and does not possess any catalytic activity. However, it has a critical role in DNA methylation through stimulation of DNMT3A/B activity [20–22].

Alterations in DNMT genes (such as overexpression and mutation) are of primary mechanisms that result in aberrant methylation patterns and consequently malignant transformation [23–25].

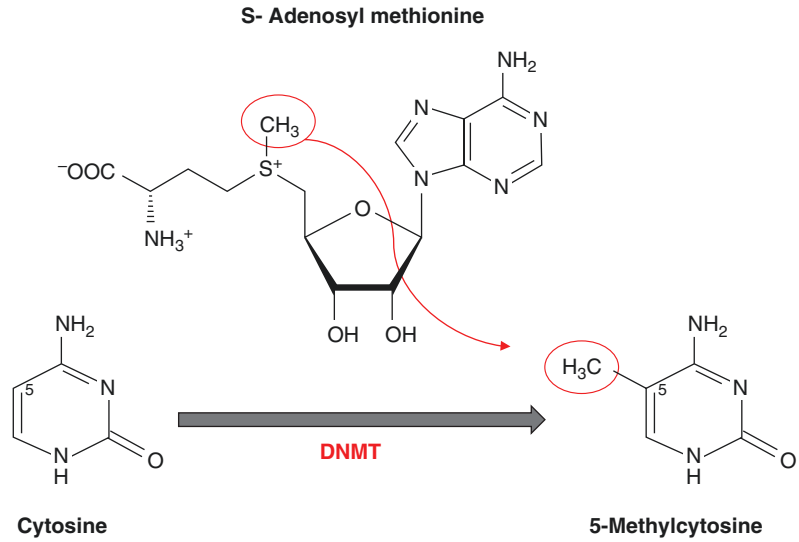

Fig. 22.1 Cytosine methylation

Table 22.1 Human DNMTs

Enzyme	Position	Function	Variation in DNMT genes and cancer development	OMIM
DNMT1	19p13.2	Methylation maintenance through binding to hemi-methylated DNA during DNA replication	*Overexpression* in cancers of breast [26], liver [27], pancreas [28], esophagus [29], AML[a] and CML[b] [30]/*Mutation* in colon cancer [31]	126,375
DNMT3A	2p23.3	De novo DNA methylation of both hemi-methylated and non-methylated DNA	*Overexpression* in liver cancer [32], CML and AML [30]/*Mutation* in AML [33–35], ALL[c] [36], and MDS[d] [37]	602,769
DNMT3B	20q11.21	De novo DNA methylation of both hemi-methylated and non-methylated DNA	*Overexpression* in cancers of breast [38], prostate [39], colon [40], AML and CML [30]	602,900
DNMT3L	21q22.3	Interacting with DNMT3A/B and increasing their catalytic activity	*Overexpression* in pure EC[e], advanced pure seminoma and pure yolk sac tumor [41, 42]	606,588

[a]*AML* acute myeloblastic leukemia
[b]*CML* chronic myeloblastic leukemia
[c]*ALL* acute lymphoblastic leukemia
[d]*MDS* myelodysplastic syndrome
[e]*EC* embryonal carcinoma

Location of human DNMT genes, their functions, and their association with tumorigenesis are indicated in Table 22.1.

22.4 Gene Silencing Mediated by DNA Methylation

Gene expression regulation in eukaryotic cells is almost a complex mechanism and interaction of a number of epigenetic components is required for a precise transcriptional regulation. Several hypotheses have been suggested as the mechanisms of gene silencing through DNA methylation (Fig. 22.2). In the first one, DNA methylation blocks promoter region and directly prevents binding of particular transcription factors (TFs) such as AP-2, c-Myc/Myn, CREB/ATF, E2F, the cyclic AMP-dependent activator CREB, and NF-kB to their recognition sites in their promoters [43, 44].

Another proposed mechanism of repression is the attachment of methyl-CpG binding domain proteins (MBDs) such as MeCP1, MeCP2, MBD1, MBD2, and MBD4 to methylated

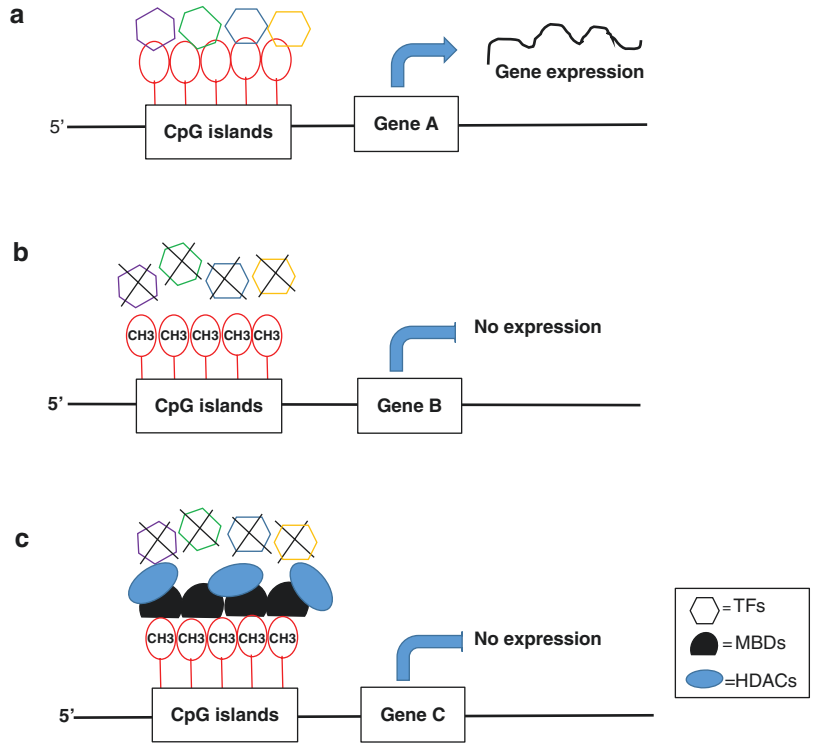

Fig. 22.2 Two proposed mechanisms of expression inhibiting mediated by DNA methylation. (**a**) When CpG islands are demethylated, transcription factors (TFs) can access to their respective recognition sites and the gene is expressed. (**b**) The expression is abolished by promoter methylation through the direct interference of binding specific TFs to the promoter region. (**c**) Methyl-CpG binding domain proteins (MBDs) bind methylated DNA, limit the accessibility of TFs to their promoter, and stimulate repressive potential of methylated DNA by employing histone deacetylases complexes (HDACs)

DNA. They repress transcription by blocking access to other elements required for gene expression and recruiting histone deacetylases complexes (HDACs), which results in chromatin denseness and gene silencing [45, 46].

22.5 Aberrant DNA Methylation and Cancer

Any disruption in proper DNA methylation—as an essential process for normal development and cell function—may lead to several disorders, including cancer. When compared with normal cells, cancer cells show a different pattern in DNA methylation, including global hypomethylation of repeated DNA sequences (such as long interspersed transposable elements (LINEs), short interspersed transposable (SINEs), and Arthrobacter luteus (Alu)) and localized disrupted hypermethylation events in CpG islands. A global reduction in methylated CpG content results in activation of silenced oncogenes and consequently increasing positive growth signals. In contrast, inactivation of tumor suppressor genes occurs due to CGI hypermethylation, which leads to a decrease in growth preventive signals. These events together provide a selective growth advantage to the cell and therefore tumorigenesis [47–49].

22.6 DNA Hypermethylation and Hypomethylation in Cancer

There is now no doubt that DNA methylation has a vital role in tumor development and is a hallmark of all types of human cancers. Studies have

shown a greater contribution of hypermethylation than hypomethylation to cancers. To date, several numbers of genes have been reported, which are influenced by aberrant methylation in malignancies. Some of these genes have been found to undergo altered methylation in one type of cancer, while some other genes are subjected to disrupted methylation in varieties of tumor types. In addition, some tumors present aberrant methylation in various genes. For instance, more than 40 genes and 60 genes have been found to have the alteration in DNA methylation patterns in lung and gastric cancer, respectively. RARB, RASSF1A, CDKN2A, MGMT, BRCA1, IGFBP3, CDH1, TIMP3, DAPK1, GSTP1, ESR1, and APC are examples of commonly hypermethylated ones in both lung and gastric cancers (Table 22.2) [88, 121].

Certain genes, which are unmethylated in normal cells, become inactivated in the cancer cells as a result of hypermethylation. Hypermethylation was first reported in the promoter of retinoblastoma tumor repressor gene (RB1) in patients with retinoblastoma [122, 123] and followed by identification of numerous tumor suppressor genes, which were silenced through hypermethylation in various cancers. These genes are involved in several crucial pathways such as angiogenesis, DNA repair, cell cycle, apoptosis, transcription, cell growth, differentiation, and cell adhesion (Table 22.2).

The cell cycle is the specific sequential events, which are regulated strictly by the complex group of components and eventually lead to cell growth, DNA replication, and cell division. Dysregulated cell cycle due to any alterations in proteins involved in cell cycle may result in the development of tumors [124]. For instance, CDKN2A (P14/P16) and CDKN2B, three important cell cycle-related tumor suppressor genes located on chromosome 9, encode cyclin-dependent kinase inhibitors and are involved in inhibiting cell cycle G1 progression. They undergo DNA methylation and become suppressed in different types of cancer (Table 22.2) [62, 125].

The genes associated with DNA repairs such as MGMT, hMLH1, BRCA1, XRCC1, and WRN are also hypermethylated in carcinomas. Genomic integrity maintenance would be assured by the accurate function of DNA repair system (Table 22.2) [126]. Defective DNA repair pathways lead to unrepaired or incorrectly repaired lesions, which eventually result in cell neoplastic transformation. MGMT which protects against the negative impact of DNA alkylation in normal tissues was indicated to be silenced through hypermethylation in various types of tumors (Table 22.2) [56, 57]. MLH1 is one of the main members of DNA mismatch repair (MMR) system and was shown that hypermethylation in this gene is frequently associated with colorectal and gastric cancers (Table 22.2) [50–52]. DNA methylation-mediated silencing in BRCA1, as a gene involved in DNA repair of double-stranded breaks, maintenance of genome integrity and transcription, has been identified in many tumor tissues. They include breast, lung, esophageal, gastric and ovarian cancer, as well as high-grade serous ovarian cancer (HGSOC) which is a subtype of ovarian cancer with distinct clinical behaviors and biomolecular features (Table 22.2) [59, 60, 127]. Aberrant promoter methylation of X-ray repair cross complementing 1 (XRCC1), which acts as a scaffolding protein for single-strand break repair (SSBR), BER (base excision repair), and NER (nucleotide excision repair) has shown association with gastric cancer (Table 22.2) [61]. WRN functions as a tumor suppressor gene and its inactivation via promoter hypermethylation leads to errors in DNA replication and chromosomal instability, as well as DNA repair. Aberrant epigenetic silencing of WRN has been reported in a wide range of tumors including colon, gastric, prostate, lung, and breast cancers (Table 22.2) [109, 110].

Cancer metastasis causes neoplastic progression through the spread of cancer cells from the primary tumor mass to surrounding tissues and forming a new tumor. Cell–cell and cell–matrix interactions are crucial in the maintenance of tissue integrity [76]. Silencing the genes involved in cell adhesion via DNA hypermethylation leads to loss of contact inhibition and consequently invasion and metastasis. Several genes connected with cell adhesion have been identified in different tumors that are silenced by DNA hypermethylation, including CDH1/E-cadherin, TSP1, PCDH10, PCDH17, CDH13/ H-cadherin,

Table 22.2 Examples of genes commonly methylated in different types of cancer

Gene	Full name	Location	Function	Tumor type
hMLH1	Mut L homologue 1	3p22.2	DNA mismatch repair	Colon [50], Gastric [51, 52], Endometrium [53], Lung [54], Ovarian [55]
MGMT	O-6-methylguanine-DNA methyltransferase	10q26.3	DNA repair	Lung [53], Brain [53], Gastric [56, 57], Lymphoma [53], Colon [53], Prostate [58]
BRCA1	Breast cancer 1	17q21.31	DNA repair, transcription	Breast [14, 59], Ovarian [59], high-grade serous ovarian cancer [60]
XRCC1	X-Ray repair cross complementing 1	19q13.31	DNA repair	Gastric [61]
CDKN1C	Cyclin-dependent kinase inhibitor 1C	11p15.4	Cell cycle	Gastric [62]
IGFBP3	Insulin like Growth Factor Binding Protein 3	7p12.3	Cell growth and cellular proliferation regulation	Gastric [63]
PTEN	Phosphatase and tensin homolog	10q23.31	Apoptosis, neurogenesis, PI3K-AKT/PKB and mTOR pathway regulation	Gastric [64]
TCF4	Transcription factor 4	18q21	Differentiation, transcription regulation	Gastric [65]
PRDM5	PR/SET Domain 5	4q27	Transcription regulation	Gastric [66]
CDKN2A/P16	Cyclin-dependent kinase 2A	9p21.3	Cell cycle	Lymphoma [14, 67], Lung [14], Gastric [62], Bladder [14], Melanoma [68], Ovarian [69], Pancreas [70], Colon [14]
CDKN2A/P14	Cyclin-dependent kinase 2A	9p21.3	Stabilizing p53, cell cycle	Colon [53], Gastric [62]
CDKN2B / P15	Cyclin-dependent kinase inhibitor 2B	9p21.3	Cell cycle	Leukemia [53, 71], Lymphoma [72, 73], Gastric [62], Squamous cell carcinoma [72]
CDH1	Cadherin 1	16q22.1	Cell adhesion, proliferation	Breast [74], Thyroid [75], Gastric [62, 76], Lung [77], Lymphoma [64]
FLNc	Filamin C	7q32.1	Cell junction	Gastric [52]
HOXA10	Homeobox A10	7p15.2	Developmental protein, transcription regulation	Gastric [62]
TIMP3	Tissue inhibitor of metalloproteinases 3	22q12.3	Cell migration, differentiation	Gastric [78], Renal [79], Brain [79], Breast [79], Colon [79], Lung [79, 80]
TSP1	Thrombospondin 1	15q14	Cell adhesion, cell cycle arrest, cell migration	Gastric [81]
HOXA1	Homeobox A1	7p15.2	Developmental protein, transcription regulation	Gastric [62]
HoxD10	Homeobox D10	2q31.1	Developmental protein, transcription regulation	Gastric [82]
NDRG2	NDRG family member 2	14q11.2	Cell differentiation, Wnt signalling pathway regulation	Gastric [83]
RARRES1	Retinoic acid receptor responder 1	3q25.32	Cell proliferation	Gastric [62], Prostate [84]
BNIP3	BCL2 interacting protein	10q26.3	Apoptosis	Gastric [85, 86]
CACNA1G	Calcium voltage-gated channel subunit alpha1 G	17q21.33	Gene expression, cell division and cell death, cell growth	Gastric [62], Leukemia [87]

Table 22.2 (continued)

Gene	Full name	Location	Function	Tumor type
DAPK1	Death-associated protein kinase 1	9q21.33	Apoptosis, translation regulation	Lung [80], Gastric [86, 88], Lymphoma [53]
GSTP1	Glutathione S-transferase Pi 1	11q13.2	Glutathione transferase activity	Prostate [53], Breast [53], Renal [53], Gastric [89]
PCDH10	Protocadherin 10	4q28.3	Cell adhesion, apoptosis	Gastric [90]
PCDH17	Protocadherin 17	13q21.1	Cell adhesion, apoptosis	Gastric [91], Colon [91]
ESR1	Estrogen receptor 1	6q25.1	Transcription regulation, signal transduction	Breast [92], Prostate [58], Colon [93]
hDAB2IP	DAB2 interacting protein	9q33.2	Angiogenesis, apoptosis, cell cycle, growth regulation	Prostate [58], Gastric [94]
RASSF1A	RASSF1A, Ras association domain family member 1	3p21.31	Cell cycle, apoptosis, signal transduction	Lung [95], Breast [95], Gastric [96], Ovarian [95], Renal [97], Nasopharyngeal [98]
RASSF6	Ras association domain family member 6	4q13.3	Cell cycle, apoptosis, cell migration, signal transduction	Leukemia [99, 100]
RASSF10	Ras association domain family member 10	11p15.2	Cell cycle, apoptosis, cell migration, signal transduction	Leukemia [99, 100]
SOCS-1	Suppressor of cytokine signaling 1	16p13.13	Signal transduction, growth regulation	Liver, Gastric [101]
APC	Adenomatous polyposis coli	5q22.2	Wnt signaling antagonist	Breast [102], Lung [102], Esophageal [103], Gastric [78], Prostate [58]
Dkk-3	Dickkopf WNT Signaling pathway inhibitor 3	11 p15.3	Developmental protein, Wnt signaling pathway	Gastric [104, 105]
ITGA4	Integrin subunit alpha 4	2q31.3	Cell adhesion	Gastric [62]
TP73	Tumor protein p73	1p36.32	Apoptosis, cell cycle, transcription regulation	Lymphoma [53], Leukemia [53], Gastric [62]
VHL	Von Hippel–Lindau tumor suppressor	3p25.3	Protein ubiquitination pathway	Renal [97]
RARB	Retinoic acid receptor beta	3p24.2	Cell growth, differentiation, gene expression regulation	Colon [53], Leukemia, Lymphoma [53], Lung [53], Prostate [58], Gastric [106–108], Breast [53]
WRN	Werner syndrome RecQ like helicase	8p12	DNA repair	Colon [109, 110], Gastric [109], Prostate [109], Lung [109], Breast [109], Thyroid [109]
EMP3	Epithelial membrane protein 3	19q13.33	Cell growth, cell proliferation, cell death, cell-cell interactions	Brain [111]
THBS1	Thrombospondin-1	15q14	Cell adhesion, angiogenesis inhibitor	Colon [112]
TPEF	Transmembrane Protein with EGF like and two follistatin like Domains 2	2q32.3	Proliferation, differentiation, apoptosis	Bladder [113], Colon [114], Gastric [115]
ARNTL	Aryl hydrocarbon receptor nuclear translocator like	11p15.3	Transcription regulation, circadian rhythms	Leukemia [116], Lymphoma [116]
CDH13	Cadherin 13	16q23.3	Cell adhesion	Breast [117], Lung [118], Lymphoma [64]
GATA4	GATA Binding Protein 4	8p23.1	Transcription regulation	Colon [119]
GATA5	GATA Binding Protein 5	20q13.33	Transcription regulation	Colon [119]
AR	Androgen receptor	Xq12	Signal transduction, transcription regulation	Prostate [120]

ITGA4, and THBS1. CDH1 is one of the most important ones and its promoter methylation is assumed to be associated with tumor invasion and metastasis, particularly in primary gastric cancer (Table 22.2) [76, 128–131].

DNA hypermethylation also affects further genes which contribute to other cellular processes, such as the genes involved in apoptosis (for example, PYCARD, CASP8, and BCL2), or transcription (like GATA4 and GATA5 that are implicated in colon cancer, and ID4 in leukemia), or angiogenesis (for instance, THBS1 and hDAB2IP) (Table 22.2).

Some examples of tumor-related genes, silenced by CGI promoter hypermethylation and their biological role are listed in Table 22.2.

Genome-wide hypomethylation of DNA, on the other hand, results in a reduction of 5-mC and has been reported in a variety of malignancies [11, 132]. It is found that reduced levels of DNA hypomethylation in proto-oncogenes and repetitive DNA sequences are correlated with loss of imprinting, reactivation of transposons and retroviruses, and chromosome instability, which all may be implicated in oncogenesis [133]. It is shown that during the cancer progression, hypomethylation level increases in the area of lesions; so that a benign cell proliferation would be changed to an invasive malignant tumor [134]. Genome-wide hypomethylation was seen in esophageal squamous cell carcinoma (ESCC) [135], metastatic hepatocellular cancer [136], cervical cancer [132], and prostate tumors [137]. Increasing the grade of global hypomethylation of DNA in several cancers, including breast, cervical, and brain, has been associated with the level of malignancy [48].

Hypomethylation of mobile DNAs causes loss of genomic integrity and stability through integration at random sites in the genome. For instance, the L1 mutational insertions leading to disrupted expression have been identified in APC and CMYC genes in colon and breast cancers, respectively [20]. Reduced level of methylation in the L1 long interspersed nuclear element also leads to transcriptional activation and is found in ESCC [135], gastric [138], colon [139], and urinary bladder cancer [140]. Moreover, hypomethylation of satellite repeats has been frequently seen in human cancers such as Wilms tumor, ovarian and breast cancer [141–144].

Loss of DNA methylation can be linked to distinct stages in different cancers. Sat2 (juxtacentromeric satellite 2) and Sat α (centromeric satellite α) are two examples of satellite sequences which are associated to primary tumor development in breast cancer, whereas they contribute to tumor progression in ovarian cancer [145, 146].

As shown in Table 22.1, variations in DNMT genes such as their overexpression or mutations are linked to cancer development. There is also a relationship between aberrant DNMTs and genome-wide hypomethylation, along with subsequent tumor occurrence [8]. For example, it is found that any reduction in DNMT1 leads to global hypomethylation and chromosomal instability [147]. Mutations in DNMT3A are also seen in acute myeloblastic leukemia (AML), following hypomethylated CpG islands of the HOXB cluster [34].

22.7 DNA Methylation as a Biomarker

The effectiveness of therapy and prognosis in most cancers are often dependent on the clinical stage at the time of diagnosis, as well as the ability to the prediction of therapeutic response through monitoring cancerous cells. Diagnostic tools have proven valuable. However, they are not always suitable for early diagnosis, comfortable enough, and risk-free for the patients. Thus, there is an eager to develop novel, less-invasive, and more powerful strategies for earlier detection, investigating treatment response, detection of residual disease, and risk assessment of relapse.

DNA methylation patterns have been provided a spectrum of opportunities to be applied as biomarkers in clinical practice. DNA methylation alterations are frequently involved in tumorigenesis/or cancer development and could be detected in surrogate tissues such as specimens like sputum, plasma, serum, stool, or urine in a non-invasive manner, which makes them the attractive

source of potential biomarkers to diagnosis and therapeutic stratification.

Factor pathway inhibitor 2 (*TFPI2*), septin 9 (*SEPT9*), glutathione S-transferase pi 1 (*GSTP1*), vimentin (*VIM*), and short stature homeobox 2 (*SHOX2*) are some of the most widely studied and well-validated DNA methylation detection biomarkers which are detectable in distal sites [148, 149]. DNA methylation of *TFPI2* is sensitively and specifically detectable in the stool DNA from stage I to III of [150], and with the less sensitivity in the serum of patients with CRC [151]. Hypermethylation of *SEPT9* gene in blood samples of the patients affected with colorectal cancer is successfully measurable with a high sensitivity and specificity, as well [152–154]. DNA methylation of *GSTP1* has been provided value as a promising biomarker in the urine samples of prostate cancer patients [155–157]. *VIM* and *SHOX2* are another valuable DNA methylation-based biomarkers for the early detection of colorectal and lung cancer, respectively. Hypermethylation of *VIM* can be detected in the stool sample of 86% of colon cancers and 76% of adenomas [158–160]. Methylation quantification of *SHOX2* in bronchial aspirates and plasma of the patients diagnosed with malignant lung disease has been proved that *SHOX2* is one of the most promising methylation biomarkers in lung cancer patients [161–165].

The DNA methylation profile can also be screened for minimal residual disease (MRD) detection, molecular prognosis, and therapy response prediction. DNA hypermethylation in the promoter of the genes *CDKN2A* (*P16*), H-cadherin (*CDH13*), Ras association domain family 1A (*RassF1A*), and adenomatous polyposis coli (*APC*) has been showing strong association with high risk of early relapse and short survival in non-small-cell lung cancer (*NSCLC*) [166, 167]. Hypermethylation of the gene O6-methylguanine-DNA methyltransferase (*MGMT*) can be used as a predictive biomarker in the patients with colorectal and brain tumor [168–174]. Methylation status of paired-like homeodomain 2 (*PITX2*) gene is a candidate prognostic biomarker in breast [175–178], prostate [179–181], and lung cancer [182].

It should be noticed that although DNA methylation profile has been provided the mentioned valuable opportunities, there are some challenges for using DNA methylation as a cancer biomarker and the following criteria should ideally be considered: First of all, the entire promoter region CpG island has to be carefully studied. Secondly, regarding inter-individual variations in DNA methylation of the specific genes, for DNA methylation analysis a reference line needs to be defined in the normal control tissue to compare the rate of aberrant methylation with cancerous tissue. In addition, the examined region should be unmethylated in normal individuals and methylated in cancerous cells. Furthermore, false-positive (maybe due to age-dependent increase in DNA methylation or other reasons) and false-negative (maybe as a result of improper selection of investigated region or other explanations) results should be considered [149].

22.8 DNA Methylation as a Therapeutic Target

In contrast with genetic alterations, epigenetic variations are reversible, which provides the opportunity for epigenetic targeting in order to cancer therapy. One of the developed strategies for epigenetic therapy is employing the agents that can inhibit DNMTs and restore the normal DNA methylation pattern [183].

The DNMT inhibitors (DNMTi) which are currently using clinically are cytosine analogs, DNA binders, S-adenosyl-l-methionine cofactor competitors, or oligonucleotides that unlike other chemotherapeutic medications do not target cells for immediate death [184]. DNMTi inhibit the function of DNMTs whether through incorporation into newly synthesized DNA and binding DNMT enzymes covalently during DNA replication, or by trapping DNMT enzymes which subsequently leads to proteasomal DNMTs degradation [184, 185].

Two most commonly used DNMT inhibitors, which are currently US Food and Drug Administration (FDA) and the European

Medicines Agency (EMA) approved for the treatment of myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), and chronic myelomonocytic leukemia (CML), are 5-azacitidine (5-Aza-CR, vidaza) and 5-aza-2′-deoxycytidine (5-Aza-CdR, decitabine) [186, 187]. Even though these compounds have also been investigated in various solid tumors, clinical trials represent that this kind of treatment has not been fruitful in solid tumors [188].

While 5-azacitidine and decitabine are particularly potent inhibitors of DNA methylation, several other agents have been proposed as being DNMTs due to some weak points (such as relative instability, low specificity, and side effects) [189]. For example, zebularine is a more stable and less toxic cytidine analogs. However, higher dose requirement for efficient therapy prevents it to be used in the clinical practice [184, 190]. Other inhibitors such as the local anesthetic procaine, epigallocatechin-3-gallate (EGCG), RG108, SGI-1027, SGI-110 (guadecitabine), hydralazine, procainamide, or psammaplin have been developed to improve the pharmacokinetic profile [191–198].

SGI-1027 inhibits DNMT1 and DNMT3a through DNA-binding mechanism, induces DNMT1 degradation, and reactivates tumor suppressor genes such as TIMP3, MLH1, and P16 by blocking DNMT1 [195, 199].

SGI-110, a dinucleotide composing of decitabine and deoxyguanosine, is one of the second-generation DNMT inhibitors that is in clinical trials (NCT01261312, NCT02901899, NCT01752933) for the treatment of AML, MDS, ovarian and advanced hepatocellular carcinoma (HCC) [184, 191, 200].

4′ -Thio-2′ -deoxycytidine (TdCyd), 5-fluoro-2′-deoxycytidine (FdCyd) are other examples of cytosine analogs that are in clinical trials for cancer therapy. The first one is in phase I trial in the patients with advanced solid malignancies (NCT02423057) [201], and the latter is in trials for the treatment of advanced solid tumors, AML, and MS (NCT00359606, NCT01041443) [202].

MG98 and miR29b are also examples of oligonucleotide-based inhibitors, which repress DNA methylation of tumor suppressor genes and prevent tumor growth [203–206].

Phthalimido-L-tryptophan RG-108 is one of the SAM competitors that show DNMT inhibition activity and TSG reactivation in the colon cancer cell line HCT116 [193, 207].

In addition, the results of many clinical trials suggest that using DNMTi in combination with other therapies (such as cytotoxic agents, immunotherapy, or other epigenetic therapies) have been associated with beneficial effects in cancer treatment.

Emerging data represent that immunotherapeutic approaches show impressive improved responses when they are simultaneously used with epigenetic agents. Using immune checkpoint blockades such as inhibitors of programmed cell death protein 1 (PD-1) and its ligand (PD-L1) or anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) immune checkpoints, combined with epigenetic targeting has shown promising in cancer immunotherapy improvement. Combining decitabine with nivolumab (anti-PD-1) in patients with non-small cell lung cancer (NSCLC) (phase II, NCT02664181); the combination of azacitidine and pembrolizumab (anti-PD-1) in metastatic melanoma (phase II, NCT02816021); azacitidine in combination with durvalumab (anti-PD-L1) and tremelimumab (anti-CTLA-4) in the patients with head and neck cancer who developed during or subsequent to anti-PD-1, anti-PD-L1, or anti-CTLA-4 monotherapy (phase I/II, NCT03019003); using guadecitabine in combination with ipilimumab (anti-CTLA-4) in metastatic melanoma (phase I, NCT02608437); the combination of azacitidine and pembrolizumab in non-small cell lung cancer patients who previously treated locally advanced or metastatic (phase II, NCT02546986); decitabine in combination with ipilimumab in relapsed or refractory MDS and AML (phase I, NCT02890329) are examples of clinical trials combining DNMTi and immune checkpoint blockades in cancer therapy [208, 209].

22.9 Common Methods for Assessing DNA Methylation

Research projects on DNA methylation have grown dramatically during the past decade and numerous strategies have been designed for detecting and validating DNA methylation status.

DNA methylation pattern detection by various methods is carried out based on three strategies:

22.9.1 Affinity-Based

The third strategy to differentiate between methylated and unmethylated cytosines is affinity-based approaches. In this technique, methylated fragments of DNA are immunoprecipitated by specific antibodies against the proteins that recognize 5-methylcytosines (methyl-CpG-binding proteins/MBDs). The procedure is processed by affinity purification on MBD beads, which allows purification of methylated fragments of DNA. Purified fragments are then subjected to further analysis such as microarray-based analyses or sequencing to identify them [210]. Affinity-based methods for screening of DNA methylation alterations are shown in Table 22.3.

22.9.2 Bisulfite-Based

Sodium bisulfite sequencing is one of the most widely used techniques for investigating and gold standard for validating the DNA methylation status. During bisulfite treatment, methylated and unmethylated cytosines on CpG dinucleotides react differentially to sodium bisulfite, so that 5-mC remains unaffected while C is converted into uracil (U). The conversion could then be identified using a variety of approaches through comparing the DNA sequence before and after bisulfite treatment. Table 22.4 provides an overview of the techniques based on sodium bisulfite treatment [214].

22.9.3 Methyl-Sensitive Restriction Enzyme-Based

In this approach, restriction enzymes distinguish sequences based on methylation status, so that only one of the two portions (either methylated or unmethylated) is digested and the other part remains unchanged after the restriction digestions. Several methyl-sensitive restriction enzyme-based methods are listed in Table 22.5, with their advantages and limitations.

It should be mentioned that nowadays a variety of massively parallel DNA sequencing platforms are available for DNA methylation analysis, which include next-generation sequencing (NGS) and single-molecule sequencing. The advent of NGS technology allows sequencing and DNA methylation analysis of millions of DNA fragments across the genome, in parallel. NGS approaches not only have increased the speed and throughput abilities of DNA sequencing, but also they are becoming a

Table 22.3 Affinity-based strategies DNA methylation: (reviewed in Olkhov-Mitsel and Bapat [211])

Method	Short description	Advantages	Limitations
Methylated DNA immunoprecipitation (MeDIP)	Methylated fractions of single-stranded DNA are immunoprecipitated using monoclonal anti-5-methylcytosine antibodies, following by amplification and sequencing (MeDIP-Seq) or hybridization to microarray platforms	Very sensitive to densely methylated sequences, commercial kits are available, global methylation analysis	Single-stranded DNA is needed, large amounts of genomic DNA is required, restricted to the antibody quality and specificity
Methylated CpG island recovery assay (MIRA)	Methyl-CpG-binding proteins (MBD2b/MBD3-Like1 protein) complex are utilized for purification of methylated DNA sequences, following by microarray-based analyses or direct sequencing	High sensitivity, sequence-independent, single-stranded DNA is not required, global methylation analysis	Restricted to the MBD binding specificity

Table 22.4 Bisulfite-based strategies (reviewed in literature [211–213])

Method	Short description	Advantages	Limitations
Whole genome shotgun bisulfite sequencing (WGSGS)	Short fragments of bisulfite-modified DNA are sequenced in parallel	High sensitivity, global DNA methylation analysis	Too expensive
Reduced representation bisulfite sequencing (RRBS)	DNA is digested using BglII or MspI to enrich the CpG sites, following by bisulfite-modification, PCR and sequencing	High sensitivity, global DNA methylation analysis, low cost, low sample input	Restricted to restriction enzymes digestion sites, all CpG islands or promoters are not captured
Denaturing HPLC (DHPLC)	Bisulfite-modified DNA is partially denatured and separated based on different G/C content by HPLC	Simple, rapid, simultaneous detection of methylated CpGs	Expensive equipment, large sample input
MethyLight	Bisulfite-converted DNA is amplified with methylation-specific primers and a TaqMan fluorescent probe	High-throughput, sensitive, quantitative, low cost	A control gene is needed for analysis, it needs a great number of specific probes to detect a region with many CpG sites
Bisulfite sequencing (BS)	Amplified fragments are sequenced to detect the methylation status of multiple CpG sites	10–30 CpG sites can be checked	Medium sensitivity, laborious, relatively expensive
Methylation-specific PCR (MSP)	Bisulfite-modified DNA is amplified with two pairs of primers (one for methylated and one for unmethylated CpGs) for detection of methylation status	Easy, rapid, cheap	A few CpGs can be investigated, qualitative, high sensitivity
Methylation-sensitive melting curve analysis (MS-MCA)	Bisulfite-modified DNA is monitored using a fluorescent dye, based on changing in the melting properties of PCR amplicons during MSP	High-throughput, quantitative	Medium sensitivity, difficult to interpret
Methylation-sensitive high-resolution melting (MS-HRM)	Bisulfite-modified DNA is monitored using a fluorescent dye, based on changing in the melting properties of PCR amplicons during MSP	High-throughput, quantitative, high sensitivity	Difficult to interpret
Sensitive melting analysis after real-time methylation-specific PCR (SMART-MSP)	Bisulfite-modified DNA is amplified using a fluorescent dye and methylation-specific primers, following by HRM analysis	High sensitivity, low false-positive frequency, high-throughput, quantitative	A control gene is needed for analysis
Methylation-specific fluorescent amplicon generation (MS-FLAG)	Bisulfite-modified DNA is amplified using methylation-specific primers and released signal is detected during PCR by PspGI-mediated digestion	Quantitative, high-throughput, low false-positive frequency	Medium sensitivity, using gel electrophoresis, low resolution
Methylation-sensitive single-nucleotide primer extension (MS-SNuPE)	Bisulfite-modified DNA is amplified using the primers that anneal to the sequence up to the nucleotide located prior to the CpG of interest. The methylated DNA results in the tag of dCTP at the end of primer, whereas the unmethylated DNA produces the dTTP to anneal to the primer	Quantitative, simultaneous detection of methylated CpGs, rapid	Medium sensitivity, using radioactive labeling

Table 22.5 Methyl-sensitive restriction enzyme-based strategies (reviewed in literature [211–213])

Method	Short description	Advantages	Limitations
Restriction landmark genome scanning (RLGS)	DNA is digested using methylation-sensitive restriction enzymes, then digested fragments are separated on a two-dimensional gel electrophoresis	Global methylation analysis	Using radioactive material and gel electrophoresis, low sensitivity, limited genome coverage
HpaII tiny fragment enrichment by ligation-mediated PCR (HELP)	DNA is digested using both HpaII and MspI restriction enzymes, followed by PCR amplification and hybridization to microarrays	Positive representation of hypomethylation and hypermethylation status of CpGs, global methylation analysis	Only CpG islands located within restriction sites are detected, low sensitivity for high CpG density regions
Methyl-Seq	DNA is digested using HpaII or MspI restriction enzymes, followed by size fractioning and next-generation sequencing	High sensitivity	High-quality DNA is required
Luminometric methylation assay (LUMA)	DNA is digested using EcoRI and HpaII, or EcoRI and MspI, followed by pyrosequencing extension	High sensitivity, quantitative, relatively small amount of DNA is required	High-quality DNA is required
Methyl-sensitive cut counting (MSCC)	DNA is digested using HpaII and MmeI restriction enzymes, followed by deep sequencing	High sensitivity	High-quality DNA is required
Comprehensive high-throughput arrays for relative methylation (CHARM)	DNA is digested using MseI and McrBC restriction enzymes and then analyzed on a specially designed array	Quantitative, global methylation analysis, not restricted to the CGIs, *regions of lower CpG density* are taken into the consideration	Medium sensitivity, restricted to enzymatic digestion
Microarray-based methylation assessment of single samples (MMASS)	DNA is digested using MseI and BstUI, HhaI, and HpaII in MMASS-v1 or AciI, HinP1I, HpyCH4IV, and HpaII in MMASS-v2	High-throughput	Medium sensitivity, high-quality DNA is required
Differential methylation hybridization (DMH)	DNA is first digested using MseI and subsequently digested using BstUI, HhaI, and HpaII, McrBC restriction enzymes. Digested DNA fragments are then amplified, fluorescently labeled, and hybridized to arrays	Semiquantitative, able to identification of hyper- and hypo-methylated CpGs, global methylation analysis	Medium sensitivity, restricted to enzymatic digestion
Methylation single-nucleotide polymorphism (MSNP)	DNA is digested using XbaI and HpaII restriction enzymes	Able to detect single-nucleotide polymorphisms (SNPs), copy number variations, loss of heterozygosity (LOH), and methylation	Medium sensitivity, restricted to enzymatic digestion
Methylation-sensitive arbitrarily primed PCR (MS-AP-PCR)	DNA is digested using RsaI, MspI, or HpaII	Simple	Low sensitivity, low-throughput, high-quality DNA is required, large sample input
Amplification of inter-methylated sites (AIMS)	DNA is digested with SmaI and XmaI, followed by PCR amplification of methylated sequences	Simple	Low sensitivity, low-throughput, using radioactive material, high-quality DNA is required, large sample input

more affordable possibility for global epigenetic profiling and are gradually replacing traditional sequencing technologies [212].

22.10 Conclusions

Epigenetic events, particularly DNA methylation, are well-recognized drivers in the pathogenesis of cancers. Emerging evidence demonstrates that altered DNA methylation, mainly promoter hypermethylation, is frequently involved in modifying the expression of key genes, which predispose to oncogenesis and tumor progression. Understanding the role and importance of aberrant DNA methylation in the pathogenesis of cancers has been increasingly resulted in exploiting DNA methylation signatures in clinical practice, including developing them as diagnostic, prognostic, and predictive biomarkers, as well as identifying novel anti-tumor targets and therapeutic strategies. DNA methylation profiling using new advancements will be promising in helping unravel the cancer complexity, improving clinical outcomes and quality of life in cancer patients, and would be a way forward to personalized, precision-based cancer therapy.

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Immunosenescence, Oxidative Stress, and Cancers

23

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23.1 Introduction

The most important risk factor for cancer development is age [1]. With increasing age, numerous alterations at multiple levels including the molecular, cellular, organ, and systemic levels are observed. On the one hand, cellular senescence seems to be an anti-cancer mechanism related to aging due to the combined effects of proliferation and environmental factors such as oxidative stress, DNA damage, and telomere shortening [2]; on the other hand, there are various interactions among physiological systems (e.g., hormonal) which can favor the development and progression of cancers with aging [2]. After several years of debate, it is now clear that the immune system plays a major role in the control of the emergence of cancerous cells [3, 4]. With aging, there are changes in the immune system leading to the state called immunosenescence which might adversely affect the anticancer activity: immune-editing, immune-surveillance, and immune-competence against cancer [5, 6]. One of the most important characteristics of immunosenescence is its implication in "inflammaging" [7–9], a state of low-grade inflammation which can also contribute to the increased cancer incidence, and, more effectively, combat the emergence of tumor cells. Experimental data implicating immunosenescence in the susceptibility to cancers and response to treatment are accumulating, but there remains much to discover. Here, we describe changes in innate and adaptive immunity with age in relation to age-related increased cancer development.

23.2 Immune System and Cancer

It took some time to understand how the immune system may interact with the cancer at various stages of its development [10–12]. Currently, this synthesis of ideas developed over the decades following the original suggestion of immune-surveillance against tumors, known as "immune-editing" that describes all facets of the interaction between the immune system and cancer. Immunity plays an important role in the host defense against tumor development. Cancer originates from self cells and, as such, usually is only weakly antigenic, which is still generally enough for their recognition as foreign. This phase of the interaction is called the elimination stage or true immune-surveillance. At this level, the immune system involves many different immune cells and is efficient at eliminating cancer cells. However, this action can result in the emergence of tumor variants and the establishment of a temporary equilibrium between the transformed cells and the efficient immune defense. At this stage, the cancer remains clinically insignificant. As the equilibrium shifts and the continuously growing, genetically unstable malignant cells generate variants, the immune response can become inhibited or exhausted, and resistant cancer cells will survive and proliferate as explained by the deficit of the built-in tumor suppressor mechanisms such as cell senescence, DNA, and damage-induced apoptosis. Eventually, the tumor escapes from immune-surveillance and becomes clinically apparent. At this stage, the tumor is orchestrating the behavior of the immune system by actively suppressing the immune response through the production of various inhibitory

substances, such as NO, IDO (indoleamine-2,3-dioxygenase), PGE2, and via other pathways. At the same time, immune suppressor cells including Tregs and MDSCs may become dominant, hence inhibiting the tumor-eliminating activity of the immune system. Thus, to eliminate the nascent tumor cells, organisms need a completely and fully functioning immune system. As we age, there are several physiological alterations in the immune system ultimately contributing to the appearance of cancers with higher incidence in the elderly.

23.2.1 Immunosenescence or Immune Aging

It is currently well established that the immune response is profoundly altered with aging [13]. Most aging-associated changes initially reported related to the adaptive immune system, but it is now accepted that the innate immune system is also affected [14–17]. Collectively, it is very difficult to establish whether the changes are only detrimental or are at least partly an adaptation to sustain decreasing immune responses by changing the threshold for immune activation. The presence of low-grade inflammation can be part of this adaptation process. This phenomenon can overcome the decreased immune reserve with aging. Nevertheless, as the immune response is implicated in cancer immunosurveillance, it can be hypothesized that even if the changes in the aging immune system may be adaptive in respect to the pathogenic environment, they can still contribute to the increased incidence of cancers [18–21]. The age-related changes in the innate and adaptive immune system in view of their implication in putative cancer development and progression will be discussed here.

23.2.2 Innate Immune System

The innate immune system plays an essential role in cancer immunosurveillance by directly eliminating the tumor cells and maintaining them in a quiescent state – but may also favor the development and progression of cancers in some ways. It should be stressed that interactions between the innate and adaptive immune system are recognized as essential for an efficient adaptive immune response. These functions are mediated by various innate cells including neutrophils, monocyte/macrophages, and NK cells. It is now recognized that most phenotypes and functions of the cells of the innate immune system are altered with aging, as briefly summarized in the following.

23.2.2.1 Neutrophils

Neutrophils are the most abundant innate immune cells. They are the first to arrive at the site of any aggression but are markedly altered with aging [17, 22]. It is interesting to note that not all their functions are changed with aging. Thus, the number of neutrophils and their capacity to adhere at inflammatory sites is not altered [23, 24]. It is also of note that while most of the effector functions are increased with aging at the basal level, they cannot be further modulated [25–28]. The most important functions increased at quiescent state are the production of free radicals and the production of proteases [25, 26] which can be important for tumor fighting and influence tumor development. Nonetheless, this can also contribute to the low-grade inflammation observed with aging, which can be detrimental. In contrast, an acute stimulation of neutrophils in the elderly reveals that they are unable to perform correctly by increasing chemotaxis, phagocytosis, and intra- and extracellular killing and to stay viable and active for a longer functional period [27]. These functions are mediated through the activation of specific receptors such as pattern recognition receptors (PRRs), Fcγ, and complement receptors. Another important function recently recognized for the elimination of foreign invaders is autophagy. Engagement of different Toll-like receptors (TLRs) such as TLR4 and TLR7 has been implicated in the activation of macroautophagy [29], which has been shown to be defective with aging [30–32], suggesting altered foreign antigen (Ag) processing. Recently, it has

been shown that the inflammasome is a complex of molecules activated by specific PRRs (NLRs and AIM2) responding specifically to challenge via the activation of inflammatory caspases such as caspase-1 and caspase-5. This ultimately results in the production of a wide range of cytokines, particularly IL-1β [33], playing a role in inflammation. There are currently very scarce data on how these inflammasomes are affected by aging [34]. After the alterations observed in neutrophil functions, it can only be suggested that their assembly and function may be altered [35].

The causes of these dysregulated effector functions remain unknown, but changes in the inflammatory environment and in the signaling pathways may contribute. Neutrophils can also be stimulated via their pattern recognition receptors by Ags that may be present in higher amounts in the periphery of aged individuals, such as DNA degradation products, altered proteins, latent/chronic viral antigen, and/or tumor-derived Ags. Recently, one of the most important discoveries was of PRRs on the surface of many immune cells including neutrophils recognizing pathogen-associated molecular patterns (PAMPs) [36]. The ever-growing family of the PRRs now includes three main types: the TLRs, the retinoic acid-inducible gene 1 protein (RIG-1)-like helicases (RLRs), and the nucleotide-binding domain and leucine-rich-repeat-containing proteins (NLRs) [37]. It is now recognized that they play an essential role in many cell functions, including neutrophil biology, allowing immune cells to discriminate between self and non-self and acting as danger-sensing receptors to alert the organism to the presence of microorganisms, transformed cells, or damaged cells.

There are currently 13 TLRs described with different recognition specificities and signaling pathways leading to well-characterized cellular responses [36]. Bacterial products are recognized by TLR2 and TLR4, while TLR3 and TLR7 recognize intracellular pathogens. Signaling is mediated either by the MyD88 pathway [38] or by the TRIF pathway [39, 40]. Activation of these TLRs results in the activation of NF-κB, a transcription factor furthering strong cytokine production [41]. Neutrophils from aged individuals display alterations in the signaling of these TLRs leading to their altered functionality [14, 27]. While the number of these receptors is not significantly changed with age, there is a significant alteration in the trafficking of signaling molecules in and out of lipid rafts. There is a need for further studies in order to truly appreciate the role of TLR in the altered functions of neutrophils with age [27].

Taken together, all available experimental evidence indicate that neutrophils participate in inflammaging (low-grade, paucisymptomatic inflammation associated with aging) but can no longer effectively counteract pathological challenges and as such may contribute to the inflammatory process becoming more chronic. Neutrophils also interact with other cells of the immune system, in addition to the adaptive arm such as B-cells for antibody production and T-cells for efficient effector functions [42, 43]. They also participate in the recruitment of monocyte/macrophages to the challenge site which take over their functions for a longer time period.

23.2.2.2 Monocyte/Macrophages

Monocyte/macrophages have been relatively poorly studied in human aging. However, currently available data indicate that there are phenotypic changes associated with altered effector functions in older individuals. Recent studies characterizing monocytes showed the existence of two distinct subpopulations: $CD14^{++}$(high) CD16- and $CD14^{+}$(low) $CD16^{+}$ [44]. These subpopulations are very distinct in their surface protein expression and their functions. The first ($CD14^{++}$(high) $CD16^{-}$) subpopulation expresses CD62L, CD64, and CCR2 with low levels of CXCR1. The second ($CD14^{+}$(low) $CD16^{+}$) lacks all these surface markers, but expresses high levels of CX3CR1. These latter cells are considered to be mainly proinflammatory, as they produce high levels of TNF-α in response to TLR2 and TLR4 ligation. By analyzing the four subpopulations of human monocytes, it was found the $CD14^{+}$ (low) $CD16^{+}$ and the $CD14^{++}$(high) $CD16^{+}$ populations were increased with aging, whereas the proportion and number of $CD14^{+}$ (low) $CD16^{-}$ were decreased compared to the young [45].

The few existing data suggest that monocyte/macrophages from aged individuals display age-related dysfunction [46–48]. These alterations include a decrease of cell surface TLR expression (TLR1 and TLR4), although this finding is controversial [31, 49, 50]. Other receptors also show an altered expression, such as the expression of the important T-cell CD80/CD86 costimulatory receptors which is decreased on monocytes upon TLR stimulation [51]. In vitro studies in humans demonstrated a higher proinflammatory cytokine profile, especially for IL-6 and IL-8 production by resting monocytes [9], despite the finding that cytokine production following stimulation with LPS is reduced. Consistent with this, another recent study found that the four monocyte subsets had lower IL-6 production upon TLR1/TLR2 stimulation, confirming earlier studies on TLR stimulation [52, 53], which indicates that monocytes are not a homogeneous population and react differently depending on the nature of the stimuli.

Many years ago, it was shown that several surface receptors such as Fcγ and FMLP had altered signal transduction upon appropriate stimulation, resulting in altered function [25, 26]. Recent data further suggest that in addition to the decrease in some TLR expression, the TLR signaling pathways show age-related alterations [27] linked to altered chemotaxis as evidenced by the reduced number of infiltrating macrophages in wounds of elderly humans. Alteration in the MAPK signaling pathways including p38 MAPK and ERK1/2 MAPKs has been reported in human monocytes with aging.

Macrophages from elderly people produce more prostaglandin E2, which suppresses T-cell activation via decreased IL-12 production [54]. Furthermore, it was demonstrated that phagocytosis, free radical production, and chemotaxis were reduced in monocytes/macrophages from healthy aged subjects [55]. No data seem to exist regarding age-related changes in the clearance of apoptotic cells, known as an important macrophage function. We can only speculate that considering the functional changes described above and the process of inflammaging, the clearance of apoptotic cells may be impaired with aging. Decrease in some receptors, as well as altered signaling leading to changes in chemotaxis and phagocytosis, supports the hypothesis that apoptotic cells are not cleared efficiently. This could lead to their persistence in becoming proinflammatory and sustaining the quiescent state stimulation of monocyte/macrophages, finally contributing to the process of inflammaging. Furthermore, these data confirm that, like neutrophils, monocytes are to some degree activated at the basal state, but are less receptive to further stimulation through their surface receptors. This baseline activation state may be very important to maintain their functions for combating/constraining constant and chronic challenges, but insufficient for eliminating new infections. Therefore, it seems that neutrophils and monocytes are probably both contributing to the low-grade inflammation with aging which not only impairs the immune environment but also creates a vicious circle which maintains their functioning at an adequate level while impairing their contribution to combating new invaders, including tumor cells. Taken together, all the experimental data available suggest that with aging, most monocyte/macrophage functions are changed with age, leading to altered tumor cell and pathogen clearing and altered regulation of the adaptive immune response and the inflammatory process, resulting in chronic low-grade inflammation and ultimately to increased age-related diseases such as infections, cardiovascular disease, and cancers. It would be of high interest to develop a clinical trial to modulate low-grade inflammation in longitudinal studies and identify potential clinical benefits.

23.2.2.3 Dendritic Cells

Dendritic cells (DCs) are the most potent antigen-presenting cells (APC) that can prime specific T-cells. There are several types of DCs [56]: Plasmacytoid dendritic cells (pDCs) are important in host defense, as they are one of the first cells to produce type I interferon, hence initiating several other responses, including NK-cell activation which amplifies host response [57–59].

The second type of DC is the conventional or myeloid-derived dendritic cell (mDC), regarded as the most important APC for T-cell activation. They express TLRs and C-type lectins for the detection of Ags and subsequently produce IL-12, IL-15, and IL-18. IL-12 is essential for induction of Th1 cell responses which will induce cytotoxic T-lymphocyte responses to clear virus-infected cells [39]. They can also activate NK cells, which directly eliminate tumor cells. In addition to presenting Ag, they also provide costimulatory signals and cytokines for optimal T-cell priming, differentiation, and proliferation [60]. Whether the numbers of DCs change during aging is still controversial.

There are several studies demonstrating alterations in pDC function in aged humans including reduced type I interferon production following TLR stimulation, e.g., via TLR7 and TLR9. It has been suggested that the increased basal oxidative stress related to aging could be the underlying cause of the decreased upregulation of the interferon regulatory factors by TLRs [61, 62]. In contrast, mDCs from aged humans showed increased expression of CD86 signaling, another sign of activation even in the "quiescent" state. However, these findings have not been corroborated by in vitro studies. Nonetheless, they do seem to retain the capacity to produce proinflammatory cytokines and to activate $CD8^+$ T-cells [63], as well as to induce IL-17 production, which is known to recruit neutrophils [64]. DCs have also been reported to have a decreased ability in naïve $CD4^+$ T-cell activation via Ag presentation [65, 66], attributed to decreased PI3K activity, a major pathway mediating cell function. Reduced PI3K was implicated in both age-related reduced DC migration and also as a negative regulator of TLR signaling. Thus, the global result of this decreased PI3K activation is a higher stimulation of the NF-kB pathway further contributing to inflammaging due to greater production of proinflammatory cytokines such as IL-6 and TNF-α in the basal state [65]. DCs have reduced Ag processing capacity concomitant with the altered expression and function of their costimulatory molecules.

23.2.2.4 Natural Killer and Alike Cells

Natural killer (NK) cells are one of the most important antitumor players in the innate immune system [10]. The NK cell population is now also divided into different subpopulations; those with a $CD16^-CD56^+$ or $CD16^+CD56^{++}$ phenotype produce high amounts of IFN-γ and are among the most cytotoxic subtypes [67]. Subset distribution changes with aging, and the number of $CD56^{dim}$ NK cells increases, while $CD56^{bright}$ cells decrease [68, 69]. Furthermore, the expression of CD57 is increased on $CD56^{dim}$ NK cells from elderly subjects, representing a highly differentiated subset of NK cells. These observations were recently extended by the finding that CD94 (member of the C-type lectin family) and KLRG1 expression on NK cells was significantly decreased in elderly subjects. Although the exact consequence of this decrease is not known, it was hypothesized that the decreased expression of these surface markers induces unregulated cell lysis contributing to chronic inflammatory conditions. Moreover, the same study revealed the presence of a greater proportion of IFN-γ-positive $CD3^-CD56^{bright}$ NK cells with aging. This may suggest a shift to a more cytotoxic, cytokine-producing, and potentially immunomodulatory NK-cell phenotype occurring as a mechanism to compensate for the decreased proportion of $CD56^{bright}$ NK cells. Aging also influences the dynamics of NK cells [67]. NK cells from the elderly have a significantly decreased proliferation and production rate, and there is an increased proportion of long-lived NK cells which can be related to the increased proportion of $CD56^{dim}$ NK cells. The increased expression of CD57 may also suggest that the NK cells of elderly people are late-stage or terminally differentiated, like many of their $CD8^+$ T-cells [70]. Taken together, the data indicate that although the number of NK cells often increases with age, there is a profound redistribution of NK cell subsets with altered receptor expression, explaining the functional alterations, leading either to decreased direct defense against virus-infected and tumor cells and/or decreased regulatory activity for other components of the innate immune response, ultimately resulting in decreased modulation of the adaptive immune

response. Recently, it has been shown that NK-cell activity is also under the control of IL15Rα/IL15, released by nonimmune cells such as muscle cells, which, by its decrease with aging can also contribute to these NK cell functional alterations [71].

Studies in very healthy elderly populations revealed that the total NK cell number tends to increase with age, while their cytotoxicity is not significantly affected [72]. However, other studies in unselected elderly populations revealed that decreased NK cell functions with aging were associated with a higher incidence of infectious diseases [73]. IL-2-induced NK cell proliferation is decreased with aging and many cytokines and chemokines produced by NK cells, such as IL-2, IL-8, are also decreased but with maintenance of IFN-γ production [74]. This decreased production of cytokines contributes to the altered activation of macrophages with aging, resulting in decreased microbicidal and tumoricidal activities. Thus, NK cells of elderly people show decreased proliferative responses to cytokines; higher total cytotoxic capacity when stimulated with certain cytokines including IL-2, IL-12, or IFN-γ; and a greater sensitivity to stimulation via CD16. The cytotoxic activity of NK cells depends on whether the whole NK cell population or activity per cell is considered. On a per-cell basis, it is decreased, which might be important for protection against developing cancer cells.

Furthermore, other receptors involved in the cytotoxic activity of NK cells, including members of the natural cytotoxicity receptor family, namely NKp30 and NKp46, decrease with aging [75]. NKp30 has also been shown to be important in the regulation of the cross-talk between NK cells and DCs. By this interaction, the NK cells can activate the DCs to more efficiently prime T-cells. DCs release Th1 cytokines which further enhance NK activation. Thus, NK cells can modulate the adaptive immune response against virus-infected or tumor cells via this interaction with DCs.

NKT cells are innate T-lymphocyte population that recognize lipid Ags presented in the context of the CD1d molecule found on monocytes, macrophages, and DCs [76]. They can increase the functions of NK cells. NKT cells are rapidly recruited from the circulation during acute inflammation and interact with various APCs expressing the CD1d molecule. Recently, it has been shown that NKT cells are able to recruit neutrophils and activate them via their IFN-γ secretion [77]. Thus, NKT cells may play an important regulatory role in the acute phase of a microbial and/or tumor cell challenge by interacting with various APCs via CD1d lipid antigenic presentation and secretion of different cytokines. There are only a few reports on NKT cell functioning in the elderly [72]. However, it can be hypothesized that the altered activation of APCs via their TLR receptors will create an unfavorable milieu for NKT activation either directly or by their cytokine secretion.

IL-17 is mainly secreted by γδ T-cells, Th-17, and NKT cells [78]. This cytokine acts indirectly on neutrophil survival through stimulation of the secretion of G-CSF. IL-17 is also released by neutrophils themselves and reinforces their survival and recruitment [79]. It can also promote tumor vascularization by angiogenic factors. These immune cells as well as IL-17 itself may have pro- and antitumor activities; currently it is not known what determines this dual effect on cancer. However, their differentiation in various subtypes, expression of specific receptors, and production of various cytokines is likely to be determined by and in turn influence the tumor microenvironment [77]. How aging affects γδ T-cells has not been well investigated to date.

23.2.3 Adaptive Immune System

Although there are changes in the innate immune response with aging as described above, it is still thought that the most important and relevant changes occur in the adaptive immune response. Among the cells composing the adaptive immune response, the T-cells are thought to be the most affected; in addition, more and more data are emerging showing that B-cells are also changed with aging. Nonetheless, it is well recognized that some of the most marked immune alterations

associated with aging concern T-lymphocyte subpopulations and functions [13]. The most recognized model for T-cell subpopulations identifies naïve ($CD45RA^+$ $CCR7^+$), central memory T_{CM} ($CD45RA^-$ $CCR7^+$), effector memory T_{EM} ($CD45RA^-$ $CCR7^-$), and T_{EMRA} or T_{TE} ($CD45RA^+$ $CCR7^-$) cells. Among these subpopulations, the highly differentiated populations of EM (effector memory: *CCR7-, CD28-, CD27-, CD45RA-*) and TEMRA-like CD4 and CD8 T-cells (T effector memory cells re-expressing CD45RA) have been shown to accumulate in older humans [13]. Currently, the suggested reason for this accumulation is a chronic antigenic stimulation, especially that caused by chronic viral infections (predominantly CMV); however, other chronic inflammatory stimulations related to specific diseases may also contribute (including diabetes mellitus type 2, atherosclerosis, and possibly Alzheimer disease) [80–83]. Interestingly, there are some reports showing that these cells also accumulate in cancer, such as at the early stage of breast cancer [84] and in renal carcinoma [85]. Furthermore, they also express the characteristic inhibitory surface receptors of exhausted and/or senescent cells like KLRG1, CD57, PD-1, and CTLA-4, as well as having reduced replicative capacity and decreased survival after TCR activation [86]. The role of these cells in cancer development is still questionable. Whether they are metabolically inert as senescent cells with short telomeres and decreased telomerase activity, or are metabolically active and able to secrete various proinflammatory cytokines and contribute to cancer development is a matter which is yet to be elucidated. The cause of this exhaustion is not known with certainty, but could either be due to direct antigenic stimulation by viral Ags such as CMV or they could be innocent bystanders affected by the chronic low-grade inflammatory environment induced by such chronic antigenic stimulation caused by constant basal proinflammatory cytokines such as TNF-α produced by the innate immune system [87]. It was shown that p38 has a role in cell activation, proliferation, and cell cycle progression [88, 89]. TNF-α can further activate p38, thus contributing to immunosenescence [87]. Interestingly, p38 is constitutively phosphorylated in EM and EMRA T-cells, contributing to their reduced telomerase activity. Thus, the proinflammatory environment causing hyperphosphorylation of signaling molecules, such as p38, may influence the development of T-cell subpopulations as found in aging and inflammatory diseases. Together, these changes may be well tumorigenic by altering adequate tumor-specific immune response; they may be good targets for therapeutic modulation, as recently demonstrated so encouragingly for PD-1/PDL-1 [90–92]. Considering these changes, it is reasonable to assume that an alteration in T-lymphocyte activation is a central issue in the age-related modifications of the immune response. Currently, the most important paradigm underlying these changes is the repetitive antigenic stimulation over the life span that could lead to partial unresponsiveness (immune exhaustion) and accumulation of memory cells. This has been shown for both $CD4^+$ and $CD8^+$ T-cells with distinct senescent status, surface molecule expression, telomere length, and functionality. This was further supported by a longitudinal study, the OCTA/NONA study, resulting in the development of the Immune Risk Profile (IRP) integrating several of these parameters [93–96]. It is of note that as appealing as the CMV paradigm may appear, it is not yet proven [97–99]. It is likely that other factors could also contribute to causing the changes in the T-cell compartment of the immune system with aging including the slight but detectable amounts of the proinflammatory cytokines concomitant with increased reactive oxygen species found in this basal proinflammatory state. Moreover, the intracellular T-cell redox environment influences T-cell function in aging [100, 101] which will be discussed later. Concomitant with these phenotypic changes, the functions of T-cells are also altered, and there is increasing evidence to implicate altered activation in the decreased T-cell functions with increasing age.

Studies of elderly humans and animals have revealed that one function of T-cells most noticeably altered is the production of interleukin-2 (IL-2) compared to younger counterparts [102]. It can be hypothesized that defects or alterations

in the proximal events during T-cell activation will strongly affect the efficiency of immune responses [102]. Thus, appropriate signal transduction cascades trigger an appropriate T-cell response, whereas alterations in the early events of T-cell signaling will result in less effective, altered overall responses [103–106]. The most important changes occur in $CD4^+$ T-cells, resulting in decreased production of IL-2 and clonal expansion. Although there are no changes in TCR number at the cell surface, the number of CD28 costimulatory molecules decreases with aging, especially on $CD8^+$ T-cells. One of the most important driving forces to decrease surface CD28 expression is TNF-α. This cytokine can also activate p38 which plays an essential role in fibroblast senescence [87]. Nearly all of the signaling pathways associated with TCR activation or IL-2 receptor responses are found to be altered with aging [107, 108]. There is an alteration in the early steps of T-cell activation including protein tyrosine phosphorylation, calcium mobilization, and the translocation of PKC to the plasma membrane. In addition, subsequent steps of the signaling pathways including the Raf-Ras-MAP kinase pathway are impaired. Decline in proximal and intermediate events of transmembrane signaling leads to the decreased activity of transcription factors, especially NF-kB and NF-AT. Not only activation signaling but also the negative regulatory network is altered with aging [108]. This altered signaling followed by decreased activation may be caused by a differential inflammatory state and subsequent T-cell phenotypic and functional change.

There are also age-related changes in the B-cell compartment [109–113]. Production of B-cells is altered with aging at different levels, resulting in decreased naive B-cells. In addition, an age-dependent loss of diversity of B-cell receptors is also observed which has been correlated to poor health and may reflect expanded clones of memory B-cells. These changes may also lead to a shift in antibody specificity and the increase of autoantibodies. These alterations in the B-cell compartment may also favor the emergence of cancers related to aging. As the B-cells respond by proliferation to the T-cell-derived cytokine and other signals even without direct antigenic stimulation [114], the collapse of antibody production in the aged may be also associated with poorer T-cell help.

Taken together, aging is associated with an exhaustion of the adaptive immune response, especially by rendering T-cells dysfunctional and unable to appropriately respond to receptor ligation. This, together with B-cell alterations, contributes to the establishment of a chronic inflammatory state, leading to higher susceptibility to diseases such as cancer and increased mortality predicted by the IRP [93].

23.2.4 Interaction Between Innate and Adaptive Immune Responses: Effect of Aging

It is evident that if any component of the immune response is not functioning optimally, the outcome cannot be optimal. Thus, the first line of defense of the organism, the innate immune response, is not only a powerful eradicator of foreign invaders but is also responsible for the activation of the adaptive immune system for long-lasting and highly specific immunity by Ag-specific, clonally expanded B- and T-lymphocytes. The reduced functioning of both monocytes/macrophages and DCs with aging will lead to reduced Ag presentation and activation of T-cell immune responses by these APCs. In addition, neutrophils secrete many molecules such as HMG-B1 and other alarmins which can directly induce DC maturation or the activation of both the innate and the adaptive immune response. It is possible that the reduced neutrophil function with aging will also affect this aspect of their role in immune response.

A very efficient network exists among the different cells participating in the innate immune response aiming to eradicate invaders, restore homeostasis by resolving acute inflammation, and ultimately to efficiently activate the adaptive immune response [16]. The individual functioning of the innate immune cells was shown to be dysregulated with aging either because of receptor-driven signaling pathway alterations or

because of an age-related proinflammatory milieu sustained by cytokines and oxidative stress [22]. These alterations will induce a disruption in their functioning and in their mutually supporting network, resulting in inefficient eradication of the challenge, contribution in chronic antigenic stimulation, and a chronic low-grade inflammation. On the other hand, they ultimately lead to the altered and inadequate activation of the adaptive immune response.

One of the important central players of the cooperation of the innate and adaptive immune response is TNF-α. This factor is at the center stage of the cytokines secreted by various cells of the innate immune system, such as monocytes stimulated by many external or internal agents leading to modulation of the T-cell response either to enhance it or dampen it via downregulation of CD28 or exhaustion of T-cells [115]. TNF-α production is increased in oxidative stress, chronic antigenic stimulation, CMV infection, and visceral adiposity [116–118]. Thus, the regulation and control of this vital molecule to maintain it under a beneficial threshold may be the key to aging and age-related pathologies such as cancer.

Alterations in the T-cell compartment can also trigger changes in the innate immune system because the accumulation of memory and terminally differentiated/exhausted T-cells secreting more proinflammatory cytokines and chemokines will chronically stimulate and attract the innate immune cells. The increased susceptibility to apoptosis of certain T-cell subsets like $CD4^+$ naive T-cells may also chronically contribute to the stimulation of innate cells.

All these data demonstrate that with aging, alterations in both arms of the immune system, as well as in their efficient cooperation, contribute to altered protection against different challenges and participate in the development and maintenance of age-related low-grade inflammation and increased susceptibility to diseases such as cancer [9]. The same interaction between the innate and adaptive immune response may favor either the eradication or the progression of cancers depending on their state of activation, the phenotype repartition, and the microenvironment.

23.3 Inflammation, Aging, and Oxidative Stress

The relationship between chronic low-grade inflammation (inflammaging) related to immunosenescence and age-associated diseases, such as cancer, remains to be elucidated. It is of note that alterations of certain proinflammatory (IL-6, TNF, IL-1) as well as anti-inflammatory cytokines (IL-10, IL-4) are observed at greater frequencies in age-associated diseases compared to healthy aging [9]. Thus, age-related immune dysregulation manifested essentially by a basic chronic low-grade inflammation and a suppression of the adaptive response may eventually lead to the development of clinically significant pathological conditions including cardiovascular disease, dementia, diabetes mellitus, osteoporosis, and cancer [8]. Age-related low-grade inflammatory process seems to accelerate the progression of chronic diseases, as well as having an immunosuppressive effect on cellular immune responses by contributing to their exhaustion. The question arises as to whether this proinflammatory activity is the *primum movens* for disease development or just a secondary reaction following latent chronic inflammatory diseases. Moreover, this low-grade inflammation may also represent an adaptive mechanism to maintain an acceptable level of response against pathogens and cells, including nascent tumor cells. However, when increasing over a certain level, it could become predominantly detrimental by favoring their proliferation and the clinical appearance of cancer.

What are the molecular events underlying inflammaging? It seems that NF-κB is at the center stage of metabolic pathways, as it controls the secretion of proinflammatory molecules, such as cytokines, chemokines, MMPs, COX2, and iNOS [119, 120]. NF-κB is also activated by many of these molecules via various pathways such as the MAPK and the IP3/Akt pathway. As might be expected from knowledge of the pathways leading to their development, NF-κB activity is highest in $CD8^+$ TEMRA cells [121]. Moreover, the FOXO family of transcription factors plays a role in longevity, cell survival, and proliferation via

the modulation of NF-κB by free radical production [122]. Thus, NF-κB modulating pathways are heavily implicated in the occurrence, as well as in the perpetuation of this low-grade inflammation.

Thus, what is the relation between inflammaging and free radicals which have been shown to increase with aging as a result of increased oxidative stress [123]? The degree of oxidative stress is the result of the disequilibrium between the production of ROS and endogenous antioxidant species. Free radicals are produced as by-products of aerobic respiration [124]. They are hormetically beneficial for signaling, enzyme activation, and microbial elimination, while over a certain threshold, they may become detrimental by causing mutations in DNA and oxidation of macromolecules [125]. The role of free radicals became the basis of one of the leading theories of aging and consequently has been related to many age-associated diseases including cancer [126, 127]. In this context, it has been known for many years that age-related increased ROS production due to mitochondrial dysfunction may cause DNA damage and favor cancer development [128]. Recently, it was recognized that local inflammatory processes such as in the intestine and stomach may lead to the development of cancers. However, the relationship between oxidative stress and inflammaging is less well established. When innate immune cells are chronically activated, they continuously release free radicals which can contribute to tumorigenesis directly as well as via the alterations they cause to the adaptive immune system, as already mentioned [129]. It is of note that free radicals can create a vicious circle by maintaining (through TLRs and inflammasome activation) the production of free radicals by other innate immune cells such as neutrophils, DCs, and monocyte/macrophages which in turn reactivate them. Thus, free radicals directly and indirectly via oxidatively modified proteins or lipids activate NF-kB, leading to pro-inflammatory cytokine production. Similarly, these free radicals and lipid peroxides also activate the Nalp3 inflammasome. These events lead to low level of activation of innate cells at the basal level and participate in its maintenance.

Oxidatively modified proteins are also continuously produced as a result of the low-grade inflammation [130, 131], accumulating in immune cells, especially in T-cells, which interfere with their functioning. Many proteins including TCR, CD45, and enzymes are targeted by free radicals and become carbonylated or glycoxidated. This accumulation is further enhanced by decreased proteasome (and other intracellular proteolytic) activity to eliminate these altered proteins [132–134]. Thus, the free radicals create an altered cellular environment, favoring the activation of innate cells and decreased functioning of adaptive immune cells.

Furthermore, these free radicals will affect the surrounding cells in infiltrating tissues by inducing cell proliferation, evasion of apoptosis, tissue invasion, angiogenesis, autophagy, and alterations in macromolecule functions either by gain of functions or by loss of functions. All these activities may contribute to some extent to tumorigenesis. Free radicals mediate these functions by stimulating different molecular pathways including the Ras, MAPK, PI3K, mTOR, and NF-kB pathways. Consequently, ROS also alter Nrf2 activity which is considered to be the master regulator of the antioxidant response [129]. Nrf2 modulates a large number of genes that control several processes including immune and inflammatory responses [135]. We have shown that with T-cell aging, the Nrf2 is altered [22], which is also hypothesized in innate immune cells, and further contributes to the inflammatory process and consequently to carcinogenesis. Thus, the immunosenescence-associated inflammaging contributes to cancer development by many pathways, especially by the increased basal free radical production, which in turn further activates these cells by propagating inflammatory signal by free radicals.

23.4 Immunosenescence and Cancer

A causative connection between inflammation and some cancers is well established [136]. Inflammation in its uncontrolled state highly

favors tumorigenesis by increasing genomic instability via the production of free radicals, persistence of proinflammatory cytokines and chemokines, and the subversion of Treg, γδT cell, and MDSC functions, as well as through angiogenesis [137]. The apparent disequilibrium between the retention of a reactive innate immune response at basal state and the more severely altered adaptive immune response with aging leads to the presence of the low-grade inflammatory status commonly present in the elderly, the inflammaging, as discussed above. Although the cause of this increased basal inflammatory state is certainly multifactorial, it is likely that one of the most important causes is chronic antigenic stimulation concomitant with increased free radical production related to oxidative stress. In recent years, the increased reactivity of the innate immune response which in the aging context is called inflammaging has been elucidated [138–140]. This phenomenon is called the trained innate memory. The basis of this phenomenon are the epigenetic and immunometabolic modifications induced by an initial challenge which render the innate cells more reactive when subsequent unrelated challenge is met [141]. This is the concept of immunological training of the innate immune system. Thus, in the context of aging, this may be an adaptation serving the better response to the lifelong antigenic challenges [141, 142, 143]. The antigenic source can be exogenous, as with persistent viral infections such as CMV [97] and subclinical bacterial infections, or endogenous like the various posttranslationally modified macromolecules such as DNA or proteins which can be modified by oxidation, acylation, or glycosylation. Such altered molecules can stimulate the innate immune response, particularly macrophages via TLRs, thus contributing to a sustained proinflammatory state which is measurable in some circumstances via increased circulating levels of IL-6, IL-1β, or TNF-α. Thus, aging is accompanied by a chronic low-grade inflammatory process and by many other changes, some related to inflammaging, some independent thereof. Hence, this may be the price that has to be paid for maintaining immunosurveillance against persistent pathogens or endogenous stressors such as cancer cells. All these changes contribute to a decreasingly effective immune environment, probably unable to appropriately respond either to new Ags such as represented by the continuous risk of exposure to new pathogens, or to chronic persisting Ags such as those from CMV or tumor cells during the life span. Therefore, inflammaging related to immunosenescence is likely to be one of the most important general driving forces for cancer development. It is of note that every individual alteration at all cellular and molecular levels also contributes to increased tumorigenesis. The most important elements for immunosenescence are the decreased neutrophil, macrophage, and DC functions but maintaining uncontrolled proinflammatory cytokine production, as well as the decreased specific adaptive immune response by T-cells to tumor Ags. TNF-α seems to play a particularly important role, as it is secreted mainly by immune cells, in contrast to IL-6. It is the consequence of and the support for inflammaging via NF-kB and AP-1 signaling.

Furthermore, an important aspect of the inflammatory response is the production of free radicals which leads to the activation of various signaling cascades resulting in effector functions and apoptosis as well as in the further production of proinflammatory cytokines. They also increase the possibility of genomic instability and epigenetic deregulation leading to enhanced mutations. These proinflammatory cytokines secreted by the cells of the innate immune system are also able to induce the production of free radicals. Thus, the deregulation of innate immune responses strongly contributes to age-related chronic inflammatory processes and associated pathologies, as well as a functionally neutral consequence of the aging process. As a result, its modulation could be beneficial in the treatment of these diseases.

Moreover, the deregulated immune response with aging also produces directly pro-tumor molecules as well as induces the accumulation of immunosuppressive immune cells either systemically or in the tumor microenvironment. Data suggest that pro-tumor *molecules* such as NO,

IDO, TGFβ, IL-10, VEGF, and PD-1,, as well as *cells* dampening the immune reactivity like MDSCs (*CD11b+, CD33+, CD34+, CD14-HLADR-*) under the high proinflammatory cytokine micro- and macroenvironment and Tregs, are increasing with age which suppress the antitumor activities of cytotoxic T-cells, NK, and NKT cells [18, 21]. These changes completely alter tumor-immune interactions necessary for cancer eradication or at least for the maintenance of the equilibrium stage.

Finally, altered immune network functioning also favors tumorigenesis. The altered presentation of antigens by DC and macrophages decreases the activation of T-cells, the functions of which are further altered by oxidative stress and proinflammatory cytokines produced by innate immune cells. In contrast, the altered T-cell phenotype and functions are further increasing the innate cell functions. Thus, a vicious circle is created leading to the appearance of tumor cells.

23.5 Modulation

Due to our increased understanding of tumor-immune interactions now, the patient's immune system, even in nonimmunological treatments, like radiotherapy, should be taken into consideration [12, 144], in order to achieve long-term tumor control or complete tumor elimination. Thus, the patient's immune system needs to become integral to cancer therapy. It is also clear that immunotherapies are mostly used in late-stage cancers when the immune system is already subverted. Consequently, immunotherapy should be initiated when the immune system is still able to react.

Dendritic cells (DCs) possess the specialized potential to present exogenously derived antigen to cytotoxic T lymphocytes in order to elicit an immune response. This process, termed cross-presentation, is crucial for the generation of immune response to viruses and tumors and in autoimmune disease. The ability of DCs to cross-present exogenous Ag to CTLs makes them an attractive target for exploitation in immunotherapy. In recent years, significant advances have been made in understanding the mechanism of cross-presentation and the DC subsets involved. The recent discovery of human cross-presenting DC has given this field a new lease of life relative to cancer immunotherapy [145]. Such an example is the injection of monoclonal antibodies (mAbs) which not only directly eliminate tumor cells but also result in the release of new tumor antigens by killing tumor cells. These can then participate in cross-presentation to T and B-cells, thus amplifying the primary treatment [146].

Modern immunotherapy clearly needs to consider many aspects of tumor biology and associated immune reactions. The heterogeneity of tumors and their microenvironment combined with the diversity of immune cells/molecules will need complex approaches to immunotherapy. The new paradigm is to use autologous tumor cells for vaccine and/or in combination with personalized peptide vaccination which would lead to eradication of tumors or at least to the retardation of their development and metastasis formation [21]. In an aging/geriatric environment, certain characteristics specific to elderly subjects, such as functional status and comorbidities, should definitely be further considered.

These last years, immune checkpoint immunotherapy targeting exhausted T-cells in earlier fatal tumor types became excessively successful to treat melanoma, renal cancer, NSCLC as well as bladder cancer [147, 148]. It was longtime debated whether because of the occurrence of all the changes in the immune response with aging the immunotherapy may be efficiently used also in elderly. The most recent clinical trials indicate that this therapy may be as almost as efficiently used to treat the above cancers in old people suffering from them as in the younger patients [149–151]. While aging has been associated with immunosenescence, we have here the evidence that certain aspects of immunity are sustained in older age, including the boosting capacity of checkpoint inhibitors. This also suggests whether older individuals or those with weakened immunity may benefit from low doses of checkpoint inhibitors in other context than cancer immunotherapy.

23.6 Concluding Remarks

There is no doubt that aging is the main risk factor for the development of many diseases including cancers, type 2 diabetes, and cardiovascular and neurodegenerative diseases. Understanding the mechanisms regulating aging is the most important for the comprehension of the occurrence of these different diseases. The low-grade inflammation seen with aging can be a common factor linking aging to these diseases; thus, it is strictly deleterious. However, from a different perspective considering inflammaging as a consequence of immunosenescence, it may be essential to survival, likely ensuring that elderly can probably react to challenge much more easily and rapidly than they would be able to with an immune response similar to young people. In fact, this could be an evolutionary adaptation to maintain a response without losing control if the immune system would have a youngish function in an aging milieu. There are several examples, such as the increase in the number of NK cells and CMV-specific late differentiated $CD8^+$ T-cells, as well as their IFN-γ secretion [72, 97, 98]. Indeed in semi-supercentenarians, it was demonstrated that the presence of a controlled inflammatory status was the most determinant for their longevity [152].Therefore, understanding the interaction between this low-grade inflammation and its shifting toward pathogenic pathways, either in cells or their microenvironment, can provide the key to unveiling why aging is the most important risk factor for these diseases. It is also evident that a unifying picture starts to emerge implicating genomic instability, metabolism, and immunity in the development of cancer and other inflammation-related diseases. However, the challenge is to discover why differentiation toward individual diseases occurs under the presence of the same elements. Notably, the occurrence of each disease predisposes to other conditions as well; the best example would be diabetes, recognized to be a very high risk for the development of cancer or rheumatoid arthritis, as well as cardiovascular diseases. The problem is to intervene in the aging process to maintain its reactivity toward different challenges and at the same time decrease the risk for the development of disease. Thus, understanding the real mechanism underlying aging may lead to delay in the onset of these pathologies, ultimately extending the healthiest life span possible with aging.

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Nutrition, Immunity, and Cancers

24

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24.1 Introduction

Changes in immunologic pathways play a leading role in all stages of cancer. Proper immune function also associates with quantitative and qualitative aspects of nutrition [1, 2]. Therefore, overnutrition and imbalanced nutrition may affect development, progression, and therapeutic response of cancer [2]. Pro-inflammatory cytokines such as tumor necrosis factor (TNF), interferon-γ (IFN-γ), and interleukins 1 and 6 (IL-1 and IL-6) are important mediators of cancer complications such as cachexia [3]. A tumor can trigger the release of cytokines such as IL-6 [4], which is associated with an increase in lipolysis and proteolysis, which in turn affect the appetite and host neuroendocrine axis and induce anorexia and cachexia [4, 5]. Several neuropeptides such as neuropeptide Y (NPY) and adipokines such as leptin have been implicated in the pathogenesis of cancer cachexia syndrome [5, 6]. Thus, an imbalance of cytokine production, and neuropeptide and adipokine dysfunction as well as changes in microbiota (particularly in GI in the consequence of cancer and tumor suppressive agents) may be a major cause of the nutritional consequences of cancer.

24.2 Role of Nutrition in Predisposition of Cancer from an Immunologic View

One of the known risk factors for cancer is obesity, especially with the modern lifestyle and low physical activity [3]. Dietary patterns have a significant effect on the cytokine profile; for instance, the high intake of saturated fats, especially in obese people, leads to infiltration of adipose tissue by macrophages producing IL-1β, IL-6, and macrophage inhibitory factor (MIF) [4–6]. Moreover, a decrease in the secretion of anti-inflammatory adipokines such as adiponectin may maintain pro-inflammatory signals and activate the production of C-reactive protein (CRP) by the liver [7, 8]. Based on previous studies, this chronic inflammatory process is related to an increased susceptibility to various types of cancer, including cancers of the gastrointestinal, respiratory, and genitourinary systems [9–11]. It has been evident that the inflammation is promoted by saturated fatty acids and their binding to the Toll-like receptors (TLR 2 and 4) activating pro-inflammatory factors such as nuclear factor-kappa B (NF-κB) [12]. Moreover, downregulation of autophagy and decreased cytoplasmic recycling of damaged organelles accelerate activation of inflammasome and complement components [13, 14]. Chronic inflammation dysregulates immune function from immunosurveillance to carcinogenic inflammasome by stimulating cellular turnover, increasing stem cell divisions, enhancing production of reactive oxygen species and metabolic rate locally [15]. Unresolved inflammation due to overnutrition provides a local immunosuppressive microenvironment by production of transforming growth factor beta (TGF-β) and myeloid-derived suppressor cells within the tumor lesion [16, 17]. Obesity also affects the microbiota leading to an intestinal dysbiosis and diminishes the bacterial and endotoxin barriers, which increases the risk of procarcinogenic metabolites presentation [18, 19]. Decreased autophagy also enhances aging process affecting immune profile by decreasing

cytotoxic T-cells, thymic atrophy, and dendritic cells' dysfunction [19–21].

Vice versa, intermittent fasting and adjusted low-carbohydrate/hypocaloric diet has beneficial effects on antagonizing the chronic inflammation process mediated by increased ketone-bodies, decreased risk factors of metabolic syndrome [1, 22–25]. Surprisingly this method can be used for boosting chemotherapy since it can increase the remodeling of the immune-cell infiltrate by an increased infiltrating cytotoxic T-cells and local depletion of regulatory T-cells [26, 27]. Treatment with one or several fasting cycles diminishes tumor growth, prevents cellular transformation, and upregulates autophagy [28–30].

Influenced by this important effect of nutrition on the immune system, characteristics of the human diet can directly stimulate gastrointestinal malignancies [31]. A diet low in fiber and vegetables may affect the regulation of carbohydrate absorption and short chain fatty acid formation, which affects the metabolism of carcinogens [32]. This process is linked to colon cancer and its progression [33]; apparently, a decrease in fiber intake may allow more time for exposure of colon cells and the immune system to the potential carcinogens, affecting intestinal transit [34]. However, recently the anti-inflammatory effects of fiber and multiple distinct phytochemicals (e.g., enterolactone, flaxseed, lignin, and spermidine) on microbiome have been reported including increased proportion of *Lactobacilli* and *Bifidobacteria* [35, 36]. Moreover, based on the evidence used to draw conclusions about a gluten free diet in patients with celiac disease leading to cancer protection, it seems reasonable to consider gluten as a booster for cancer in celiac patients [37, 38]. Meat consumption is a risk factor for some cancers, especially colon, rectum, and prostate. Red meat consumption increases the risk of colon cancer by causing increased production of heterocyclic amines [39, 40]. On the other hand, a change in the normal diet and deficiency of vitamins or minerals may affect the adequacy of either innate immunity (phagocytic activity, chemotaxis of neutrophils, or release of cytokines from monocytes) or adaptive immunity (immunoglobulin production of B-cells or cell-mediated immunity) [41–44]. Many of the consequences of malnutrition in the regulation of signal transduction and immunoregulatory gene expression were first recognized in the early 1800s as nutrigenomics [44, 45]. The majority of these changes are reversible after administration of adequate nutrition supplements [46]. The following list of specific dietary factors has been studied in relation to the immune aspects of cancer.

24.2.1 Protein–Calorie Balance

The formation of lymphocytes, eosinophils, and vital immune system proteins such as thymic hormones, antibody (Ab) responses to T-cell dependent antigens (Ags), and Ab affinity are affected by protein–calorie imbalance [47]. It has long been recognized that caloric restriction with a well-balanced diet avoiding certain nutrient deficiencies can increase longevity and has cancer preventive effects in mammals [48].

24.2.2 Essential Fatty Acids

Essential fatty acids, mainly suggested by consumption of nuts, in our body can regulate the production of prostaglandins, prostacyclins, thromboxanes, and leukotrienes, causing a significant effect on the host immune system and regulation of inflammation and C-reactive proteins [49].

24.2.3 Antioxidants (Selenium, Vitamin E, and Vitamin C)

These nutrients have strong antioxidative effects and may reduce the risk of cancer by neutralizing reactive oxygen species or free radicals that can damage DNA [50, 51].

24.2.3.1 Vitamin A

Vitamin A plays an important role in protection against measles, white blood cell (WBC) function, resistance to carcinogens, and skin and mucous membrane defenses. Vitamin A precursor carotenoids, such as lycopene, have a potential effect on cancer prevention [52, 53].

24.2.4 Vitamin D

25-hydroxyvitamin D has been of interest based on ecologic studies on populations with greater exposure to ultraviolet light who had a lower risk of breast cancer, colon cancer, and prostate cancer. This vitamin regulates humoral Ab response, enhances organ specific cytotoxic T-cells, and supports a Th2-mediated anti-inflammatory profile of cytokines; therefore, its anticancer properties are strongly suggested [54–56].

24.2.5 Vitamin B6

Pyridoxine and its metabolite PLP (pyridoxal-5′ phosphate) induce immunosurveillance activation and Th1 cytokine-mediated immune responses. Epidemiologic studies and laboratory animal models have shown that vitamin B6 modulates the risk of cancer. It is not clear how vitamin B6 mediates this effect, but it has been reported that high dietary vitamin B6 attenuates and low dietary vitamin B6 increases the risk of cancer [55, 57–59].

24.2.6 Folate

Folate is important for DNA methylation, repair, and synthesis, which is also crucial for lymphocyte development [60, 61]. Epidemiologic studies have shown that low folic acid intake is associated with a higher risk of various cancers, most notably colon, breast, and probably cervical cancer. The fact that methylenetetrahydrofolate reductase, an enzyme predicted to reduce the risk of colon cancer, is associated with folate status supports the role of folate deficiency in cancers [62, 63].

24.2.7 Calcium

Many studies show that calcium may reduce the risk of colorectal cancer via direct and indirect effects. Calcium has a direct effect on proliferation, stimulating differentiation, and apoptosis in the colonic mucosa [64, 65]. Its indirect effect is binding to toxic secondary bile acids and ionized fatty acids to form insoluble soaps in the lumen of the colon [66].

24.2.8 Nutrition Overdose in Cancer

In addition to deficiency, an overdose of some micronutrients can also have an immunosuppressive effect, especially megadoses of vitamin E [67]. High doses of certain minerals such as chromium, copper, iron, manganese, and zinc also may induce cancer and immune dysfunction [68].

In summary, attenuated innate and adaptive immunity as a result of an inadequate diet leads to a higher risk of cancer and lower homeostasis for cancerous antigens, which could be resulted from reducing nutrient intake, increasing losses, and interfering with utilization due to altering metabolic pathways. Thus, nutrition may have a significant role in immune prevention and immune surveillance of cancer.

24.3 Aging as a Confounder of the Triangle of Nutrition, Immunity, and Cancer

Aging may be a confounder of the triangle of nutrition, immunity, and cancer (Fig. 24.1); however, neither the relationships nor the mechanisms of interaction are known. Unfortunately, only a few studies have considered that nutrition and immune function simultaneously decrease in elderly individuals [69]. It is known that increased age adversely affects the function of the immune system as well as nutrient intake habits [70]. Therefore, both immunosuppression (mainly due to decreased effectiveness of T and natural killer cells) and nutritional deficiencies (as defined by the 1989 recommended dietary allowances) in the elderly may have independent correlations with an increased risk of infection and neoplasia development [42].

One of the probable mechanisms that may affect both immunity and nutrition in old people is turn-

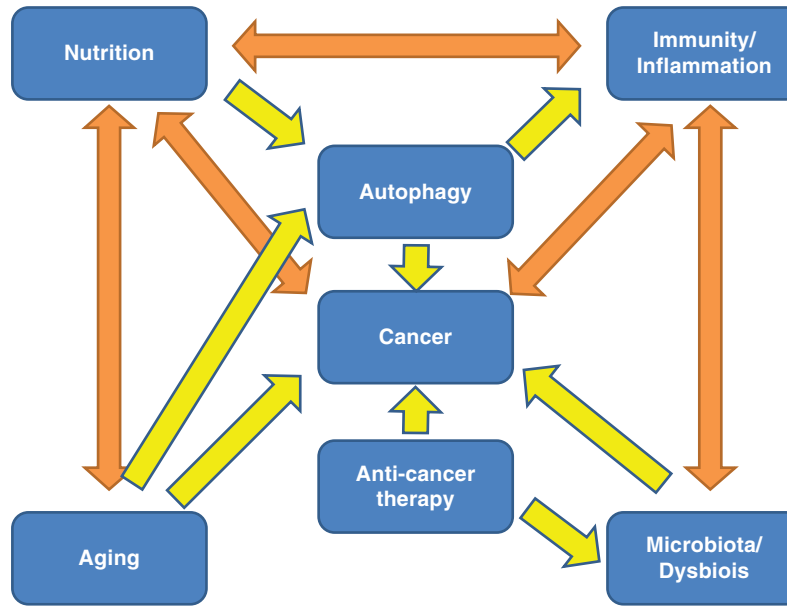

Fig. 24.1 Schematic overview of complex network of diet-immunity-cancer

over fluctuations of cellular components in lysosomes or autophagy. Advanced age leads to a reduction in the autophagy of loading viral Ags and cross presentation of tumor Ags into MHC class I molecules, as well as pathogen killing [71–73]. Similarly, the capability of autophagy for energetic balance recycling of amino acids to maintain protein synthesis under starvation conditions and the capacity of intracellular lipid stores or glycogen mobilization are disturbed [74, 75]. However, only minimal information has been produced concerning human cancer initiation as a direct result of a specific dietary etiology in the elderly.

24.4 Microbiota as a Confounder of the Triangle of Nutrition, Immunity, and Cancer

Studies examining the composition of alimentary elements on the intestinal microbiome and the role of dysbiosis in different diseases states have uncovered associations with inflammation and tumorigenesis [76, 77]. Moreover, the impact of immunosuppressive and anticancer agents on the microbiota profile has been recorded [78–81]. High protein diet can increase the microbial diversity and proportion of *Bifidobacteria*, *Lactobacilli,* and *Eubacterium Rectale* but can decrease *Bacteroides* species. Similarly most of natural sugar can enhance incidence of *Bifidobacteria* rather than *Bacteroides*. Moreover high fat diet inhibits propagation of the lactic acid bacteria but provide an environment in favor of *Clostridiales* and *Bacteroides.* Probiotics also can change the microbiota by overpresentation of *Bifidobacteria, Lactobacilli*, *aerobes/anaerobes,* and lower presentation *coliforms, Helicobacter pylori, Escherichia coli* [82, 83].

Immunosurveillance profile (low short chain fatty acids, low lipopolysaccharide levels, low IL-6, and high IL-10) is associated with specific microbiome molecular patterns which usually can be linked with Mediterranean diet with dominancy of *Bifidobacteria, Lactobacilli, Eubacteria, Bacteroides,* and *Prevotella.* Studies that involve intake of a specific dietary component demonstrate how certain bacteria tend to respond to the nutrient-specific challenge. Protein, fats, digestible and non-digestible carbohydrates, and probiotics all induce shifts in the microbiome with secondary effects on host immunologic and metabolic markers suggesting maintaining healthy gut microbiome is critical to human health [84, 85].

24.5 Role of Cancer in Predisposition to Malnutrition from an Immunologic View

Despite the role of nutrition in either preventing or causing cancer in humans, malnutrition is a common problem (global percentage of 56.5%), and weight loss is often predictive of shortened survival in these patients [86]. In advanced stages of cancer, up to 35% of related deaths may be linked to improper diet [87, 88]. Moreover, a proportion of patients with malignancy develop cachexia, a progressive involuntary weight loss status that is attributed to clinic-pathologic factors of the tumor (origin, metastasis, and size), host immunity, and antitumor treatment (Fig. 24.2) [89]. During the development of cancer-associated cachexia, several Th2-dominant condition mediators such as IL-2 and TNF (prognostic markers) are implicated in appetite loss and metabolic disturbances, as well as leptin, IL-1, IL-6, IFN-γ, leukemia inhibitory factor, NPY, and proteoglycan 24 K [90, 91]. These immunologic and metabolic changes induce cancer cachexia syndrome, which is characterized by patient tissue wasting, anorexia, appetite loss, prolonged fatigue and lethargy, insulin resistance, microcytic anemia, hyperlipidemia, and hypoalbuminemia [92, 93]. Metabolic features of this syndrome include increases in the heterogeneity of energy requirement, substrate cycling and turnover, Cori cycle activity, and hepatic protein synthesis, as well as decreases in peripheral muscle protein synthesis, serum protein lipase activity, and plasma concentration of branched chain amino acids. In general, the severity of malnutrition and cachexia in digestive neoplasias is in highest percentages (from 79% in esophageal cancer to 40% in rectum cancers) due to the involvement of all predisposing factors described in Fig. 24.2 during the development of cancer and in chemotherapy or tumor resection. It should be noted that antitumor agents with their side effect on cells with high turnover may exacerbate malnutrition [94]. This could be explained by the competition between cancerous regions and normal cells of the gastrointestinal system to use nutrients to repair the adverse effects of antitumor drugs (hypermetabolic state) [95]. Briefly, impaired caloric intake, side effects of therapy, changes in taste and mood, pain and other adverse consequences of eating, obstruction, fistula, and malabsorption all promote malnutrition in cancer patients; therefore, well-nourished cancer patients with intact gastrointestinal integrity have lower morbidity and mortality than others [96]. It should be noted that cachexia after cancer differs from cachexia following starvation. Increased protein and glucose turnover, high whole body synthesis and catabolism, accelerated hepatic protein production (especially acute phase

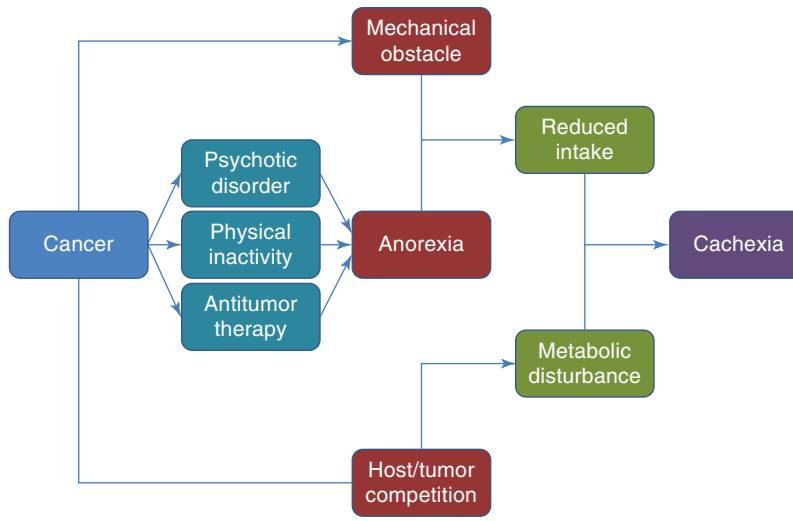

Fig. 24.2 The casual pathways of cachexia occurrence after malignancy

agents), increased serum free fatty acid levels, and depletion of fat stores were reported only in cancer patients. However, metabolic abnormalities and, paradoxically, impaired immune response are probable consequences of cancer cachexia, as explained in the previous section. Increased levels of immunosuppressive mediators (e.g., TGF-β), decreased C3 and delayed hypersensitivity response, and diminished numbers and activity of NK cells are the most common changes in the immune system of patients with cancer cachexia, leading to more infectious complications and poor prognosis [96]. Neutrophil chemotaxis, monocyte phagocytosis and cytotoxicity, number of T-cells, and proliferation of lymphocytes are also defective in patients with lung cancer. Phagocytic and bactericidal activities of neutrophils were low in hepatocellular carcinoma patients. In addition, surgical stress in cancer patients enhances Th2 and compromises the Th1/Th2 balance and expression of HLA-DR on monocytes, which is considered to be a central marker of immune paralysis after surgical trauma [97, 98]. Most of these immune parameters are also reduced during radiotherapy and chemotherapy because of their side effects on bone marrow. However, these factors are reversible after nutrition improvement [99].

24.6 Role of Nutritional Support in Immune Restoration of Cancer Patients

Adjuvant therapy of cancer patients by different nutritional support strategies (dietary counseling, oral nutritional supplements, enteral tube feeding, and parenteral tube feeding) is the mainstream recommendation to increase their quality of life and to obviate the risks associated with gastrointestinal complications and reverse malnutrition. However, there is no comprehensive approach based on the needs of cancer patients with cachexia or those with increased nutrient requirements. Several studies have shown the effectiveness of nutritional supply in groups of patients with malignancy that resulted in weight gain, increased appetite, increased energy and protein intake, reduced gastrointestinal toxicity, and enhanced immune function [100]. In the clinical setting with standard treatment protocols, it turns out that the implementation of nutrition support in patients with cancer is most effective when it is limited to special, well-described circumstances. Nonetheless, the potential advantages of some specific nutrients have been described and are outlined below [101].

24.6.1 Arginine

Arginine is a semi-essential amino acid with immunomodulatory potentials such as stimulated thymic growth and mononuclear cell response to mitogens, which enhances lymphokine-activated killer cell generation via a nitric oxide-mediated mechanism and stimulates the release of polyamines by the small intestine. In one randomized trial of malnourished patients with head and neck cancer, follow-up at 10 years indicated better survival in those who received supplemental arginine preoperatively [102].

24.6.2 Glutamine

Glutamine is the most abundant amino acid in the human body and the preferential fuel of rapidly dividing cells such as lymphocytes and macrophages [103]. However, supplementing glutamine in the diets of patients with cancer may be counterproductive because glutamine (which is essential for fast growing cells in culture) may promote accelerated tumor growth. A meta-analysis of studies that used parenteral glutamine postoperatively showed it was associated with a shorter hospital stay and a lower incidence of infectious complications [104].

24.6.3 Branched Chain Amino Acids

L-valine, L-leucine, and L-isoleucine can improve the immune response and maintain serum albumin level in the course of hepatocellular carcinoma recurrence [105].

24.6.4 Nucleotides, Long-Chain

Omega-3 polyunsaturated fatty acids and eicosapentaenoic acid. These lipid agents have anti-inflammatory, anticachectic, immunomodulating, and antitumor effects [106–108].

24.6.5 Fructooligosaccharides

This group of functional fibers associated with increased lactic acid bacteria acts as an immunomodulator by stimulating IgA synthesis, promoting mucin production, modulating inflammatory cytokines, and decreasing Ag absorption [90].

24.6.6 Bioactive Compounds

Agaricaceae fungus consisting of ergosterol, oleic acid, and triterpenes may inhibit neovascularization induced by tumors and therefore attenuate cancer progression [109].

24.6.7 Antioxidants (Vitamin E and Vitamin C)

Since chemotherapy may induce mucositis and bleomycin in particular induces chromosomal damage in lymphocytes, the administration of vitamins C and E may reduce the side effects of therapy [110].

24.6.8 Vitamin A

This fat-soluble vitamin can increase the numbers of NK cells or regulatory lymphocytes in cancer patients [89]. A recent study showed that all-trans retinoic acid can potentiate the chemotherapeutic effect of cisplatin by inducing differentiation of tumor initiating cells in liver cancer [111].

24.7 Concluding Remarks

In summary, due to the safety and cost-effectiveness of oral dietary therapies, nutrition counseling and the implementation of nutritional supplements should be the initial approaches to nutritional support [112]. Even though parenteral nutrition may also lead to weight gain and improvement in nitrogen balance in patients with cancer, it does not clearly improve serum albumin levels or alter whole body protein turnover even with prolonged administration. Therefore, when nutrition support is chosen as a therapy, the use of enteral nutrition is preferred if the gastrointestinal tract is functional [113, 114]. The use of parenteral nutrition should be limited to malnourished cancer patients who are receiving active anticancer treatment, whose gastrointestinal tract is not functional or who cannot tolerate enteral nutrition, and who are anticipated to be unable to meet their nutrient requirements for 14 days or more [113]. Moreover, it is proposed that preoperative and postoperative immune-nutrition intervention by total parenteral nutrition using a lipid-based regimen is the method of choice in cancer patients who have undergone major surgery to reduce immune dysfunction without enhancing tumor growth (increased augmentation of lymphocyte blastogenesis and production of helper T-lymphocyte lymphokine IL-2, increased ICAM-1 level, and decreased IL-4 and IL-10 values) [114, 115]. This observed preference of parenteral nutrition is marginal, and enteral methods are always the preferable route for cancer patients with an intact digestive system. It is also reported that complement components and lymphocyte response may be better with enteral rather than parenteral nutrition [115, 116].

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Inborn Errors of Immunity and Cancers

25

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25.1 Introduction

Immunodeficiency disorders are classified as either primary (genetic) or secondary (acquired). Primary immunodeficiencies (PIDs) are a heterogeneous group of disorders that predispose to frequent and severe infections, autoimmune disorders, and in certain diseases, cancers. The genomic revolution has identified hundreds of new genetic etiologies of immune dysfunction, including defects in regulators of known immune pathways, scaffolding proteins of immune receptors, transcription factors, and genes involved in DNA replication and repair [1]. The International Union of Immunological Societies Expert Committee in 2015 has reported more than 300 genetically defined single-gene inborn error of immunity [2].

The actual incidence and prevalence of PIDs remain unclear given lack of specific, dedicated epidemiologic studies; however, recent epidemiologic studies have suggested that PIDs are more common than generally thought. In a report by Bousfiha et al. [3] published in 2013, it was estimated that six million people may be living with a PID worldwide, whereas only 27,000–60,000 have been identified to date. The overall risk of developing cancers in children with PIDs is reportedly 4–25%, with lymphomas representing up to 60% of all cancer types [4–6]. As therapeutic strategies improve, we should anticipate that the emergence of cancers will be unmasked by increasing longevity.

Increasing evidence suggests that defective immunosurveillance mechanisms, interacting with oncogenic viruses, chronic antigen stimulation, defective DNA damage response, and genetic alterations of oncogenic and tumor suppressor genes, are the major factors driving the development of cancer in patients with PIDs [7–11]. While further elucidation of the precise molecular pathogenesis of cancers in the context of immunodeficiency syndromes offers an exciting prospect for the development of targeted cancer therapies, we report here the most recent clinical observations on the incidence and types of cancers, which should alert clinicians to the potential importance of more vigilant screening

in immunodeficient patients. It should be noted, however, that surveillance protocols should be applied judiciously, without indiscriminate and frequent use of certain radiological procedures, due to increased risk of radiosensitivity in some syndromes [12]. Furthermore, early intervention with hematopoietic cell transplantation, which is indicated in certain PIDs, may decrease not only the infection but also the cancer risk [13].

25.2 Predominantly Antibody Deficiencies

25.2.1 Common Variable Immunodeficiency

Common variable immunodeficiency (CVID) is the second most common PID (second to selective IgA deficiency), which affects both children and adults, with an estimated prevalence of 1 in 25,000 individuals [14]. CVID is a clinically and genetically heterogeneous group of diseases characterized by hypogammaglobulinemia of two or more isotypes (IgG, IgA, or IgM), impaired functional antibody responses, and consequently increased susceptibility to chronic recurrent bacterial infections [15]. Furthermore, affected individuals are predisposed to autoimmune and granulomatous diseases as well as hematological and certain solid malignancies in 1.5–20.7% of subjects [15–17].

Non-Hodgkin lymphomas (NHLs) represent the most common malignancies with up to a 259-fold increase in risk compared to the general population [18–20]. NHLs in CVID are mostly extranodal, well differentiated, and of B-cell origin [18]. In older studies, there was an increased risk of gastric cancer (up to 47-fold) [19–21], probably related to the increased frequency of pernicious anemia or *Helicobacter pylori* infection [22]. However, a 2010 study of 476 patients revealed that gastric cancer was diagnosed in only 0.6% of patients, suggesting a potential downward trend. In this study, 6.7% of patients developed NHL and 0.8% developed Hodgkin lymphoma (HL). Other solid malignancies, including breast, colon, oral, and other cancers, collectively accounted for cancer in up to 4% of patients [16].

There are multiple genetic components involved in CVID pathogenesis [23, 24]. Monogenic causes of CVID have been found in approximately 2–10% of cases. Genes that have been implicated in monogenic CVID include *CD19* (OMIM∗107265), *CD20* (*MS4A1*, OMIM∗112210), *CD21* (*CR2*, OMIM∗120650), *CD81* (OMIM∗186845), isotype switching and somatic hypermutation (*ICOS*; OMIM∗604558), tumor necrosis factor (TNF) receptor superfamily, member 13B (*TNFRSF13B* or *TACI*; OMIM∗604907), member 13C (*TNFRSF13C* or *BAFF-R*; OMIM∗606269), and member 7 (*TNFRSF7*, OMIM∗186711), TNF ligand superfamily, member 12 (*TNFSF12* or *TWEAK*, OMIM∗602695), *IL21* (OMIM∗605384), IL21 receptor (*IL21R*, OMIM∗605383), cytotoxic T lymphocyte-associated 4 (*CTLA-4,* OMIM∗123890), protein kinase C, delta (*PRKCD*, OMIM 176977), phospholipase C, gamma-2 (*PLCG2*, OMIM∗600220), nuclear factor-kappa B, subunit 1 (*NF-κB1*, OMIM∗164011) and subunit 2 (*NF-κB2*, OMIM∗164012), NLR family pyrin domain containing 12 (*NLRP12,* OMIM∗609648), lipopolysaccharide-responsive beige-like anchor protein (*LRBA, OMIM∗606453)* among so many others [24]. There is accumulating evidence that at least a subgroup of patients with CVID has a complex rather than a monogenic inheritance. Further genetic complexity may come from transcriptional and epigenetic disturbances.

The immunologic defects in CVID are multifaceted. Despite normal numbers of B-cells in the majority of affected individuals, their inability to undergo terminal differentiation into immunoglobulin-secreting plasma cells forms the core common defect [25]. T-cell abnormalities are also frequently encountered in patients with CVID, including impaired T-cell proliferative responses, partly due to defects in T-cell receptor signaling [26, 27]; decreased numbers of CD_4^+ T-cells in conjunction with normal to increased numbers of CD_8^+ T-cells, giving rise to reversed CD_4:CD_8 ratio [28, 29]; imbalanced T-helper cell responses, representing a shift toward a Th1 phenotype [30, 31]; increased suppressor T-cell activity [30]; and diminished

expression of the costimulatory molecule CD40 ligand [32]. Several studies have also reported disturbed frequencies and functional characteristics of Treg, which contributes to the aberrant immune responses observed in CVID [33]. Moreover, the absolute and relative NK, invariant NKT, and plasmacytoid dendritic cell numbers are reported to be decreased in patients with CVID [34, 35].

The complex derangement in numerical and functional characteristics of B, T, NK, and dendritic cells results in impaired humoral and cellular immune responses. As a result, patients often develop chronic inflammatory and autoimmune diseases, as well as recurrent bacterial infections. These factors, along with persistent antigenic stimulation, mainly from chronic *Helicobacter pylori* [36], human herpesvirus 8 [37], cytomegalovirus [38], human papillomavirus (HPV) [39], and Epstein–Barr virus (EBV) [40] infections, may ultimately drive tumorigenesis; however, their relative contribution and the precise underlying mechanisms remain to be elucidated [15]. Furthermore, given the possible role of an autocrine B-cell activating factor (BAFF) signaling circuit in promoting tumor cell survival and proliferation [41, 42], it is possible that aberrant BAFF-R signal transduction resulting from CVID-related mutations might enhance malignant transformation [15]. Finally, defective DNA repair, as evident by enhanced radiosensitivity, has been reported in patients with CVID [43], with those having the highest rate of chromosomal aberration developing lymphoma [42, 43].

25.2.2 X-Linked Agammaglobulinemia

X-linked agammaglobulinemia (XLA) is the prototypic humoral immunodeficiency arising from a defect in B-cell maturation, affecting the transition of B-cell progenitors into mature B lymphocytes and leading to the consequent failure of immunoglobulin production. It is estimated to afflict three to six out of every million males of all racial and ethnic groups. As the maternally derived antibodies are degraded, most patients with XLA begin to experience recurrent infections by the end of the first year of life [21, 44]. Approximately 10–15% of individuals with XLA have higher concentrations of serum immunoglobulin than expected or are not recognized to have immunodeficiency until after the age of 5 years. XLA is mainly characterized by recurrent bacterial infections, in particular with extracellular encapsulated bacteria, most commonly localized in the respiratory tract. Diarrhea and skin infections are also frequently seen [21, 44, 45]. Despite general resistance to viral infections, affected individuals are susceptible to severe and chronic enteroviral infections [46].

The gene defective in XLA, Bruton's tyrosine kinase (*BTK*; OMIM∗300300), encodes a cytoplasmic tyrosine kinase of the Btk/Tec family [47]. The crucial role of BTK in B-cell growth and differentiation has been documented by a developmental block at the pro-B-cell to pre-B-cell transition with a reduction in mature B-cells [45], whereas T lymphocyte subsets are normal and may show a relative increase. In B-cells, B-cell antigen receptor (BCR) cross-linking activates BTK downstream of the Src family kinases [48, 49], where it is a critical component in BCR-coupled calcium signaling cascade [50, 51]. BTK also acts as a mediator of oxidative stress-induced apoptosis of irradiated neoplastic B-cells and B-cell precursors [57], probably via the negative regulation of the antiapoptotic signal transducer and activator of transcription 3 (STAT3) function [52]. In addition, BTK interacts with and functions downstream of Toll-like receptor (TLR)-8 and TLR9, linking BTK to the innate immune system [52–54].

Although the overall chance of developing malignancies in XLA is low, there are reports of a 30-fold increased risk of colorectal cancer in patients with XLA [55]. Aberrant immunological function and/or persistent asymptomatic inflammation in the colon is generally thought to contribute to the increased risk of colorectal cancer. However, it has been shown that *BTK* loss of function is associated with excessive Wnt-β-catenin signaling [56], which is known as a major contributor to the development of colorectal carcinoma [57]. In addition to colorectal cancer,

cases of pituitary adenomas [21], gastric adenocarcinoma [58], squamous lung cancer [59], and extranodal cytotoxic T-cell lymphoma [60] have been reported.

25.2.3 Selective IgA Deficiency

Selective IgA deficiency (IgAD) is the most common PID with a prevalence that varies from 1 in 143 to 1 in 18,550 in different ethnic groups [61, 62]. It is defined as occurring when serum IgA levels are equal to or below 0.07 g/L with normal IgM and IgG levels in individuals 4 years of age or older in whom other causes of hypogammaglobulinemia have been excluded [63]. As many as 85–90% of patients with IgAD are asymptomatic, which could be explained by a compensatory increase in IgM production and subsequent increase in secretary IgM in the mucosal lumen [64]. However, IgAD can present with a broad spectrum of clinical manifestations, including recurrent sinopulmonary and gastrointestinal infections, allergic disorders, GI diseases (especially celiac disease), progressive neurodegenerative disorders, autoimmunity, and malignancy, with gastric carcinomas and lymphomas being frequently associated with the disease [64–68].

In IgAD, the common finding is a defect in the maturation of B-cells producing IgA [67]. The genetic basis of IgAD is complex and has remained unclear. Autosomal recessive, autosomal dominant, and sporadic transmission patterns have all been observed. In view of the lack of an identified primary genetic defect and the variation in the inheritance patterns, it is likely that IgAD represents a heterogeneous group of genetic abnormalities such as CVID. In support of this notion is the observation that mutations in transmembrane activator and calcium modulator and cyclophilin ligand interactor (*TACI*) gene (*TNFRSF13B*; OMIM∗604907), which appear to act as a disease-modifying mutation, have been found in IgA deficiency and CVID [69]. Moreover, a novel shared risk locus associated with lower inducible costimulator (ICOS) and higher cytotoxic T lymphocyte-associated protein-4 (CTLA-4) expression has been recently defined in both diseases [70]. Both major histocompatibility complex (MHC) and non-MHC associations have been identified. Among the former, the ancestral HLA-A1, B8, DR3, and DQ2 (8.1) have been associated with susceptibility to IgAD [71]. Non-MHC genetic associations include *IFIH1* (OMIM∗606951), *CLEL16A* (OMIM∗611303), *PVT1* (OMIM∗165140), and *ATG13-AMBRA1* (OMIM∗615088-OMIM∗611359) [72].

The association of malignancy, especially of the lymphoreticular and gastrointestinal systems, with IgAD has been documented mainly in adults [73, 74] with an estimated twofold increased risk compared to general population [75]. However, in a combined Danish and Swedish study of 386 patients with IgAD, the incidence of cancer was not increased. Yet, the investigators in the same study found that relatives of the same patients had slightly elevated cancer rates. In contrast to adults, children with IgAD appear not to be at risk of malignancy [76, 77], which has only been reported in case reports [78–80].

25.3 Immunodeficiencies Affecting Cellular and Humoral Immunity

25.3.1 Coronin-1A Deficiency

Coronin-1A deficiency is a rare PID manifested by either $T^{-}B^{+}NK^{+}$ severe combined immunodeficiency or variable degrees of T-cell lymphopenia associated with severe viral infections, EBV-associated lymphoproliferative disease (LPD), and shortened telomeres. Thus far, nine patients with coronin-1A deficiency have been reported in the literature, of who five developed B-cell lymphomas before reaching the age of 2 years [81–86].

Coronin-1A, expressed predominantly in hematopoietic cells, is a member of the Coronin family of actin-associated proteins. Coronin-1A can link the plasma membrane to the actin cytoskeleton and is essential for signal transduction, migration, phagocytosis, and vesicle trafficking [82, 87–91]. Coronin-1A deficiency is caused by mutations in *CORO1A* gene (OMIM∗605000).

There seems to be a genotype–phenotype correlation in the patients reported so far. Complete absence of Coronin-1A is associated with severe combined immunodeficiency, whereas hypomorphic mutations causing diminished but still detectable protein expression lead to a milder immunological phenotype [81–85]. Recently, a truncating mutation in CORO1A was identified in two young adult siblings with a history of disseminated varicella, cutaneous warts, and $CD4^+$ T-cell lymphopenia, which permits normal protein expression and survival into young adulthood. This mutant was shown to prevent oligomerization and was associated with increased filamentous actin accumulation in T-cells, severely defective thymic output, and impaired T-cell survival but normal calcium flux and cytotoxicity [86].

25.3.2 MST1 (STK4) Deficiency

MST1 deficiency, also known as STK4 deficiency, is a novel autosomal recessive PID, which has been only reported in 12 patients from five different families [92–95]. MST1 deficiency is characterized by profound CD4 lymphopenia, accompanied by multiple bacterial and viral infections, mucocutaneous candidiasis, and autoimmune complications. EBV-associated LPD developed in 4 out of 12 patients during the course of their illness [92–95].

MST1 deficiency is caused by homozygous mutations resulting in premature stop codon in macrophage stimulating 1 (*MST1*) gene, also known as serine/threonine kinases 4 (*STK4*) (OMIM∗604965) [92–95]. MST1 deficiency results in a naïve T-cell survival defect (due to defective IL-7R/Bcl2 pathway and increased Fas expression) [92, 93], as well as impaired lymphocyte trafficking (both non-functional expression of the homing receptors CCR7 and CD62L and impaired LFA-1 activation and cell polarization have been proposed) [93, 95]. Notably, MST1 deficiency has overlapping features with other PIDs involving defects in actin cytoskeletal reorganization, including DOCK8 deficiency and WAS. Recent studies have also suggested a role for MST1 in regulation of autophagy, though with conflicting evidence regarding its precise role [96–98].

25.3.3 Purine Nucleoside Phosphorylase Deficiency

Purine nucleoside phosphorylase (PNP) deficiency is a rare, autosomal recessive, combined immunodeficiency disorder, with an estimated frequency of 4% among patients with SCID [99]. The disease usually manifests during the first year of life; however, the onset of symptoms may vary, with some patients having no apparent clinical immunodeficiency until later in childhood [100–102]. Common clinical manifestations in patients with PNP deficiency include recurrent bacterial, viral, and opportunistic infections; prolonged diarrhea; failure to thrive; neurologic abnormalities, including nonprogressive cerebral palsy, ataxic diplegia, or disequilibrium; and autoimmune disorders, including autoimmune hemolytic anemia, idiopathic thrombocytopenia, autoimmune neutropenia, lupus, and central nervous system vasculitis [99, 100, 103–106]. Due to profound T-cell abnormalities, patients are extremely susceptible to viral infections and may develop disseminated or even fatal disease [99, 101]. A high frequency of malignancy is also noted, including pharyngeal tumors, lymphoma, and lymphosarcoma [99, 107, 108]. In a report of 33 patients with PNP deficiency, 4 had developed lymphoma or lymphosarcoma and 1 had a pharyngeal tumor [99].

Several disease-causing mutations have been identified in the *PNP* gene (OMIM∗164010), producing proteins with differing degrees of enzymatic activity that inversely correlate with clinical severity (i.e., more functional proteins are associated with milder forms of disease, while less functional proteins lead to severe phenotypes) [102, 109, 110]. PNP is an enzyme in the purine salvage pathway that reversibly converts inosine to hypoxanthine and guanosine to guanine. Of all accumulated PNP substrates, only deoxyguanosine can be phosphorylated further in the mammalian cells. Thus, in PNP deficiency,

there is accumulation of abnormally high levels of lymphotoxic dGTP [111, 112]. This, in turn, inhibits ribonucleotidase reductase activity, depletes dCTP, and inhibits DNA synthesis and repair [111, 112]. Moreover, mitochondrial dGTP is also likely to inhibit mitochondrial DNA repair and initiate the apoptotic protease cascade triggered by cytochrome C release [113–115].

The most characteristic immune abnormality in PNP deficiency is a profound defect in T-cell number and function; however, abnormal B-cell functions, including defective antibody production, are common and in part due to abnormal T-cell help [99, 116]. However, an intrinsic defect in B-cell function has not been excluded. The T-cell specificity of PNP lies in the high deoxyguanosine phosphorylating activity in the T lymphocytes, as compared with B lymphocytes or other tissues [117, 118], and the inherent susceptibility of immature thymocytes to apoptosis during T-cell selection [119, 120].

25.3.4 Wiskott–Aldrich Syndrome

Wiskott–Aldrich syndrome (WAS) is a rare X-linked immunodeficiency with highly variable manifestations characterized by thrombocytopenia with small platelets, eczema, and humoral and cellular immunodeficiency with increased susceptibility to pyogenic and opportunistic infections. Patients with WAS may also present with an increased incidence of autoimmunity and malignancies [121–126].

The disease is caused by mutations in the *WAS* gene (OMIM∗300392), which is expressed exclusively in hematopoietic cells. More than 300 unique mutations spanning the *WAS* gene have been described. The effect of a given mutation on WASp expression correlates with the disease severity: mutations that cause decreased WASp levels result in the mild variant X-linked thrombocytopenia (XLT), characterized mainly by thrombocytopenia [127, 128], whereas mutations that abolish WASp expression or result in the expression of a truncated protein are associated with classic WAS. In addition, a third disorder termed X-linked neutropenia (XLN), characterized by neutropenia and variable myelodysplasia, has been attributed to activating mutations in the GTPase-binding domain of WASp [129–131].

The WASp is a multifaceted protein which exists in complex with several partners involved in relaying signals from cell surface receptors to the actin cytoskeleton; lack of WASp results in cytoskeletal defects that compromise multiple aspects of normal cellular activity including proliferation, phagocytosis, immune synapse formation, adhesion, and directed migration [124]. It is therefore not surprising that lack of WASp results in a wide range of defects in cellular function involving all hematopoietic cell lineages [124].

Malignancies are relatively common in older patients (adolescent and young adults), especially in those with autoimmune manifestations, and are frequently associated with a poor prognosis [122, 125, 132]. The most frequent malignancy reported is B-cell lymphoma, which often occurs in EBV-positive patients [122, 126]. In a report of 154 patients with WAS, 21 (13%) developed malignancies, mostly of lymphoreticular origin, with the average age at onset of 9.5 years [122]. Nonlymphoid malignancies, including glioma, acoustic neuroma, testicular carcinoma, and Kaposi sarcoma, have infrequently been reported [122, 133]. The development of hematological malignancies in WAS patients is at least partly due to NK cell and cytotoxic T lymphocyte dysfunction [134–136], absence of invariant NKT cells [137, 138], and chronic stimulation of autoreactive cells and ineffective clearance of virally infected cells [139, 140]. It has been reported that despite normal expression levels of lytic molecules, the cytotoxic $CD8^+$ T-cells from WAS patients failed to effectively kill B-cell lymphoma target cells due to inefficient polarization of cytotoxic granules toward the target tumor cells [134]. Recently, activating mutations in WASp (which give rise to XLN) have been found to lead to genetic instability through dysregulation of actin polymerization. Enhanced and delocalized actin polymerization throughout the cell was shown to inhibit myelopoiesis through defective mitosis and cytokinesis, with micronuclei formation indicative of genomic instability [141].

Despite lack of direct evidence, genomic instability might contribute to the development of malignancies in WAS patients [124].

Early HSCT is the treatment of choice for patients with classic WAS, preferably from a matched related donor [142]. Furthermore, immune reconstitution in WAS patients following HSCT leads to a decrease in cancer risk [142]. Gene therapy is an alternative to HSCT in the treatment of WAS [143]; however, the long-term outcome needs to be further monitored. This could be explained by the fact that the viruses used for therapy integrate in the host genome, with preferential insertion at transcription start sites, promoter and enhancer regions of active genes, and at conserved noncoding DNA, resulting in a high rate of transformations and the development of secondary malignancies [144, 145]. Acute T-cell leukemia due to vector insertion in the vicinity of the T-cell oncogene *LMO2* has been reported in one patient [146, 147].

25.3.5 Dedicator of Cytokinesis 8 Deficiency

Dedicator of cytokinesis 8 (DOCK8) deficiency, initially described as a form of autosomal recessive hyper-IgE syndrome [148], is now regarded as a combined immunodeficiency disorder presenting early in life with: (1) recurrent sinopulmonary infections; (2) cutaneous viral, bacterial, and fungal infections; (3) severe atopy, asthma, and allergies; (4) immune-mediated pathologies including autoimmune hemolytic anemia and vasculitis; (5) neurological complications; (6) malignancies; and (7) extremely high serum IgE levels and eosinophilia [148–152]. Cutaneous viral infections are the most distinctive clinical feature and often identified as recalcitrant, extensive lesions caused by herpes simplex virus, HPV, Molluscum contagiosum virus, and varicella zoster virus [149–152]. Moreover, EBV and/or cytomegalovirus infections are documented in up to 40% of patients [151, 152]. Increased frequencies of malignancies, including squamous cell carcinoma (SCC), cutaneous T-cell lymphoma/leukemia, Burkitt's lymphoma, anaplastic B-cell lymphoma, acute myeloid leukemia (AML), as well as adrenal leiomyoma and microcytic adnexal carcinoma, have been reported in up to 17% of DOCK8-deficient patients [149, 150, 152–154].

The disease is due to biallelic mutations in the *DOCK8* gene (OMIM∗611432), which encodes DOCK8, a member of the DOCK180-related family of atypical guanine- nucleotide-exchange factors (GEFs) highly expressed in lymphocytes [155]. DOCK8 was initially shown to bind to the Rho GTPases Cdc42, Rac1, RHOJ, and RHOQ in a yeast two-hybrid system but not in GST pulldown assay [156]. Following the generation of *Dock8*-knockout mice, Dock8 was found to have Cdc42-specific GEF activity [157]. Ham et al. [158] reported that DOCK8 exists in a macromolecular complex with the Wiskott–Aldrich syndrome protein (WASp), an actin nucleation-promoting factor activated by Cdc42, as well as with talin, a protein required for integrin-mediated adhesion. Subsequently, Janssen et al. [159] demonstrated that the WASp-interacting protein (WIP) bridges DOCK8 to WASp and actin in T-cells, and that the Cdc42-specific GEF activity of DOCK8 is essential for the integrity of the subcortical actin cytoskeleton as well as for TCR-driven WASp activation, F-actin assembly, immune synapse formation, mechanotransduction, T-cell transendothelial migration, and homing to lymph nodes, all of which also depend on WASp. These findings indicate the role of DOCK8 in TCR-driven actin dynamics and formation of the immunologic synapse, which are required for full T-cell activation, proliferation, and acquisition of effector functions. Additional roles of DOCK8 have also emerged, including linking the TLR9-MyD88 cascade to the transcription factor STAT3 that is essential for B-cell proliferation and differentiation [160, 161], regulating Src-dependent NK cell cytotoxicity and cytokine production in response to target cell engagement or receptor ligation [162], and controlling IL-2 signaling, crucial for maintenance and competitive fitness of regulatory T-cells, via a STAT5-dependent manner [163].

DOCK8 deficiency impacts both innate and adaptive immune responses. Immunological

features of DOCK8 deficiency, besides high serum IgE levels and eosinophilia, include lymphopenia (progressive with age) affecting $CD4^+$ and $CD8^+$ T-cells (especially the $CD4^+$ T-cells) and, to a lesser extent, NK and B-cells [149–152], plus a virtual lack of circulating $CD19^+CD27^+$ memory B-cells [160]. Studies in DOCK8-deficient patients have demonstrated decreased T-cell activation and proliferation in response to mitogens [149–152], but not to specific antigens [151]; however, these functional studies are inconclusive due to the difficulty in isolating naive T-cells from the peripheral blood. In murine models of Dock8 deficiency, despite the twofold reduction in peripheral naïve T-cells, the Dock8-deficient mice generated a normal primary $CD8^+$ immune response to viral infection and the defect was mainly localized to decreased survival of $CD8^+$ memory T-cells [164], which can explain why DOCK8 deficient patients are susceptible to recurrent infections. DOCK8-deficient humans and/or mice also exhibit abnormalities in cytokine secretion associated with a T-helper 2-biased immune response [149, 151, 164], low serum IgM levels and impaired antibody responses [160, 165], decreased $CD4^+$ T-helper type 17 cells, and impaired NK cell cytotoxicity [149, 150, 158, 166].

Increased susceptibility to malignancy in DOCK8-deficient patients can be explained by failure of $CD8^+$ T- and NK cell-mediated tumor immunosurveillance, as well as chronic antigenic stimulation. Moreover, there is evidence that DOCK8 itself might have direct tumor suppressor activity [167–170], and that loss of DOCK8 expression might contribute to carcinogenesis [171]. Reduced DOCK8 expression has been demonstrated in the vast majority of primary lung cancers, irrespective of the histological type, compared with normal lung tissue. Epigenetic mechanisms, including DNA methylation and histone deacetylation, were indicated to be involved in DOCK8 downregulation in lung cancer cells [167], as with other candidate tumor suppressor genes, such as p16, RASSF1A, and MYO18B [172–175]. Moreover, homozygous deletions of the *DOCK8* gene have been shown in breast and gastric cancer cell lines. These results suggest that genetic and epigenetic inactivation of DOCK8 is involved in the development and/or progression of lung cancers and other cancers by disturbing the regulatory functions of DOCK8 in cell migration, morphology, adhesion, and growth of cells [167].

25.3.6 RHOH Deficiency

Ras homolog family member H (RHOH) deficiency is a novel form of PID recently identified by genome-wide linkage analysis in two young adult siblings born to consanguineous French parents [176]. Since childhood, both patients displayed a phenotype resembling epidermodysplasia verruciformis (EV), characterized by persistent cutaneous infections with EV-specific HPV (EV-HPV) genotypes. The older sibling had also developed Burkitt's lymphoma in childhood, granulomatous lung disease, and psoriatic-like lesions, whereas the younger sibling had molluscum contagiosum, psoriatic lesions, and gingivostomatitis, indicating that the phenotypic spectrum of the disease is not restricted to susceptibility to HPV [176].

RHOH deficiency results from homozygous loss-of-expression mutations (Y38X) in the *RHOH* gene (OMIM∗602037) located on chromosome 4p13, which encodes an atypical Rho GTPase (RHOH) expressed predominantly in hematopoietic cells. RHOH is GTPase deficient and remains constitutively in the active, GTP-bound state, suggesting that its activity is likely regulated by the level of the protein expressed in the cells rather than guanine nucleotide cycling [177]. It has been shown to counteract Rac GTPase activities in lymphoid cell lines and cytokine-stimulated hematopoietic progenitor cells, resulting in reduced proliferation, increased apoptosis, and defective actin polymerization [177–180].

Immunologic evaluation of RHOH-deficient patients revealed no major abnormality in the frequencies of B-cell subsets, NK cells, NKT cells, monocytes, or polymorphonuclear cells, as well as in antibody production. Despite maintaining normal T-cell counts, both patients displayed a

restricted T-cell repertoire, lack of circulating naive T-cells consistent with the defect in thymic T-cell development observed in *Rhoh*−/− mice [179], expansion of effector memory T-cells (more likely to be consequences of chronic infection), altered expression of T-cell tissue-homing markers with strikingly lower than normal proportion of skin-homing $\beta7^+$ T-cells, and impaired T-cell proliferative responses to anti-CD3 but variable responses to mitogens and recall antigens [176]. It is evident that on TCR stimulation, murine RhoH undergoes tyrosine phosphorylation and mediates recruitment of Zap70 and Lck to the TCR/linker of activation in T-cell (LAT) signalosome [181]. This finding has been confirmed in RHOH-deficient T-cells of patients, showing little or no ZAP70 phosphorylation in the presence or absence of CD3 stimulation [176]. The combination of T-cell defects common to both mice and humans, including impaired T-cell responses, a lack of naive cells, and smaller than normal proportion of $\beta7^+$ T-cells, might explain the pathogenesis of susceptibility to cutaneous EV-HPVs.

The *RhoH/TTF* (translocation three four) gene was first identified by fusion to the *BCL6/LAZ3* oncogene resulting from *t*(3;4)(q27;p11) translocation in an NHL cell line [182–184]. Another chromosomal alteration involving the *RhoH/TTF* gene in a patient with multiple myeloma and *t*(4;14)(p13;q32) translocation has also been identified [184]. Moreover, aberrant somatic hypermutations in *RHOH* gene have been previously reported in various B-cell malignancies, including diffuse large B-cell lymphomas [185], AIDS-related NHL [186], primary central nervous system lymphomas [187], and, rarely, Burkitt's lymphoma [185]. However, it remains unclear whether these mutations translate into abnormal levels of RhoH expression in lymphomas and what pathophysiological contribution hypermutation in the RhoH gene plays in lymphomagenesis.

25.3.7 MCM4 Deficiency

MCM4 deficiency is an autosomal recessive disorder manifested with short stature, adrenal failure, selective NK cell deficiency (low number and function), and predisposition to viral infections [188–191]. To date, only one patient with MCM4 deficiency has been reported to develop EBV-associated LPD [188].

MCM4 deficiency is caused by homozygous mutations in minichromosome maintenance complex component 4 (*MCM4)* gene (OMIM∗602638). MCM4 is part of the replicative helicase MCM2-7 complex, which is essential for normal DNA replication and genome stability in all eukaryotes. MCM4 is crucial for preserving the DNA integrity during the proliferation of NK cells. The immunologic defect in MCM4 deficient patients is lack of transition of $CD56^{bright}$ NK cells to $CD56^{dim}$ NK cells, which account for about 90 % of circulating NK cells. Other immune cell lines are normal in number and function.

It is also possible that MCM4 deficient patients may have an increased risk of neoplastic change due to defective DNA damage response pathways. It has been demonstrated that Chaos3 mouse model, which by virtue of an amino acid alteration in MCM4 that destabilizes the MCM2–7 DNA replicative helicase, is at increased risk of genomic instability and cancer development [192].

25.3.8 Signal Transducer and Activator of Transcription 3 Deficiency

Hyper-IgE syndrome (HIES) is a complex PID characterized by recurrent staphylococcal infections beginning early in infancy, predominantly involving the skin and lungs, chronic eczema, and markedly high serum IgE concentrations [193–195]. Skin infections due to *S. aureus* lack the usual local or systemic features of inflammation, forming so-called cold abscesses [196]. Recurrent sinopulmonary infections, resulting in bronchiectasis and pneumatocele formation frequently superimposed with bacterial and fungal infections, are the major causes of morbidity and mortality in patients with HIES [197]. Despite having extremely high serum IgE levels and eosinophilia, patients with HIES are usually free

from other allergic manifestations, recognized as a marked difference from DOCK8 deficiency [193, 195]. In patients with HIES, serum IgG, IgM, and IgA levels are usually normal; however, most have impaired antigen-specific antibody response to immunization [198]. Diminished circulating memory B-cells and defects in the differentiation of Th17 cells have also been demonstrated [198–200]. The multisystem nature of the disease extends beyond the immune system and accounts for the characteristics craniofacial, musculoskeletal, dental, and vascular abnormalities [201–204].

Dominant negative mutations in *STAT3* (OMIM∗102582) have been identified as the major molecular etiology of autosomal dominant and sporadic cases of HIES [205, 206]. STAT3, one of the seven STAT proteins in the human, is a transcription factor and plays a critical role in responses to many cytokines and growth factors through the shared signal-transducing molecule gp130 [194, 195]. It is crucial for cell proliferation, survival, migration, apoptosis, and inflammation in various tissues [207], probably explaining the diverse clinical findings in patients with HIES.

STAT3 deficiency is associated with an increased risk of LPD, most notably HL and NHL (relative risk: 259), with the majority of B-cell origin and aggressive histology [208–210]. Other cancers described in patients with HIES include leukemia and cancers of the vulva, liver, and lung [211]. The underlying mechanisms, however, remain unclear. The higher risk of tumor formation has been attributed to defective immunosurveillance and chronic B-cell stimulation, resulting in an increased turnover of B-cells and accumulating genetic aberrations, giving rise to malignant B-cell clones [212].

25.3.9 Chromosome 22q11.2 Deletion Syndrome

Chromosome 22q11.2 deletion syndrome is relatively common (estimated in 1 in 4000 births) [213], and about 6% of newly diagnosed cases are familial [214]. The presenting symptoms of chromosome 22q11.2 deletion syndrome vary depending on age. While developmental delay and speech issues are the usual presenting symptoms in older children and adults, cardiac anomalies, hypocalcemia, and infection are the major manifestations in infants. Cardiac defects are seen in approximately 80% of patients; and conversely, tetralogy of Fallot and interrupted aortic arch type B have a strong positive predictive value for chromosome 22q11.2 deletion syndrome [215, 216]. Palatal dysfunction, feeding problems, facial dysmorphism, renal anomalies, and gastrointestinal manifestations also are seen in most of these patients [217]. Patients are also at an increased risk of atopy and autoimmune disease development [218, 219].

The immune system is affected in approximately 75% of the patients [217, 219, 220]. The severity ranges from absent thymic tissue and no circulating T-cells to completely normal T-cell counts. Many infants with low T-cell counts will demonstrate improvement in the first year of life, but after that, T-cell counts decline [221]. Patients may also suffer from variable degrees of B-cell defects [222, 223]. In a cohort of 687 patients with chromosome 22q11.2 deletion syndrome, six cases of malignancy were identified. This gives an overall frequency of 0.9% (900 per 100,000) in this large pediatric group of patients, whereas the overall risk of malignancy in children under the age of 14 years is 3.4 per 100,000 children [224]. As reported in the literature, patients with chromosome 22q11.2 deletion syndrome have a clearly increased risk of lymphoma, particularly B-cell lymphoma [225–228]. There have also been reports of myelodysplasia, acute lymphoblastic leukemia, SCC, astrocytoma, neuroblastoma, hepatoblastoma, renal cell carcinoma, and rhabdoid tumors [224, 229–233].

25.3.10 DNA Repair Defects

B and T lymphocyte development depends largely on multiplex genetic rearrangements, i.e., V(D)J recombination, class switch recombination, and somatic hypermutation, which are

carried out by multiple DNA repair and damage response protein complexes [234]. Variations in the DNA repair genes might compromise the delicate balance between the generation of genetic variation and replication fidelity of DNA [235, 236].

PIDs associated with defects in DNA repair, collectively termed genomic instability syndromes, are generally associated with cellular radiosensitivity, developmental defects, and predisposition to cancer [236–238]. Syndromes known to be associated with malignancies, including ataxia-telangiectasia, Nijmegen syndrome, Bloom syndrome, DNA ligase IV deficiency, Artemis deficiency, cartilage hair hypoplasia, PMS2 deficiency, and FAAP24 deficiency are summarized in Table 25.1.

Although these defects are associated with an increased risk of lymphoid malignancies, mainly NHL, nonlymphoid tumors affecting the brain, skin, breast, and gastrointestinal tract have also been reported [238–244]. This is partly due to the fact that diverse DNA repair processes are not specific to antigen receptor diversification. DNA double-strand breaks, arising from multiple sources, including exposure to ionizing radiation, can potentially lead to replication errors, loss or rearrangements of genomic material, and eventually cell death or carcinogenesis. The DNA damage response pathway, responsible for sensing and repairing the damaged DNA, comprises the most powerful tumor surveillance mechanism [243]. The observation of an increased risk of cancer development in heterozygote carriers provides additional insight into their tumorigenic potential [244–247]. Additionally, defects in immunosurveillance mechanisms per se, similar to certain PIDs not associated with DNA repair defects, contribute to cancer development.

25.4 Congenital Defects of Phagocyte Number or Function

The underlying mechanism of cancer development in PIDs caused by defects of phagocytic cells is quite different from that observed in other immunodeficiency disorders. Here the implicated genes are important for proper myeloid cell development; thus cancers form due to dysregulated myelopoiesis. This is distinct from cancers that occur in some other conditions including impaired immunosurveillance and presence of specific viruses.

25.4.1 Severe Congenital Neutropenia (Kostmann Syndrome)

Severe congenital neutropenia (SCN) is a rare PID characterized by a maturation arrest of myelopoiesis at the level of the promyelocyte/myelocyte stage with peripheral blood absolute neutrophil counts (ANCs) below 0.5×10^9/L, in addition to early-onset superficial and systemic bacterial infections [254, 255]. The skin and mucous membranes are usually affected by ulceration, gingival hyperplasia, periodontitis, and abscess formation [256]. Patients may also suffer from neurological disorders including developmental delay, mental retardation, epilepsy, and decreased cognitive function [257, 258].

SCN follows an autosomal dominant or recessive pattern of inheritance or can occur sporadically. It is a genetically heterogeneous disorder caused by a variety of mutations in several different genes. Nonetheless, the different genetic forms of SCN share a rather similar clinical phenotype. Mutations in the neutrophil elastase (*ELA2*) gene (OMIM∗130130) are found in approximately 50% of all cases, i.e., those with dominant autosomal or sporadic SCN [256, 259]. ELA2 is a serine protease, exclusively expressed in neutrophils and monocytes, and is stored in the primary granules of neutrophils [260]. Interestingly, mutations in the *ELA2* gene are also responsible for the clinical phenotype of cyclic neutropenia. The pathophysiological mechanisms responsible for the development of different phenotypes, congenital or cyclic neutropenia, are not yet understood [261]. Most patients with autosomal recessive disease, which comprises approximately 30% of SCN, have mutations in the HS-1-associated protein X (*HAX1*)

Table 25.1 Clinical and immunological features of DNA repair defects associated with cancers

Gene	Disease	Inheritance	Clinical features	Pathogenesis	Immune defects	Associated cancers
ATM (OMIM∗607585)	Ataxia-telangiectasia	AR	Cerebellar ataxia, oculocutaneous telangiectasia, chromosomal instability, radiosensitivity, thymic aplasia, recurrent sinopulmonary infections, cancer predisposition (up 40%)	Disorder of cell cycle checkpoint and DSB repair, role in V(D)J, CSR	Often decreased IgA, IgE, and IgG subclasses, increased IgM, antibodies variably decreased, progressive T-cell lymphopenia, normal B-cell count	Lymphomas, lymphoid leukemias (mainly T-cells), epithelial tumors, gastric carcinoma [238, 239, 248]
NBN (OMIM∗602667)	Nijmegen breakage syndrome	AR	Severe microcephaly, bird-like face, mental and growth retardation, chromosomal instability, radiosensitivity, recurrent sinopulmonary infections, strong predisposition to lymphoid malignancy	Disorder of cell cycle checkpoint and DSB repair, role in V(D)J, CSR, SHM	Often decreased IgA, IgE, and IgG subclasses, increased IgM, antibodies variably decreased, decreased B- and T-cell counts	Hodgkin and non-Hodgkin lymphomas, leukemias (mainly B-cells), brain tumors [240]
BLM (*OMIM*∗210900)	Bloom syndrome	AR	Short stature, bird-like face, sun-sensitive erythema, erythema, marrow failure, chromosomal instability, cancer predisposition	Role as a RecQ-like helicase	Low IgM and IgA, normal B- and T-cell counts	Leukemias, lymphomas, carcinomas [249]
LIG4 (OMIM∗601837)	DNA ligase IV deficiency	AR	Microcephaly, facial dysmorphisms, radiation sensitivity, may present with RS-SCID, Omenn syndrome, or with a delayed clinical onset	Impaired NHEJ, role in V(D)J, CSR	Decreased serum Igs, decreased B- and T-cell counts	EBV-positive B-cell lymphomas, T-cell ALL [241, 250]
DCLRE1C (OMIM∗602450)	Artemis deficiency	AR	Radiation sensitivity, may present with RS-SCID or Omenn syndrome	Role in V(D)J, CSR	Decreased serum Igs, markedly decreased B- and T-cell counts	EBV-positive B-cell lymphomas [251]
RMRP (OMIM∗250250)	Cartilage hair hypoplasia	AR	Short-limbed dwarfism with metaphyseal dysostosis, sparse hair, bone marrow failure, autoimmunity, predisposition to cancers, impaired spermatogenesis, neuronal dysplasia of the intestine	Role in ribosomal RNA processing, mitochondrial DNA replication, and cell cycle control	Normal or reduced serum Igs, variably decreased antibodies, normal B-cell count, decreased or normal T-cell count, impaired lymphocyte proliferation	Non-Hodgkin lymphomas, basal-cell carcinoma [242]

(continued)

Table 25.1 (continued)

Gene	Disease	Inheritance	Clinical features	Pathogenesis	Immune defects	Associated cancers
PMS2 (OMIM∗600259)	PMS2 deficiency (class switch recombination deficiency due to impaired mismatch repair)	AR	Recurrent infections, café au lait spots, cancer predisposition	Defective CSR-induced DSBs in Ig switch regions	Low IgG and IgA, elevated IgM, abnormal antibody responses, normal B-cell count, decreased, switched, and non-switched B-cell counts	Leukemias, lymphomas, colorectal carcinoma, brain tumors [243]
FAAP24 (OMIM∗610884)	FAAP24 deficiency	AR	Fatal EBV-associated LPD in early childhood	Increased endogenous DNA damage and failure to efficiently invoke cell cycle checkpoint responses	Failure of peripheral mononuclear cells to limit outgrowth of autologous EBV-infected B-cells, as well as failure of T-cell lines established from lymph node biopsy to kill autologous EBV-infected B-cells and K562 target cells	EBV-associated LPD [252, 253]

gene (OMIM∗605998) [262]. HAX-1, a mitochondria-targeted protein containing Bcl-2 homology domains, is an apoptosis-regulating protein [262]. Mutations in the glucose-6-phosphatase catalytic subunit 3 (*G6PC3*) gene (OMIM∗611045) have been identified in a group of autosomal recessive SCN patients with additional syndromic features including cardiac and urogenital anomalies and increased venous marking [263]. Patients with X-linked SCN harbor activating mutations in Wiskott–Aldrich syndrome (*WAS*) gene (OMIM∗300392), leading to a constitutively active form of the WAS protein and unregulated actin polymerization [131]. Inactivating mutations in the proto-oncogene growth factor-independent 1 (*GFI1*) gene (OMIM∗600871) are also associated with SCN [264]. In addition, SCN without a maturation arrest has recently been associated with p14 protein deficiency [265]. Finally, acquired nonsense mutations in colony-stimulating factor 3 receptor (*CSF3R*) gene (OMIM∗138971) have also been found to affect 20% of SCN patients [266].

SCN patients are at an increased risk of myelodysplasia (MDS) and AML development with a cumulative incidence of leukemia of 22% after 15 years of G-CSF treatment [267, 268]. Independent of the genetic subtype, conversion to leukemia in patients with SCN is often associated with one or more somatic cellular genetic abnormalities (e.g., monosomy 7, *RAS* mutations, trisomy 21, or *CSF3R* mutations), which may be diagnostically useful to identify subgroups of patients at high risk of developing leukemia [261]. Other risk factors for progression to MDS and/or AML are the severity of neutropenia, younger age at diagnosis, and prior exposure to G-CSF [269]. Interestingly, marrow cells from nearly 80% of patients with SCN who transform to leukemia show point mutations in *CSF3R*, suggesting that these mutations play an important role in leukemogenesis [270].

Hematopoietic stem-cell transplantation (HSCT) is the only definitive treatment for patients with bone marrow failure, MDS, or leukemia; however, it seems that patients with SCN may be at increased risk of transplant-related mortality for unknown reasons. As a result, there is no clear consensus on when a patient with SCN should undergo HSCT [271].

25.4.2 Shwachman–Diamond Syndrome

Shwachman–Diamond syndrome (SDS) is a rare autosomal recessive, systemic disease characterized by exocrine pancreatic insufficiency, impaired hematopoiesis, and leukemia predisposition [272]. Other clinical features include skeletal, immunologic, hepatic, and cardiac disorders [271]. There is considerable phenotypic variability between individuals, and making the diagnosis can be challenging, particularly in older patients in whom symptoms such as steatorrhea may have resolved [271] or may not be present [273]. The most common hematologic abnormality in patients with SDS is neutropenia, which can be chronic or intermittent. Anemia and thrombocytopenia are also common manifestations. Patients with SDS are susceptible to recurrent infections [274] likely due to neutropenia. Other immune defects have also been reported. These include neutrophil chemotactic defects [275, 276], decreased proportions of circulating B-cells, low immunoglobulin levels, decreased in vitro B-cell proliferation, lack of specific antibodies, or decreased total circulating T lymphocytes, as well as decreased proliferative responses [277, 278].

Approximately 90% of patients with clinical features of SDS have mutations in the Shwachman–Bodian–Diamond syndrome (*SBDS*) gene (OMIM∗607444) [279], with the encoded protein being essential for normal ribosome maturation, though its precise molecular function remains unclear [280, 281]. In addition to a stem-cell defect [282], patients with SDS have also a serious, generalized marrow dysfunction with an abnormal bone marrow stroma in terms of its ability to support and maintain hematopoiesis [281, 283].

Similar to other marrow failure syndromes, patients with SDS have an increased risk for MDS and AML [284], with an estimated risk of 19% at 20 years and 36% at 30 years [269]. There

are also case reports of solid tumors in patients with SDS [285–287]. The reason behind this malignant predisposition is not known. However, several theories have been proposed, including chromosome instability [288, 289], accelerated apoptosis linked to increased expression of the Fas antigen and to hyperactivation of the Fas signaling pathway [290], and abnormal gene expression patterns as evident by upregulation of several oncogenes, including *LARG*, *TAL1*, and *MLL*, and downregulation of several tumor suppressor genes, including *DLEU1*, *RUNX1*, *FANCD2*, and *DKC1*, which might result in continuous stimulation favoring evolution or progression of malignant clones [291]. Accordingly, all patients with SDS should be monitored with peripheral blood counts every 3–4 months and marrow evaluation on a yearly basis, and if indicated, HSCT should be done prior to the development of overt leukemia.

25.4.3 GATA2 Deficiency

GATA2 deficiency causes a wide spectrum of phenotypes including disseminated mycobacterial infections (typically Mycobacterium avium complex), opportunistic fungal infections, disseminated HPV infections, and pulmonary alveolar proteinosis, with an increased risk of myelodysplasia, cytogenetic abnormalities, and myeloid leukemias [292–295]. Germline mutations in *GATA2* have been initially associated with several clinical entities, including MonoMAC syndrome (Monocytopenia and Mycobacterium avium complex infections) [293, 296], DCML (dendritic cell, monocyte, and lymphocyte) deficiency [294], familial MDS/AML (without other hematopoietic defects) [297, 298], and Emberger's syndrome characterized by congenital deafness and primary lymphedema of the lower limb [299], which are now collectively defined as GATA2 deficiency. Despite pleiotropic clinical manifestations, the high propensity for the development of MDS constitutes the most common clinical denominator. This form of immunodeficiency occurs either as an autosomal dominant form or sporadically, and can present from early childhood to late adulthood [296]. Reportedly, near 380 GATA2-deficient patients have been reported, with a roughly estimated prevalence of myeloid neoplasia of at least 75% [300].

While considerable efforts have been made to identify the mutations that characterize this disorder, pathogenesis remains a work in progress. Heterozygous disease-causing germline mutations in *GATA2* gene (OMIM∗137295) indicate dominant interference of gene function by either dominant negative effects or haploinsufficiency [293, 301, 302]. The GATA family of transcription factors, which contain zinc fingers in their DNA-binding domain, have emerged as candidate regulators of gene expression in hematopoietic cells. GATA2 functions in the regulation of hematopoiesis and, in particular, is required for maintenance and survival of the hematopoietic stem-cell pool [303, 304]. GATA2 also functions in the formation of early blood and lymphatic vessels [305, 306]. The role of *GATA2* mutation in disease manifestation is incompletely understood but likely complex and thought to be linked to the generation or maintenance of progenitors required for the affected cell subsets [301].

Immunological characterization of patients with the MonoMAC syndrome/DCML deficiency revealed profoundly decreased or absent monocytes, NK cells, and B-cells as well as a severe decrease in circulating and tissue dendritic cells (DCs). In most cases, GATA2 deficiency is accompanied by a severe reduction in peripheral blood NK cells, specifically the $CD56^{bright}$ subset, with marked functional impairment [293], which predispose to significant HPV and other viral infections, as well as HPV-associated SCC. Bone marrow failure resulting from loss of stem cells may underlie the multilineage cytopenias described in most patients; however, the underlying mechanisms for cytogenetic abnormalities or the leukemic transformation need to be further clarified [300]. In addition to MDS/AML and SCC, two cases of invasive melanoma have been reported, suggesting an association between decreased GATA2 expression and melanoma progression [307].

25.5 Defects in Intrinsic and Innate Immunity

25.5.1 Epidermodysplasia Verruciformis

Epidermodysplasia verruciformis (EV) is a chronic, genetically inherited skin condition characterized by increased susceptibility to cutaneous infection with certain HPV genotypes, referred to as EV-HPVs [308, 309]. EV begins during infancy or early childhood, and the more benign lesions manifest as flat, wart-like, hypopigmented, or hyperpigmented papules, or pityriasis versicolor-like plaques, whereas lesions with greater potential for malignant transformation present more variably as verrucous and seborrheic keratosis-like lesions, occurring mainly on sun-exposed areas [308–310]. Approximately 30–60% of individuals eventually develop skin malignancies, eventually in the fourth to fifth decades, with Bowen carcinoma in situ being the most frequent tumor, followed by invasive SCC and, less frequently, basal-cell carcinoma [310–313].

EV is inherited primarily in an autosomal recessive pattern [314], although both X-linked recessive and autosomal dominant modes of inheritance have been reported [315, 316]. Genome-wide linkage studies have identified two EV susceptibility loci *EV1* and *EV2*, on chromosomes 17 and 2, respectively [317]. Mutations in the *EVER1* (OMIM∗605828) and *EVER2* (OMIM∗605829) genes, which are part of the EV1 locus, have been identified in approximately 75% of patients with EV [308].

The EVER proteins, localized in the endoplasmic reticulum of human keratinocytes [318], interact with ZnT-1 [319], a zinc transporter regulating cellular zinc homeostasis. Loss of EVER zinc homeostasis enhances the expression of viral genes, specifically the pro-oncogenic *E6* and *E7*, contributing to HPV-mediated carcinogenesis. Besides keratinocytes, EVER proteins are expressed in T and B lymphocytes, NK cells, endothelial cells, myeloid cells, and DCs. Zinc has been shown to contribute to TCR signaling by increasing ZAP70 phosphorylation [320]. Mutated, dysfunctional *EVER* genes would disrupt zinc homeostasis and consequently produce a defect in cell-mediated immunity, which could compromise viral clearance and lead to malignant transformation [319, 321].

25.5.2 Warts, Hypogammaglobulinemia, Infections, and Myelokathexis Syndrome

Warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome is a rare, dominantly inherited PID characterized by warts, hypogammaglobulinemia, infections, and myelokathexis, which refers to neutropenia resulting from abnormal retention of mature neutrophils and increased neutrophils apoptosis in the bone marrow [322–324]. The incidence of WHIM syndrome has been estimated to be 0.23 cases per million births [325]. The clinical onset usually occurs during infancy or early childhood with recurrent gastrointestinal, respiratory, and cutaneous bacterial infections and increased susceptibility to HPV infection, causing numerous, recalcitrant skin and genital warts [323, 324]. Genital warts (condylomata acuminata) may undergo dysplastic changes conferring to an increased risk of malignancy [322–324]. Contrary to the long-held belief, HPV is not the only unique viral susceptibility in WHIM syndrome. More recently, EBV-associated LPD [326, 327] as well as herpes zoster [328], herpes simplex virus [328, 329], and molluscum contagiosum [326] infections have been reported, indicating a generalized susceptibility to *Herpesviridae* viruses.

WHIM syndrome is primarily caused by gain-of-function mutations in the gene encoding the chemokine receptor CXCR4 (OMIM∗162643) [330], a member of the G-protein-coupled receptor superfamily specific for the CXC chemokine stromal cell-derived factor 1 (SDF-1) [331], also known as CXCL12. All *CXCR4* mutations reported to date disrupt receptor downregulation leading to enhanced and prolonged chemotactic responsiveness to SDF-1 [332, 333].

Immunological and hematological abnormalities in WHIM syndrome include peripheral

neutropenia, B lymphopenia with a particular reduction in the number of switched memory B-cells ($CD27^+$ IgD^-), T lymphopenia with decreased number of naïve T-cells, and a relative expansion of memory T-cells with a restricted repertoire, deficiency of plasmacytoid DCS, and hypogammaglobulinemia [334–337]. The mechanisms by which dysregulated CXCR4 signaling affects leukocyte homeostasis and predisposes to a selective susceptibility to HPV infection and carcinogenesis are still unknown. It remains possible that defective trafficking of effector cells (T-cells and NK cells) and antigen-presenting cells might contribute to defective cutaneous immunity, explaining the abnormal susceptibility to viruses affecting the skin [212].

25.6 Diseases of Immune Dysregulation

25.6.1 X-Linked Lymphoproliferative Disease

X-linked lymphoproliferative disease (XLP), formerly known as Duncan disease, is a rare and often fatal inherited immunodeficiency disorder initially described by Purtilo et al. [338], with an estimated incidence of one to three per million male births [339]. It is characterized by severe immune dysregulation in males with a variable clinical presentation, often following EBV infection, manifesting as fulminant infectious mononucleosis and/or acquired hemophagocytic lymphohistiocytosis (HLH), dysgammaglobulinemia, and malignant lymphoma [340–343]. Other, albeit less common, clinical features of XLP include aplastic anemia, lymphocytic vasculitis, pulmonary lymphoid granulomatosis, arthritis, colitis, and psoriasis [343–345].

Most cases of XLP are caused by germ line mutations in the Src homology 2 domaincontaining gene 1A (*SH2D1A*; OMIM∗300490) encoding the 128 amino acid signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) [346–348]. In humans, SAP is expressed predominantly in NK, NKT, and T-cells [349–351]. It has been shown to serve as an adaptor molecule downstream of several SLAM immunomodulatory receptors family [352]. The SLAM–SAP association potentiates the development of NKT cells, T–B-cell conjugation required for the development of germinal centers and immunoglobulin production, and EBV-directed cytotoxicity by T- and NK cells. In addition, it is required for normal T-cell homeostasis mediated by reactivation-induced cell death (RICD) [353, 354]. A second XLP-like disorder caused by mutations in the X-linked inhibitor of apoptosis protein (*XIAP*; OMIM∗300079) was described in 2006 [355]. XIAP directly limits the activity of several critical death-inducing caspases, either by direct enzyme inhibition or through ubiquitin-mediated proteasomal degradation. XIAP overexpression, or increased activity, is associated with cancer progression, resistance to therapy and poor prognosis [356]. XIAP deficiency is predominantly associated with recurrent EBV-associated HLH; however, no lymphoma occurrence has been reported in affected patients till now [171, 343, 355, 357].

SAP-deficient patients are at increased risk of lymphoma development, as well as other LPD. Approximately 30% of patients develop lymphoma at a mean age of 15 years at diagnosis [343, 358]. Expectedly, the majority are of B-cell origin, arising in extranodal sites, most commonly localized in the ileocecal region, with Burkitt's lymphoma comprising approximately 50–60% of total lymphomas [343, 359, 360]. Notably, not all cases of lymphomas arise due to malignant transformation of EBV-infected B-cells, as up to one-third of patients with lymphoma are EBV seronegative [343, 358, 360], indicating that the genetic defect per se can result in lymphoma. It is likely that defective antitumor immunosurveillance due to poor $CD8^+$ T- and NK cell cytotoxic responses and lack of NKT cells contributes to lymphomagenesis [212].

25.6.2 IL-2-Inducible T-Cell Kinase Deficiency

IL-2-inducible T-cell kinase (ITK) deficiency is a novel PID characterized by severe EVB-

associated immune dysregulation, with a clinical picture similar to that seen in X-linked lymphoproliferative disease (XLP) [361]. ITK deficiency was originally described in 2009, where two ITK-deficient female siblings from a consanguineous Turkish family developed uncontrolled EBV infection resembling hemophagocytic lymphohistiocytosis (HLH) with eventual progression to HL [362]. In a report of three cases from a single Arab family, the first presentation was HL, whereas fulminant hemophagocytosis and severe mononucleosis appeared after remission of lymphoma [361]. Adding to the complexity of the disease, seven additional ITK-deficient patients, of who four developed HL, were identified following the screen of patients with autoimmune lymphoproliferative syndrome or suspicion of congenital forms of HLH [363, 364]. More recently, the clinical spectrum of ITK deficiency has been further extended to include late-onset isolated involvement of the lungs and the mediastinal lymph nodes with a polyclonal proliferation of small B-cells not suggestive of any malignant lymphoma [365].

In ITK deficiency, germ line loss-of-function mutations in the *ITK* gene (OMIM∗186973) result in pronounced instability or truncation of the ITK protein [361]. ITK, a member of the Tec family tyrosine kinases, is expressed in T as well as NK cells, invariant NKT cells, and mast cells [366]. ITK plays a critical modulatory role in the T-cell receptor (TCR) signaling cascade. In mice, it functions in the positive/negative selection of thymocyte development, as well as regulation of conventional vs. innate-type $CD8^+$ T-cell development [367, 368]. Moreover, $Itk^{-/-}$ $CD8^+$ T-cells fail to mount effective primary or memory immune responses to a variety of viral infections [368–370]. ITK deficiency affects the expansion of cytotoxic T lymphocytes, causes a delay in the expression of cytolytic effectors during activation, and leads to an intrinsic defect in degranulation [371]. In the absence of ITK, enhanced development of $CD4^+$ gammadelta T-cells can induce the secretion of IgE by wild-type B-cells [372]. Itk is also crucial for invariant NKT-cell development and function in mice [366]. Similarly, a characteristic reduction in naive $CD45RA^+$ T-cells and NKT cells has been reported in ITK-deficient patients [373]. Moreover, ITK has been shown to differentially regulate NK cell-mediated cytotoxicity, which might be impaired in the absence of ITK protein [374].

The development of LPD in ITK-deficient patients almost always follows primary EBV infection and is diagnosed as HL, as opposed to Burkitt's lymphoma or other NHL seen in XLP [375]. It is speculated that perturbed innate and adaptive antitumor immunosurveillance, including lack of NKT cells and impaired NK- and T-cell-mediated cytotoxicity, plays contributory roles in the development of EBV-associated LPD in ITK-deficient patients [212].

25.6.3 XMEN Disease

X-linked immunodeficiency with magnesium defect, EBV infection, and neoplasia (XMEN) disease has been recently identified in nine male patients (two of which were siblings) [376–378]. The major clinical features of XMEN disease include persistent elevation in EBV-viral load, EBV-associated LPD, often with splenomegaly, dysgammaglobulinemia, and decreased CD4:CD8 ratio. In addition, XMEN patients may have susceptibility to sinopulmonary and ear infections, viral pneumonias, and other viral infections, but these are generally mild and infrequent. EBV-associated lymphoproliferation ultimately emerges in late childhood and is the most common cause of severe morbidity and mortality in this patient population [376–378].

XMEN disease is caused by loss-of-function mutations in *MAGT1* (OMIM∗300715), which encodes a membrane-associated transporter that selectively conducts Mg^{2+} across the membrane, with almost no permeability to other cations including Ca^{2+} [379, 380]. Immunological investigations in patients with MAGT1 deficiency revealed CD4 lymphopenia, leading to an inverted CD4:CD8 ratio and reduced number of recent thymic emigrant T-cells, indicating that impaired thymopoiesis may contribute to CD4 lymphopenia. No major disturbance was observed in other lymphocyte populations. MAGT1-deficient

T-cells showed impaired proliferation and activation upon in vitro stimulation with anti-CD3 antibody. In contrast, T-cell activation in response to phorbol myristate acetate and ionomycin was intact, showing that the patients had a proximal TCR signaling defect prior to the induction of the Ca^{2+} flux. MAGT1-deficint B-cells showed normal activation upon BCR stimulation [376]. Recapitulating the patients' phenotype by knocking down MGAT1 in normal T-cells, as well as rescuing patients' T-cells with ectopic expression of MAGT1, established that MAGT1 is required for TCR-stimulated Mg^{2+} influx that transiently raises free $[Mg^{2+}]_i$ in order to temporarily coordinate T-cell activation [376, 381].

XMEN patients have uncontrolled EBV infection and a predisposition to lymphoma. This has been attributed to a selective loss of NKG2D expression (posttranscriptional, accelerated protein turnover) and the resultant impaired cytolytic responses of NK and cytotoxic $CD8^+$ T lymphocytes [377], which are essential for control of viral infections and tumor immunosurveillance [382]. Hence, MAGT1 not only mediates TCR-induced Mg^{2+} flux but also regulates the basal-free $[Mg^{2+}]_i$ homeostasis required for NKG2D cytolytic activity. This has been verified by cultivation of NK and cytotoxic $CD8^+$ T lymphocytes from XMEN patients in Mg^{2+}-supplemented medium, causing a dose-dependent increase in free $[Mg^{2+}]_i$, which did recover the cytotoxicity defect partially in cytotoxic $CD8^+$ T lymphocytes and almost completely in NK cells [377]. Most notably, magnesium supplementation in vivo concurrently reduced EBV-infected cells, which may provide an adjunctive treatment to prevent early lymphoma development and mortality in XMEN patients.

25.6.4 CD27 Deficiency and CD70 Deficiency

CD27 deficiency and CD70 deficiency are two related, newly identified PID predominantly manifesting with EBV-related diseases, hypogammaglobulinemia, and additional viral infections [383–387]. CD27 deficiency has been reported in 17 patients, of who five developed EBV-associated LPD and six developed lymphoma (HL, T-cell lymphoma, or diffuse large B-cell lymphoma) [386]. CD70 deficiency has been recently identified in four patients, of who three developed EBV-associated HL. The immunological phenotype of CD27- and CD70-deficient patients includes largely normal counts of T, B, and NK cells but reduced proportions of memory B-cells, impaired $CD8^+$ T cytotoxic responses to EBV, and variably reduced NK cell function [383, 384, 387].

CD27, a member of TNF receptor superfamily, is expressed on human naïve and some memory T-cells, germinal center and memory B-cells, plasma cells, and a subset of NK cells [388–392]. CD27 binds to its specific ligand CD70, which is structurally related to TNF and is only transiently expressed on activated dendritic, T-, and B-cells [390, 393, 394]. CD27 deficiency is caused by homozygous/compound heterozygous mutations in *CD27* gene (OMIM∗615122), resulting in absent/reduced CD27 expression [386]. CD70 deficiency is caused by homozygous frameshift or in-frame deletions in *CD70* gene (OMIM∗602840), causing an abolished CD70 surface expression or binding to its cognate receptor CD27 [387].

CD27-CD70 interaction regulates the survival, function, and differentiation of T-, B-, NK, and plasma cells [383, 390, 395]. In T-cells, CD27-CD70 interaction is critical for cell proliferation, long-term maintenance of antigen-specific T-cells, antiviral responses, antitumor immunity, and alloreactivity [396, 397]. In B-cells, ligation of CD27 by CD70 results in enhanced plasma cell formation and increased IgG production [397]. CD27–CD70 interaction is also essential for augmented IFN-γ secretion by NK cells [398, 399] and development of iNKT cells [384]. These data support the notion that CD27 deficiency and Cd70 deficiency can increase susceptibility to malignancies.

25.6.5 CTPS1 Deficiency

CTP synthase 1 (CTPS1) deficiency is a novel autosomal recessive PID characterized with

early-onset severe viral (EBV and VZV) and bacterial infections, LPD and EBV-associated NHL. CTPS1 deficiency has been reported in eight patients from five different families, of who two patients developed EBV-driven B-cell NHL. The immunologic features of CTPS1 deficiency include variable lymphopenia (exacerbated during infection episodes) with inversed CD4:CD8 T-cell ratio and poor proliferation to antigen, decreased expansion of NK cells, and low numbers of iNKT and MAIT cells, with normal to elevated immunoglobulin levels [400].

CTPS1 deficiency is caused by homozygous mutations in cytidine 5-prime triphosphate synthase 1 (*CTPS1*) gene (OMIM∗123860). CTPS1 is required for the de novo synthesis of the CTP nucleotide, a precursor of the metabolism of nucleic acid. CTP synthase activity is a potentially important step for DNA synthesis in lymphocytes, as evident by enhanced expression of CTPS1 following TCR activation, as well as impaired capacity of activated T- and B-cells to proliferate in response to antigen receptor mediated activation in the absence of CTPS1 [400]. The finding that CTPS1 deficiency causes no extra-hematopoietic manifestations favors a redundancy with CTPS2 activity in other cell lineages and tissues.

25.6.6 RASGRP1 Deficiency

RASGRP1 deficiency is a novel autosomal recessive PID, which has been reported in only four patients presented with EBV-associated LPD and EBV-driven HL [401–403]. The immunologic features of RASGRP1 deficiency include lymphopenia notably characterized by decreased counts of B-cells, naïve $CD4^+$ and $CD8^+$ T-cells, NK cells, MAIT and absence of iNKT cells, and impaired T-cell proliferation in response to antigens and mitogens [401–403].

RASGRP1 deficiency is caused by homozygous mutations in RAS guanyl releasing protein 1 (*RASGRP1*) gene (OMIM∗603962). RASGRP1 is a guanine-nucleotide-exchange factor (GEF) preferentially expressed in T- and NK cells, which in turn activates the cascade of Raf-MEK-ERK kinases (also termed as the MAP kinases/MAPK cascade) [404, 405]. Notably, RASGRP1-deficient T-cells exhibit defective MAPK activation and decreased CD27-dependent proliferation toward CD70-expressing EBV-transformed B-cells, which is a crucial pathway required for expansion of antigen-specific T-cells during anti-EBV immunity, as well as failure to up-regulate CTPS1, which is an important enzyme involved in DNA synthesis [401].

25.6.7 RLTPR (CARMIL2) Deficiency

RLTPR deficiency is a novel autosomal recessive PID characterized by recurrent bacterial, fungal, and mycobacterial infections, viral warts, Molluscum contagiosum, malignancies, as well as atopy [406–408]. To date, 14 patients have been reported in the literature, of who one was diagnosed with leiomyosarcoma and four were diagnosed with EBV-positive disseminated smooth muscle tumors [406, 408]. Immune phenotypic and functional studies are indicative of impaired naïve-T cell activation, proliferation, effector function, and insufficient gain of T-cell memory, with particular absence of regulatory T-cells [408].

RLTPR deficiency is caused by mutations in RGD motif, leucine rich repeats, tropomodulin domain, and proline-rich containing (*RLTPR*) gene (OMIM∗610859), also known as *CARMIL2* (capping protein regulator and myosin 1 linker 2). RLTPR deficiency selectively impairs the activation of the canonical NF-κB pathway in a CD28-dependent manner, and leads to defective cytoskeletal organization and migration. Of note, RLTPR deficiency is not associated with EBV-induced B-cell proliferation, but rather with slowly proliferating smooth muscle tumors associated with EBV infection [408].

25.6.8 Autoimmune Lymphoproliferative Syndrome

Autoimmune lymphoproliferative syndrome (ALPS) is a rare disease characterized by defective

Fas-mediated apoptosis that disrupt lymphocyte homeostasis [409]. The prevalence and true incidence of ALPS are unknown, likely since many instances remain undiagnosed or misdiagnosed. Though considered a rare disease, ALPS is now more commonly diagnosed given recognition of adult-onset disease and patients with a mild phenotype [410]. Apoptotic defects lead to LPD manifesting with lymphadenopathy, hepatomegaly, splenomegaly, autoimmune disease, and secondary malignancies. Autoimmunity, affecting over 70% of patients, is mainly directed against blood cells [411]. Other autoimmune manifestations are rare and include autoimmune nephritis, hepatitis, arthritis, uveitis, iridocyclitis, and vasculitis [412]. Autoantibodies are more common than obvious clinical disease and present in up to 92% of patients [413]. Signature laboratory abnormalities in ALPS include an increased number of characteristic T-cell population termed double-negative T (DNT) cells (though not pathogonomic), as well as in vitro evidence of defective Fas-mediated lymphocyte apoptosis. Furthermore, elevated circulating levels of soluble FAS ligand (sFASL), IL-10, vitamin B12, IL-18, and IgG may be useful to aid in diagnosis [414–420].

Germline or somatic mutations in genes regulating the Fas apoptotic pathway, including *FAS* (*TNFRSF6*, or *CD95*; OMIM∗134637), *FASL* (*TNFSF6*, or *CD95L*; OMIM∗134638), and *CASP10* (OMIM∗601762), have all been linked to ALPS [421]. Over the past decade, improvements in genomic technologies have led to the description of a number of ALPS-like autoimmune and LPD which are often misdiagnosed as ALPS, including *RAS*-associated leukoproliferative disease (RALD) [422]; caspase-8 deficiency state (CEDS) [423]; *p110delta* activating mutation causing senescent T-cells, lymphadenopathy, and immunodeficiency (PASLI or activated PI3K delta syndrome) [424]; *CTLA-4* haploinsufficiency with autoimmune infiltration (CHAI) [425]; gain-of-function signal transducer and activator of transcription 3 (*STAT3*) mutations [426]; and lipopolysaccharide-responsive vesicle trafficking, beach and anchor containing (*LRBA*) deficiency with autoantibodies, regulatory T-cell defects, autoimmune infiltration, and enteropathy (LATAIE) [425, 427]. XLP, a genetic immunodeficiency caused by mutations or deletions in the *SH2D1A* gene, can be included in the spectrum of ALPS-like disorders, since these patients frequently display defective apoptosis in response to TCR restimulation [428, 429]. Mutations in the ALPS and ALPS-related genes often manifest with variable penetrance [430]. Thus, patients with ALPS often have family members with the same genetic mutation with no clinical phenotype or very mild symptoms. The penetrance of the mutation is not related to the type of mutation but probably depends on unknown genetic and environmental modifiers. Hence, the clinical significance of isolated detection of a heterozygous Fas mutation in a healthy relative of a patient with ALPS is not yet clear.

Apoptosis is critical in tumor scrutiny as FAS, a putative tumor suppressor, is silenced in many tumors [431–433]. As anticipated, patients with ALPS have an increased risk of malignancies, most commonly both HL and NHL [434, 435]. This risk is estimated to be up to 60 to 150 times that of the general population and is more prevalent in *FAS* mutant [410, 434]. An increased risk of cancer has also been observed in unaffected family members who may inherit the same mutation but fail to develop an overt ALPS phenotype [434]. Sporadic NHL harbors somatic mutations of the *FAS* gene in 11% [436] of cases and in the *CASP10* gene in 14.5% of cases [437]. Furthermore, in HL, somatic *FAS* gene mutations are found in Reed–Sternberg cells in 10–20% of cases [431, 438].

25.6.9 Autoimmune Polyendocrinopathy with Candidiasis and Ectodermal Dystrophy

Autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy (APECED), formerly known as autoimmune polyendocrine syndrome type I (APS-1), is a rare autosomal recessive disease, most commonly seen in Iranian

Jews, Sardinians, and Finns. The diagnosis of APECED is reached if patients manifest at least two of the following conditions: (1) chronic mucocutaneous candidiasis (CMC), (2) hypoparathyroidism, or (3) Addison's disease. Additional autoimmune components may appear throughout life and include gonadal failure, diabetes mellitus type 1, hypothyroidism, pernicious anemia, hepatitis, alopecia, vitiligo, and/or ectodermal dystrophies. Although the endocrine features are clearly autoimmune, the underlying immunodeficiency predisposing to CMC has been a long-standing puzzle. Recently, autoantibodies against the Th17-related cytokines IL-22, IL-17A, and IL-17F, which are implicated in protection against fungi at epithelial surfaces, were discovered in the sera of APS-1 patients [439, 440], suggesting that the underlying immunodeficiency in patients with APECED has an autoimmune basis.

The disease is characterized by loss of tolerance against self-antigens [441, 442], which is caused by mutations in the autoimmune regulator (*AIRE*) gene (OMIM∗607358) [443, 444]. AIRE acts as a crucial transcription regulator that prompts immunological central tolerance by inducing the ectopic thymic expression of many tissue-specific antigens, among other functions [445, 446]. Although the syndrome is a monogenic disease, the great variability that characterizes APECED implies that additional factors modulate the clinical expression of the disease.

Several cases of oral and esophageal SCC have been reported in APECED patients with CMC [447–450]. In a cohort of 92 Finnish patients, six had developed oral or esophageal SCC by the mean age of 37, representing 10% of patients older than 25 years [447]. The partial T-cell defect of APECED seems to favor the growth of *Candida albicans* and predispose to chronic mucositis and the development of SCC. Besides chronic inflammation and increased cell turnover, *Candida albicans* biotypes are capable of producing the carcinogenic nitrosamine N-nitrosobenzylmethylamine [451, 452], and can also act to promote oral carcinogenesis in rats when a known carcinogen, 4-nitroquinoline-1-oxide, is repeatedly applied [453].

25.7 Concluding Remarks

The expanded life expectancy of patients with PIDs has increased the overall risk for developing cancers. However, the management of cancers in such patients remains challenging, in part due to the rarity, the increased risk for infection and other complications, as well as the rather slow pace of scientific advancement related to these conditions. Continued progress in understanding the interplay between chronic antigen stimulation, oncogenic viruses, genetic factors, and impaired host immunity during tumor formation in various PIDs may lead to earlier diagnosis of the disease, choosing the best treatment modalities available and development of novel therapeutic strategies to decrease morbidity and mortality brought about by malignancies.

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Allergies and Cancers

26

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26.1 Introduction

Worldwide, especially in industrialized countries, allergies and cancer cause high morbidity, mortality, and financial burden to healthcare systems. An overall of 14.1 million people were diagnosed with cancer, and 8.2 million died from cancer in 2012, with increasing incidences

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especially in developing countries [1]. In developed countries, for instance, in Germany and in the USA, cancer is the second leading cause of death after cardiovascular diseases [2, 3]. Cancer rates are rising due to an increasingly aging population and changes in lifestyle [1]. Allergies are even more prevalent, but mortality is much lower. In Germany about 30% of all adults have been diagnosed with some type of allergy during their life time, and about 300 million people are suffering from asthma worldwide [4, 5].

Interest in possible relationships between these prevalent diseases arose in the 1950s. Following studies revealed a decreased prevalence of allergies among cancer patients [6]. Since then a lot of research has been done, but still no generally accepted statement about the correlation has been established. As the immune system is involved in both allergic and neoplastic diseases, a connection might be obvious; nonetheless, the nature of this connection is dichotomous. On the one hand, allergies are regarded as a hyperactive state of the immune system, which leads to better detection and destruction of tumor cells. On the other hand, allergic reactions go along with inflammatory processes, which might initiate and support tumor growth [7]. Hence, there are different hypotheses on the relationship, which appears to be complex and not universally applicable for every type of cancer or allergy. This chapter will give an overview about studies examining these relationships and describes possible mechanisms that could explain them.

26.2 Molecular Mechanisms of Allergy

By definition, allergy is an immunologic reaction to normally innocuous environmental antigens (Ags), so-called allergens, and it is mostly equated with type I hypersensitivity (immediate-type hypersensitivity) according to the classification by Coombs and Gell. This type is mediated by immunoglobulin (Ig) E in response to T helper cell type 2 (Th2) polarization of $CD4^+$ T-cells [8]. Usually IgE is associated with defense against helminthic infections [9]. Atopy is the hereditary tendency to immediate-type reactions and increased production of IgE, however, not every allergic disease has to be atopic [10]. There are different genes associated with atopy, but environmental factors are of great importance as well. During fetal and postnatal periods, the immune system is rather Th2 polarized which shifts toward Th1 during the first years of life [9]. According to the hygiene hypothesis, infectious diseases in childhood are important for Th1 bias. This corresponds with an increasing incidence of allergic diseases in industrialized countries where excessive hygiene leads to an inadequate Th1/Th2 balance [11].

Allergic reactions are induced by low doses of allergens. Allergens are proteins, many of which are enzymes, and their allergenicity is determined by protease activity, surface features, or glycosylation patterns. Soluble allergens enter the body, orally or by inhalation, where they are taken up by antigen-presenting cells (APCs) such as dendritic cells (DC) which present them to naïve $CD4^+$ T-cells via major histocompatibility complex (MHC) class II [12]. In the presence of interleukin (IL)-4, naïve $CD4^+$ T-cells differentiate into Th2 cells which are characterized by the production of mainly IL-4 and IL-5. On the contrary, Th1 cells which develop under the influence of IL-12 from the same precursor cells predominantly produce interferon (IFN)-γ and IL-2. Further factors determining polarization are the Ag's route of entry, Ag dose, and the way of Ag presentation [13, 14]. Th2 cells organize the further allergic response towards the specific allergen, as shown in Fig. 26.1. Secretion of IL-4 or IL-13 by Th2 cells causes the isotype switch to IgE in B-cells. Additionally, a costimulatory signal, namely the engagement of CD40 on the surface of B-cells and CD40 ligand on the surface of Th2 cells, is required for the stimulation of the B-cell [15]. Subsequently, sensitized B-cells differentiate into plasma cells and produce allergen-specific IgE. Moreover, B-cells themselves are also able to take up soluble Ags via specific B-cell receptors and present them to naïve $CD4^+$ T-cells inducing Th2 differentiation [9]. IL-5, IL-6, and IL-9 may enhance IgE production, whereas Th1 cytokines like IFN-γ and IL-12 act as inhibitors [14].

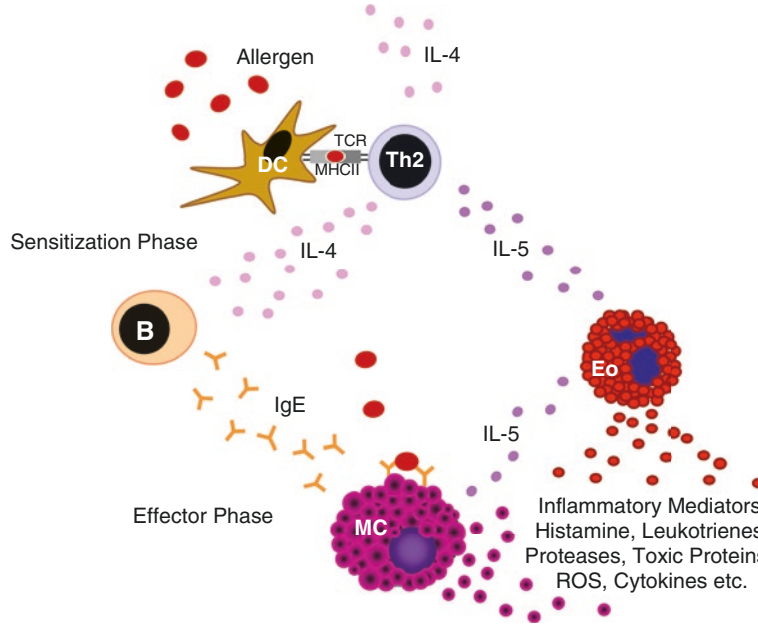

Fig. 26.1 Type I allergic reaction. *B* B-cell, *DC* dendritic cell, *eo* eosinophilic granulocyte, *IgE* immune globulin E, *IL* interleukin, *MC* mast cell, *Th2* T helper cell type 2, *ROS* reactive oxygen species. For further explanation, see text.

Most of the IgE engage to the high affinity receptor FcεRI on the surface of mast cells even in absence of Ag. If allergens bind to specific IgE, FcεRI is cross-linked, followed by an inflammatory reaction [15]. Mast cell mediators such as histamine, lipid mediators, and cytokines are released during the effector phase of an allergic reaction and induce typical allergic symptoms. FcεRI is also expressed on basophils, which are also able to release allergic mediators being stored in granules [16]. As basophils are able to produce IL-4 as well, they can amplify IgE production [17]. When specific IgE was once built, further exposition to the corresponding allergen elicits an allergic reaction without renewed sensitization [9].

Production of IL-5 by Th2 cells and mast cells activates eosinophils to secrete inflammatory mediators as well as highly toxic proteins and free radicals from their granules [8, 9]. Hours after the early phase of the reaction, the late phase may take place, which is characterized by infiltration of further inflammatory leukocytes and eventually a chronic inflammation may be established [18]. The cells involved in allergic reactions reside predominantly in tissues close to the body surface as their actual function is to defend against multi-cellular parasites, which invade primarily into skin and mucosa-associated lymphoid tissue. Therefore, these cells are specialized to evoke Th2 immune responses [8].

26.3 Types of Allergic Diseases

Allergic asthma is a chronic inflammatory disease of the airways caused by inhaled allergens. Symptoms are breathlessness, wheezing, and coughing due to bronchial constriction and increased mucus secretion. It is often accompanied by hyper reactivity of the airways to other stimuli [10, 19]. Allergic rhinitis or hay fever is an allergic inflammation of the nasal mucosa, which results in sneezing, itching, runny or blocked nose, and is often combined with allergic conjunctivitis [20]. Atopic dermatitis or eczema is a manifestation of atopy, which occurs predominantly among children, showing symptoms like itching, red rashes, and small vesicles on the skin [20, 21]. Food allergies mostly cause diarrhea or vomiting, but they may also affect the respiratory tract and others [8]. Anaphylaxis is a systemic reaction against an allergen with life-threatening symptoms like hypotension or airway constriction [20].

26.4 Molecular Basics of Carcinogenesis

Cancer is a genetic disease in consequence of a number of mutations in somatic cells. Unlimited growth of the mutated cells leads to formation of neoplasms. Tumor cells are capable of invading into tissues, and eventually of disseminating and building metastases in distant regions of the body. The clinical phenotype is varying as well as the implications, depending on the type of cancer and the affected patient. Although the incidence of cancer increases with age, tumors occur in every age group [22].

The development of cancer, carcinogenesis, is a multistep process which requires progressive alterations in the genome of normal cells. Mutations can occur spontaneously or can be generated by so-called carcinogens [23]. A carcinogen is an environmental factor like a chemical compound, a biological substance, a virus or radiation that is able to interact with DNA and cause damages or alterations in the genome. Usually cells have several mechanisms to repair DNA damages. During the process of repair, the cell cycle is stalled, preventing that this mutation is multiplied. If no repair is possible, the cell is destroyed by apoptosis [24]. An abolition of these mechanisms is a precondition for oncogenesis. Therefore, mutations have to occur in genes that are responsible for the control of cell proliferation, differentiation, or apoptosis [25]. Such critical genes can be divided into two groups: oncogenes and tumor suppressor genes [26]. Products of oncogenes are, e.g., transcription factors, growth factors or their receptors. Tumor cells are characterized by gain-of-function-mutations in oncogenes resulting in overexpression of oncogene proteins and subsequent increased growth [27]. Tumor suppressor genes, or rather their products, have a repressive effect on cell growth. Loss-of-function-mutations in tumor suppressor genes result in unimpeded proliferation or evasion of apoptosis [25].

However, one single mutation is not sufficient for the formation of a cancer cell. Carcinogenesis is a multistep process involving several events that incapacitate control of the cell cycle, thereby creating a cell with growth advantages [28]. The initiation process of carcinogenesis, characterized by somatic changes, is followed by the process of promotion. Different promoters like chemical irritants, hormones, or inflammation induce proliferation of the damaged cells and further mutations, as the genome of cancer cells is very unstable [25, 29]. The next step is tumor progression. By means of alteration of cell adhesion molecules and protease activity, cancer cells are capable of leaving the primary tumor and infiltrating into tissues. Subsequently tumor cells spread through blood or lymphoid vessels, and build metastases in distant parts of the body while they are displacing healthy tissue [30].

26.5 Types of Cancers

Pancreatic cancer is one of the cancer types with the poorest prognosis, as mortality rates almost correspond to incidence rates [31]. The most common type is adenocarcinoma, which affects the exocrine component of the pancreas, but other components of the pancreas may also be affected. Main causes are smoking, diabetes mellitus, and chronic pancreatitis [22]. Lung cancer is the third leading type of cancer among men and women, and the leading cause of death from cancer among men. More than two thirds of the cases are caused by cigarette smoke [31]. Cancers of the colon and rectum represent the second most common type of cancer. Besides the hereditary component, dietary habits are a major risk factor [3, 31]. Skin cancer includes malignant melanoma, basal cell carcinoma, squamous cell carcinoma, and some others [22]. The first one causes more deaths; however, the others are more prevalent, yet with higher curing rates [31]. Meningioma and glioma are the two most common types of brain cancer, whereby the causes are largely unknown [32]. Lymphatic and hematopoietic cancers are, e.g., leukemia, Hodgkin's lymphoma, or non-Hodgkin's lymphoma. Leukemia is characterized by an abnormal proliferation of leukocytes and can be classified into acute or chronic and myelogenous or lymphocytic forms [22]. Acute lymphocytic leukemia is

the most common tumor disease in childhood, whereas the etiology is still not identified [31]. Among reproductive cancer, prostate cancer in men and breast cancer in women are the leading types of cancer. Furthermore, breast cancer is the most frequent cancer-induced cause of death among women. Other common reproductive tumors are tumors of the uterus, cervix, and ovaries [31].

26.6 Anti-tumor Immunity

In 1970, Burnet and Thomas established the hypothesis of cancer immuno-surveillance. It states that, to a certain degree, the immune system is able to detect and destroy tumor cells before they can arise to clinically detectable malignancies. Meanwhile this hypothesis has been expanded to the theory of immuno-editing, which is comprised of three phases: the elimination phase, the equilibrium phase, and finally the escape phase [33].

The elimination phase complies with the process of immuno-surveillance. Immune cells of innate and adaptive immune response identify tumor cells by so-called tumor Ags [34]. If these are presented to an activated $CD8^+$ T-cell, the tumor cell is directly destroyed by release of cytotoxic proteins. Moreover, antigen-specific B-cells produce specific antibodies, which can opsonize tumor cells, and lead to either antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) [35].

Besides this adaptive immune reaction, there are cells of the innate immune system involved in immuno-surveillance, which execute antigen-independent immune responses. Among them are natural killer (NK) cells and NK T-cells, which are able to recognize and directly kill tumor cells [25]. In addition, these two cell types produce IFN-γ that is probably the most important cytokine in anti-tumor immunity [33]. It acts indirectly by modulating the immune response, e.g., by activation of macrophages or augmentation of T-cell response and NK cell activity, and it is able to increase immunogenicity of tumor cells. Moreover, IFN-γ itself has anti-proliferative, apoptotic, and angiostatic capacities, which directly affect tumor cells [36, 37]. However, cancer cells are capable of defending against these immune mechanisms. Either they lack certain MHC peptides, making them unrecognizable to T-cells or they do not express costimulating signals, which lead to T-cell tolerance [38]. Hence, if the immune system is not able to kill the entire tumor cells, the process of immunoediting reaches the equilibrium phase, characterized by dynamic dying and generation of further mutated cancer cells [34]. Following Darwin's rules, those cells, which show surviving advantages through reduced immunogenicity, resist the immune attacks. Thus, tumor cells also are shaped and sculpted by immune cells, leading to cell populations that are capable of evading any immune reactions [33]. In this case, surviving tumor cells enter the escape phase. Besides the absent immunogenicity, tumor cells are also able to suppress immune reactivity so that they can proliferate continuously and eventually develop a malignant tumor [38].

Altogether, the immuno-surveillance hypothesis describes that the immune system is in fact able to fight tumor cells, but also promotes carcinogenesis by sculpting poorly immunogenic mutants.

26.7 Relationship Between Allergies and Cancers in General

The first studies relating to possible associations between allergies and cancer date back to more than half a century [39, 40]. However, until now the results have not been consistent, despite the various researches in this regard [41].

Regarding cancer in general, there seems to be a balance between studies reporting positive and negative correlations with different types of allergies. While analyses of the Cancer Prevention Study II indicate a slightly decreased risk for people suffering from hay fever or asthma [42], data from the First National Health and Nutrition Examination Survey (NHANES I) show an up to

50% increased risk of developing any type of cancer [43]. Together with several other studies [19, 21, 39, 44–56], no general conclusion can be drawn which identifies the role of allergies in cancer epidemiology. As the term cancer includes diseases of diverse etiologies and a variety of affected tissues, it is necessary to distinguish between different cancer sites as well as specific types of allergy. In the following, those associations that are supported by the majority of studies are presented.

26.7.1 Cancers Positively Correlated with Allergies

With one exception, all of the evaluated studies suggest a positive association between a history of asthma and lung cancer. Without controlling for smoking, a study of 78,000 asthmatic patients found an increased risk for women as well as for men [49]. Another study observed a positive association with asthma, yet no associations with hay fever only, both asthma and hay fever, and any of these conditions [42]. A further survey calculated a lower, but still elevated risk for asthma when controlling for smoking. An additional analysis of the effect of respiratory drugs taken for the treatment of asthma showed no connection to cancer development [19]. In a Taiwanese study asthma was the only type of allergy associated with risk of lung cancer [48]. In contrast, El-Zein et al. [57] reported an inverse relationship between lung cancer and asthma and other allergic diseases.

The prevalence of skin cancer was predominantly examined among subjects suffering from atopic dermatitis, for other types of allergy there is only little evidence available. Atopic dermatitis was associated with a clearly increased risk of keratinocyte carcinoma, which made up half of all observed excess cancers in this study. Among 6275 hospitalized patients with atopic dermatitis, not a single case of malignant melanoma was found [50]. Another study involving patients with atopic dermatitis found an increased risk of melanoma as well as of non-melanoma skin cancer [51].

26.7.2 Tumor-Promoting Effects of Allergies

The positive association between specific types of cancer and allergies is mainly explained by exemplary description of the relationship between asthma and lung cancer. Increased susceptibility to inhaled carcinogens due to impaired mucociliary clearance and pulmonary obstruction and, above all, inflammatory processes are regarded to be responsible for the increased prevalence of lung cancer among asthmatic patients [49, 58–60]. As described before, allergic reactions go along with chronic or subchronic inflammation. There is evidence that tumors predominantly arise at sites of inflammation, and that inflammatory cells and mediators are found in all tumors [61].

Inflammatory reactions are usually triggered by infections. Macrophages, which have detected infectious agents, release chemokines that attract other inflammatory leukocytes, such as neutrophils and further macrophages. Additionally they release cytokines, which increase vascular permeability to facilitate migration of attracted cells into afflicted tissues. Leukocyte recruitment is mediated by adhesion molecules and extracellular proteases, which relieve movement into the tissue [29]. Since inflammatory responses are supposed to remove the causes as well as to rebuild damaged tissues, an environment rich in growth promoting, but also rich in damage causing factors is required. Consequently, the conditions for carcinogenesis are established.

Reactive oxygen species (ROS) released by macrophages are capable of causing DNA damages, thus promoting tumor initiation. Permanent cell regeneration raises the probability of carcinogenic mutations [29]. Cancer promotion is supported by growth factors like TGF, IL-1, IL-6, or IL-8. Furthermore, several inflammatory mediators have angiogenic properties or stimulate the production of angiogenic factors. For dissemination, cancer cells exploit the mechanisms that leukocytes utilize for extravasation into inflamed tissues. These are activation of selectin molecules, interactions between integrins and adhesion molecules of the immunoglobulin superfamily, and secretion of proteinases [29].

Apparently, an inflammatory microenvironment is essential for tumor progression, but vice versa, tumors themselves also secrete inflammatory mediators, which recruit leukocytes and mediate inflammation [38, 62]. Accordingly, Dvorak [63] described tumors as "wounds that do not heal," indicating that pathogen-induced inflammation is usually self-limiting, while cancer-related inflammation is triggered permanently [29]. Oncogenic mutations that initiate carcinogenesis may also lead to the establishment of an inflammatory environment. The activation of the Ras oncogene by mutation, for instance, leads to the expression of proteins that induce the production of inflammatory mediators [38, 61]. The main mediator cells of tumor-induced inflammation are tumor-associated macrophages (TAM). They are able to release almost all of the cytokines and chemokines required for tumor progression and their abundance has been shown to correlate with a poor prognosis [29, 64].

One of the key molecules in the connection between inflammation and carcinogenesis is the transcription factor nuclear factor (NF)-κB. NF-κB is an endogenous tumor promoter as it is activated immoderately by carcinogenic mutations. In addition, it is a coordinator of inflammation by regulating expression of several pro-inflammatory and survival factors [59, 64].

26.7.3 Cancers Negatively Correlated with Allergies

The association between a history of allergy and pancreatic cancer seems to be quite definite. Five surveys could demonstrate an inverse association. Holly et al. reported a decreased prevalence of any self-reported allergy among pancreatic cancer patients. This correlation was available for multiple allergens like house dust, plants, mold, animals, and food. Furthermore, with increasing numbers of allergies and increasing severity of symptoms the risk of cancer development decreased. It should be noted that even after receiving a hypo-sensitization therapy, allergic patients still showed a reduced risk [65]. Hay fever was correlated with a reduced risk of pancreatic cancer in Turner's prospective study [42]. Eppel et al. [66] found a risk of pancreatic cancer in allergic patients that was scaled down by more than 50%, but not for asthma patients. Similar results were reported by Cotterchio et al. [67] a few years later, but another study could demonstrate an inverse correlation with asthma, which was strongest with the intake of asthma medication [68]. Another study that additionally investigated a possible association between variants in IL-4 and IL-4 receptor α genes and cancer prevalence found a negative correlation for any allergy, hay fever, and reaction to animals. However, variants in the above-mentioned genes were not correlated to cancer [69]. A more recent study detected a significantly increased survival of non-resected pancreatic cancer patients with self-reported allergies. In the cohort that has undergone a resection, results were non-significant [70].

Cancers of the colon and rectum are less prevalent among individuals that show a history of allergy. Several studies identified allergies to be inversely associated with colorectal cancer. The probability of developing colorectal cancer with any self-reported allergy in an Italian study was lowered, whereas the association was stronger when allergy was diagnosed at age 35 or older. Regarding colon and rectum cancer separately, the risk of rectum cancer development was lower than colon cancer, whereas the latter was not statistically significant [71]. Another case-control study obtained a protective effect of any allergy on cancer development. Self-reported allergy was inversely associated with both colon and rectum cancer [72]. The risk of colorectal cancer calculated by Turner et al. was reduced by more than 20% among patients suffering from both asthma and hay fever, and less reduction was observed among patients suffering from hay fever only [42]. In addition, a large prospective study in Hawaii and California showed an inverse relation between atopic diseases and colorectal cancer [73]. A prospective study from Iowa involving only women noted an inverse correlation for allergy in general which was the strongest in patients with skin allergies. Moreover, the risk was decreasing

with an increasing number of allergies [74]. Allergic rhinitis was negatively associated with rectal cancer among Taiwanese patients, and the association was stronger for males than for females [48]. Combining the cohorts from the Cancer Prevention Study (CPS) I and II, Jacobs et al. [75] calculated a relative risk of 0.83 for colorectal cancer mortality when having both asthma and hay fever. A current meta-analysis of prospective studies confirmed a 12% decreased risk for colorectal cancer and any allergic condition [76].

Most studies agree about a decreased risk of tumors of the brain, specifically glioma, being associated with atopic diseases. In a hospital-based case-control study, the prevalence of glioma was reduced in combination with physician-diagnosed history of any allergy and asthma as well as with self-reported allergy to chemicals. Meningioma risk was not associated with any type of allergy. In addition, the risk of acoustic neuroma was positively associated with hay fever, allergy to food, and allergy to other substances [77]. One further case-control study found hospitalized glioma cases to be less likely to suffer from asthma, as well as hay fever, atopic dermatitis, or allergy in general. Moreover, there was a stronger risk reduction in conjunction with use of any allergic medication like nasal spray or antihistamines [78]. Wigertz et al. contrasted the prevalence of allergy among glioma and meningioma cases with non-cancerous individuals. They showed a decreased risk of glioma among subjects with asthma, atopic dermatitis, and hay fever. Treatment of hay fever with nasal spray or eye drops was associated with lower risks than non-treated disease. Meningioma risk was only reduced among atopic dermatitis patients [79]. In children having asthma, a 45% risk reduction could be observed [80]. One case-control study used immunoglobulin E (IgE) levels for the measurement of allergy besides a self-reported history of allergy. As IgE levels did not significantly confirm self-reported allergies, odds ratios for the risk of glioma development varied but both implicated a decreased risk [81]. A few years later the same research group reported similar risks for meningioma development [32]. A more recent study confirmed this with an odds ratio of 0.46 for allergen-specific IgE levels and glioma [82]. Besides glioma and meningioma, data from the INTERPHONE study also indicate allergies to protect from acoustic neuroma [83]. Data from the Glioma International Case-Control Study involving more than 8000 participants from 14 countries recently indicated a 30% lower risk for glioma among patient with respiratory allergies [84].

26.8 Tumor-Protecting Effects of Allergies

The majority of the presented studies attribute negative associations between allergies and cancers to an enhanced immuno-surveillance among allergic patients due to a hypersensitive and hyperactive immune system. This implies that immune cells of allergic subjects are more effective in detecting and destroying cancer cells [48, 53]. The pivotal cells of immuno-surveillance are NK cells by virtue of their capacity to carry out ADCC and to produce IFN-γ [37]. There is evidence for increased numbers and activity of NK cells in subjects suffering from asthma or allergic rhinitis [85–87]. Additionally, it could be proved that there is a negative correlation between cancer incidence and natural cytotoxicity which would further explain an improved potential for immuno-surveillance among allergic individuals [88].

Besides the classical cells of immuno-surveillance, other immune cells may be anti-tumor effectors as well. Below, critical cells and mediators of allergic reactions and their possible anti-tumor activities are given. While in non-allergic individuals their activity may be negligible due to low occurrences, their actions may be increased among allergic subjects, explaining a negative correlation between allergies and cancer incidence.

Allergic disorders are marked by increased levels of eosinophils, a condition named eosinophilia, as eosinophils are important effector cells in allergic reactions [89]. A role for eosinophils in immuno-surveillance of tumors

was considered since they were observed in different tumor infiltrates. Indeed, higher numbers of tissue or blood eosinophils correlated with better prognosis, e.g., improved survival rates in lung and colon cancer [90, 91]. Although eosinophils might contribute to tumor growth by release of VEGF, thereby initiating angiogenesis, in vitro and in vivo studies substantiated rather anti-tumor activities [6, 92].

Eosinophils are recruited by secretion of IL-5 from Th2 cells and eotaxin-1, a specific chemokine. Particularly IL-5 induces differentiation from $CD34^+$ precursor cells, stimulates synthesis of granule proteins, and activates eosinophil effector functions [93, 94]. These effector functions are mainly mediated by the release of their granule proteins, which are highly toxic towards pathogens, as well as towards tumor cells. In vitro studies could prove the direct cytotoxicity of eosinophil cationic protein (ECP) [90, 91, 94]. ECP causes lysis of tumor cells by creating pores in the cell membrane [95]. Further granule proteins like major basic protein or eosinophil peroxidase have indirect anti-tumor properties in terms of triggering the release of histamine from mast cells. Besides the IL-5 dependent activation, eosinophils are also responsive to specific IgE. As they express IgE receptors on their surface, binding of IgE leads to tumor-specific antibody-dependent cellular phagocytosis (ADCP) [6].

A study that involved lung cancer patients, investigated anti-tumor activities of eosinophils in vitro. For this purpose, eosinophilia was induced by IL-2 treatment in cancer patients. Eosinophils were then purified from blood samples and added to tumor cells. ADCC and direct lysis by eosinophils from IL-2 treated patients were highly increased compared to those of non-treated patients or healthy donors, which did not harm tumor cells at all [90]. This suggests that in fact there are differences in cytotoxic potentials between allergic and non-allergic individuals. The influence of IL-2 was to ascribe to secondary cytokine production because IL-2 has no direct effect on eosinophils, but stimulates lymphocytes. Thus, eosinophil activation was most likely mediated by IL-5.

Another study confirmed the involvement of eosinophils in anti-tumor immunity in methylcholanthrene-induced fibrosarcoma models. Among IL-5 transgenic mice, which show increased levels of eosinophils, tumor growth and incidence were reduced whereas among eotaxin-deficient mice incidence was increased. An even greater increase of incidence was observed in eosinophil-deficient mice. This provides evidence that, at least chemically induced cancers, may be effectively fought and inhibited in growth by eosinophils [93].

IgE is the key mediator of allergic reactions. Binding of IgE to the high affinity receptor FcεRI on the surface of mast cells and basophils leads to ADCC, whereas binding to the low affinity receptor CD23 on the surface of macrophages or eosinophils leads to ADCP [6]. Usually IgE is predominantly present in tissues bound to its receptors, but in allergic patients, serum IgE levels are up to tenfold higher than normal [96]. In addition to defense against helminths and hypersensitivity towards allergens, IgE Abs may also be directed against tumor Ags, thereby mediating anti-tumor activities. In vitro studies could demonstrate IgE-mediated effector activities against human ovarian carcinoma cells [96, 97]. Furthermore, treatment of mice with IgE targeted on tumor cells resulted in decreased growth of induced cancer. The effect was significantly stronger for IgE than for treatment with IgG. Besides the curative potential of IgE, a protective long-term immunity against the specific tumor cells was observed as well [98]. The incidence of survival was monitored within a case-control study among glioma patients. Those who had elevated levels of IgE were observed to survive on average 9 months longer compared to patients with moderate or borderline IgE levels. Additionally, elevated IgE levels were more common among control subjects than in patients, which might support the assumption of an anti-tumor capacity of IgE [99]. Among pancreatic cancer patients, IgE levels were detected to be fivefold higher than in control groups, whereas levels of other Igs were similar. Tumor-specific IgE was found to mediate ADCC against tumor cells, whereas IgE isolated from healthy controls

did not [100]. Recapitulating, IgE is an effective mediator of anti-tumor cytotoxicity as well as phagocytosis of tumor cells.

Mast cells, generally associated with allergic reactions are also frequently found in tumors, attracted by the tumor cells themselves, where they assume an angiogenic role [101]. In most cancer types, mast cells therefore act as tumor-promoters by producing angiogenic and lymphangiogenic factors. However, in some cancers, e.g., breast cancer, they might rather be tumor-suppressors by producing anti-tumorigenic molecules such as TNF and IL-9, whereas in yet others they are apparently innocent bystanders [102].

Typical Th2 cytokines are IL-4, IL-5, IL-13, and IL-10. The role of IL-5 in recruiting and activating eosinophils has already been described. IL-10 and IL-13 exhibit rather tumor-promoting than anti-tumor activities [92, 103]. IL-4 is known as Th2 differentiation factor and mediator of IgE isotype switch in B-cells [104]. However, IL-4 also shows anti-tumor activities. First, IL-4 induces the infiltration of macrophages and eosinophils, which mediate cytotoxicity towards tumor cells [105]. Second, IL-4 is one of the most potent inhibitors of angiogenesis by blocking migration of endothelial cells. The resulting restricted tumor growth could be proved for local as well as for systemic application of IL-4 in vivo [106]. Moreover, IL-4 receptor has been shown to be expressed on different human tumors and immunogenicity of melanoma cells could be increased by IL-4 by means of enhanced MHC class II expression [107]. In animal studies, induction of an allergic reaction in IL-4 transgenic mice additionally suppressed melanoma growth through the activation of NK cells, which subsequently activate the STAT6 pathway [108].

Tumor growth factor (TGF)-β, which is involved, e.g., in the inflammatory processes in asthma, plays a dichotomous role in cancer, depending on the stage of the tumor as well as the cellular context. Especially during early stages of tumorigenesis, TGF-β can act as a tumor suppressor through its cytostatic and pro-apoptotic effects [109].

As described, many crucial components of allergic reactions were separately shown to have anti-tumor activities, but only little research has been done yet to evaluate the combined effects of these cells. One study evaluated growth of inoculated tumor cells in mice that were sensitized against ovalbumin. Tumor cells in allergic mice showed the same proliferation rate like those in non-allergic mice, whereas apoptosis was increased [110]. Consequently, tumor progression was decreased in allergic mice, which might support the relationship between allergy and some types of cancer in humans.

26.9 Concluding Remarks

Even despite extensive research, the relationship between allergies and cancer remains elusive. As there are studies, which show negative as well as positive correlations, one has to take a closer look at the specific type of cancer and the location it arises. Allergies are accompanied by inflammatory reactions, which constitute an optimal environment for carcinogenesis, thus promoting the development of tumors at this specific site. Additionally, systemic effects in terms of enhanced immunosurveillance can likewise be evoked, thus preventing from cancer at other areas. The presented examples of a positive correlation between asthma and lung cancer as well as atopic dermatitis and skin cancer and a negative correlation between allergies and pancreatic cancer, colorectal cancer and glioma fit this classification. Nonetheless, there is still a need for well-conducted epidemiological studies, as well as for investigations on the molecular level to clearly define the relationship between allergy and cancer.

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27 Envisioning the Application of Systems Biology in Cancer Immunology

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27.1 A Primer on Systems Biology

Biomedicine has evolved extremely fast in the last decade. Many challenging new insights into the nature of biological systems and the avenue of new experimental techniques have synergized during this period to change our perception about Biomedicine. Biological systems are nowadays envisioned as complex networks composed of dozens to thousands of proteins, genes, and miRNAs, which interact to control cellular- and tissue-level phenotypes. One can say that Biology is the Science of the ultimate complexity because in one sense every single cell contains as much complexity as entire solar systems or galaxies. In this context of increasing complexity, Systems Biology has emerged a decade ago.

Systems Biology is a methodological approach that combines quantitative experimental data, mathematical modeling, and other tools from computational biology to address biological and biomedical questions from a systemic perspective. It is almost a mandatory research strategy when: (a) analyzing massive amounts of high-throughput quantitative experimental data, (b) trying to understand the function and regulation of biochemical networks enriched in regulatory motifs like feedback loops, and (c) integrating biological data from diverse sources across temporal and spatial scales. Within the methodology, the use of mathematical or computational modeling is an essential step, necessary to integrate and analyze data, formulate and explore biological hypothesis, or perform quantitative predictions with a therapeutic aim [1]. It has a clear interdisciplinary nature because it involves expertise in biomedicine, quantitative experimental techniques, data engineering, mathematical modeling, and bioinformatics, only to mention some of the scientific profiles of researchers that can get involved in a systems biology project.

Due to this multiplicity of disciplines, over the years the concept of Systems Biology has become fuzzy and difficult to define precisely. At the moment, Systems Biology describes at least three different approaches, all of them relying on the use of quantitative experimental data and mathematical modeling. We describe them briefly in the following subsections.

27.1.1 The "Omics" Paradigm and the Use of Statistical Models

In the last few years, it has become technically and economically affordable to perform quantitative, high-throughput experiments to measure the concentrations or activation state of proteins and other biomolecules like RNAs or metabolites. This has given rise to numbers of new experimental fields (e.g., genomics, transcriptomics, proteomics, and metabolomics, collectively known as "Omics" techniques). When applied to samples obtained from large cohorts of patients suffering complex multifactorial diseases, especially cancer, these techniques have already generated massive amounts of clinical and biomedical data. These data are a precious resource to discover the molecular mechanism behind the emergence of a disease. From an applied perspective, these techniques can be used to generate new protocols and tools for early diagnosis, or more efficient and personalized therapeutic treatments. However, the data alone are not sufficient: human intuition and direct interpretation are not well-suited tools for the analysis of massive volumes of high-throughput data. Complex mathematical models, which rely on the intensive use of

advanced statistical and computational methods, are necessary to interpret and analyze the amount and type of data generated through the "Omics" paradigm.

These statistical models have been successfully exploited in the search of biomarkers for cancer progression, metastasis, or resistance [2]. In this case, patients in a clinical study are classified in groups according to the progression status of the tumor. Expression profiles of proteins, RNAs, or other biomolecules, obtained from patient samples, are analyzed using statistical models to find one or more disease-associated genetic signatures. These genetic signatures account for groups of genes having an expression pattern that, considered globally, can be used to discriminate between patient groups. The ultimate aim is to use these genetic signatures for improving diagnosis and/or prognosis. For some tumor entities, genetic signatures have been already found that could be successfully associated with progression, and are currently used in prognosis tests [3, 4]. However, one has to say that the statistical elucidation of this kind of signatures should never be the end point of a research process. It has to be followed by additional in vitro/in vitro experiments and clinical studies to find a mechanistic interpretation for them [5].

27.1.2 Mathematical Modeling and Systems Theory: Dissecting the Complexity Emerging Out of the Structure of Biochemical Networks

Accumulating experimental evidences indicate that, at the molecular level, cells are organized in large and complex regulatory networks that involve genes, interacting proteins, and different kinds of coding and noncoding RNAs and metabolites. When trying to find a mechanistic interpretation for the behavior behind these large networks, simple human intuition and direct data analysis fail because they involve too many interacting variables [1, 6]. Furthermore, these networks contain a plethora of cross-talking regulatory motifs, like feedback and feedforward loops that show often counterintuitive behavior. In Engineering and Physics, mathematical modeling has been used for a century to investigate the dynamics, regulation, and controllability of other physical or artificial systems containing similar regulatory motifs. It is therefore not a surprise that biological data-based mathematical modeling has emerged as a powerful tool, able to dissect the nature of biochemical networks, interpret the complex nonintuitive relations between their compounds, and provide support in the design of hypothesis and experiments. This strategy has been used with remarkable success in the last years in molecular oncology and cancer signaling. It has proved to be useful in: (a) the detection and analysis of the nonlinear behavior emerging from the combination of feedback, feedforward, and other regulatory motifs in biochemical networks [7, 8]; (b) the integration of diverse sources of high-throughput data accounting for the regulation and dynamics of large cross-talked biochemical networks, with hundreds of compounds [9]; (c) the derivation, analysis, and validation of hypotheses concerning the structure and regulation of cancer-related pathways [10, 11], or (d) the design and assessment of conventional, targeted, or combined anticancer therapies [12, 13].

27.1.3 Bridging Biological Scales Through the Integration of Biological Data in Multiscale Models

Evidences are growing in recent years pointing to the fact that, in many cases, the influence of the surrounding media in the tumor cannot be separated from the tumor biology [14]. The microenvironment interacts with the tumor and affects its progression via a number of selective forces including hypoxia, lack of nutrients, or immune-driven apoptosis, while the tumor can modify the features of its microenvironment to subvert the body protective mechanisms [15]. This notion is the motivation behind the many efforts to develop data-driven mathematical models of cancer progression, able to account for the spatial organization of tumors and

the interaction with the surrounding microenvironment [16]. The so-called cancer multiscale models are mathematical constructs that are able to simulate global spatio-temporal features of tumors like growth, angiogenesis, as well as therapy or hypoxia-mediated apoptosis and necrosis [17].

27.2 One Step Further: Integrating the Different Perspectives of Systems Biology into a Unified Framework

Although each one of these mathematical model-based approaches has proved to be quite successful in accelerating the discovery in tumor basic biology and clinics, they have limitations that cannot be ignored. Statistic models are extremely useful tools to analyze enormous amount of patient data and find expression patterns associated with given clinical phenotypes; however, those statistical expression patterns alone suffer with the lack of support provided by a truly mechanistic interpretation of the data, the sort of analysis that provides biological causation. Mathematical models of biochemical networks can provide insights into the biological mechanisms underlying cancer progression, but are not able to account for the effects of the tumor–microenvironment interaction. Current multiscale models are accurate in describing biomechanical forces, cell phenotypes, and spatial interactions between tumor cells and their surroundings. However, they lack a precise description of the intracellular mechanisms driving those phenotypic features, as well as a connection to the clinical understanding of the tumor biology.

These limitations are the motivation why researches have tried to integrate the different scopes into a unified conception of Systems Biology in recent years [8, 18–21]. The idea is to develop a unique framework that integrates tools and methods from statistics, bioinformatics, computational biology, and mathematical modeling with the aim of integrating biomedical data across biological and spatiotemporal scales. This approach must be able to: (a) link massive clinical patient data with the function and (dis)regulation of biochemical networks; (b) provide a strategy to combine different kinds of quantitative high-throughput biological data into integrative pictures of cancer; (c) connect cancer genotypes and phenotypes from a mechanistic, causal, data-driven perspective; (d) provide tools to detect and investigate regulatory, feedback loop-like structures that extend across multiple biological organization levels like paracrine and autocrine loops; and (e) determine the consequences of this multilevel cross-talk in the context of cancer and the immune response. In our vision, this ultimate version of the Systems Biology method involves iterative integration of data from clinical trials and in vitro/in vivo biomedical research using techniques of data analysis, bioinformatics, and mathematical modeling and simulation. The proposed workflow is sketched in the following paragraphs (Fig. 27.1).

Step 1 In clinical cohorts of, for example, cancer patients vs. healthy individuals, high-throughput data of tissue and/or plasma concentrations for proteins, RNAs or other molecules are collected together with biometric data from the patients. The data are processed, integrated, and analyzed using statistical models aiming to group them according to their gene expression vs. the progression status profiles. In this way, one can obtain cancer-associated genetic signatures relevant to the phenotype under investigation (e.g., chemoresistance, aggressiveness, and metastatic potential). These signatures account for a group of genes, proteins, miRNAs, or other molecules, for which a robust statistical correlation is found between their combined expression pattern and the investigated cancer phenotype [5].

Step 2 Relevant biomedical and clinical knowledge is gathered from databases, computational algorithms, and publications inspected via manual curation or text mining. This information is used to find feasible biochemical interactions (i.e., protein–protein interactions, transcriptional regulation…) between compounds of the genetic signature, but also with other kinases, transcrip-

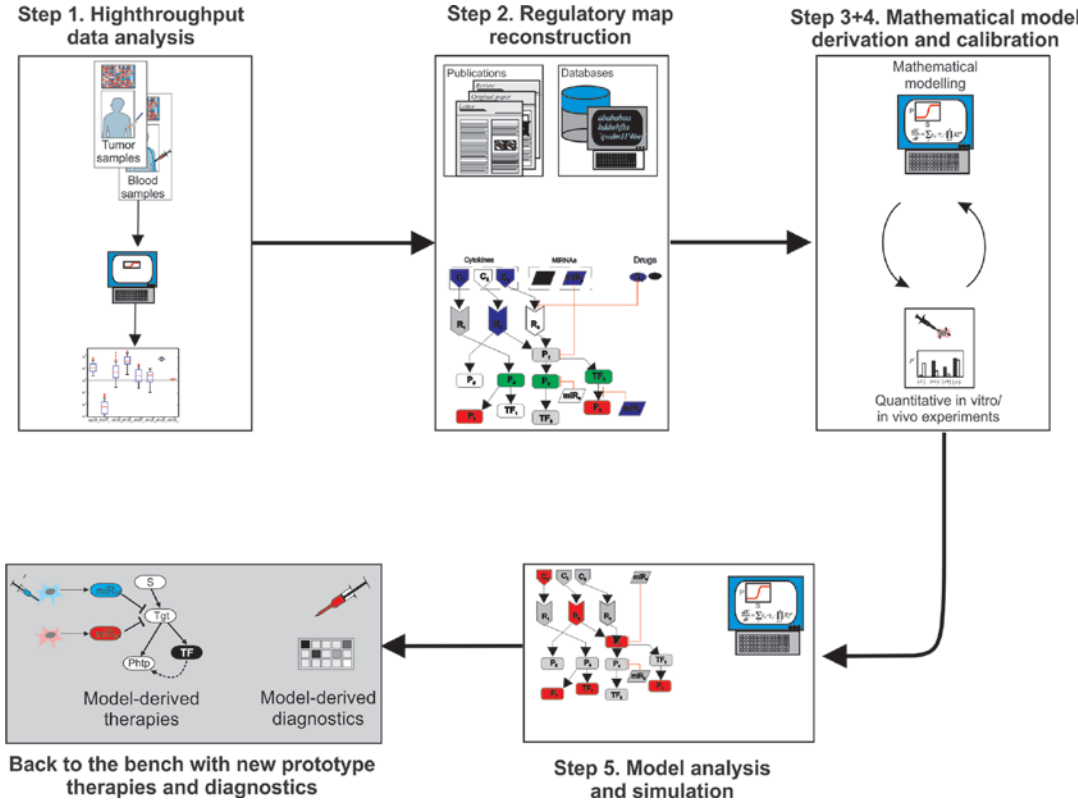

Fig. 27.1 Sketch of an advanced systems biology workflow

tion factors, or microRNAs, all of them relevant to the investigated cancer phenotype. In this way, we can construct a network of cross-talked intracellular pathways relevant to the investigation of the aimed cancer phenotypes. Furthermore, similar networks can be constructed for the cell types in the tumor microenvironment related to the phenotype investigated. Since tumor cells and cells in the microenvironment secrete cytokines and other molecules signaling each other, the obtained network is one of cell-to-cell communication, accounting for the tumor–microenvironment interaction in the cancer phenotype under investigation. The network obtained is often called regulatory map, nothing but a visualization of the state of the art of the biochemical and biomedical knowledge about the cancer phenotype under investigation. Tools from network biology can be used to dissect the topology of the regulatory map and isolate regulatory motifs relevant for the derivation of hypothesis and experiments [8, 22].

The method involves iterative integration of data from clinical trials and in vitro/in vivo biomedical research using techniques of data analysis, bioinformatics, network biology, and mathematical modeling.

Step 3 The parts of the network relevant to the biomedical scenarios which are related to the investigated cancer phenotype are translated into a mathematical model. The model consists of mathematical equations, in an adequate modeling formalism, accounting for the evolution on time of the expression and/or activation status of the network compounds, as well as their connection to the phenotypes. Many modeling formalisms are available, all of which are with advantages and disadvantages [6]. To circumvent some of these disadvantages, one can combine them in hybrid models. For example, we have combined interconnected submodules in ordinary differential equations and Boolean logic. Ordinary differential

equations are excellent tools to analyze the nonlinear behavior of signaling pathways with multiple, nested feedback and feedforward loops, while logic models are an ideal representation of massive transcriptional networks. The combination of both model types allowed us to analyze the large-scale, nonlinear transcriptional and posttranscriptional networks and their connection to cancer cell phenotypes [23].

Step 4 Additional quantitative in vitro/in vivo experimental data are used to improve the biological characterization of the model, that is, to make it more accurate in terms of prediction of the relevant biomedical scenarios. This is often called model calibration and allows assigning appropriate values to model parameters and other model features. Alternatively, this process also allows for the validation of hypothesis concerning the structure and regulation of the network in the biomedical context analyzed; in this case, iterative cycles of modeling and experimentation can be used to formulate, refine, prove, or disprove hypothesis concerning the existence and relevance of given biochemical interactions [24]. With the use of the mathematical model, one can analyze spatio-temporal regulatory features of the network that elude the elucidation via conventional experimentation, like self-sustained oscillations, or bistability.

Step 5 In recent years, various studies have proved that a well-calibrated, data-driven mathematical model can be used with predictive purposes in the context of molecular oncology. The underlying idea is to use model simulations and other tools to assess existing therapies in a personalized manner, design new therapies, or detect sets of biomarkers for cancer prognosis. In a final step, one has to go back to the bench and design additional in vivo/in vitro experiments to confirm the model predictions. Alternatively, the model predictions can be combined with virtual screening and other techniques from computational biology and immunoinformatics, and used in the process of drug discovery or vaccine development. For example, potential drug targets, identified via mathematical modeling, can be used as most promising candidates in a drug screening procedure via protein docking-based techniques [21].

27.3 Does Cancer Immunology Need a Systems Biology Approach?

In our opinion, the immune system is one of the most complex realizations of a biological system. The immune system is actually a multiscale system [25] (Fig. 27.2). It involves many types of cells, whose fate, proliferation, or activation status is controlled by feedback loop-regulated pathways. These pathways very often cross-talk, creating complex networks. Furthermore, the activation status of given immune cells depends on other immune cells by direct contact or through secretion of local or global signaling molecules, especially cytokines. In this way, the immune system is enriched in cell-to-cell communication circuits and autocrine loops. When we further consider the interaction between the immune system and a tumor, the picture becomes more systemic-like. Tumor cells and the immune cells in the surroundings communicate through chemical signals and affect each other's fate. Tumors secrete antigens (Ags) detected by immune cells like dendritic cells, while cells from the immune system secrete cytokines and antibodies (Abs) targeting the tumor cells. In addition, features of the microenvironment in which the tumor is hosted can affect the response of the immune cells. Finally, all these processes are happening at the same time, and affecting each other at different biological and temporal scales. Altogether, this suggests the use of a systemic strategy to tackle the complexities of the tumor–immune system interaction. In the following section, we discuss some published results that illustrate how systems biology can be used in the context of oncology and tumor immunology.

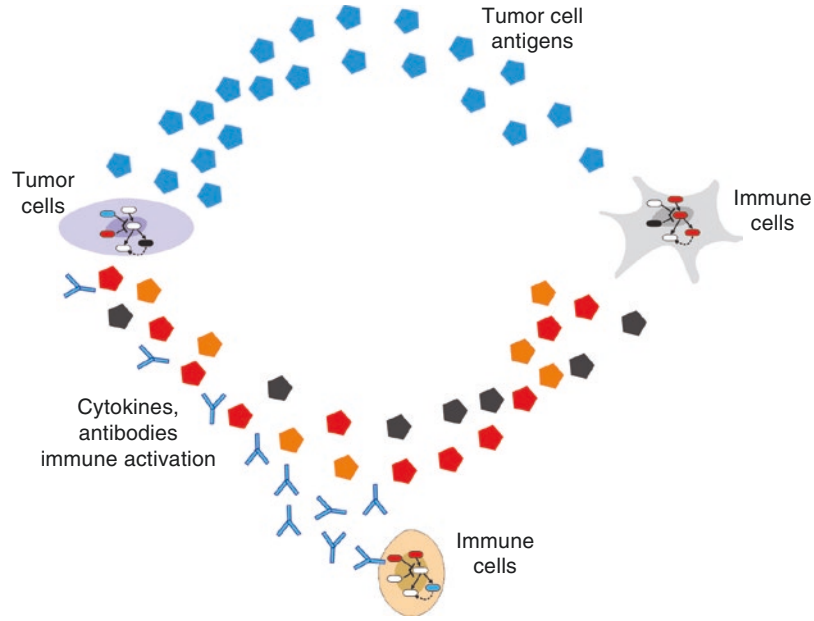

Fig. 27.2 Tumor-immune cells interaction envisioned as a multilevel system

27.4 A Quick View on Current Results

27.4.1 Computational Biology, Bioinformatics, and High-Throughput Data Analysis Used in the Design of Immune Therapies for Cancer

Availability of next-generation sequencing along with –omics data shifted the paradigm for the cancer treatment and opens the doors toward possible cancer immunotherapy [26]. Like traditional vaccines that stimulate host immune system to recognize and destroy pathogens, cancer vaccines are aimed to generate immune response to differentiate tumor cells from the normal cells for their possible elimination. For several of the pathogen origin cancers, such as cervical cancer caused by Human Papillomavirus; hepatocellular carcinoma caused by Hepatitis B and Hepatitis C virus; Hodgkin's lymphoma by Epstein-Barr virus; T-cell leukemia by Human T-cell leukemia virus; and Kaposi's sarcoma by Kaposi's sarcoma herpes virus, there have been considerable success in designing cancer vaccines in the past and many of them are currently in use or in the advance stages of clinical trials. Most of these vaccines are designed in a similar way to the traditional epitope-based vaccine-designing approaches. However, for the nonpathogen origin cancer, the major challenge for the immune system is to distinguish cancer cells from the health cells in order to activate B-lymphocytes to produce Abs or T-lymphocytes. In order to trigger antibody-dependent cellular cytotoxicity or phagocytosis to kill cancer cells, these Abs need to recognize specific proteins normally on the outer membrane of the cancer cells [27]. T-lymphocytes have the capacity to selectively recognize peptides (antigens) derived from self/ nonself proteins attached with major histocompatibility complexes on the antigen-presenting cells (APCs). Use of cytotoxic T-cells (CTLs), dendritic cells (DCs), and monoclonal antibodies are now well-established strategies to design potential cancer immunotherapeutics [28].

The major challenge in the development of cancer vaccines is the recognition of "self" Ags by the immune system for which the system is already tolerized. Therefore, the potential approach is to identify nontolerogenic, tumor-associated antigens (TAAs) suitable to develop

Ag-specific anticancer vaccines from a large pool of "self" Ags [29]. In spite of success in other infectious diseases, the use of small self-peptides as Ags in cancer vaccines did not attain much interest in the past because of their poor immune response and minimal therapeutic benefits. Most of these free peptides are likely to have short half-life and poor pharmacokinetics properties and thus rapidly cleared before they are loaded on the dendritic cell surfaces in the complex with MHC molecules to stimulate $CD8^+$ and $CD4^+$ T-cells for the initiation of adaptive immune responses [30]. However, the coadministration of suitable dendritic-cell-activating adjuvant along with short TAAs peptides was shown to boost immune responses in advanced melanoma [31] and vulvar intraepithelial neoplasia patients [32]. These studies generated the hope to design effective therapeutic cancer vaccines.

In order to avoid the "self" recognition that normally results in the weakened immune responses for cancer vaccines; researchers have validated the use of DNA vaccines in preclinical studies where the tumor-derived sequences were initially fused with the genes encoding microbial proteins [33]. This strategy helped T helper cells in the induction of Abs against tumor Ags along with epitope-specific antimicrobial $CD8^+$ T-cells. Another example PROSTVAC, a DNA vaccine for prostate cancer, which includes recombinant vaccinia virus encoding prostate TAAs along with adhesion molecules and DCs stimulator, is already in the clinical trial phase III [34]. Besides, several monoclonal antibodies (mAbs) and other small molecules such as kinase inhibitors, angiogenesis inhibitors, proteasome inhibitors, and molecular receptor blockers are also combined with immunotherapy for developing targeted anticancer therapies [35]. Many Abs boost the immune response against cancer cells. Ofatumumab and ipilimumab are two such mAbs recently approved by the US FDA. While ofatumumab targets CD20 protein which inhibits early-stage B-lymphocyte activation in chronic lymphocytic leukemia [36], ipilimumab specifically targets cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) that provide inhibitory signal for activated T-cells [37]. Unconventionally, mAbs are also shown to target intracellular oncoproteins; this finding opens a new possibility to predict potential targets for TAAs discovery [38, 39].

Still, the detection of effective nontolerogenic TAAs from extra/ intracellular oncoproteins is one of the major challenges in cancer immunotherapy. To recognize TAAs, one has to carefully investigate sites for cancer-specific point mutations, chromosomal aberrations, splicing variants, alternative reading frames along with overexpressed gene/proteins and other regulatory elements (Transcription factors, miRNAs, etc.) [40–42]. For many of these data mining approaches, well-established computational pipelines already exist in the public domain. For therapeutic cancer vaccines, the idea is to either amplify or induce new immunogenic responses in the cancer patients based on $CD8^+$ or $CD4^+$ T-cell responses by recognizing differentially expressing TAAs from microarray data repositories [43]. One of such databases is Oncomine, which has huge repository of gene expression profiles from microarray studies to identify differentially expressing genes in various stages of major types of cancer [44]. These data analysis pipelines facilitate the discovery of novel cancer biomarkers and drug/vaccine candidates. In the following section, we will describe the use of bioinformatics tools and computational pipelines to discover potential cancer vaccine candidates with a case study.

27.4.1.1 Case Study: Computational Approaches to Design DNA Vaccine for Cervical Cancer Caused by Human Papillomavirus

Cervical cancer is the most common and slow-growing malignant cancer present in the tissues of the cervix or cervical area in women. Persistent infection with human papillomavirus (HPV) is considered to be one of the major etiological factors for cervical cancer [45]. More than 100 different types of human papillomaviruses (HPV) have been identified [46] and categorized into high-risk and low-risk strains. A total of 16 different high-risk strains have

already been identified, among them strain 16 and 18 are together responsible for approximately 70% of all cervical cancer cases [47]. Two HPV vaccines GARDASIL and CERVARIX are currently in use as prophylactic vaccines and offer no therapeutic benefit for patients already infected with the virus or those with precancerous lesions or cervical cancer [48]; also, they are not completely effective against all high-risk strains of this virus. In contrast, therapeutic vaccines generate a T-cell immune response to eliminate existing viral infection. Epitope-based vaccines provide a specific strategy for prophylactic and therapeutic application of pathogen-specific immunity. The identification of epitopes suitable for diagnostic use and for therapeutic or prophylactic intervention is clearly a crucial prerequisite of these strategies. Selection of immunogenic, consensus, and conserved epitopes from proteins of major high-risk strains may provide an experimental basis for the design of very specific T-cell and DNA vaccines effective against all high-risk strains [49]. Likewise, many metastatic tumors also exhibit heterogeneity at the genomic level and some subclones able to escape the immune system [50, 51]. It is therefore important to design tumor vaccine in such a way that it is effective against all the subclones. Herein, the authors will highlight computational pipeline adopted in one of their previously published research work which was used to design in silico DNA vaccine against human papillomavirus (HPV) by using consensus epitopic sequences of L2 capsid protein from all high-risk HPV strains [52]. In addition, various computational parameters are optimized to increase the immunogenicity of the vaccine by considering multiepitopic sequence, codon optimization, CpG motifs optimization, inclusion of promoters and other immune-stimulatory molecules. A generalized computational pipeline for the design of DNA vaccine is highlighted in Fig. 27.3.

The work initiates with the detection of differentially expressing genes/ proteins in cancer (non-pathogenic) or identification of conserved immunogenic regions from pathogens involved as the major etiological agents. From the conserved regions, MHC class I and class II epitopes are predicted followed by inclusion of proteosomal/lysosomal cleavage sites. Various computational approaches may be followed to filter the immunogenic peptide such as 3D structure modeling to calculate the solvent accessibility of cleavage sites, post-cleavage conservancy of epitopes, and then long half-life for proper immunogenicity using molecular dynamics simulations. The selected peptide can then be back translated and optimized for codons and CpG motifs. In silico cloning experiments may also be performed for the selection of good expression systems to be used for vaccine development.

27.4.2 Retrieval of Sequence Data and Identification of Conserved Regions in the Protein

In case of previously designed HPV vaccines, researchers thoroughly investigated L1 and L2 capsid protein form the virus to detect potential vaccine candidates. Although previous in vitro neutralization studies demonstrated high cross-reactivity with L2 antisera, yet, some of the high-risk HPV strains failed to neutralize due to high rate of mutation in the L2 capsid protein. To overcome this problem, we first retrieved L2 capsid protein sequences for all the high-risk HPV strains from NCBI (http://www.ncbi.nlm.nih.gov) and UniProt (http://www.uniprot.org) database. To identify conserved regions in the protein, we performed multiple sequence alignment using ClustalX software. Based on the multiple alignment files, we identified conserved regions in the L2 capsid proteins using Shannon entropy function available on Protein Variability Server (http://imed.med.ucm.es/PVS). From the alignment file, Shannon entropy was calculated as:

$$H = -\sum_{i=1}^{M} P_i \log_2 P_i$$

where P_i is the fraction of residues of amino acid type *i,* and M is the number of amino acid types.

To identify the conserved regions in the L2 capsid proteins of all high-risk HPV strains, the

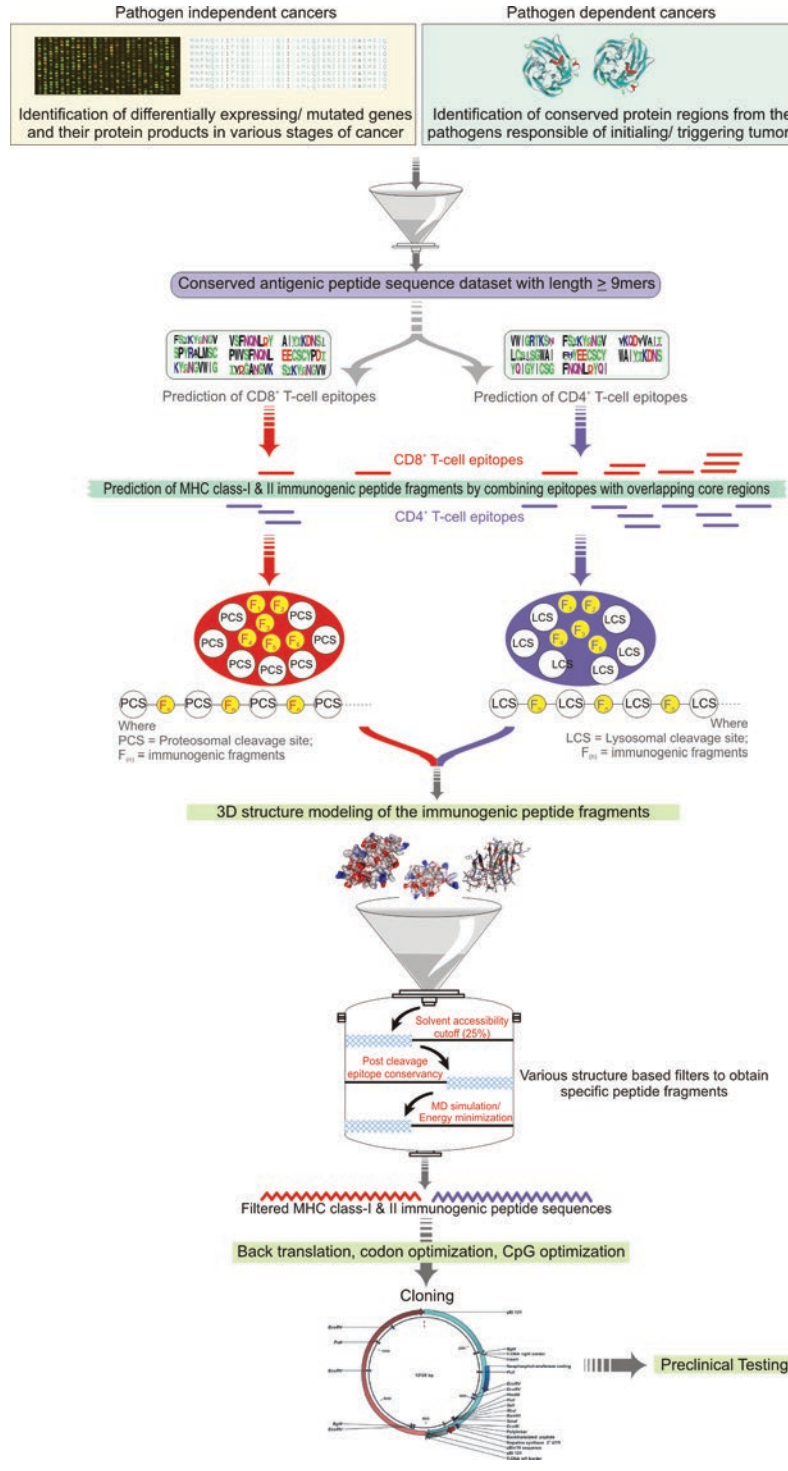

Fig. 27.3 Generalized workflow for computer-aided epitope-based DNA vaccine design

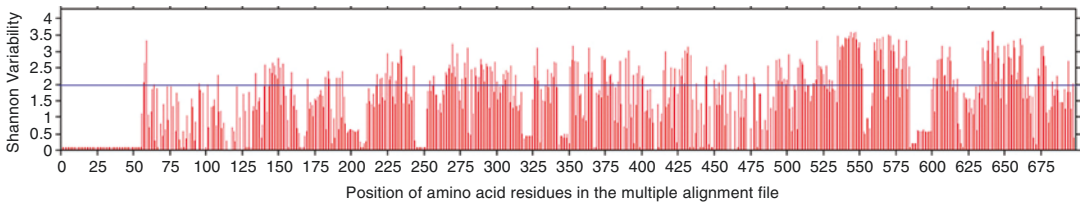

Fig. 27.4 Figure showing the Shannon variability score of individual positions in the multiple alignment files of L2 capsid protein from high-risk HPV strains. Red bars indicate the variability score of amino acid residue *i* at the given position in the multiple alignment file. Blue line represents the cut-off Shannon variability score. All the red bars below the blue line are potential conserved sites for analysis

cut-off score of Shannon entropy was set to 2.0 (Fig. 27.4). The fragments with Shannon variability score <2.0 and continuous length of >9 amino acid residues were further selected for the epitopes identification.

27.4.3 Prediction of MHC Class I and Class II Epitopes

Epitope mapping is always the key step in the vaccine designing. Epitopes are usually thought to be derived from nonself protein Ag that interacts with Abs or T-cell receptors and thereby activating an immune response. Besides from nonself proteins, epitopic sequences from host can also be recognized by MHC molecules. For the effective vaccine, it is important for the epitopes to invoke strong response from T and B-cells. Large numbers of bioinformatics algorithms were designed for this purpose, to name a few are Position Specific Scoring Matrices (PSSMs) based SYFPEITHI [53], Artificial Neural Network (ANN) [54], Stabilized Matrix Method (SMM) [55], and Average Relative Binding (ARB) [56]. According to Tong and colleagues, computational methods for MHC peptide binding can be based on (a) sequence binding pattern using binding motifs, decision trees, or machine learning algorithms line artificial neural networks, hidden Markov models and support vector machines, and (b) three-dimensional peptide/MHC interactions using homology modeling and docking studies [57]. Feldhahn and colleagues implemented FRED, a framework for T-cell epitope detection, which predicts the binding of epitopes to MHC class I and class II HLA alleles using several binding affinity algorithms (SYFPEITHI, SVMHC, BIMAS, and NetMHCpan) and predictions on the features of antigen processing and proteosomal cleavage [58]. The same team has developed EpiToolKit, a web-based platform that implements a variety of computational methods for immunomics [59], including tools for HLA genotyping based on next-generation sequencing data, polymorphic and nonpolymorphic epitope prediction, as well as epitope selection and ranking based on scoring matrices and predictions for proteosomal degradation [60].

In case of DNA vaccine designing against HPV causing cervical cancer, we used RankPep server (http://imed.med.ucm.es/Tools/rankpep.html) for the prediction of consensus binding epitopes (9 mers) for both MHC class I and class II molecules with default parameters. In total, we used 75 MHC class I and 49 for MHC class II matrices for the prediction of potential epitopes from all the consensus L2 capsid proteins.

27.4.4 Reverse Translation of Immunogenic Peptide Fragments

To back translate peptide sequence into the DNA sequence, large numbers of bioinformatics tools are available in the public domain. Because of the degeneracy of the genetic code, the back translation is ambiguous as most amino acid residues are encoded by multiple codons. To design optimal DNA sequence, most of these tools use codon frequency table specific for the organism of interest. We used Backtranseq program of mEMBOSS 6.0.1 for this purpose.

27.4.5 Optimization of Codons and CpG Motifs

Codon optimization is the process to enhance the efficiency of DNA expression vector to express the foreign gene in the host's cell environment. DynaVacs server (http://miracle.igib.res.in/dynavac) was used to compute optimal codon for each of the amino acid residue encoded by the stretch of DNA. The server optimizes codons according to the codon usage table derived from the Kazusa Codon Usage Database (http://kazusa.or.jp/codon). We used codon frequency table for *Homo sapiens* that rank codons by analyzing their frequency of occurrence in 93,487 coding sequences [61]. Immunogenicity of Ag-specific DAN vaccine was previously shown to significantly increase by the optimization of CpG motifs [62]. We again used the DynaVacs server for CpG optimization [63]. In this process, the consensus motif XCGY (where X is any base but C, and Y is any base but G) was incorporated in the sequence as triplet (XCG or CGY) by substituting the less frequent codons that codes the same amino acid residues.

27.4.6 Insertion of Cleavage Motifs and Finalization of DNA Sequence

For the purpose of generating specific epitopes, proteasomal and lysosomal cleavage motifs were also included before and after each MHC class I and class II epitope, respectively. These cleavage motifs are targeted by the proteasomal and lysosomal cleavage machineries to generate immune responses in the host. The corresponding nucleotide sequence of 12-residues long peptide HEYGAEALERAG was added as proteosomal cleavage motif before and after the optimized DNA sequence of each MHC class I epitope. HEYGAEALERAG motif contains all five cleavage sites Y3-G4, A5-E6, A7-L8, L8-E9, and R10-A11 defined for eukaryotic proteasomes in which A5-E6 is the major cleavage site [64]. Similarly, nucleotide sequence of 5-residues long peptide KFERQ was added as lysosomal cleave motif before and after the DNA sequence of each MHC class II epitopes. KFERQ specifically acts as a recognition motif toward heat shock proteins and facilitates further steps for the degradation of proteins by lysosomes [65] to generate MHC class II epitopes. At the end, start and stop codons were added to finalize the DNA vaccine. Arrangement of the epitopes is very crucial and one of the deterministic factors for the efficacy of the DNA vaccine. The folding of the protein product in the host will largely depend on the arrangement of these epitopes and also determine the solvent accessibility of the cleavage motifs. Various computational tools can be used for this purpose including molecular dynamics simulation approaches. The overall arrangement of the DNA vaccine construct is shown in Fig. 27.5.

27.4.7 In Silico Cloning Experiments of DNA Vaccine Construct

Several expression systems have been successfully designed in the past, for the cloning of number of genes encoding surface antigens from pathogens for vaccine development. A good DNA vaccine vector should be designed with minimal functions so that the only gene expressed in mammalian cells is the antigen-encoding gene. We performed the cloning experiments using clc-DNA Workbench 5.0.1. For our purpose, pVAX1

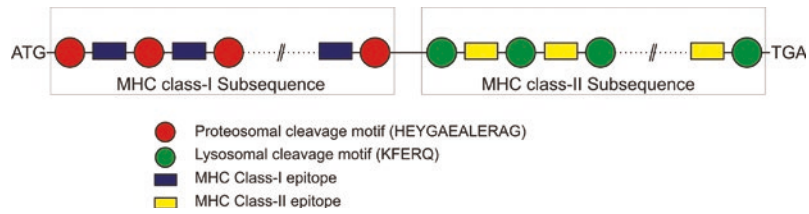

Fig. 27.5 Arrangement of various segments of DNA vaccine constructs. The arrangement of epitopes in the sequence is very crucial to increase the efficacy of DNA vaccine

vector was selected as an expression system. pVAX1 is a nonfusion vector specifically designed to stimulate cellular as well as humoral immune responses [66] and requires that the inserted gene of interest contain Kozak translation initiation sequence (Kozak), an initiation codon (ATG), and a termination codon (TAA, TGA, or TAG). When these designed DNA vaccines are injected into host, antigenic protein gets translated and alerts the body's immune system to generate immunization memory cells.

The methodology described above highlights how various bioinformatics algorithms and computational tools can be combined to design novel and effective vaccine candidates before being subjected to in vitro confirmatory studies.

27.4.8 Personalized Detection of Tumor Epitopes Using Sequencing Data

Tailor-made therapeutic vaccination is an emerging field in cancer immunotherapy, in which the vaccine is customized based on the genomic profiling of the patient, taken by whole-exome and RNA-sequencing of blood and tumor samples. Integrating this information with bioinformatics pipelines to acquire the unique mutation profile of each individual patient, tumor neoepitopes are predicted, synthesized, and injected into the patient intravenously, often using patient blood-derived mature dendritic cells [67]. Usually, only 7–9 neoepitopes are loaded on to the dendritic cell for vaccination [68]. That makes the selection of the right neoepitopes based on patient's genomic profile crucial for the success of the immunotherapy. Hundal and colleagues developed pVAC-Seq, a software tool for predicting tumor neoantigens using genomics and transcriptomics data [69]. In this tool, the prediction of somatic variants using whole-exome sequencing data and that of gene expression profile using tumor transcriptomics data are used as input to generate wild-type and mutant epitope sequences. The obtained mutant epitopes are scored based on binding affinity, coverage, and Variant Allele Frequency. Jaitly and colleagues presented a pipeline for detecting patient-specific epitopes using whole-exome sequencing data and transcriptomics data [70]. In their method, they analyzed whole-exome sequencing data to predict and annotate somatic variants. Next, they predicted epitopes containing mutations after combining genomics and transcriptomics data based on patient's MHC class I HLA haplotypes. The predicted epitopes are ranked based on (a) the number of patient alleles they target, (b) the expression level of the gene the epitope is associated with, and (c) their binding affinity to their targets. The final selection of promising tumor epitopes relies on the use 3D docking simulations of the epitope to the MHC.

27.4.9 Detection of Gene Signatures Associated with Immunotherapy Responsiveness

As we mentioned before, gene expression profiles of patient samples acquired making use of RNA sequencing, proteomics, or other high-throughput data generation techniques can be analyzed using advanced statistical techniques to find genetic signatures. These signatures are intended to provide more accurate diagnosis or guidance on the feasibility of given therapies. In recent years, a number of publications illustrate the use of this approach in onco-immunology. In an analysis of a large amount of colon cancer patient samples and clinical records, Mlecnik and coworkers found that the use of a scoring system to quantify the immune cell infiltration in colon cancer, which is based on gene expression profiling and in situ immunohistochemical staining, had superior abilities to predict tumor recurrence and patient survival than the analysis of microsatellite instability, the current standard marker for colon cancer prognosis [71]. In line with this, Charoentong and coworkers made use of large amounts of sequencing data in public repositories to create a web-based tool able to characterize in detail the tumor immune profile in 20 highly prevalent solid tumor entities (https://tcia.at/) [72]. In their platform, they implement the

so-called Immunophenoscore, a computational tool that utilizes tumor-sequencing data to generate a machine learning-based score accounting for tumor immunogenicity. Similar approaches can be used to predict the efficiency of anticancer immunotherapy. Buschow and collaborators used microarrays from the blood transcriptome of patients treated with therapeutic dendritic cell vaccination in melanoma to predict the efficacy of the treatment [73]. They found a gene signature correlated with the treatment efficacy, in which low Raf Kinase inhibitory protein (RKIP) expression levels in blood samples taken after the first months of vaccination correlated with a poor overall survival.

Sequencing data analysis can be combined with network analysis to generate mechanistic hypothesis on genes signatures obtained by correlation and other statistical methods. Dreyer and coworkers developed and loaded into a web platform a comprehensive regulatory network of signaling pathways important in malignant melanoma (www.vcells.net/melanoma). The regulatory network was designed to facilitate the mining of RNA sequencing data from tumor samples and cell lines. When they used the network to analyze RNA sequencing datasets from malignant melanoma patients treated with anti-PD-1 immunotherapy, they could isolate a core regulatory network differentially regulated in pretreatment tumor samples of responding patients vs. nonresponding patients to anti-PD-1 immunotherapy. This core regulatory network includes multiple genes involved in mesenchymal cell-related phenotypes, especially epithelial to mesenchymal transition, commonly associated with resistant to multiple anticancer therapies. The analysis linked upregulation of factors like SLUG, AP-2, and the E2F family to a decrease in the responsiveness to anti-PD-1 therapy in melanoma. Interestingly, Khan and coworkers found an E2F-centered core regulatory network associated with high aggressiveness and poor survival in bladder and breast tumors, two other highly immunogenic solid tumors [74]. Further, Lai and coworkers found that several miRNAs, including MiR-205-5p and miR-342-3p, can cooperate repressing E2F1, one of the transcription factors upregulated in the mentioned core network accounting for resistance to anti-PD-1 therapy in melanoma [75]. This suggests an intriguing hypothesis, that of using miRNA-based therapy as coadjuvant of immunotherapy in melanoma [76].

27.5 Mathematical Models Used in Basic Oncology Research

27.5.1 Pathways and Networks

The successful use of systems biology to elucidate the regulation and function of cancer-related pathways is well proved by a large body of literature published in the last decade. In this context, mathematical modeling has been used to investigate the time-dependent behavior of biochemical systems, to integrate multiple data sources, or to validate the existence of new regulatory or transcriptional interactions in given regulatory pathways. A question in biochemical networks for which data-driven mathematical modeling is necessary is the elucidation of the nonlinear properties emerging from the combination of regulatory motifs containing positive/negative feedback and coherent/incoherent feedforward loops. When biochemical pathways or networks hold these regulatory structures, they often display behavior that evades direct reasoning. Many papers, which use data-driven modeling approach, succeeded proving how signal amplification [11], sustained oscillations [77], or bistability [78] emerge as hallmarks of signaling and transcriptional networks.

To mention an example on immune-related pathways, Das and colleagues [79] integrated different modeling approaches with in vitro experiments to elucidate the interplay between Ras activation and SOS proteins in the activation of T- and B-lymphocytes. What makes their work interesting is that both proteins, Ras and SOS, are integrated in a positive feedback loop that participates in the Ag receptor stimulation of lymphocytes. In this feedback loop, Ras gets strongly activated upon membrane receptor stimulation, a process which is mediated by members of the SOS family. In turn, SOS activity at the plasma

membrane is allosterically upregulated by active RasGTP. To validate the existence of this positive feedback loop and its functional consequences, the authors combined model simulations and time-dependent in vitro experiments with human and chicken lymphocytic cell lines. They found that under some stimulatory conditions, the biochemical system displays bistability. That is, for intense enough stimulation, the pathway works like and all-or-nothing system: transient but intense stimulus can trigger a sustained activation of the system and the downstream pathway. When we consider a population of lymphocytes, this property may induce the emergence of a bimodal response, with a subpopulation of lymphocytes getting full and sustained activation, while others remain inactive. From an immunological perspective, the authors hypothesize that this system induces the emergence of a short-term mechanism of molecular memory. This mechanism can improve the activation of T-lymphocytes which were stimulated in previous serial encounters with rare antigen-bearing cells.

In the study by Das et al. [79], the focus was to elucidate the dynamics of a small signaling system containing regulatory loops. In other cases, one tries to address how several pathways crosstalk to each other and integrate their signals to achieve the regulation of given phenotypic responses. This has also been explored using mathematical models of large regulatory networks in the context of cancer [80] and immunology [81]. For example, Carbo and collaborators [82] used a systems biology approach to investigate the regulation of the pathways underlying $CD4^+$ T-cell differentiation. By collecting and organizing the state of the art of biomedical knowledge, they constructed a comprehensive regulatory map of the critical pathways regulating the differentiation of naïve $CD4^+$ T-lymphocytes into Th1, Th2, Th17, or iTreg. The regulatory map was translated into a mathematical model in ordinary differential equations, and characterized using perturbation experiments, in which different concentrations of relevant cytokines were used to stimulate the shift between different signaling and transcriptional pathways and therefore the distinctive differentiation of the naïve T-cells. Once the model was calibrated and validated, model simulations and sensitivity analysis were combined to determine the model parameters controlling the activation of different pathways. They found that the pathway regulating the nuclear receptor PPARc function plays a major role controlling the shift between the Th17 and iTreg transcriptional and phenotypic programs. Based on these findings, they foresee a therapeutic potential to the regulation of PPARc signaling in the context of chronic inflammatory and infectious diseases. In this way, the authors show how a full systems biology strategy can be extremely useful to dissect the signaling and transcriptional networks controlling differentiation and plasticity of in immune cells.

27.5.2 Genotype–Phenotype Mapping

Mathematical models can be used to bridge the gap between intracellular pathways and the cellular phenotypes they regulate. In this case, the idea is to develop mathematical models that consider how genetic or epigenetic changes in critical cancer-related pathways can affect the fate of tumor cells, and trigger (or disrupt) phenotypic responses at the cellular level. Some authors call this the genotype–phenotype mapping [83]. This idea has been applied to investigate the deregulation of critical cancer regulatory networks during tumorigenesis and emergence of chemoresistance phenotypes in melanoma [8] and colorectal cancer [84]. Santos and coworkers extended this idea and integrated tumor sample gene expression data with kinetic modeling simulations to investigate the genotype–phenotype mechanisms promoting resistance to immunotherapy in metastatic melanoma [85] (Fig. 27.6). The mathematical model used accounts for the interplay between cytotoxic T and tumor cells in melanoma micrometastases (Fig. 27.6a, b). Further, the model includes equations reflecting key processes belonging to the innate immune response at the tumor site, like natural killer cells activation, and

equations describing the immunity elicited by the immunotherapy, here dendritic-cell or melanoma antigen vaccination. Systematic perturbative model simulations accounting for sensitivity and resistance to anticancer vaccination were performed and clustered in model-derived phenotypic signatures. They further matched these phenotypic signatures with existing transcriptomics data from tumor samples of melanoma patients treated with immunotherapy. To make the comparison possible, they annotated the differentially expressed genes and aggregated the expression of those with similar gene ontologies into metagenes. The model-derived phenotypic signatures were in agreement with metagenomic signatures obtained from the clinical data (Fig. 27.6c). Among other predictions, the model-derived phenotypic signatures pointed to the existence of an unexpected new mechanism of immunotherapy resistance, in which genes linked to antigen presentation get intermediate expression levels, in a way melanoma micrometastases are able to minimize the complementary antitumor immune responses elicited by both cytotoxic T and natural killer cells (Fig. 27.6d).

27.5.2.1 Multiscale Modeling

In a more refined version of the previous strategy, systems biology and data-driven modeling can be used to account for spatial features of tumor organization and the interaction of the tumor with the surrounding microenvironment. This is the rationale for the so-called cancer multiscale models, which has been successfully used in the last years to investigate the detailed dynamics of tumor growth or angiogenesis [17]. In the recent literature, there are several excellent reviews about the topic [83], as well as a number of

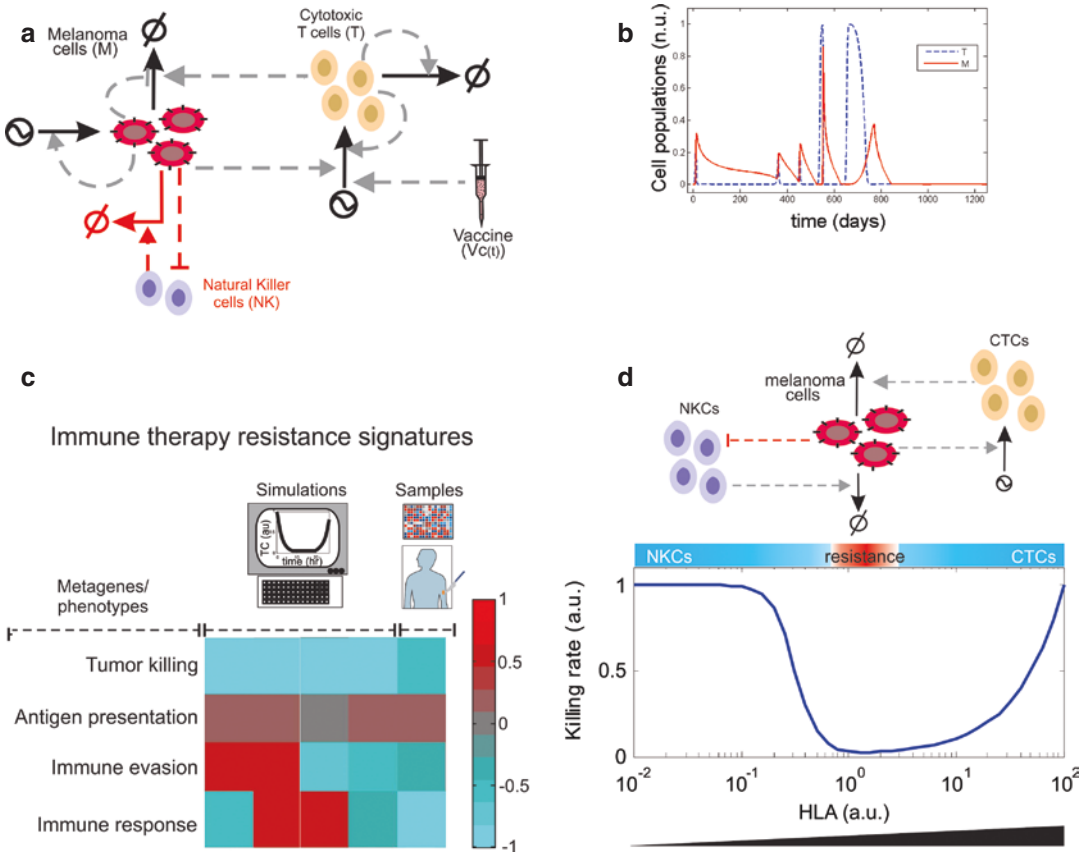

Fig. 27.6 Model-based genotype–phenotype mechanisms promoting resistance to immunotherapy in metastatic melanoma according to Santos et al. [85]

examples of cancer multiscale models [16, 86], many of which referred to angiogenesis.

To mention an example with a cancer immunology focus, Pak and coauthors [87] derived a mathematical model to investigate features of the delivery of recombinant immunotoxins, a family of new molecules with anticancer activity. They are composed of an Ab fragment targeting specific tumor cell Ags, and a protein toxin fragment, which is released and triggers cytotoxic effects upon recognition, internalization, and processing of the molecule. The authors derived a mathematical model that links recombinant immunotoxin dosing and changes in tumor volume. In the model, a tumor is divided into a series of spherical subunits that contain a blood vessel and a number of tumor cells surrounding it, which can be present as normal, intoxicated, or dead tumor cells. For each one of these structures, the model contains a set of differential equations accounting for the dynamics of immunotoxin, from its release from the blood vessel until its internalization in a tumor cell, which becomes intoxicated. In this way, the model accounts for the amount of immunotoxin released, present, and degraded in each tumor subunit. The other part of the model describes the dynamics of tumor cell populations existing in the subunit. This part of the model considers processes like cell growth, immunotoxin-related death, and cell migration to occupy the space cleared after the death of highly intoxicated cells. Using model simulations, Pak and colleagues found that Ag shedding, a key mechanism in the dynamics of tumor-specific surface Ags, is critical for the success of the therapy. Using model simulations, they found that Ag shedding homogenizes the distribution of the immunotoxin in solid tumors; therefore increasing the efficiency of the therapy.

27.5.3 Mathematical Models Used to Assess and Design Therapies

Previous results illustrate the potential of systems biology and data-driven modeling to explore the structure, function, and regulation of biochemical networks, as well as their interplay with cancer-related cell and tissue phenotypes. In addition, Systems biology can play a major role in translational medicine, providing tools for clinical data integration, as well as for design, assessment, and personalization of anticancer therapies [88, 89]. In the following, we illustrate these possibilities with several recent examples.

27.5.3.1 Assessment of Conventional Therapies

A very promising use for systems biology is the personalized assessment of anticancer therapies. The literature contains many recent works illustrating how date-driven modeling can be used to maximize the efficiency of current therapies, but also to detect patient subpopulations for which they are not suitable. For example, mathematical models can be used to determine under which conditions a conventional therapy: (a) is toxicologically safe [90, 91], (b) does not induce further resistance [8, 92], and (c) can be combined with other therapies [8]. Furthermore, data-driven models can be used to establish the drug dosage and timing that optimize the anticancer effect and/or reduce toxicity [93].

For example, Engel and collaborators [90] made use of data-driven mathematical modeling to look for the optimal administration dose and timing of several conventional anticancer drugs minimizing the risk of acute neutropenia, a side effect of anticancer therapy in malignant lymphoma and other cancers. What makes therapy-associated acute neutropenia important for cancer patients is that they get a drastic reduction of neutrophil blood levels, which make them more vulnerable to bacterial infections and increases the risk of life-threatening sepsis. Engel and coworkers derived, characterized, and tested a quantitative data-based ODE model that describes the generation, proliferation, and differentiation of neutrophils and other human granulocytes. The model was extended to account for the changes in the granulocyte dynamics suffered by patients with lymphoma treated with cytostatic drugs and recombinant GSCF, an adjuvant therapy that stimulates granulocyte production and accelerates the recover from neutropenia. The model

was characterized with patient data obtained from several large randomized clinical trials, in which efficacy and safety of multidrug chemotherapies were. The obtained model describes precisely the time-response of white blood cell levels for ten different therapeutic regimes. Furthermore, the authors suggest that the model can be used as a predictive tool, able to assess the safety of other nonexplored conventional anticancer drugs regimes. Although the model was characterized with data from patients suffering malignant lymphoma, they claim that the model can be adapted to assess the risk of therapy-associated neutropenia in other tumor entities.

This idea can be extended to other conventional anticancer therapies. For example, Ribba and colleagues [91] developed a multiscale model to investigate the effect of some tumor features in the efficiency of radiotherapy. The authors constructed a model for colorectal cancer progression that links cell cycle progression, DNA damage level, and other signaling pathways to the sensitivity of individual cells to the irradiation doses. Their model integrated four modules, implemented using different modeling frameworks. Some of the key features of the model are: (a) it includes regulatory pathways controlling cell cycle, cell division, and apoptosis; (b) these pathways are connected with the fate of individual tumor cells and actually control tumor cell death and proliferation; (c) the model also considers the spatial structure of the tumor, that is, how cells get distributed and interact with the tumor microenvironment through gradients, growth- and antigrowth-factors, and hypoxia; and (d) additional model equations describe how different irradiation dosing (time and dose) triggers DNA damage in proliferative tumor cells. When they simulated radiotherapy administration with their model, they found that the efficacy of conventional irradiation protocols can be improved if the cell cycle-regulated dynamics of tumor growth is considered when planning the schedule of irradiation sessions. This result is in line with others suggesting similar optimal schedule of chemotherapy sessions, something known as cancer chronotherapy [94]. In line with these results, mathematical modeling has also been applied to the assessment and personalization of immunotherapies in solid tumors. Kronik and coworkers employed computational modeling and simulation to personalize the design of a therapeutic vaccine against metastatic prostate cancer [95]. In their approach, clinical trial data are used to generate personalized instances of a computational model, which predicts the levels of known prostate cancer antigens in the curse of vaccination, a parameter used as a surrogate biomarker of the therapy efficacy. These authors further elaborated these ideas and deployed a similar strategy to personalize adoptive T-cell therapy in the context of metastatic melanoma [96]. In their approach, data on the growth rate or residual tumor size of the individual patient are used as input in the computational model and used to personalize the T-cell dosage or the schedule of the therapy administration.

27.5.3.2 Design of New Chemo and Immune Therapies

Systems biology has become a valuable approach to boost the procedure of drug discovery and the design of combined therapies that integrate conventional and targeted chemotherapy. The underlying idea is to combine predictive model simulations, sensitivity analysis, and other advanced model-based computational methods to help detect single or combined potential drug targets. These model-obtained potential drug targets can later direct the search from new drugs [21, 97, 98]. In a quite remarkable example of this strategy, Schoeberl and colleagues combined high-throughput and time series data with mathematical modeling of the receptor tyrosine kinase signaling family to detect new anticancer drug targets [12, 99]. They derived, calibrated, and validated an ODE mathematical model describing the known features of the ErbB/PI3K signaling network in the context of cancer progression. Predictive model simulations were combined with computational sensitivity analysis to identify which members of the ErbB family have a major effect in the activation of AKT signaling in cancer cell lines. They later synthesized a human monoclonal antibody that inhibits the phosphorylation and subsequent activation of their top one

model-detected drug target, the ErbB3 receptor. The model predictions were validated by showing that this antibody stops the growth of human tumor xenografts in mice models. Interestingly, the team is entirely composed of researchers from a biotech company devoted to the use of systems biology in drug discovery (Merrimack Pharmaceuticals, Cambridge, USA). This strategy has also delivered some interesting results in the context of immune anticancer therapies.

Kim and Lee [100] used data-driven modeling of the lymph node-tumor interaction to analyze whether preventive vaccination with cytotoxic T lymphocytes (CTLs) can be employed to promote the clearance of microtumors before clinical detection (Fig. 27.7). Toward this end, they derived a hybrid mathematical model composed of two interconnected modules. The first module describes the dynamics of CTL activation, including the tumor antigen production at the tumor site, its detection by antigen-presenting cells, and the subsequent maturation and their migration to the lymph node. Furthermore, the module includes the activation of cytotoxic T-lymphocytes by the matured antigen-presenting cells and its subsequent proliferation, maturation, and migration, as well as the emergence of memory T-cells. The second module describes the interplay between active cytotoxic T-lymphocytes and tumor cells, including tumor cell detection, recruitment of additional CTLS and CTL-mediated tumor cell death. The model was characterized using data from breast cancer. The authors used the mathematical model to determine a threshold in the size of the anti-cancer memory CTL pool able to promote an effective clearance of microtumors. Furthermore, the model predictions attribute an important role in the success of the immune response to the rapidity in which CTLs detect the tumor site. Paradoxically, the model simulations suggested that tumors with fast growth rate are more prone to CTL destruction due to the faster production of tumor antigens and hence, faster detection by cytotoxic T-lymphocytes. The model describes the dynamics of CTL activation, including tumor Ag production, its detection by antigen-presenting cells and the activation of cytotoxic T-lymphocytes by the matured antigen-presenting

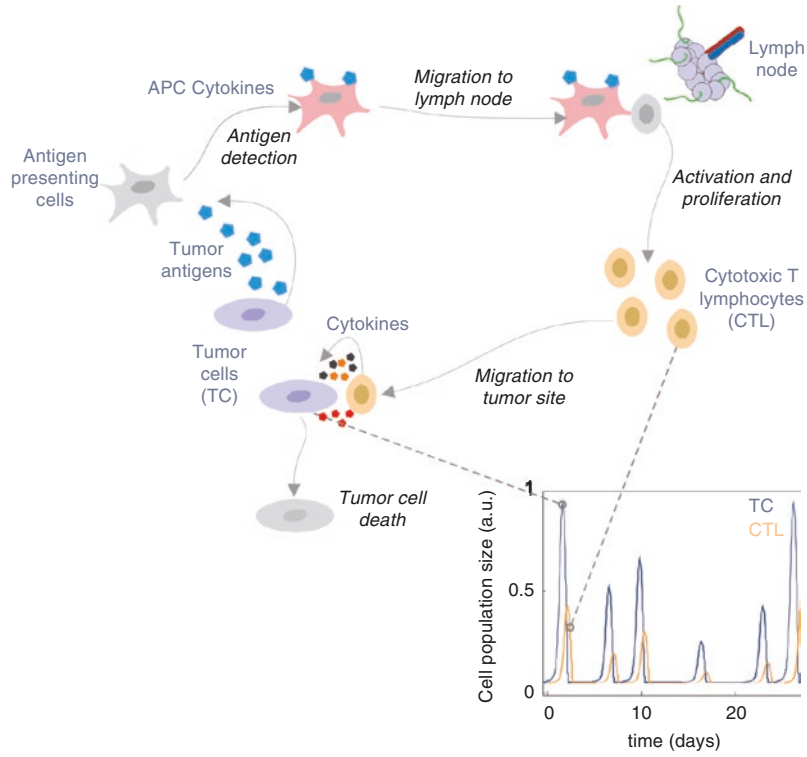

Fig. 27.7 Data-driven modeling of the lymph node–tumor interaction and the clearance of microtumors with cytotoxic T-lymphocytes (CTL) vaccination

cells. In addition, the model describes tumor cell detection by CTLs and CTL-mediated tumor cell death. The model can simulate variations over time of the populations of different immune cells and the tumor cells. In a mouse model of breast cancer, Pennisi and collaborators derived a hybrid computational model describing the immune response to lung metastases elicited by an anticancer vaccine [101]. By making computational simulations, they succeeded in finding vaccine administration protocols maximizing the suppression of lung metastases while reducing the amount of vaccine doses. In an effort that continued over time, de Pillis and collaborators have proven that mathematical modeling is a useful tool to work in the design and assessment of anticancer dendritic cell vaccination [102, 103], as well as in its combination with chemotherapy [104].

27.5.3.3 Unconventional Therapies

A fascinating option with data-driven mathematical modeling is to explore therapies inspired in not yet experimentally proven concepts and ideas. In this sense, modeling is used to formulate new hypothesis on the origin and progress of cancer, as well as to foresee how one could derive new therapies based on this. In the recent literature, there are some examples of this procedure [105, 106]. In a series of recent papers, Gatenby and coworkers hypothesized that adaption to chemotherapeutic agents has an energetic cost for cancer cells, and this can be exploited to design anticancer therapies [106, 107]. In fact, the starting point of their hypothesis is that chemoresistant cells need additional energetic resources to keep working the resistance mechanisms against drugs. Their adaptive therapy relies on considering the existence of several coexisting subpopulations of cancer cells in the tumor, with different genetic and phenotypic backgrounds regarding chemoresistance. In their hypothesis, one can favor the proliferation of chemosensitive cells by manipulating the timing and dose of conventional chemotherapy, in a manner in which these cells can effectively compete with chemoresistant ones for space and resources and delay the development of a fully resistant tumor. To substantiate their hypothesis, they have derived a series of in vitro data-driven mathematical models, which describe the growth of tumors composed of chemosensitive and chemoresistant cancer cell subpopulations. For the most updated version of the model, they performed in vitro experiments under conditions of normal growth and genotoxic drug administration using either normal MCF-7 cell lines or mutant cell lines overexpressing proteins involved in the efflux of anticancer drugs. Using data from these experiments, they characterized the rates of growth and drug sensitivity of both tumor cell subpopulations in the model. Later, model simulations were performed to analyze the tumor growth rate when different versions of their adaptive therapy were used; they compared the results with the tumor growth rate under conventional genotoxic chemotherapy. They found that the combination of their adaptive therapy (which tunes the timing and dose of conventional chemotherapy) with the administration of nonchemotherapeutic membrane pump substrates (a kind of "competitive" inhibitors of drug efflux) and 2-deoxyglucose (an inhibitor of glucose transporters and glycolysis) provokes a fourfold increase in the progression-free survival in their computational models. Serre and coworkers derived and validated against clinical data a pharmacodynamics mathematical model intended to predict the combination of immune checkpoint inhibitors (anti PD-L1 and anti CTLA-4) and conventional radiotherapy in the treatment of aggressive tumors [108]. The authors envision the mathematical mode like a computational tool to optimize and help in the synchronization of the schedules for immunotherapy and radiotherapy. Hatzikirou and collaborators derived and calibrated a mathematical model that accounts for the interplay between bacterial infection and TNFα-driven immunity in the context of experimental therapies against aggressive solid tumors [109]. Their results, partially validated with in vitro experimentation, suggested that intermediate bacterial loads combined with low-level TNFα therapy could trigger a favorable anticancer immune response in tumor-bearing individuals.

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Glossary (Extended Definitions Are Available in the Encyclopedia of Systems Biology [110])

Pathway Biochemical system with a unique input signal, in which network compound interactions follow a rather sequential cascade of events.

Network Complex and highly interconnected biochemical system composed of dozens to hundreds of interacting proteins, metabolites, RNAs, as well as several concurrent input signals.

Cross-Talk Property of a biochemical system integrated by several pathways, in which signals from one pathway modulate the activity of the other.

Regulatory Map Graphical depiction, following a code of symbols, of the compounds, interactions, input signals, and phenotypic output of a biochemical network. One can say that a regulatory map is a visualization of the state of the art of the biomedical knowledge about the biochemical network.

Positive Feedback Loop Biochemical system in which the activation of a biochemical event positively regulates a biochemical process upstream the system. Under some conditions, this kind of system induces signal amplification, bistability and hence the conversion of a transient signaling into a sustained one.

Negative Feedback Loop Biochemical system in which the activation of a biochemical event negatively regulates a biochemical process upstream the system. Under some conditions, this kind of system induces homeostasis, but it can also provoke the emergence of sustained oscillations in the concentration or activation of the network compounds.

Feedforward Loop Biochemical system in which a downstream network compound is simultaneously regulated by, for example, a transcription factor and a protein whose expression is regulated by the transcription factor. The feedforward loop is coherent when the downstream network compound is consistently regulated by both interactions (both interactions activate or both inhibit) and incoherent when the regulation is opposite.

Model Calibration Computational procedure in which quantitative data are integrated with the mathematical model. The aim is to give values to the model parameters, in a way that model simulations are able to reproduce the experimental data available.

Predictive Model Simulation Computational procedure in which the model can be used to extrapolate the behavior of the system investigated under experimental conditions not yet tested.

Model Validation Procedure by which predictive model simulations are compared with new experimental data, not used in model calibration. A model is considered validated when there is an agreement between the predictive simulations and the experimental data used.

ODE Model Mathematical model of biochemical systems that describe spatio-temporal changes of protein concentrations and other biological molecules using kinetic equations. These equations describe the variation on time of the populations or concentration of the considered biomolecules.

Boolean/Logic Model Class of discrete computational models used to model biochemical systems, in which the network compounds can have one of the two possible states at any time: 1 or ON, which means that the compound is expressed or active; and 0 or OFF, nonexpressed or inactive.

Agent-Based Model Class of discrete computational models used to model biochemical systems and cell-to-cell interactions. A cellular automaton is the computational representation of a regular grid of cells. Each cell can have a finite number of states (similar to the ON/OFF of Boolean models), and transitions in states affected by the states of the surrounding cells in the grid.

Bistability Property of biochemical networks containing positive feedback loops, by which small perturbations drastically change the behavior of the system, for example, inducing a transition between quick signal termination after transient stimulation and persistent activation.

Self-Sustained Oscillations Property of some biochemical systems containing negative feedback loops, in which the concentration of the network components oscillates regularly in time, even under constant external stimulation.

Sensitivity Analysis Computational tool used to analyze mathematical models. This tool provides information about the model parameters for which a variation in their value significantly affects the behavior of the system.

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Principles of Immunological Diagnostic Tests for Cancers

28

Amber C. Donahue and Yen-lin Peng

Contents

28.1 Introduction

Through the use of deliberate mutation of immunoglobulin genes, the immune system has evolved the ability to produce antibodies (Abs) able to bind targets with exquisite specificity (i.e., recognition of ONLY the target) and impressive affinity (i.e., strong binding to the target). These abilities explain why Abs remain an invaluable tool for the detection and measurement of biological phenomena and already represent some of the treatment modalities of the present and near future. While most of the work with Abs is currently ex vivo, their use in vivo has already shown significant progress and benefits. Antibodies are currently used for biosensing of

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specific targets in the body, in order to deliver radioactive isotopes or cytotoxic drugs (reviewed in Ricart and Tolcher [1]). Antibodies have also been used for visualizing specific biological processes such as tumor shrinking and tumor growth [2–5] or to aid in the imaging of tumors. These types of applications for antibodies will likely become more common as immunoglobulin engineering becomes more sophisticated, increasing the potential of using Abs in vivo for the targeting of specific lesions or tumors or even for the neutralization of specific biological processes. In the meantime, Abs are widely used in multiple formats and platforms to aid in the detection of a wide range of cancers. This chapter will introduce the structure of the immunoglobulin protein, including the most commonly used altered and engineered variants created by researchers, and provide detail on how these various Abs can be labeled to allow their detection. A number of different applications then become possible. The principles of these applications and the ways in which they can be combined to create diagnostic tests will be outlined, including how diagnostic assays are increasingly being designed to include the detection of large numbers of targets simultaneously, a technique known as multiplexing.

28.2 Overview of Antibodies

Antibodies, or soluble forms of immunoglobulin (Ig), possess a vast array of possible specificities and a structure that is one of the more stable among mammalian proteins. Researchers have capitalized on the large pool of specificities provided by naïve B lymphocytes as well as on the refinement of specificities for the recognized motif, or epitope, provided by the process of somatic hypermutation during clonal expansion of activated B-cells. However, the ex vivo generation of Abs is becoming the standard for the purposes of research, diagnostics, and therapy. This allows for an increased amount of versatility through a large number of sources and formats. Clinicians and researchers have the choice of intact Ab molecules or fragments, as well as polyclonal or monoclonal antibodies (mAbs) from a number of different species. Each of these various Ab molecules can also be chemically linked to a multitude of reporter molecules, allowing the use of Abs in a wide range of assay platforms. The most common of these platform variants is described below.

28.2.1 Monoclonal Vs. Polyclonal Antibodies

A polyclonal Ab preparation consists of a mixture of immunoglobulin molecules with multiple specificities, all of which are directed against the target. Most polyclonal Ab mixtures are created by the injection of a purified full-length recombinant protein into an animal, which can lead to the generation of Abs that recognize many portions of the protein. In other instances, a short peptide comprising a more specific region of interest is used, creating a number of different Abs that recognize a very specific region or "epitope." In most cases, the rabbit is used to generate polyclonal Ab mixtures. Many other species can also be used to create these Abs, contributing to the multiplexing flexibility of Abs. The injected peptide or protein, known as an immunogen, is selected to include a very specific, and preferably unique, region of interest in a target molecule. When the injected animal's immune system recognizes the peptide or recombinant protein as foreign, the resulting immune response will generate multiple immunogen-specific Abs, which can then be isolated from the animal to yield a polyclonal antiserum. In some cases, this antiserum is purified further using affinity chromatography [6].

Because of a higher degree of confidence in their affinity and specificity, mAbs are often chosen over polyclonal preparations when possible. Kohler and Milstein developed the first mAbs in the mid-1970s by expanding on the techniques used to generate polyclonal Ab preparations. As with polyclonal Ab stimulation, an immune response is elicited to an injected immunogen. In this case, however, multiple antibody-producing daughter B-cells are isolated from the spleen of the injected animal after several days. Myeloma

cells are then fused with the harvested antibody-producing B lymphocytes to generate hybridomas. These hybridomas can produce large amounts of the Abs expressed by the original activated daughter B-cells and are capable of proliferating in culture indefinitely. Single hybridomas are separated and expanded in culture to create monoclonal populations. The Abs produced by the monoclonal populations are then screened for affinity and specificity [7, 8].

Several technologies for more cost-effective, rapid, and simpler generation of mAbs have since been developed. Chimeric or "humanized" Abs have been made possible by recombinant techniques, combining human Ab DNA with the sequence encoding the binding site of a mouse mAb [9]. Recent years have also seen the emergence of bacterial expression of antibodies, which allows for the selection of advantageous Ab specificities via phage display. The displayed Ab fragments are generated from the plasma cells of human donors or from the spleen of an immunized animal. Increasingly, however, these phage libraries and other screening tools are generated by genetic engineering (discussed in greater detail in Donzeau and Knappik [9]). The highly specific high-affinity mAbs required for therapies, diagnosis, and basic research are created using these methods.

28.2.2 Antibody Fragments

Depending on the requirements of the assay platform, Abs can be used in a number of different formats, including the intact immunoglobulin molecule as well as multiple types of smaller fragments (Fig. 28.1). The Fab fragment includes the entire light chain, as well as the variable and first constant region of the heavy chain, and can form stable H/L heterodimers without being covalently linked. In some cases, Fab fragments can remain joined through a C-terminal disulfide bond (Fig. 28.1c) [9]. Fab fragments can be created by papain digestion of intact immunoglobulin molecules, or more recently, through genetic manipulation. The F(ab')$_2$ fragment is similar, in that it also retains the disulfide bond which covalently links the two chains of the Fab fragment (Fig. 28.1b). In the case of the F(ab')$_2$ fragment, however, a portion of the flexible hinge region remains intact following its creation by digestion of intact Abs with pepsin. Additional small fragments and multivalent engineered Abs can also be created through genetic engineering and may enjoy increasing use in diagnostic assays and possibly cancer therapy in the coming years.

28.2.3 Reporter Labeling

There are a number of reporter molecules available for use in visualizing and even quantifying the binding of an Ab to its target [10]. One such class of reporters is the group of laser-activated fluorescent molecules called fluorophores or fluorochromes, commonly used in flow cytometry (see Sect. 28.8). Other reporters can be enzymatic and therefore depend on chemical reactions to be detected. For these reporters, the Abs are chemically linked, or conjugated, to an enzyme such as alkaline phosphatase (ALP) or horseradish peroxidase (HRP). An intense color is generated by the product created when these enzymes are incubated with chromogenic substrates, allowing measurement with a spectrophotometer. It is also possible to incubate these enzyme-linked Abs with a chemiluminescent substrate, the product of which gives off light, which can then be quantified by a number of different instruments and even captured on film.

A common third approach, often used to allow greater flexibility for the multiplexing of targets, includes biotin-conjugated Abs [11]. Biotin recognizes streptavidin with a high level of specificity and affinity, forming one of the strongest known noncovalent bonds. Streptavidin can be linked either to fluorophores or to enzymes like HRP and ALP, providing the flexibility to use a particular biotinylated Ab across multiple assay platforms. Similarly, within a single platform, the same biotinylated Ab can be used in multiple wells or tubes and, if necessary, be identified by different colors by using varied streptavidin-conjugated reporters, as with the multiple fluorophores used in flow cytometry [12].

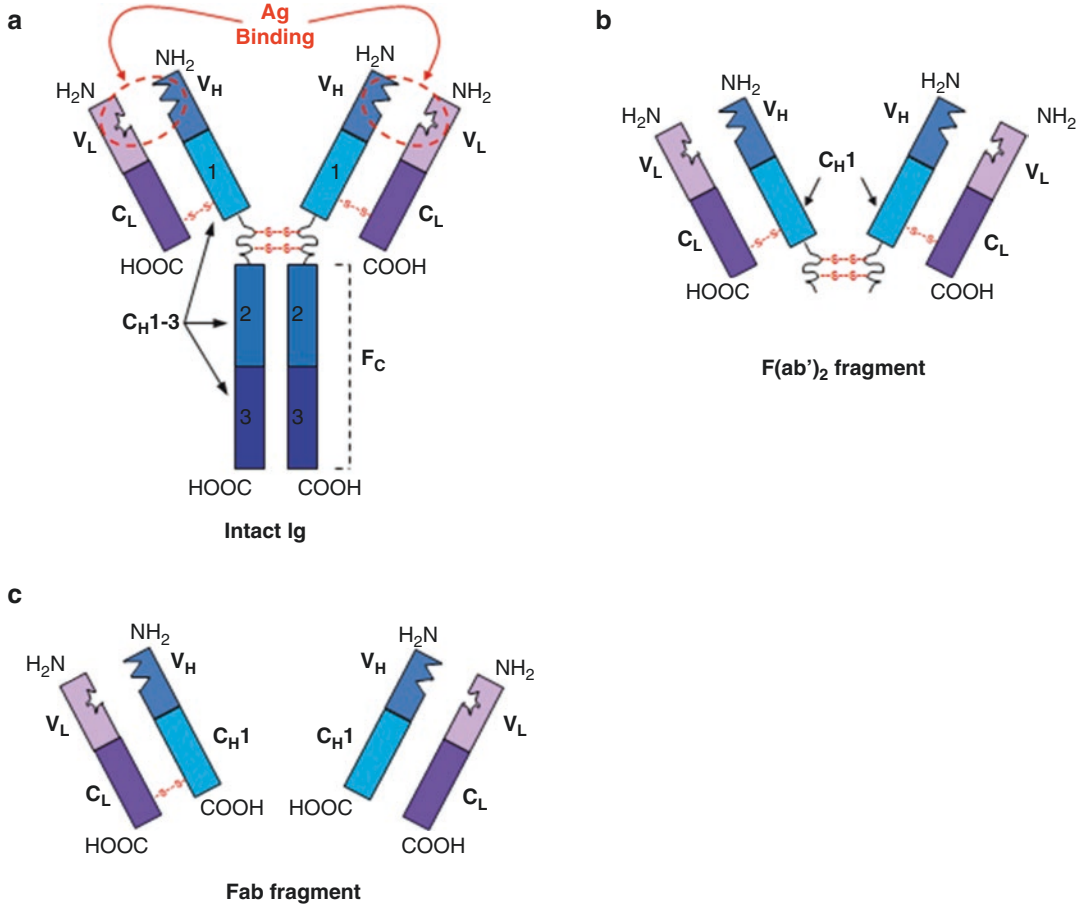

Fig. 28.1 Intact immunoglobulin and common antibody fragments. (**a**) Schematic representation of an intact immunoglobulin molecule. Each heavy chain (*blue*) consists of three constant domains (C_H1–3) and the variable domain (V_H). C_H1 and C_H2 are linked by the flexible hinge region, which forms two disulfide bonds with the hinge region of the complementary heavy chain. Each light chain (*purple*) consists of one constant domain (C_L) and one variable domain ($V\ L$) and is associated with the heavy chain through a disulfide bond proximal to the carboxy-termini of the two chains (COOH). The antigen-binding regions of the molecule (Ag Binding) are found at the amino-termini of the V_H/V_L pairs (NH_2) and are circled in red. The Fc portion of the molecule, consisting of C_H2–3, is indicated. Domain labels are constant throughout the figure. (**b**) The $F(ab')_2$ antibody fragment. Enzymatic digestion of intact immunoglobulin with pepsin results in the cleavage of the molecule at the hinge region, maintaining the disulfide bonds and yielding the $F(ab')_2$ fragment. (**c**) Papain cleaves the hinge region of intact immunoglobulin just above the disulfide bonds, generating two Fab fragments. Fab fragments can also be created through genetic manipulation. The heavy and light chains can associate noncovalently (*right*) or may maintain a disulfide bond near the carboxy-termini (*left*)

28.2.4 Primary and Secondary Antibodies

Some diagnostic assay formats require the use of Ab pairs for detection (see Fig. 28.6b for a schematic representation). The first, or primary, Ab is specific for the target. A secondary reporter-conjugated Ab can be used in cases where the primary Ab does not include a reporter. Antispecies Abs, which are directed against immunoglobulin molecules produced by a different species, are commonly used as secondary Abs. For example, mouse immunoglobulin is injected into a goat to produce an immune response, resulting in a polyclonal goat anti-mouse Ab preparation that can be labeled with a

reporter molecule. The goat anti-mouse Ab preparation is used to detect the presence of the primary mouse mAb wherever it may be bound to the target. However, in order to avoid possible cross-reactivity and to minimize the complexity of the assay, simpler assays in which the primary Ab is directly conjugated to a reporter are preferred when the assay system permits.

28.3 Immunoprecipitation

For many years, specific Abs have been used as a means to bind and concentrate targets in solution [13]. This process, known as immunoprecipitation (IP), involves the mixing and incubation of the specific Ab with a solution containing the molecule of interest (Fig. 28.2). After sufficient time to allow the Ab to bind the target, the Ab itself can be captured through binding to beads coated with bacterial protein A, protein G, or a mixture of both. The solution can then be centrifuged to pellet the beads at the bottom of the tube, allowing the supernatant to be transferred or discarded. Through this process, the target has been isolated and greatly concentrated and is now more readily detected.

When searching for comparatively rare proteins, which are present at much lower concentrations, a larger number of cells or volume of bodily fluids like plasma are required. This larger amount of material often presents problems for the detection system, which can be solved through the capture and concentration of the target by IP. In other cases, IP is used to diminish the amount of background detected by the assay system. The background can be minimized either by pulling the target out of the sample mixture for detection or by specifically depleting the mixture of an unwanted protein(s) that has been found to conflict with the detection of the target. IP is often used as a first step before detection by immunoblotting.

28.4 Immunoblotting

Also known as Western blotting, immunoblotting (IB) makes use of specific Abs for the detection of proteins of interest [14]. Sodium dodecyl sulfate (SDS) and heat are used to denature the proteins in a sample, which can range from a bodily fluid such as plasma, to a solution of cellular proteins released from cells by treatment with a lysis

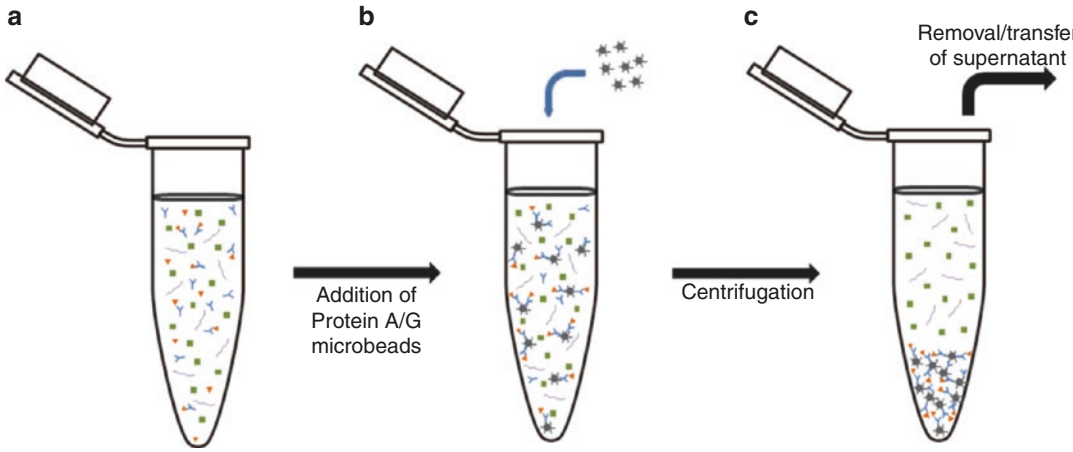

Fig. 28.2 Immunoprecipitation. (**a**) Cell lysate or other biological sample is incubated with specific antibody (*Ab*), which binds to the target in solution. (**b**) Microbeads coated with bacterial protein A, protein G, or a combination of both are added to the solution. The Abs, whether bound to target protein or free, will be bound by the bacterial proteins coating the bead. (**c**) Following centrifugation, the beads and their cargo of Ab and target protein will form a pellet at the bottom of the tube. The supernatant, now depleted of the target protein, can be transferred to another tube or discarded. These schematic representations of Abs and their targets will be used for all subsequent figures

buffer. These proteins are separated according to mass via polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a membrane for detection (Fig. 28.3). The specific primary Ab is washed over the surface of the membrane for a prolonged incubation period, allowing it to bind the target protein, followed by incubation with a secondary enzyme-conjugated anti-species Ab. After the addition of a chemiluminescent substrate, a band of light will be generated at the position where the primary and secondary Abs are bound to the membrane. The amount of protein present dictates the amount of primary and secondary Ab bound to the membrane, which in turn dictates the intensity of the light generated. This light signal is traditionally detected by exposure to autoradiography film, but advances in low-light camera-based systems have led to increasing use of these documentation methods. On a traditional immunoblot exposed to film, lower intensity signals correspond to fainter, thinner bands, while larger amounts of signal create fatter, darker bands (Fig. 28.3).

Due to the fact that it provides an opportunity to physically view the interactions of an Ab with the proteins present in a sample matrix, immunoblotting is still widely used in a research setting despite being an older technique. This characteristic can help researchers determine the specificity of an Ab during the development of a cancer test, even if another technique will ultimately be used for detection. However, despite the fact that the method is comparatively time consuming and labor intensive, there are still

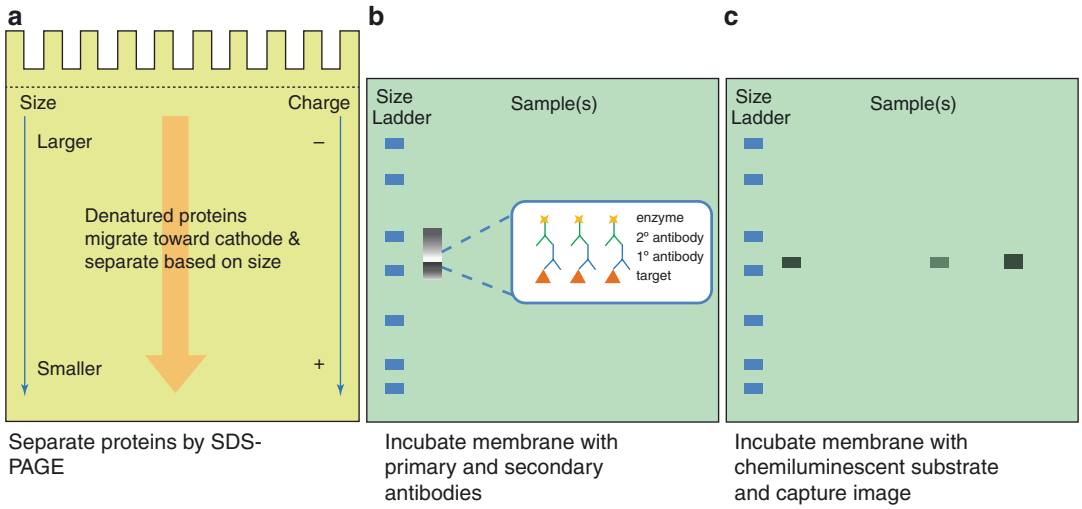

Fig. 28.3 Immunoblotting. (**a**) Samples are denatured in lysis buffer, loaded onto a polyacrylamide gel, and separated by electrophoresis (*PAGE*). The presence of sodium dodecyl sulfate (*SDS*) in the buffer masks the native charges of the proteins and lends an overall negative charge, allowing the proteins to migrate toward the cathode according to size, with smaller proteins traveling farther through the matrix than large proteins (SDS-PAGE). Proteins can also be analyzed by their native conformations under nondenaturing conditions in the absence of SDS (not shown). (**b**) Separated proteins are transferred to a nitrocellulose or polyvinylidene fluoride (*PVDF*) membrane via the application of electrical current. The membrane is then probed with primary Ab specific for the target protein or residue, followed by an enzyme-conjugated secondary anti-species Ab (more detail on secondary antibodies and reporters is given in Fig. 28.5). A molecular weight standard containing multiple proteins of known molecular weights is usually included in each experiment (size ladder), to provide an estimation of the distribution of the sample proteins. The proteins in these ladders are often dyed, sometimes with multiple colors, to allow visualization on the membrane. (**c**) The target is visualized by incubating the membrane with the chemiluminescent substrate of the reporter enzyme, which emits light. The signal is captured by exposure to autoradiography film or by a camera-based gel-documentation system. The quantity of target can then be extrapolated from signal intensity and/or band size, with larger bands corresponding to more bound target, although this measure is not truly quantitative, but relative to the other samples in that experiment only

some cancer-related diagnostic tests which make use of Western blotting. Examples include confirmatory tests for Ri, Hu, or Yo, which are found in paraneoplastic syndromes associated with a number of cancers. The proteins of interest in these Western-based tests are actually Abs themselves. The Ri immunoblot detects the anti-Ri Ab present in patients with paraneoplastic myoclonus/opsoclonus syndrome, which is most often associated with gynecological cancers, breast cancer, and small cell lung cancer. The Yo, or Purkinje cell, Ab is also found in patients with breast, ovarian, and other gynecological cancers, in this case suffering from paraneoplastic cerebellar degeneration. Hu antineuronal nuclear Abs are detected by Western blot in a small percentage of patients with small cell lung cancer and are associated with paraneoplastic sensory neuropathy and encephalomyelitis. The highly specific Abs used in these Western blots provide confirmation of the identity of the Hu, Yo, and Ri Abs initially detected by first-line screening tests.

28.5 Radioimmunoassays

One of the first highly sensitive methods for measuring the levels of proteins such as hormones in the blood was the radioimmunoassay (RIA) [15]. In a classic RIA, a known quantity of purified target protein is radiolabeled, most often with a gamma radioisotope of iodine. This "hot" protein is mixed with a specific Ab that has been immobilized on a surface, and then, the biological sample containing unlabeled or "cold" protein is added to the mixture (Fig. 28.4). In a standard competition assay, the cold protein will then compete with the radiolabeled protein for binding to the Ab, leading to the displacement of a fraction of the radiolabeled protein. The amount of target protein present in the sample can then be extrapolated by measuring the amount of displaced radioactivity.

RIA technology allowed some of the first specific and sensitive tracking of important hormones like insulin in human blood [16] and is still used in some cancer-related diagnostics today, including thyroid hormone testing. Some thyroid hormone tests, including reverse T3, free T4, and especially thyroid-stimulating hormone (TSH), are still offered via RIA. These thyroid hormone tests are included as diagnostic tests in the preliminary characterization of thyroid nodules as malignant or benign and in the diagnosis of TSH-secreting pituitary adenomas. In the interest of laboratory safety, however, technology has moved away from techniques requiring the handling of radioactivity, and the RIA method has largely been replaced by enzymatic immunoassays.

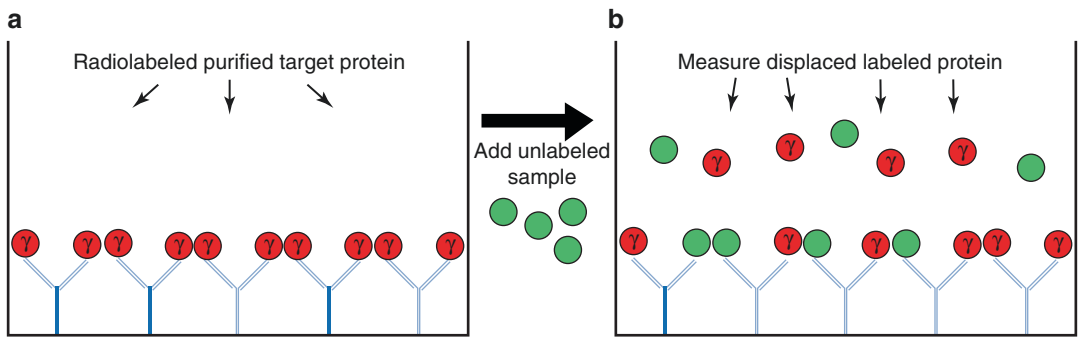

Fig. 28.4 Radioimmunoassay. (**a**) Purified target protein is radiolabeled, often with the gamma isotope of iodine (γ) and incubated with immobilized specific antibody (*Ab*). Sample containing unlabeled target protein is then added to the well. (**b**) The unlabeled target protein competes with the purified radiolabeled protein for binding to the Abs, displacing some of the radiolabeled protein when present at high enough concentrations. The unbound protein is removed from the well, and the radioactivity of the displaced radiolabeled protein is measured to give an indirect measure of the amount of unlabeled target protein present in the sample

28.6 Enzymatic Immunoassays

Enzymatic immunoassays (EIAs) are the archetypal antibody-based detection format and a foundation of basic cellular biology research. The best known EIA format is the enzyme-linked immunosorbent assay (ELISA) [17], which has been used for the detection of targets in both cell lysates and nearly every bodily fluid, ranging from whole blood to sputum to cerebrospinal fluid. Most commonly, ELISA assays are performed in microtiter plates containing 96 or more wells, providing the opportunity to test a large number of samples in a single run. Further, as the treatment of each well is often identical, the format of the ELISA assay lends itself to a high degree of automation using liquid handling robots and plate washers. Since the ELISA often contains multiple lengthy incubation steps, the ease with which it can be automated provides valuable time and labor savings in a high-throughput cancer diagnostics laboratory.

ELISA formats can range from simple to complex, incorporating from one to four Abs (Fig. 28.5) [17]. At the most basic end of the spectrum is the "direct" ELISA, which uses a single reporter-labeled primary Ab to detect the target that has been adsorbed to the surface of the well or plate (Fig. 28.5a). More commonly used, however, is the "sandwich" ELISA, which can use from two to four Abs as shown in Fig. 28.5b. In many cases, the sandwich format is preferred due to the greater level of specificity conferred by requiring two different specific antibodies to bind the target before detection is achieved. The first Ab which binds the target is referred to as the "capture" Ab and is bound to the plate/well either through direct adsorption or through interaction with a corresponding anti-species Ab that is bound to the plate instead. The capture Ab will bind the target during incubation with the lysate or bodily fluid, after which the irrelevant proteins are washed away, leaving the enriched and purified target. The second, or "detection," Ab is now incubated in the well and allowed to bind to the target wherever it has been captured in the well. The detection Ab can be directly labeled with a reporter or can be detected itself by a secondary reporter-conjugated anti-species Ab. The important consideration to remember when designing a sandwich ELISA is that if a secondary anti-species Ab will be used for detection, the capture and detection Abs must have been generated in different species, to prevent the binding of the secondary detection Ab to both.

The flexibility made possible by the sandwich ELISA allows the detection of specialized protein motifs. Examples include the differentiation between isoforms created by alternative splicing [18] or detection of posttranslational modifications such as phosphorylation, acetylation, glycosylation, methylation, ubiquitination, and even protein cleavage [18–23]. The turnover rate of

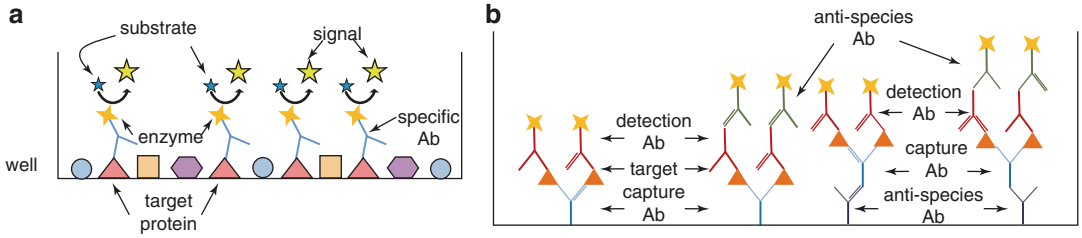

Fig. 28.5 ELISA. (**a**) The simplest ELISA consists of proteins adsorbed to the surface of a well and incubated with specific enzyme-conjugated Abs. After binding of the Abs to the target protein, the well is washed, and the colorimetric or chemiluminescent substrate is added. The reporter enzyme acts on the substrate, generating signal in the form of color or light, respectively. (**b**) The sandwich ELISA and its possible variations. The specific capture Ab can be directly coated onto the surface of the well or be bound itself by an anti-species Ab. After capture of the target protein, the target is bound by the detection Ab, which can be conjugated to a reporter itself or bound by a reporter-conjugated secondary anti-species Ab. Each of these permutations is represented

important proteins, the activation status of specific pathways, and other important cellular activities can be inferred from the posttranslational modifications of important cell signaling proteins. For detection of these modifications, the target protein can be bound by the capture Ab, the unbound background protein is washed away, and then a detection antibody specific for the modification of interest can be used to determine whether the protein contains that posttranslational change. The opposite approach can also be taken, in which a detection Ab specific for the target protein can be used to probe the proteins pulled out of solution by a capture Ab specific for phosphotyrosine, for example. In some cases, the posttranslational modification at a specific amino acid residue is even included in the immunogen, in order to generate an Ab specific ONLY for the version of the protein containing a phosphorylated residue at a given position rather than the nonphosphorylated version.

It is also theoretically possible, though generally technically difficult, to use a sandwich ELISA to detect the protein product of a gene fusion, such often happens in cancer. One such example is the BCR-ABL fusion protein, which is the result of the so-called Philadelphia chromosome, or the reciprocal translocation t(9;22);(q34;q11), that occurs most often in chronic myeloid leukemia (CML). In this example, a capture Ab specific for the BCR protein would immobilize both wild-type (WT) and fused BCR, while only the fusion protein would be bound by the anti-Abl detection Ab.

The ability to detect multiple targets side by side in a single aliquot of sample can provide a great deal of important information, as well as maximize the information derived from the often inadequate and precious samples received in cancer diagnostic laboratories. Newer ELISA technologies have emerged in the last decade that make multiplexing possible through the use of multispot wells. In this assay layout, a number of different capture Abs are bound to the bottom of each well in discrete spots, ranging from 2 to 4 up to 100 (Fig. 28.6a). Flexibility has been further increased by breakthroughs in chemical linkers, which allow assay designers to mix and match the capture Abs in a given well and do it in-house (Fig. 28.6b). These linker-conjugated capture Abs are used with specialized plates, in which the binding partner of each chemical linker has already been spotted in a specific position on the bottom of the well. Each capture Ab

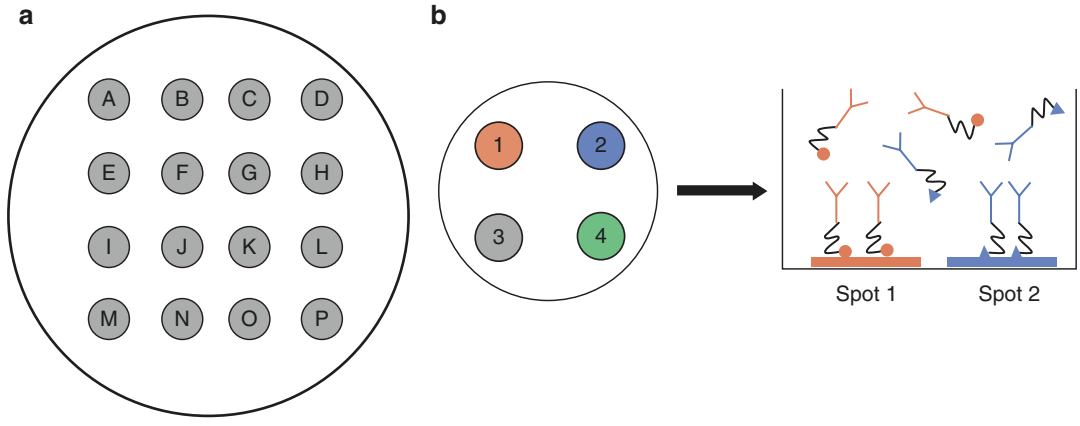

Fig. 28.6 Multispot ELISAs. (**a**) A schematic representation of a 16-spot multispot ELISA well. Each spot, or letter, corresponds to a different capture Ab that is carefully applied to the plate in one discrete area, usually by robot. A single sample can then be incubated in the well and 16 different sandwich ELISAs performed simultaneously on one small volume of analyte. (**b**) Chemical linkers can create multispot assays without robotic spotting of the capture antibodies, allowing mixing and matching of desired analytes. Each capture Ab is conjugated to one of several chemical linkers and incubated simultaneously in the well. Each linker binds only to its corresponding spot, isolating each capture Ab in one specific region of the plate. Multiple sandwich ELISAs can then be performed as in (**a**)

will therefore only bind to one particular spot within the well, and the sample can then be added to the well and interrogated for the presence of many target proteins at once.

These sorts of multiplexed ELISA platforms generally require camera-based detection systems that include sophisticated software capable of discriminating and parsing the signal generated by multiple spots in a single small well. Adding an ever greater level of control over the process, some more advanced ELISA platforms now include computer-controlled initiation of the chemiluminescent reaction. In this system, the reporter is a true electrochemiluminescent (ECL) reagent, requiring an electrical current to undergo the chemical reaction, and the assay is performed in a specialized plate containing a small electrode in each well. The computer controls the application of current, usually breaking the plate down into sections read in sequence. These sorts of adaptations to the ELISA platform represent some of the advances made in the last decade and will likely see increasing uptake in the design of cancer tests.

This versatility in the sandwich ELISA platform, as well as the flexibility provided by the large number of available reporter/detection formats, suggests that similarly ingenious ELISAs will continue to be developed. Most commonly in cancer diagnostics, however, more straightforward sandwich ELISAs are used for the purposes of quantitative detection and monitoring of relevant proteins. An example is the HER2 ELISA, which measures the level of HER2/neu present in the serum of breast cancer patients. With the inclusion of a standard curve on the ELISA plate, the amount of HER2/neu protein present in the well can be quantified, and the concentration of the protein circulating in the body can be extrapolated. These data can be used by the clinician to assess the patient's prognosis and to determine the likely response of the patient to a given therapy. Further, if a baseline concentration of the circulating protein is established prior to administering therapy, subsequent longitudinal measurements can be compared to that baseline and used to monitor the efficacy of therapy.

28.7 Immunocytochemical and Immunohistochemical Assays

Immunohistochemistry (IHC) and immunocytochemistry (ICC) are similar techniques used by researchers and pathologists to recognize particular cell types or to determine the location of important proteins within the cell. These proteins can include indicators of apoptosis or proliferation, as well as tumor markers. IHC and ICC assays can provide a wealth of information to the trained observer (Fig. 28.7) [24, 25]. The cells being studied can be found in an intact tissue section as is the case in IHC or taken from suspension or from a smear as in ICC. As with an ELISA, these cells are incubated with the primary Ab specific for the protein of interest and can be detected either through direct conjugation of that primary Ab or by the binding of a secondary reporter-conjugated anti-species Ab. ICC and IHC can use both enzymatic and fluorescent reporters; the use of fluorescent reporters is also sometimes referred to as immunofluorescence, differentiating the technique slightly due to the requirement for a fluorescent or confocal microscope, as opposed to the light microscope that can be used to visualize enzymatic reporters. Additional common antibodies or dyes are often used to identify structures within the cell, such as the nucleus. The prepared samples are viewed using advanced microscopy techniques and often computer-based image analysis systems as well.

In recent years, advances in automation have generated higher throughput solutions for IHC and ICC. One such advance, tissue arrays, allows the placement of multiple patients' samples on a single slide, which leads to a significant increase in the uniformity and speed of slide preparation. Further, increasingly sophisticated software and new automation systems reduce the amount of time that is required to screen slides, thereby greatly increasing throughput. An example is the InScape system, which includes the scanning of the slide to create a high-resolution digital image, and automated determination of results using marker-based algorithms after the region of interest is chosen by a pathologist. The result is then

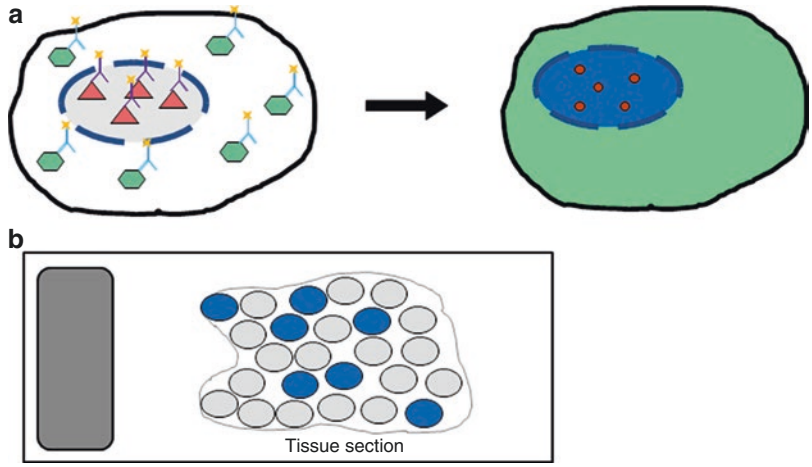

Fig. 28.7 Immunocytochemistry and immunohistochemistry. (**a**) Simplified schematic of ICC, depicting a single cell probed for two specific proteins. One protein is found to be localized to the cytoplasm (*green*), while the other protein is localized to the nucleus (*red*). This nuclear localization is confirmed by a co-stain which identifies the nucleus (*blue*). (**b**) Simplified schematic of IHC, depicting a slide-mounted tissue section. Only a few cells in the tissue section express the protein for which the sample has been stained (*dark blue*). IHC and ICC can make use of both colored stains and fluorescent markers and often require microscopes with multiple excitation and/or emission filters (not shown)

verified by the pathologist, saving a great deal of time in the analysis of IHC stains.

ICC and IHC continue to be valuable tools for pathologists due to the ability of the technique to map the location of the target protein to a specific position within the cell. Some types of proteins, such as transcription factors, are regulated wholly or in part by localization. For example, many transcription factors are found in the cytoplasm when inactive and shuttled to the nucleus following activation. Mutations in some proteins that lead to improper localization within the cell have been demonstrated to contribute to malignancy. ICC/IHC assays for the visualization of the localization of these proteins, as well as assays that detect the presence or absence of posttranslational modifications, different isoforms, and even mutant proteins, are all valuable diagnostic and prognostic tools for pathologists.

One of the best known and most commonly used IHC tests in cancer diagnostics is the staining of breast cancer sections for the presence of the estrogen receptor protein (ER). As a predictive marker, ER is currently the most useful test for establishing patient prognosis. In addition, it continues at this time to be the best predictor of patient response to hormone therapies. ER is often ordered in tandem with IHC staining for the progesterone receptor (PR) as well, which provides similar, if less statistically significant predictive information.

28.8 Flow Cytometry

One of the most powerful techniques to make use of the versatility of Abs is flow cytometry [26]. An ever-increasing number of fluorophores are available as reporters, allowing high orders of multiplexing with newer instruments; in some cases, up to 11 different parameters can be recorded simultaneously. These reporter fluorophores absorb the energy provided by laser light at a specific "excitation" wavelength and then emit energy at a different "emission" wavelength. This emitted light is captured by the cytometer using an elegant and elaborate series of optical filters and photomultipliers (Fig. 28.8). In newer cytometers, multiple lasers are used to increase the available excitation spectrum and thus take advantage of the range of available fluorophores; these cytometers therefore require computer-controlled timing of the lasers

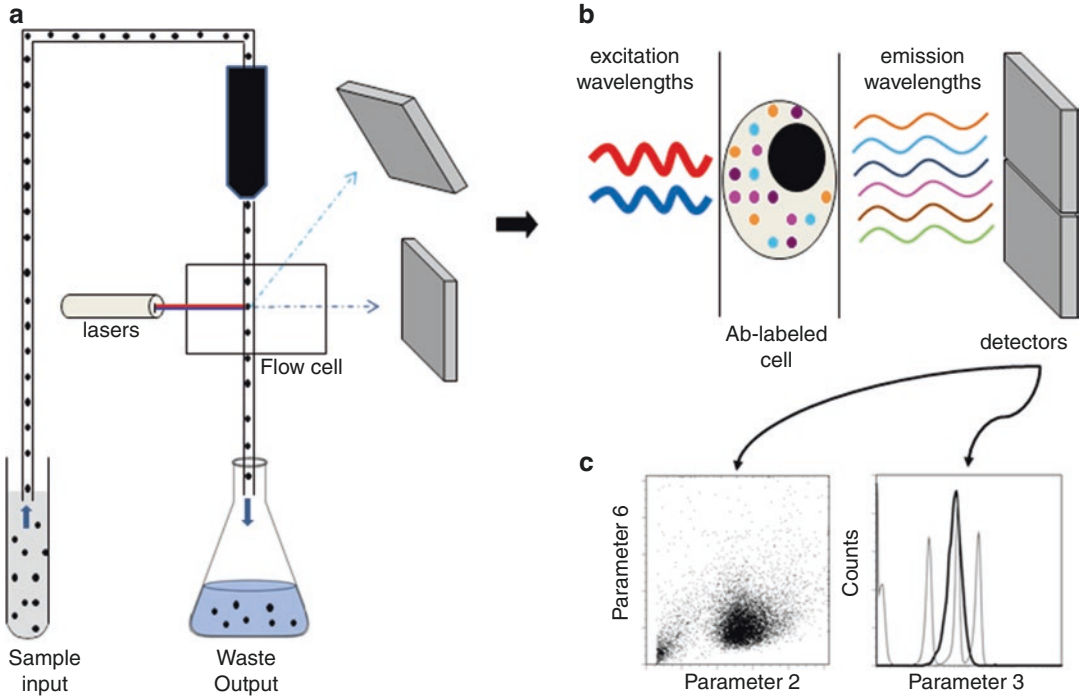

Fig. 28.8 Basic principles of flow cytometry. (**a**) Cells, which have been incubated with fluorophore-conjugated Abs, are drawn from the sample tube into the machine, where they pass the beam(s) of laser light in single file and continue on to a waste receptacle. (**b**) As the cells pass the interrogation point, any bound fluorophores are excited by the laser light. The excited fluorophores then emit light at slightly different wavelengths, which are captured by detectors after passing through a complex system of optics (not shown). (**c**) Software manipulation of the recorded light signals results in data that can be analyzed in many ways and combinations. Each target assayed, or parameter, can be analyzed in tandem with any other in a dot plot (*left*; see Fig. 28.9 for more details) or analyzed singly in the form of histograms and then compared to the histograms of other samples (*right*)

and optical filters. The combination of these numerous reporters with the adaptability provided by streptavidin conjugation of the fluorophores and pairing with biotin-conjugated Abs provides an impressive number of possible analyte combinations that can be studied for a particular cell type or biological fluid.

Initially, and perhaps still predominantly, flow cytometry was used as a platform for the study of intact cells, intended to measure the levels of proteins present on the surface of the cell. The multiplexing ability provided by the range of fluorophores and number of possible parameters allows the analysis of several surface markers simultaneously and has made possible the characterization of the numerous subsets of cell types present in the human body. However, advances in the technology in the last few decades have also allowed the detection and quantitation of both intracellular and soluble proteins using flow cytometry, as well as cellular DNA content, greatly expanding the possibilities afforded by this platform.

The events occurring inside a given cell can provide valuable insights, including whether the cell is activated, in the process of proliferating or in the process of dying under particular conditions. In more traditional cell biology research, these questions would generally be answered using Western blotting or perhaps even ELISAs. Despite being powerful methods which characterize the response of a population of cells to a given condition, both techniques actually offer the average response of the entire population tested. Even the most carefully purified cell preparations generally contain a mixture of different cell types, and this heterogeneous population may very well express the protein of interest at different levels or even exhibit a differential reaction to the conditions

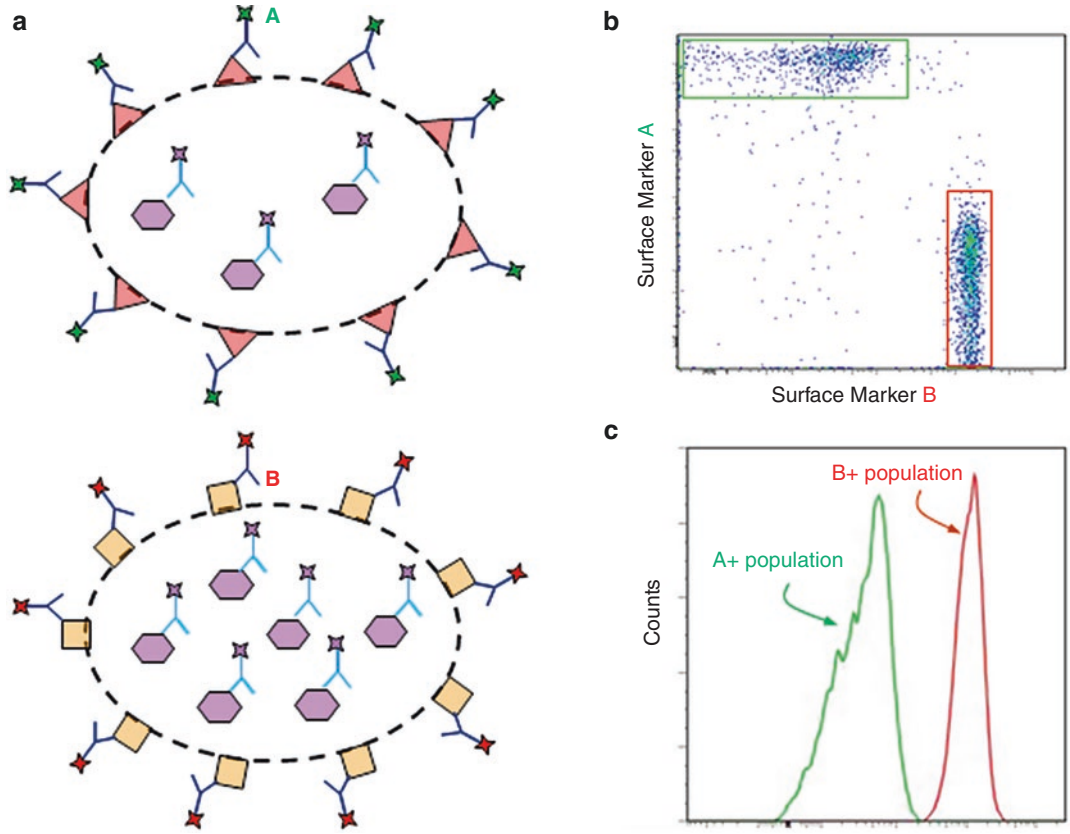

Fig. 28.9 Surface and intracellular cytokine staining of permeabilized cells. (**a**) Mixed cell populations are labeled with Abs specific for surface markers that identify subsets such as different lineages, different activation states, and others. Two different cell subsets are indicated here by binding to two different surface marker Abs, represented here by green ("A," *upper*) and red ("B," *lower*) reporters, which will be seen by the cytometer as different parameters. The cells are then permeabilized to allow passage of Abs across the membrane, represented by the dashed line surrounding the cell. Permeabilized cells are incubated with Abs specific for the intracellular target (*purple* reporter), which will be seen by the cytometer as a third parameter that is the same for all cells. (**b**) After sample acquisition by the flow cytometer, the different cell subsets are differentiated by their expression of the surface markers for which they were stained. Comparison of two parameters is generally done with a dot plot, in which each dot represents a single cell; the dot plot shown here is colored like a heat map to indicate areas of greater and lesser cell density. Surface marker "A" (*green* reporter; y-axis) is present at high levels on the upper cell, while surface marker "B" (*red* reporter; x-axis) is absent, indicating that these cells will fall in the top left corner of the dot plot. Conversely, the lower cell shows high levels of marker "B" and low levels of marker "A," placing them in the lower right corner of the dot plot. These expression patterns create two distinct populations in the dot plot. "Gates" can then be drawn around the populations (*rectangles*), telling the software to consider only those cells falling within the gate in downstream analyses. (**c**) The cells within each gate are analyzed for levels of the intracellular protein (*purple* reporter). Levels are suggested by the intensity of the staining for the third parameter ("Intracellular Marker," x-axis). The diagram in (**a**) depicts the upper cell as having a lower level of the target intracellular protein, and this is reflected by the green histogram falling farther to the left on the scale than the red histogram, indicating a higher intensity of staining in the surface marker B-positive cells than in the marker A-positive cells

being studied. This heterogeneity can make it difficult to interpret results and represents a major roadblock for the study of rare cell types, which are in short supply and often difficult to adequately purify. For these reasons, the ability of flow cytometry to discriminate between lineages by surface marker expression, and combine this with intracellular cytokine staining in preparations of fixed and permeabilized cells, is an important advance in studying intracellular events in mixed populations of cells (Fig. 28.9) [27–32]. These sorts of intracellular cytokine staining protocols have allowed

the study of cell signaling cascades in intact normal cells [33], as well as characterization of aberrant signaling in mutation-bearing cancer cells and in cancer cells exposed to emerging therapies.

Further advances in flow cytometry have even made it possible to mix samples from two different sources, including from two discrete patients or from a single patient pre- and posttreatment, using a "barcoding" method [34]. Each sample is mixed with a different fluorescent dye that emits at a distinct "signature" wavelength, which, when the samples are mixed, allows discrimination of each through sorting based on the detection of the signature. Although a boon for researchers, this technique has yet to become standard practice in clinical oncology diagnostics laboratories. Flow cytometry itself, however, is firmly entrenched, primarily as a valuable tool for hematopathologists, who use flow cytometry to examine the populations of circulating cells in the blood in order to discover subsets of abnormal cells, such as those present in hematological malignancies like leukemias and lymphomas. Flow cytometry panels for differential diagnosis of leukemia/lymphoma can contain upward of 20 cell surface markers, and algorithms characterizing the patterns of these markers on the surface of cell populations in the blood help pathologists identify the particular type of leukemia or lymphoma present.

28.9 Bead-Based Assays

As with the detection of intracellular proteins, the study of soluble proteins present in bodily fluids and in cell culture supernatants was traditionally performed by immunoblots or ELISA. But again, as with intracellular proteins, flow cytometry now represents an additional platform for the detection of soluble proteins through the use of bead-based assays. In a design that combines the best features of IP and sandwich ELISAs, Abs are coated onto microbeads rather than plates, and these beads can then be incubated with the sample fluid putatively containing the protein of interest. Following capture by the beads, the protein can then be bound by a specific detection Ab. As with sandwich ELISAs, the bead-based assay can use up to four Abs, but again, fewer Abs are generally preferred (Fig. 28.10). One successful

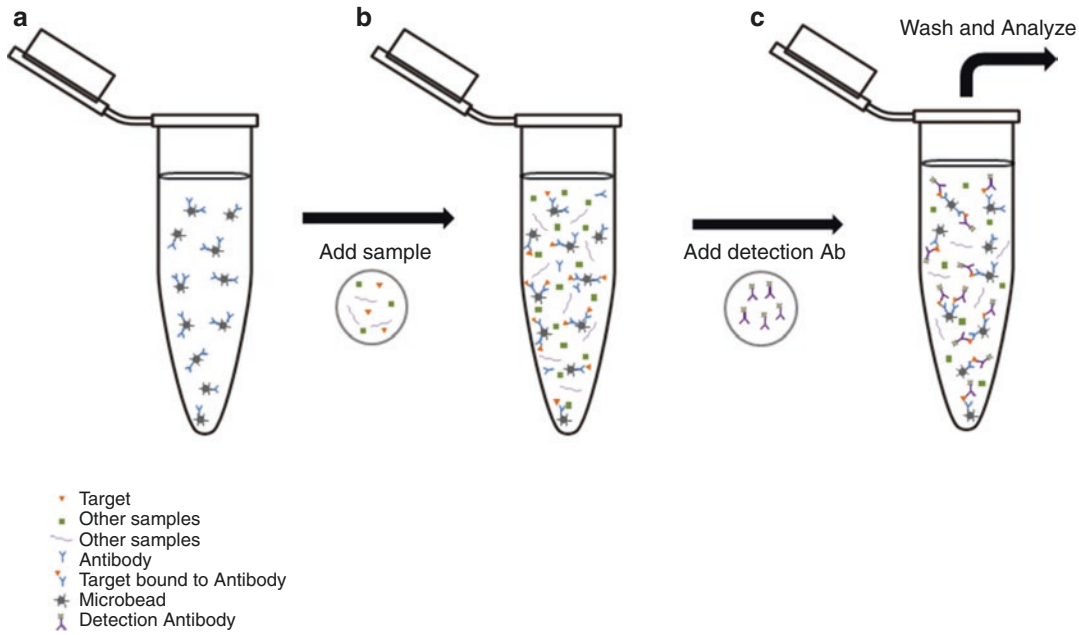

Fig. 28.10 Bead-based flow cytometry assays. (**a**) Capture Abs are coated on microspheres. (**b**) The beads are incubated with proteins in solution (e.g., lysate, cell culture supernatant, or plasma) and bind only the target protein. (**c**) The target protein is bound by fluorophore-conjugated detection antibody, the sample is washed to remove unbound detection antibody, and the beads are analyzed by flow cytometry

application of this technology is the detection of soluble proteins released into the bloodstream by dying leukemia cells [35–37]. Despite the similarities of the technique to the sandwich ELISA, the bead-based assay benefits from greater multiplexing possibilities, including the Luminex and cytometric bead array technologies.

As stated above, the most advanced cytometers can measure upward of 11 or more parameters. This often presents calibration issues due to the slight spectral overlap of the fluorophores available. One approach to avoiding this problem is to use a single fluorophore to measure different analytes, rather than a large number of different "colors." The cytometric bead array (CBA) makes use of beads of different sizes, one size for each of the different capture antibodies to be used. All detection antibodies can then be conjugated to the same reporter fluorophore, because the discrimination between the different proteins detected will be provided by the size of the bead, which is one of the parameters measured as the particle flows past the cytometer's detector. These different bead sizes will result in easily distinguishable populations and thus analytes, as shown in Fig. 28.11a, while the level of protein captured and detected by a given antibody pair will be quantified by the intensity of the reporter's fluorescence (not shown). In this way, the CBA assay allows the measurement of multiple analytes side by side in the same sample.

Beyond just determining the relative amounts of protein captured by the CBA assay, however, researchers have applied a standard curve to the assay, allowing the quantitation of detection Ab molecules bound to a bead. Each experiment includes a tube containing four groups of beads, each with a different known level of bound reporter fluorophore. The data derived from this sample are used to generate a standard curve, plotting the known number of reporter molecules against the mean fluorescence intensity (MFI) measured by the cytometer. Using this curve and the MFI value recorded for a given sample, the number of bound reporter-conjugated detection Abs can be calculated. This technique provides an even more accurate quantitation of the level of the target protein present in the matrix and can even be applied to the more traditional nonbead-based flow cytometry methods of intracellular and surface protein detection.

The Luminex technology makes use of a combination of the advantages of both microbead assays and flow cytometry, creating a method ostensibly able to analyze up to 100 targets in one well (see Luminex Corporation for examples). Luminex makes use of polystyrene microspheres impregnated with carefully controlled levels of both red and infrared dyes. These different titrations create different color signatures for each population of beads, much like the barcoding technique described above (Fig. 28.11b). These different beads can then be coated with discrete capture Abs, mixed together, and incubated with the biological matrix. Following capture of the target proteins, detection Abs are added, all conjugated to the same reporter fluorophore as in the case of the CBA assay. The data are then collected using the basic principles of flow cytometry, in that the dyes inside the beads are excited with a red laser to reveal the "signature" identifying which target should be captured by that particular bead, and a green laser is used to excite the reporter fluorophore to allow the measurement of the levels of protein actually captured [38]. The multiplexing capabilities of this platform provide the potential for Luminex to provide as much information about a sample as some types of antibody microarrays or multispot ELISAs (see below) and is therefore currently more often used in a cancer research or clinical trial setting.

28.10 Antibody Arrays

The antibody microarray makes possible the detection of a very large number of analytes in a complex sample, similar to its predecessor, the DNA microarray [39, 40]. Most antibody microarray formats are essentially ELISAs on a necessarily grand scale, as shown in Fig. 28.12. These arrays are valuable both for basic research and in the search for diagnostic and prognostic markers of cancer. A small volume of biological material can yield a substantial amount of information using this technique, and often of greater importance, relationships, and patterns within the data

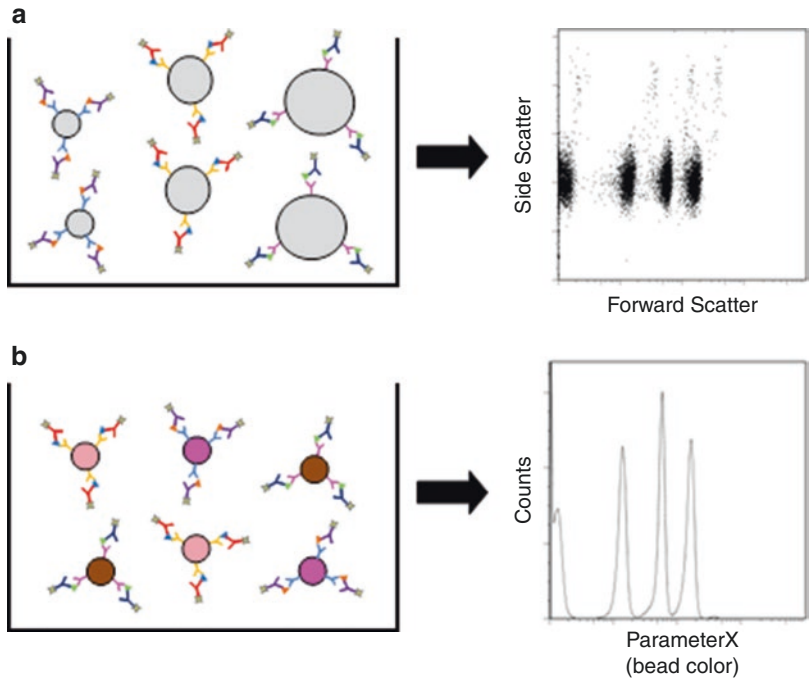

Fig. 28.11 Cytometric bead array and Luminex technologies. (**a**) The CBA platform consists of the Abs specific for each target being conjugated to beads of a different size. The beads are incubated with the sample at the same time, allowing capture of the target proteins. The beads are then incubated with detection Abs for each target, all conjugated to the same fluorophore (*left*). When analyzed, the different bead sizes are recognized by the cytometer via the forward and side scatter parameters and are identifiable as discrete populations that can be analyzed separately via gating (*right*). (**b**) Luminex technology makes use of beads of the same size which have been impregnated with dyes of slightly different wavelengths. Each set of beads is coated with a different capture Ab, incubated with sample to capture target protein, and detected with a fluorophore-conjugated detection Ab (*left*). The cytometer-based analysis instrument detects the slight variations in the color of the bead (Parameter X), creating discrete populations based on bead color which can be gated (*right*). The reporter fluorophore intensities within each population can then be analyzed, yielding information about the concentration of each target analyte

can be recognized and characterized in a single snapshot experiment. Antibody microarrays can be designed in a number of different formats, including the variable of whether it is protein or antibody bound to the array itself.

In its infancy, antibody array technology most closely paralleled that of DNA microarrays by spotting the surface of the array with probes consisting of mAbs. Universally labeled proteins are then incubated with the array, and the captured protein is identified by its binding position on the array (Fig. 28.12a) [39]. The protein-labeling process includes either direct labeling with reporters or indirect detection using biotin or digoxigenin. Through the use of multiple reporters, it is also possible to compare two samples by incubating them together in a classic competition assay (Fig. 28.12a). This antibody array format is generally referred to as a direct array and is the best option for assaying truly large numbers of analytes in a single array, as the only major limitations are space and the availability of specific antibodies for the desired targets. To date, most arrays offered commercially contain analytes numbered in the hundreds. The primary technical hurdles encountered when using direct Ab arrays include limited specificity and sensitivity and filtering out background signal. In addition, there is

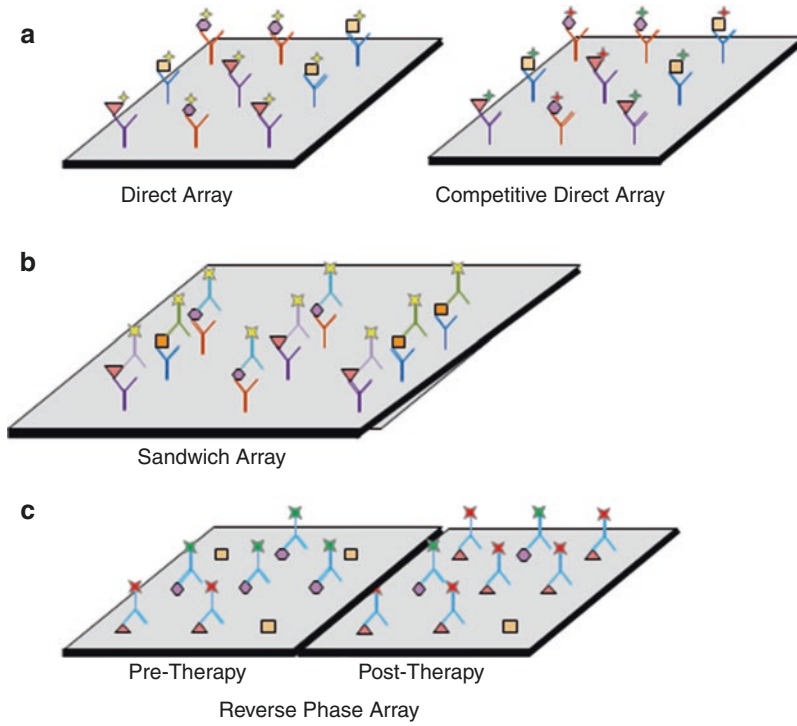

Fig. 28.12 Antibody array formats. (**a**) Direct antibody arrays involve the spotting of specific Abs onto a surface. The array is then incubated with reporter-labeled proteins (*left*). The identity of a target protein that binds to the array is determined by matching the location of the signal to the known layout of the Abs. In a competitive direct array, the proteins in two separate samples are labeled with distinct reporters (*red* and *green*) and incubated with the array simultaneously (*right*). The target proteins will compete for binding to the Abs on the array, and the relative signal intensities will indicate which sample contained greater quantities of each protein assayed. (**b**) The sandwich antibody array is highly similar to the sandwich ELISA depicted in Fig. 28.5b, simply with a large number of capture Ab specificities combined into a single assay and requiring one small volume of analyte. (**c**) The reverse-phase array consists of the proteins in a sample being adsorbed to the array surface, followed by detection with reporter-conjugated Abs as in Fig. 28.6a. Although the number of targets that can be analyzed simultaneously is limited here, the value of the reverse-phase array is that it allows multiple samples to be analyzed side by side. The example represented here is pre- and posttherapy, and the changes in protein expression resulting from the treatment are clear

always the concern that the direct labeling of the proteins may interfere with recognition of the protein by the Ab due to the physical masking or alteration of the epitope.

With these limitations in mind, additional antibody microarray formats were developed to include both capture and detection antibodies (Fig. 28.12b) [41]. Specificity is greatly enhanced when relying on the recognition of the target protein by two different Abs for detection, as one source of background is minimized. In addition, the problem of possible epitope masking is also solved by removing the necessity of labeling the proteins. One limitation of this sandwich approach, in both basic ELISAs and the antibody array, is the occasional lack of good matched antibody pairs. Another concern is the problem of cross-reactivity among the detection antibodies, which generally serves to limit the number of possible targets when using a sandwich microarray in contrast to a direct array. However, as the targets of greatest interest or benefit for a given model or cancer type are determined, highly customized arrays are being developed for diagnostic, prognostic, and research uses. For example, some arrays are designed to study groups of putative or known breast cancer markers, while others are used to screen the effects of drug candidates on their target cells.

There is also, as might be expected, an antibody microarray design in which it is the protein mixture that is immobilized on the surface of the array (Fig. 28.12c) [41]. These protein spots can then be probed with reporter-conjugated-specific Abs. This reverse-phase array allows the immobilization of multiple samples' proteins on a single array, providing side-by-side analysis, and simplifies the analysis of insoluble proteins. This assay format is also plagued by nonspecific interactions, however, and restricted to a smaller number of detection Abs by the limited reporter multiplexing options. In spite of these technological restrictions, the reverse-phase Ab array is also a valuable tool for clinicians and researchers alike.

28.11 Concluding Remarks

Many of the most spectacular breakthroughs in the field of cancer diagnostics in recent years have been on the molecular side of the coin, with the advent of next-generation or advanced sequencing leading the charge. In the shadow of such advances, many of the techniques described in this chapter tend to look outdated and simplistic. Despite this (likely unfair) comparison, many of the diagnostic assays based on the platforms discussed herein continue to be the foundation of cancer patient workups and represent many of the gold standards in diagnosis, prognosis, and treatment decision-making. One chief reason for the importance of these assays is that molecular assays do not tell the whole story. For example, it has been amply demonstrated that the level of mRNA, though often useful as a marker in and of itself, does not always directly correlate to the level of the protein that will be translated. Similarly, molecular assays reveal nothing about the posttranslational modifications that can dictate subcellular localization or activation of a protein, which can be a more telling measure of aberrant function than the sequence of the gene. The ability to study the actual protein of interest itself is an important aspect of learning as much as possible about the malignancy, to better fight and defeat it. To this end, researchers have harnessed the power of the immune system to create clever tools for the study of proteins via the exquisite sensitivity of Abs, and these tools continue to be absolutely invaluable in the diagnostic workup of cancer patients.

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Immunohistochemistry of Cancers

29

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Contents

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29.1 Introduction

Immunohistochemistry (IHC) is the art of using antibodies (Abs) to detect specific antigens (Ags) in tissues. Histopathologic evaluation of diseases has been altered and enhanced by the advent of IHC and some sophisticated techniques have been replaced by IHC due to its easy and versatile immunohistochemical techniques. Of course, disorganized application of IHC could be misleading.

Immunohistochemistry is based on specific Ab–Ag interactions. The Abs which are used to detect Ag(s) are called primary Abs. Primary Abs are linked to enzymes (main part of chromogenic system) via another Ab called link Ab. This linkage to enzymes is mediated by polymers or some molecules such as streptavidin–biotin complexes. Peroxidase is the enzyme mostly used in immunohistochemistry. Alkaline phosphatase is also used (but less frequently). Some mechanisms are shown in Fig. 29.1.

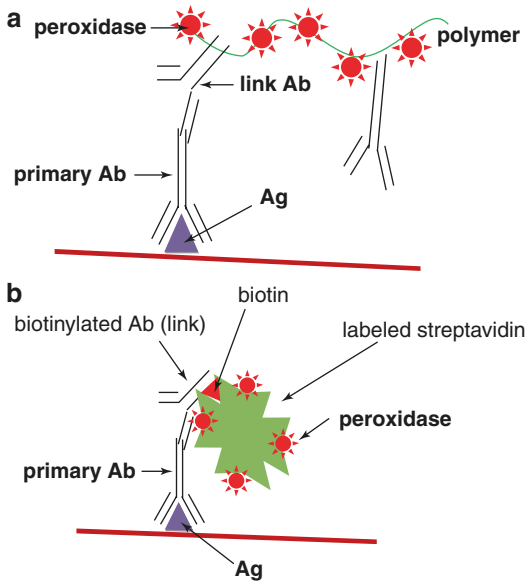

Fig. 29.1 Schematic mechanisms of two immunohistochemistry methods. (**a**) Secondary antibodies and enzymes link to polymer molecule. (**b**) Biotinylated secondary antibody and labeled streptavidine

Immunohistochemistry has wide application including research uses, diagnostic purposes, prognostic and therapeutic aims. IHC is a nice technique for tracking of proteins and haptens, so it is used to define expression of specific genes at the level of proteins. It is also very useful in diagnostic pathology including definition of cellular lineage (epithelial, vascular, lymphoid, etc.) or subtyping of some specific lesions and malignancies such as malignant lymphomas. Prognostic and therapeutic applications have gradually become widely popular such as the definition of hormone receptor status of breast cancer (ER, PR, and AR) and oncogene products (e.g., Her2, EGFR, and c-kit) which could be a part of guidelines for targeted therapy of the tumors.

29.2 Immunohistochemistry of Skin Tumors

29.2.1 Markers of Normal Skin

Skin tissue is composed of epidermal and adnexal components as well as mesenchymal dermal components. All epithelial cells in epidermis, folliculosebaceous unit, and sweat glands reveal pankeratin markers such as AE1/AE3 (Fig. 29.2a). Keratinized squamous cells (SC) and proliferative keratinocytes express cytokeratin (CK) 6/16, non-keratinized SC reacts with CK4/13 and basal keratinocytes exhibit reactivity for CK 5/14/15 (Fig. 29.2b). Squamous cells in palm and sole are reactive for CK 1/9/10 [1, 2]. Eccrine and apocrine glands comprise sweat structures of the skin. Normal eccrine glands show reactivity with CD7, CD20 (Fig. 29.2c, d), CEA, and S100, while apocrine glands exhibit immunostaining for CEA and GCDFP15 [3, 4]. Sebaceous glands exhibit reactivity for CK10 as well as EMA rimming cytoplasmic lipid vesicles (Fig. 29.2e) [5]. Normal melanocytes express S100, HMB45, and MART-1/melan-A, but do not react with tyrosinase [6]. Langerhans cells are stained with CD1a (Fig. 29.2f), S100, langerin, and CD31 [7]. Displaying neurotactile differentiation, Merkel cells of normal skin are reactive for CK20, MOC-31, neurofilament, and CD56 [8–10]. Markers of the normal epidermal components are depicted in Fig. 29.3. The immunoprofile of normal skin components and respective cancers is summarized in Table 29.1.

29.2.2 Epithelial Tumors

Squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) are derived from spinous layer and basal layer of the epidermis, respectively. Well-differentiated SCC expresses high molecular cytokeratin, while those with poor differentiation express low molecular cytokeratin. Cytokeratin, p63, and vimentin are present in the sarcomatoid variant of SCC [11]. EMA, one of human milk fat globule proteins not expressed in normal keratinocytes, is expressed on malignant squamous cells. Basal cell carcinoma expresses BerEP4 (Fig. 29.4), but do not demonstrate reactivity with EMA and p63, distinguishing it from SCC [12].

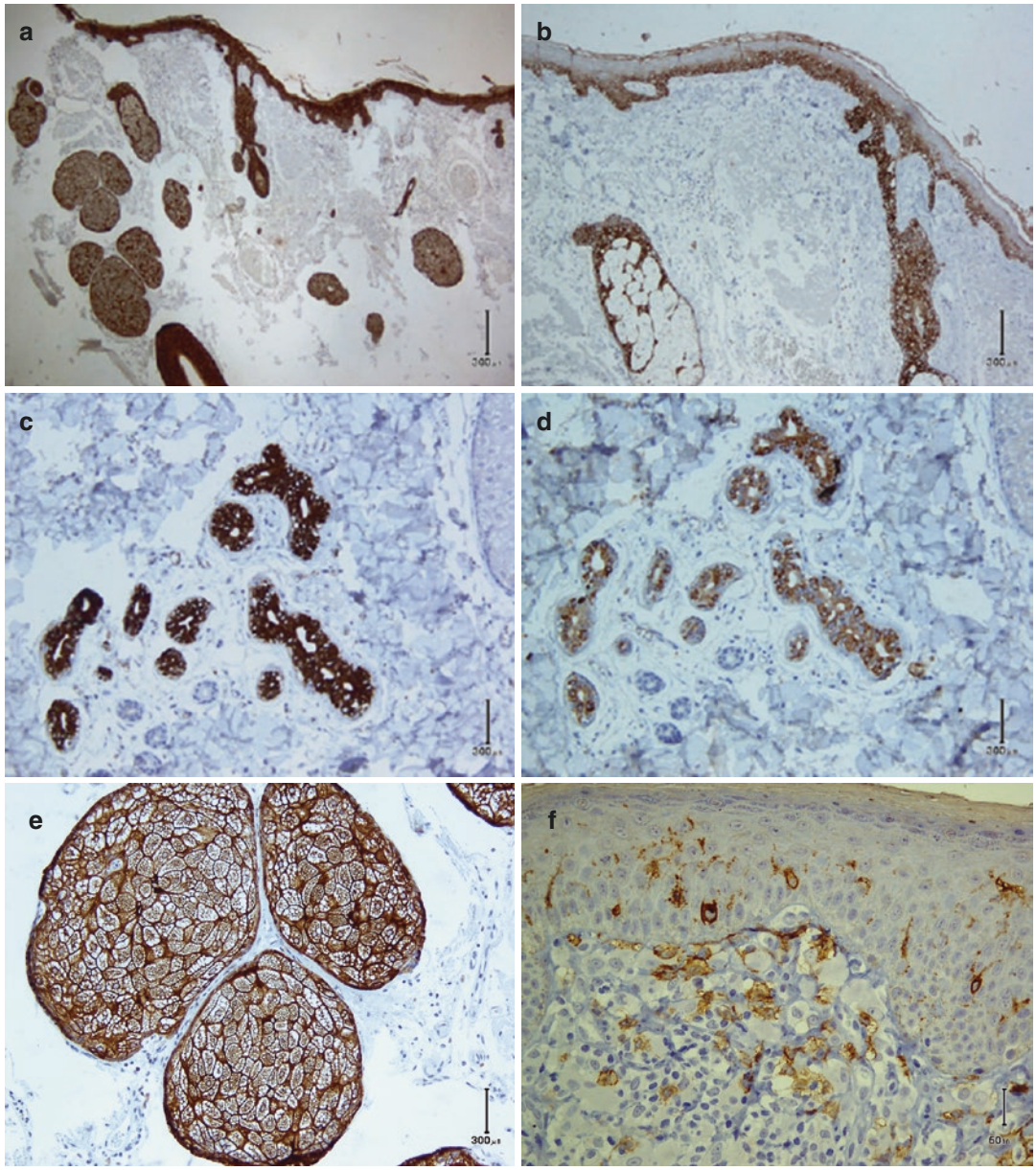

Fig. 29.2 Normal skin. (**a**) Pankeratin of AE1/AE3 stains epidermis, folliculosebaceous unit epithelium, and sweat glands. Basal keratinocytes is highlighted by CK5 (**b**). Sweat glands are immunostained by CK7 (**c**) and CK20 (**d**). EMA (**e**) reacts with sebaceous glands rimming cytoplasmic vacuoles and CD1a highlights dendritic Langerhans cells in epidermis (**f**)

29.2.3 Sweat Gland Tumors

Malignant eccrine tumors are distinct from benign eccrine tumors by displaying reactivity with EMA. Eccrine tumors display CEA, CD15, and p63, which are also common with apocrine tumors. Differentiating markers of apocrine tumors are TAG-72 (CA72.4) and GCDFP15 (Fig. 29.5), which are not expressed on eccrine tumors [13]. S100 is demonstrated in 50% of eccrine tumors, but not in apocrine tumors. A remaining challenge is distinguishing primary

eccrine carcinoma from metastatic carcinoma by immunoprofile of CK5/6 and p63, which are positive in eccrine carcinoma, but not in metastatic carcinoma [14]. Paget disease is an intraepidermal extension of neoplastic cells into the epidermis, which shares similar histopathologic features with malignant melanoma and Bowen disease. Immunohistochemistry study can be a helpful method in differentiating these tumors as denoted in Table 29.2 [15]. CK20 and GCDFP-15 are useful markers in distinguishing primary and secondary perianal Paget's diseases, respectively [16].

29.2.4 Trichogenic Tumors

Tumors with trichilemmal differentiation display reaction with CK14/15/19, BerEP4, and p63 but not react with EMA (except proliferating trichilemmal tumor), CEA, S100, CD15, CA72.4, HMB45, and GCDFP15 [3]. Trichilemmal carcinoma displays reactivity with CEA and S100 and proliferating trichilemmal carcinoma (malignant proliferating tumor) shows reactivity with EMA and CD34 [18]. Desmoplastic trichoepithelioma shares histopathologic similarities with infiltrating BCC and microcystic adnexal carcinoma. The immunoprofile of these tumors are demonstrated in Table 29.3.

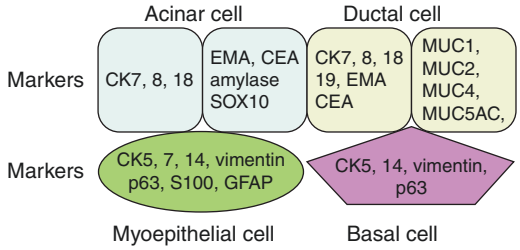

Fig. 29.3 Immunohistochemistry antibodies in schematic normal epidermal components

29.2.5 Sebaceous Tumors

Sebaceous tumors exhibit reactivity with CK5/14/15, CK8/18, EMA, CD15, anti-adipophilin (ADP), and androgen receptor. CK15 is positive in sebaceoma but does not exhibit reactivity in sebaceous carcinoma [22]. Sebaceous tumors do not express CEA, S-100, CA72.4, and GCDFP-15 in comparison with sweat gland tumors, which are positive for these markers [4, 23]. Sebaceous carcinoma is differentiated from BCC by showing reactivity for EMA (Fig. 29.6) and negative reaction to BerEP4, vice versa of BCC [24]. Proliferating markers are good markers to differentiate sebaceous adenoma from sebaceous carcinoma (Table 29.4).

29.2.6 Melanocytic Tumors

Being a sensitive but a nonspecific marker of melanoma, S100 is a calcium-binding protein given its name because of solubility in 100% saturated ammonium sulfate solution. Other S100-positive tumors include undifferentiated carcinoma, nerve sheath and glial tumors, adipose tumors and histiocytic and Langerhans cell proliferations [27, 28]. Considering as highly specific marker of melanocytes, the gp100 group include HMB-45

Table 29.1 Immunoprofile of normal epidermis, folliculosebaceous, and sweat gland structures in comparison with respective tumors

Cell	Antibodies	Tumor	Markers
Keratinocyte	CK6/16	Squamous cell carcinoma	EMA, p63
Basal keratinocyte	CK5/14/15	Basal cell carcinoma	BerEP4
Eccrine cell	CK7, CK20, CK5/14, CK1/10, CEA, S100	Eccrine carcinoma	EMA, CEA, CD15, p63, S100
Apocrine cell	CEA, GCDFP15	Apocrine carcinoma	EMA, CEA, CD15, p63, CA72.4, GCDFP15
Trichogenic cell	CK14/15/19	Trichilemmal carcinoma	CEA, S100
		Proliferating trichilemmal carcinoma	EMA, CD34
Sebaceocyte	CK5/14/15, CK8/18	Sebaceous carcinoma	EMA

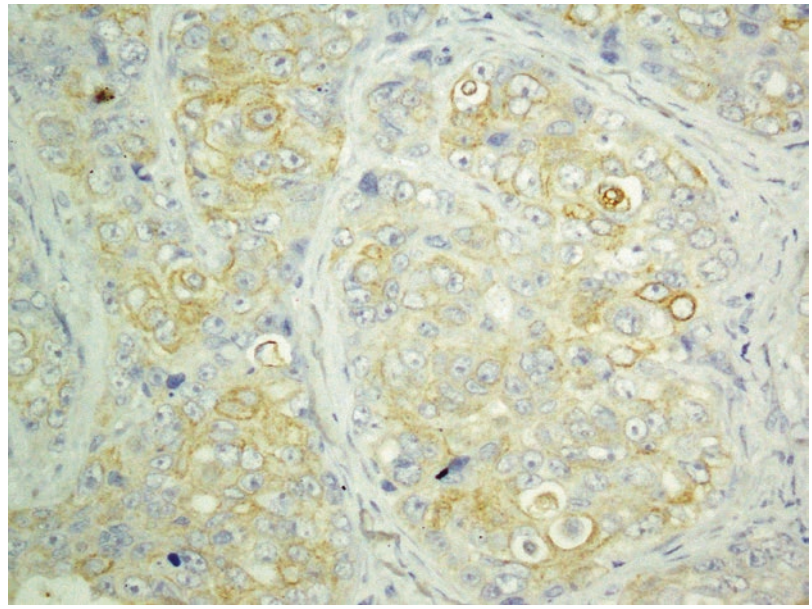

Fig. 29.4 Immunoreaction of basal cell carcinoma with BerEP4

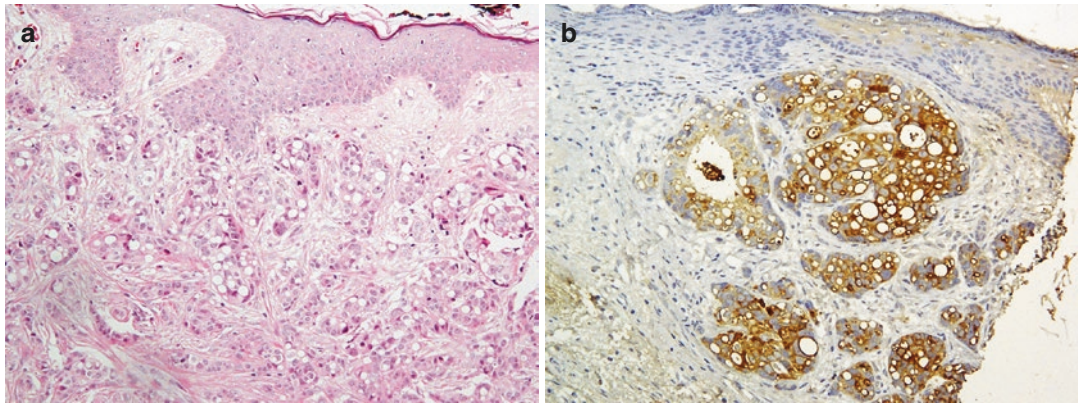

Fig. 29.5 Primary skin apocrine carcinoma (**a**) immunostained by GCDFP15 (**b**)

Table 29.2 Immunophenotype of mammary and extramammary Paget disease (PD), Bowen disease, and malignant melanoma

Makers	Mammary PD	Extramammary PD (apocrine carcinoma in situ)	Bowen disease (SCC in situ)	Melanoma (in situ)
CK7	+	+	–	–
CEA	+	+	–	–
CAM5.2	+	+	–	–
GCDFP15	+	+	–	–
MUC1	+	+	–	–
MUC5AC	–	+	–	–
CA15-3	+	–	–	–
CA72.4	–	+	–	–
KA-93	–	+	–	–
CK5/6	–	–	+	–
S100/HMB45/MART	–	–	–	+

References: [15–17]

Table 29.3 Immunoprofile of desmoplastic trichoepithelioma (DTE), infiltrating basal cell carcinoma (IBCC), and microcystic adnexal carcinoma (MAC)

Tumor	DTE	IBCC	MAC
Panel antibodies	EMA, CK5/6, CD10 (stroma), CK15, CK20, p63, Bcl-2, BerEP4	CK5/6, CD10 (epithelial), p63, Bcl-2, BerEP4, stromelysin-3, p53	EMA, CK7, Ck5/6, CK15, p63, SMA

References: [19–21]

and MART-1/melan-A with 60% and 80% sensitivity, respectively. Melanoma Antigen Recognized by T-cells-1 (MART-1) is a protein, which serves as a potential target for cytotoxic T lymphocytes recognized by two monoclonal antibodies (mAbs), A103, and melan-A [29]. Desmoplastic/spindle cell variant of melanomas do not show reactivity with HMB45 and MART/melan-A. Instead, these melanomas are more reactive with S100, p75-NGF-R, and tyrosinase [30]. Small-cell melanoma is another variant of the melanoma, which could be distinguished from other small-cell undifferentiated tumors of the skin and subcutaneous tissue by panel Abs (Fig. 29.7). The immunoprofiles of these tumors are summarized in Table 29.5.

29.2.7 Prognostic Markers of Melanoma

Detection of BRAF p. V600E mutation by immunohistochemistry in melanomas could be used as a first step to identify patients with melanoma as candidates for BRAF inhibitors. Displaying by immunohistochemistry, melanoma progression is correlated with MERTK expression: highest in metastatic melanomas, followed by primary melanomas and nevi [33, 34]. Other prognostic markers correlated with melanoma progression and prognosis include MIB-1 (Ki-67), Bcl2, p53, p16, cyclin-D1, cyclin-D3, osteopontin, NM23, E-cadherin, beta-catenin, Wnt5a/frizzled, Cdc42, and CXCR4 [35–41]. Novel makers including PD-L1, RORα, and RORγ have also been introduced recently, but further studies are needed to prove their role in malignant melanoma [42–44].

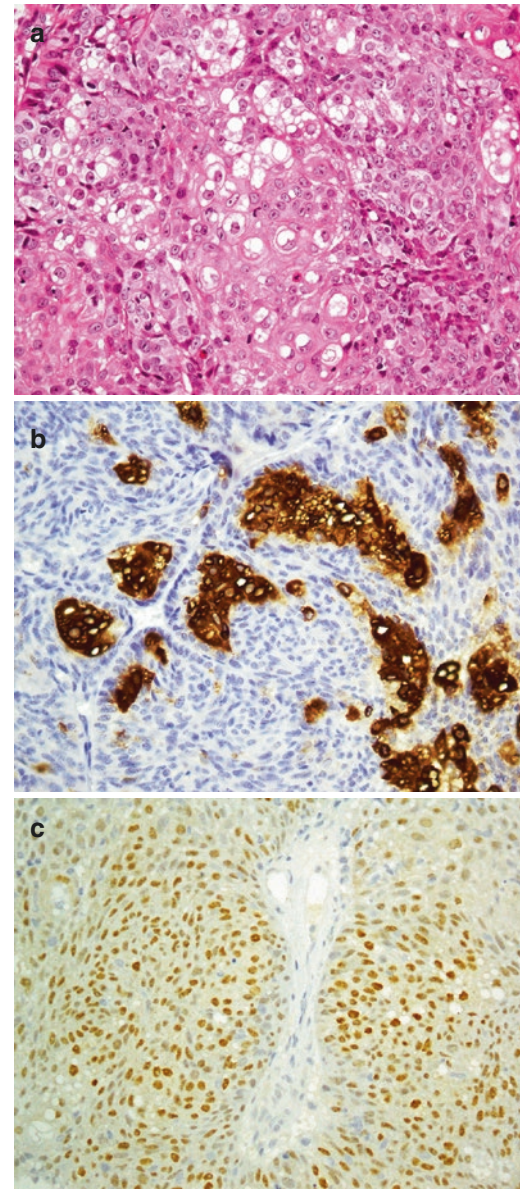

Fig. 29.6 Sebaceous carcinoma (**a**). Sebocytes are stained with EMA (**b**). Nuclear reactivity of tumor cells for androgen receptor (**c**)

Table 29.4 Immunoprofile of sebaceous adenoma (SA) and sebaceous carcinoma (SC)

Tumor	Ki67	p53	Bcl2	P21
Sebaceous adenoma	10%	11%	56%	34%
Sebaceous carcinoma	30%	50%	7%	16%

References: [25, 26]

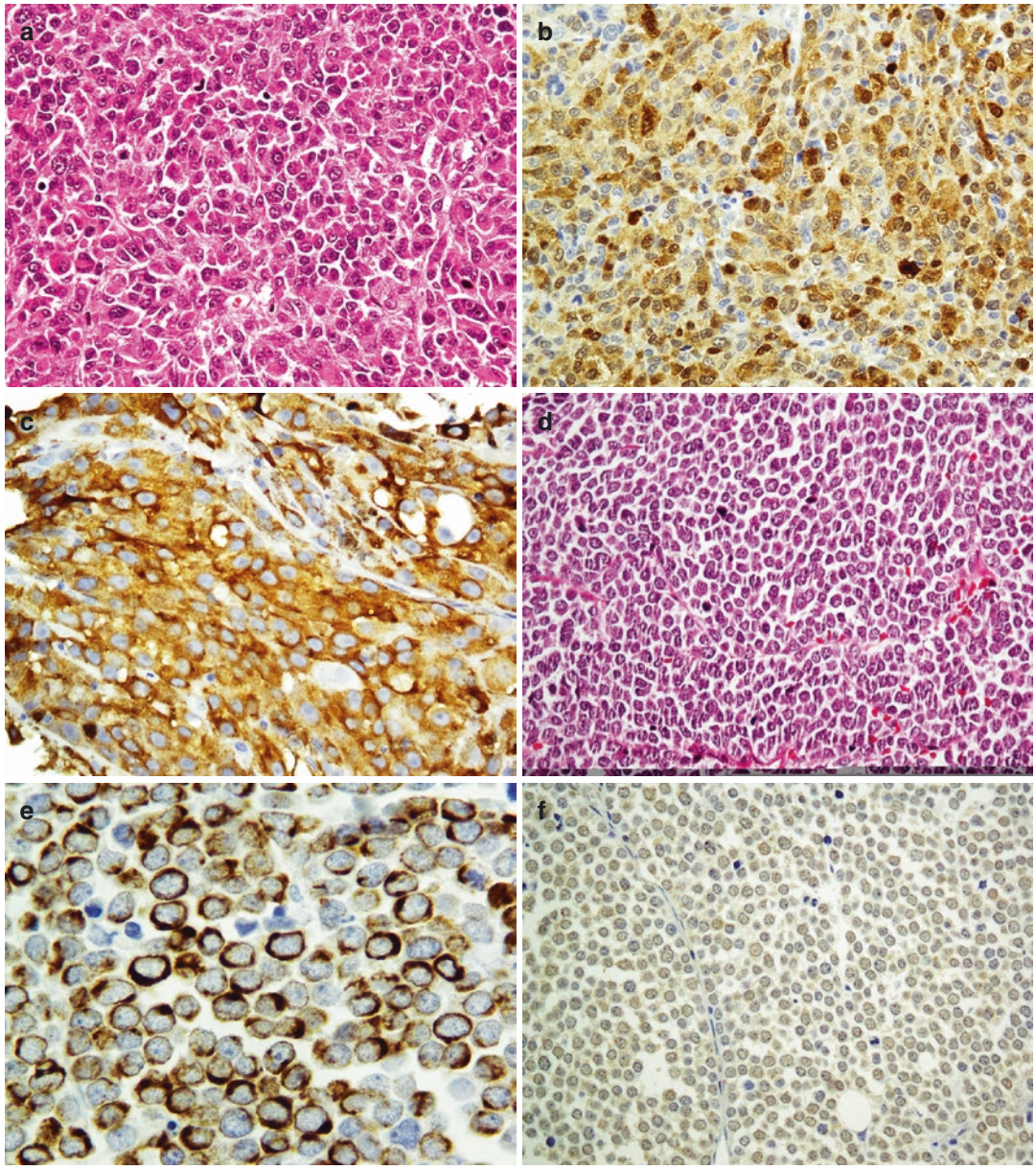

Fig. 29.7 Small round cell tumor in skin. Malignant melanoma (**a**) reacts with S100 (**b**) and Melan-A (**c**) antibodies. Merkel cell carcinoma (**d**) immunostained by CK20 as paranuclear dots (**e**) and shows weak reaction with CD99 (**f**)

29.2.8 Specific Mesenchymal Tumors of the Skin

Mesenchymal tumors are discussed in soft tissue tumors, but some tumors which are more seen in skin are discussed here. Kaposi sarcoma, which originates from endothelial cells, is an intermediate malignant potential vascular tumor of the skin positive for a highly sensitive and specific Ab called HHV8-Latent Nuclear Antigen-1 [45]. Dermatofibrosarcoma protuberance is an intermediate tumor of fibrohistiocytic cell origin which is diffusely positive for CD34 (Fig. 29.8) and negative for factor XIIIa separate from

Table 29.5 Immunopanel of small-cell melanoma (SCM), Merkel cell carcinoma (MCC), small-cell squamous carcinoma (SSCC), small-cell eccrine carcinoma (SEC), peripheral neuroectodermal tumor/extraskeletal Ewing's sarcoma (PNET/ES), lymphoma, rhabdomyosarcoma (RMS), and metastatic pulmonary small-cell carcinoma (MPSC)

Panel antibodies	SCM	MCC	SSCC	SEC	PNET/ES	Lymphoma	RMS	MPSC
S100/HMB45/MART	+	–	–	–	–	–	–	–
CK20/CD56/SYN/CGN	–	+	–	–	–	–	–	–
CK/EMA	–	–	+	+	–	–	–	+
CD15/MOC31/TAG-72	–	–	–	+	–	–	–	–
CD99/CD56/SYN/CGN	–	+	–	–	+	–	–	–
LCA/CD3/CD20	–	–	–	–	–	+	–	–
DES/MSA/MYG	–	–	–	–	–	–	+	–
CEA/TTF-1	–	–	–	–	–	–	–	+

Note: *CGN* chromogranin A, *DES* desmin, *MYG* myogenin, *MSA* muscle-specific antigen, *LCA* leukocyte common antigen, *SYN* synaptophysin
References: [27–32]

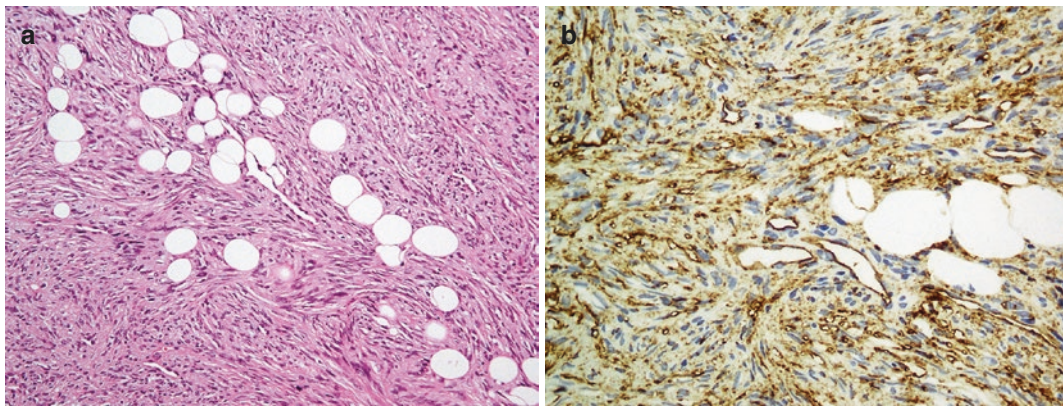

Fig. 29.8 Dermatofibrosarcoma protuberans. Spindle fibrohistiocytic cells, entrapping subcutaneous fat tissue (**a**) highlighted by CD34 (**b**)

dermatofibroma which is in reverse of DFSP (CD34−, factor XIIIa+) [46]. Considering it as a superficial variant of malignant fibrous histiocytoma, atypical fibroxanthoma is a fibrohistiocytic tumor exhibiting reactivity with vimentin, CD10, and CD99 (Fig. 29.9) [47]. Among tumors with smooth muscle differentiation, leiomyoma and leiomyosarcoma are reactive for SMA, desmin, and caldesmon similar to extracutaneous equivalents [48–50]. Neurothekeoma (NTKs) is a distinctive neoplasm of the skin showing schwannian and neuroectodermal differentiation which typically labels with S100 (conventional variant), CD99, and NKI-C3 (cellular variant) [51].

29.3 Immunohistochemistry of Head and Neck Tumors

29.3.1 Tumors of the Nasal Cavity and Paranasal Sinuses

Tumors of nose and paranasal sinuses can be categorized in two groups of small-cell carcinomas and undifferentiated carcinomas. Small-cell carcinomas of nasal cavity and paranasal sinuses include olfactory neuroblastoma (ONB), melanoma, lymphoma, rhabdomyosarcoma, small-cell neuroendocrine carcinoma, and ES/PNET (Table 29.6). Undifferentiated carcinomas include sinonasal undifferentiated carcinoma,

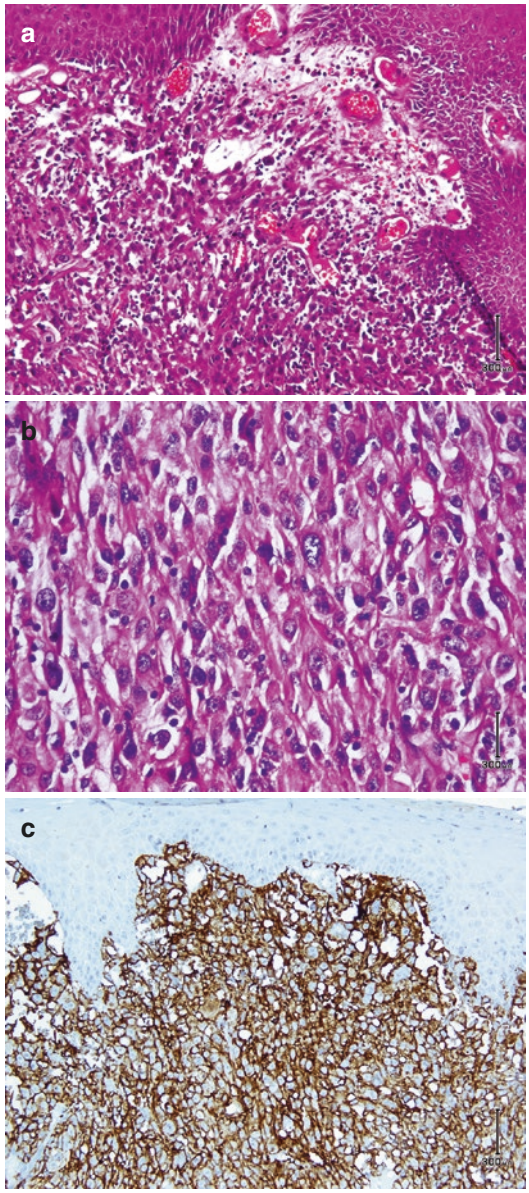

Fig. 29.9 Atypical fibroxanthoma. Atypical pleomorphic cells with vesicular nuclei in dermis (**a**, **b**) are immunostained by CD10 (**c**)

undifferentiated nasopharyngeal carcinoma (Fig. 29.10), and undifferentiated neuroendocrine carcinoma [57, 58]. All poorly differentiated and undifferentiated carcinomas express cytokeratin [52]. Undifferentiated nasopharyngeal carcinoma reacts with EBV and undifferentiated neuroendocrine carcinoma is positive for neuroendocrine markers and S100 [59]. NUT midline carcinoma (NMC) is an aggressive tumor with translocation of *NUT* (Nuclear protein in testis) gene resulting in the formation of *BRD4-NUT* fusion gene. Recently, new mAbs against the NUT Ag have been designed which will improve the diagnosis of NMC [60]. Immunohistochemistry of poorly differentiated and undifferentiated carcinomas are denoted in Table 29.7.

29.3.1.1 Theranostic Application

In olfactory neuroblastoma, immunoreactivity with Bcl-2 may predict response to neoadjuvant chemotherapy and seems to be associated with worse survival [63] (Fig. 29.11).

29.3.2 Tumors of Larynx, Nasopharynx, and Oropharynx

Squamous cell carcinoma (SCC) is the most common malignancy in the head and neck. Typically, head and neck SCCs are positive for cytokeratin cocktails, AE1/AE3, and pancytokeratin. Human papilloma virus (HPV) is detected in some SCCs of oropharynx and known as a risk factor of head and neck SCCs [64, 65]. Being as a variant of SCC, basaloid squamous cell carcinoma (BSCC) is another tumor with predominance of basaloid components. Basaloid

Table 29.6 Immunohistochemistry of small-cell carcinomas of nasal cavity: olfactory neuroblastoma (ONB), rhabdomyosarcoma (RMS), Ewing sarcoma/peripheral neuroectodermal tumor (ES/PNET), and small-cell neuroendocrine carcinoma (SNEC)

Tumor	ONB	Melanoma	Lymphoma	RMS	SCC	ES/PNET	SNEC
Immunoreactive markers	SYN	HMB45, S100, vimentin	LCA, vimentin	Desmin, Myogenin, vimentin	AE1/AE3, EMA, SYN	CD99, SYN	Cytokeratin, neuroendocrine markers

References: [50, 52–56]

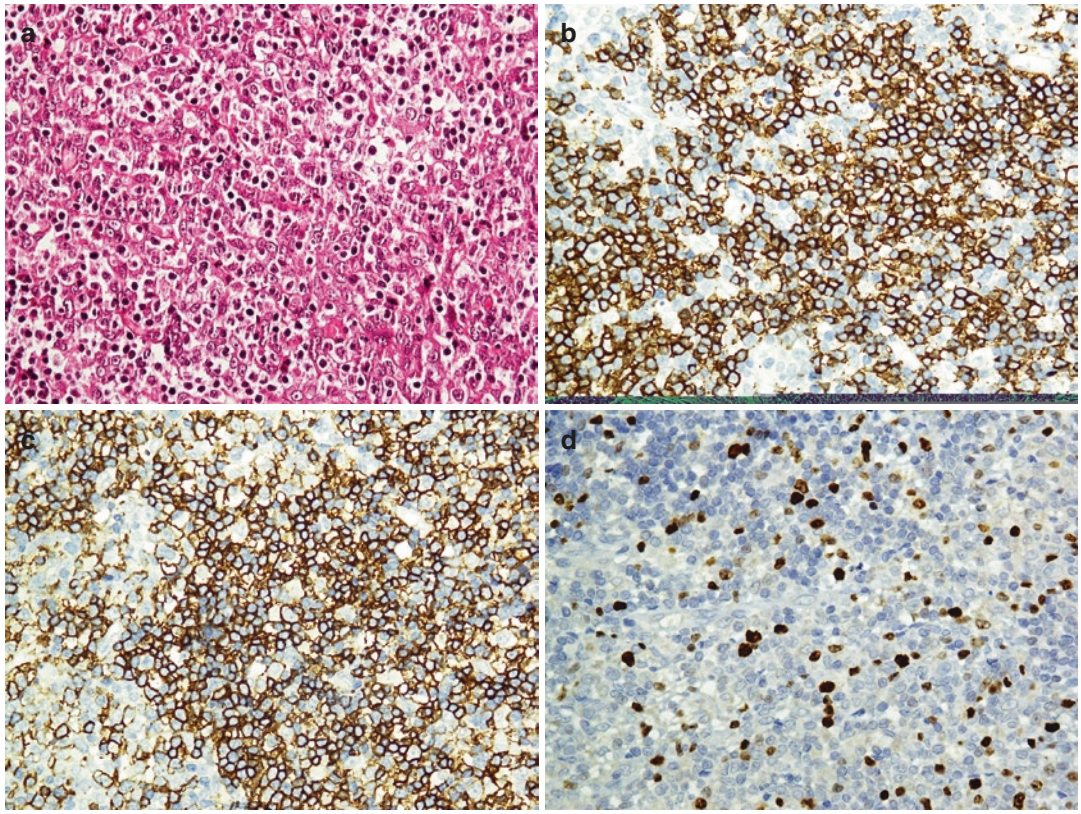

Fig. 29.10 Undifferentiated nasopharyngeal carcinoma shows infiltration of large undifferentiated cells with intermixed small lymphocytes (**a**). Cytokeratin antibody highlights malignant cells (**b**) and intermixed lymphocytes react with LCA (**c**). Ki-67 antibody reacts with about 20% of malignant cells (**d**)

Table 29.7 Immunohistochemistry of poorly differentiated and undifferentiated carcinomas of nasal cavity: sinonasal undifferentiated carcinoma (SNUC), undifferentiated neuroendocrine carcinoma (UNEC), and undifferentiated nasopharyngeal carcinoma (UNPC)

Markers	SNUC	UNPC	UNEC (Fig. 29.11)
Cytokeratin	+	+	+
EBV	–	+	–
Neuroendocrine	–	–	+
CD99	–	–	+/–
S100	–	–	+

References: [52, 61, 62]

squamous cell carcinomas express p63 which is relatively specific, but also found in other squamous tumors (Fig. 29.12). Neuroendocrine markers are negative in BSCC [66]. Spindle squamous cell carcinoma (SSCC) is a cytokeratin-negative SCC of which spindle cell component is uniformly and strongly positive for vimentin [67]. Undifferentiated nasopharyngeal carcinoma shows reactivity to EBV immunostaining as well as some SCCs and BSCCs [68, 69].

29.3.2.1 Prognostic Marker

As a transcription repressor of E-cadherin, Snail-1 is expressed in more than half of the cases of SSCC but not in SCC. In addition, it can be a novel marker for the prediction of metastasis [70].

29.3.3 Tumors of the Salivary Glands

Salivary glands are tubuloacinar exocrine glands having two layered epithelia, which comprise of luminal (acinar and ductal cells) and abluminal (myoepithelial and basal cells). Luminal cells are positive for low molecular cytokeratin, whereas

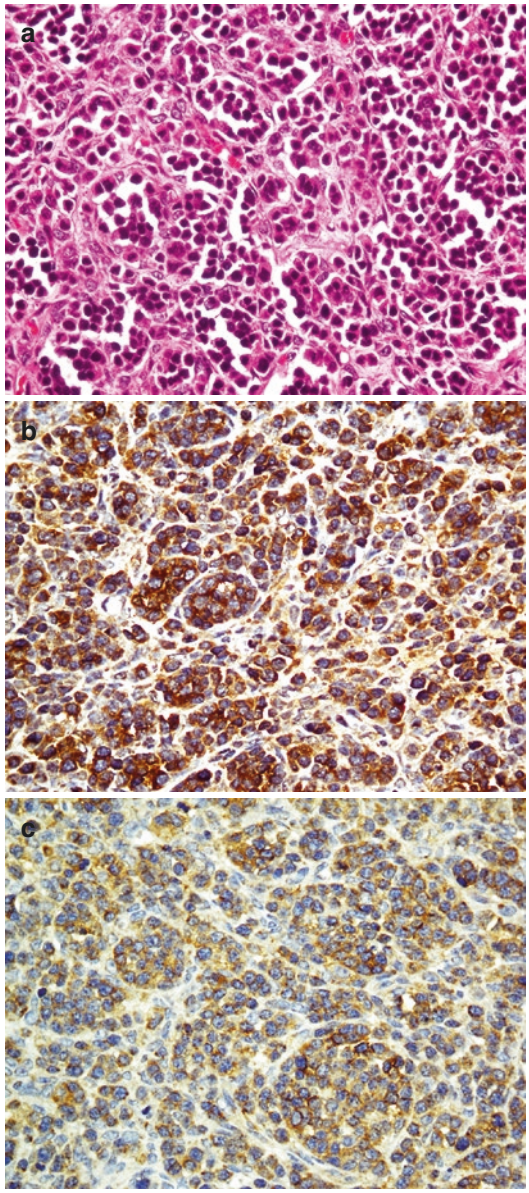

Fig. 29.11 Neuroendocrine carcinoma (**a**). Tumor cells are immunostained with synaptophysin (**b**) and NSE (**c**)

myoepithelial and basal cells react with high molecular cytokeratin and myoepithelial markers. The majority of salivary gland carcinomas can be diagnosed by routine Hematoxylin and Eosin (H&E) stained slides and immunohistochemical (IHC) staining has only a limited role in the diagnosis of salivary gland tumors [57, 71]. Figure 29.13 summarizes the various components of the normal salivary glands with an emphasis on the immunohistochemistry Abs.

29.3.4 Immunohistochemistry of Salivary Gland Tumors

The most common malignant tumors of salivary glands consist of acinic cell carcinoma, adenoid cystic carcinoma (Fig. 29.14), basal cell adenocarcinoma, epithelial-myoepithelial carcinoma, mucoepidermoid carcinoma (Fig. 29.15), myoepithelial carcinoma, polymorphous low-grade adenocarcinoma, and salivary duct carcinoma. All tumors are cytokeratin-positive; however, different immunoprofile patterns exist [72]. C-kit (CD117) is positive in acinic cell carcinoma and adenoid cystic carcinoma [73, 74]. Acinic cell tumor and mucoepidermoid carcinoma demonstrate reactivity with membrane-bound mucin (MUC) [75, 76]. Myoepithelial carcinomas are positive for both epithelial and myoepithelial markers but do not exhibit reaction with EMA and CEA [77]. Malignant monophasic salivary gland tumors include acinic cell carcinoma, myoepithelial carcinoma, mucoepidermoid carcinoma, and polymorphous low-grade adenocarcinoma. Immunophenotype profile of monophasic and biphasic tumors are denoted in Tables 29.8 and 29.9. Application of CK7 and CK20 is a useful panel in distinguishing primary salivary gland carcinoma ($CK7^+$, $CK20^-$) from metastatic carcinoma ($CK7^-$, $CK20^+$) [86].

29.3.4.1 Prognostic Marker

In mucoepidermoid carcinoma, MUC1 expression is correlated with tumor progression and worsened prognosis, whereas MUC4 expression is related to a better prognosis [76] (Fig. 29.17).

29.3.5 Tumors of Thyroid and Parathyroid Glands

The functional unit of thyroid is the follicle, which is composed of follicular cells and C cells.

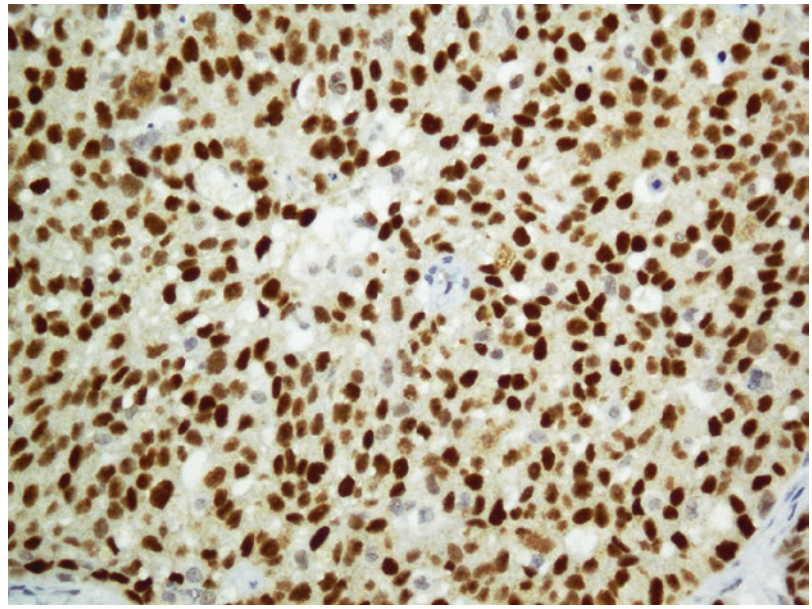

Fig. 29.12 p63 immunoreaction in basaloid squamous cell carcinoma

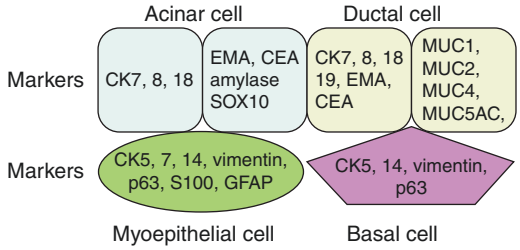

Fig. 29.13 Normal salivary gland components with immunohistochemistric antibodies

Follicular cells exhibit reactivity with thyroglobulin, TTF1, PAX8, AE1/AE3, EMA and CK7, CK8/18/19, whereas C cells are positive for calcitonin, TTF1, CK7, synaptophysin, and chromogranin. Being as a nuclear transcription factor, TTF1 is expressed on follicular and C cells. A follicular cell-specific marker is thyroglobulin, which does not react with C cells (Fig. 29.16). As a member of the paired box (PAX) gene family, PAX8 is a sensitive marker of thyroid tumors similar to TTF1. Among intermediate filaments, CK19 is more expressed in papillary carcinoma than other tumors [87]. Parathyroid hormone (PTH) and parafibromin are markers of parathyroid tumors. Parafibromin is uniformly expressed in parathyroid adenomas, whereas its expression is often reduced in parathyroid carcinomas. Table 29.10 shows an immunopanel of thyroid and parathyroid tumors.

29.4 Immunohistochemistry of Lung Tumors

Lung tumors are classified as small-cell and non-small cell lung cancers. Among non-small cell lung cancers, adenocarcinoma is the most common form and would be discussed in this book. Other variants including SCC and bronchoalveolar carcinomas also share similar IHC patterns.

29.4.1 Adenocarcinoma

The most frequent IHC pattern for lung is positivity for CK7, TTF1, and Napsin A, along with negative staining for CK20, CDX2, and MUC2. It is highly advocated to consider the fact that there are recently increasing reports of primary pulmonary adenocarcinomas with intestinal differentiation, which are CK7- and TTF1-negative, but CK20-positive which can be highly misinterpreted

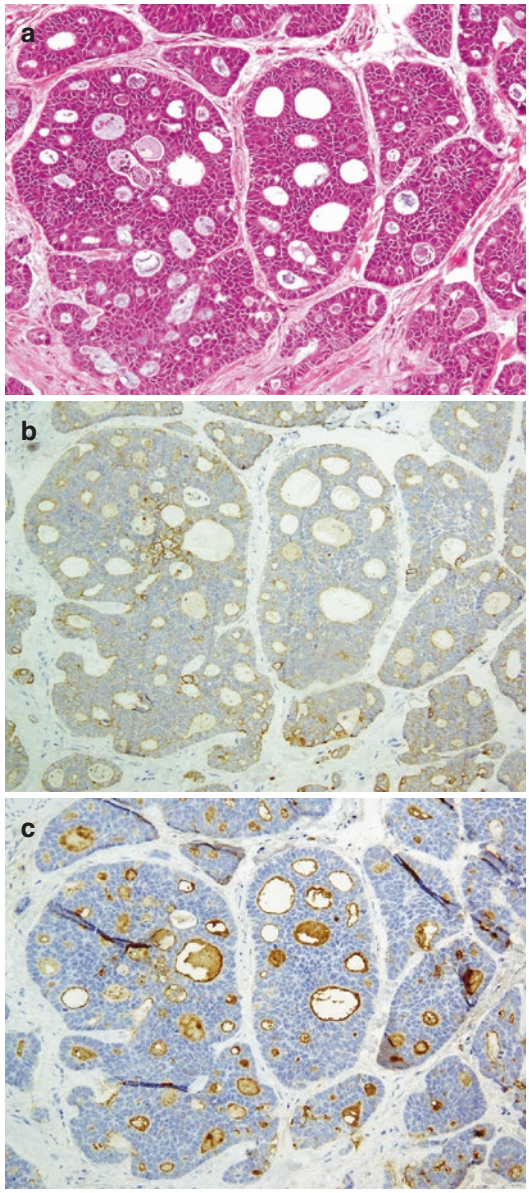

Fig. 29.14 Adenoid cystic carcinoma with typical cribriform pattern (**a**) shows immunoreaction with EMA (**b**) and CEA (**c**)

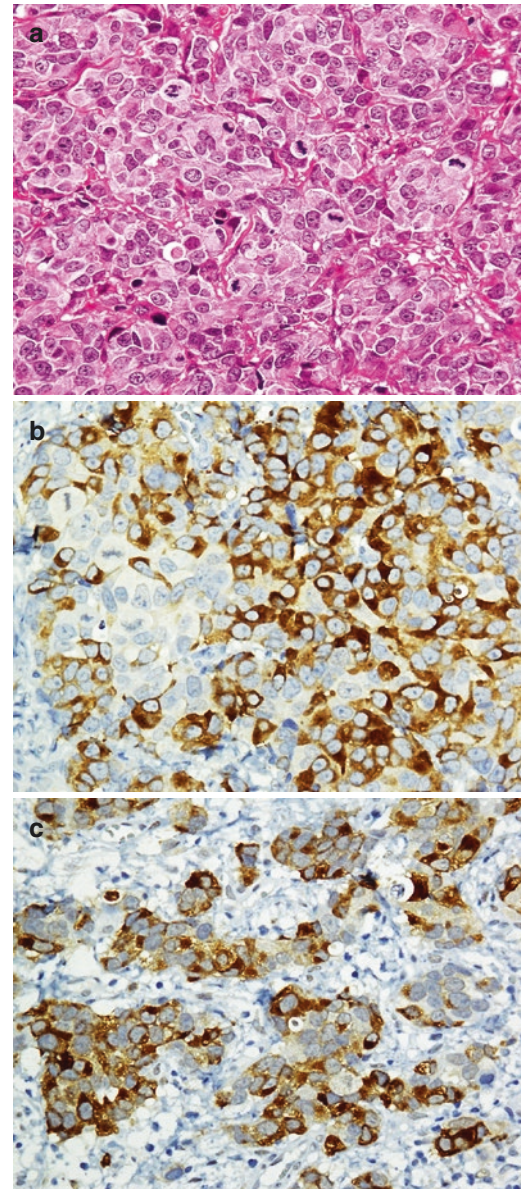

Fig. 29.15 Poorly differentiated mucoepidermoid carcinoma with polygonal atypical epidermoid cells (**a**) exhibits immunostaining with CK7 (**b**) and EMA (**c**)

Table 29.8 Immunophenotype of monophasic malignant salivary gland tumors: acinic cell carcinoma (AC), myoepithelial carcinoma (MC), mucoepidermoid carcinoma (MEC), and polymorphous low-grade adenocarcinoma (PLGC)

Tumor	AC	MC	MEC	**PLGC**
Epithelial markers	CAM5.2, CK7/8/18, EMA, CEA, MUC3	AE1/AE3, CAM5.2, CK14, 34βE12	CAM5.2, CK7/8/14/18/19, EMA, CEA, MUC1/4/5AC, 5B	CAM5.2, CK7, 14, EMA
Myoepithelial/ basal markers	N	p63, calponion, SMA, myosin	p63 (epidermoid component)	p63
Other markers	c-kit, S100	Vimentin, S100, GFAP	–	S100

References: [72, 73, 75–79]

Table 29.9 Immunophenotype of biphasic malignant salivary gland tumors: adenoid cystic carcinoma (ACC), basal cell adenocarcinoma (BCA), epithelial-myoepithelial carcinoma (EMC), and salivary duct carcinoma (SDC)

Tumor	ACC	BCA	EMC	SDC
Epithelial markers	CAM5.2, CK7/14/19, EMA, CEA	AE1/AE3, CAM5.2, CK7, EMA, CEA	AE1/AE3, CAM5.2, CK14	AE1/AE3, EMA, CEA
Myoepithelial/basal markers	p63, calponin	p63, calponin, SMA	p63, calponin, SMA	p63
Other markers	c-kit, S100	c-kit, S100	S100	AR, GATA3, Her2/neu

References: [72, 73, 75, 80–85]

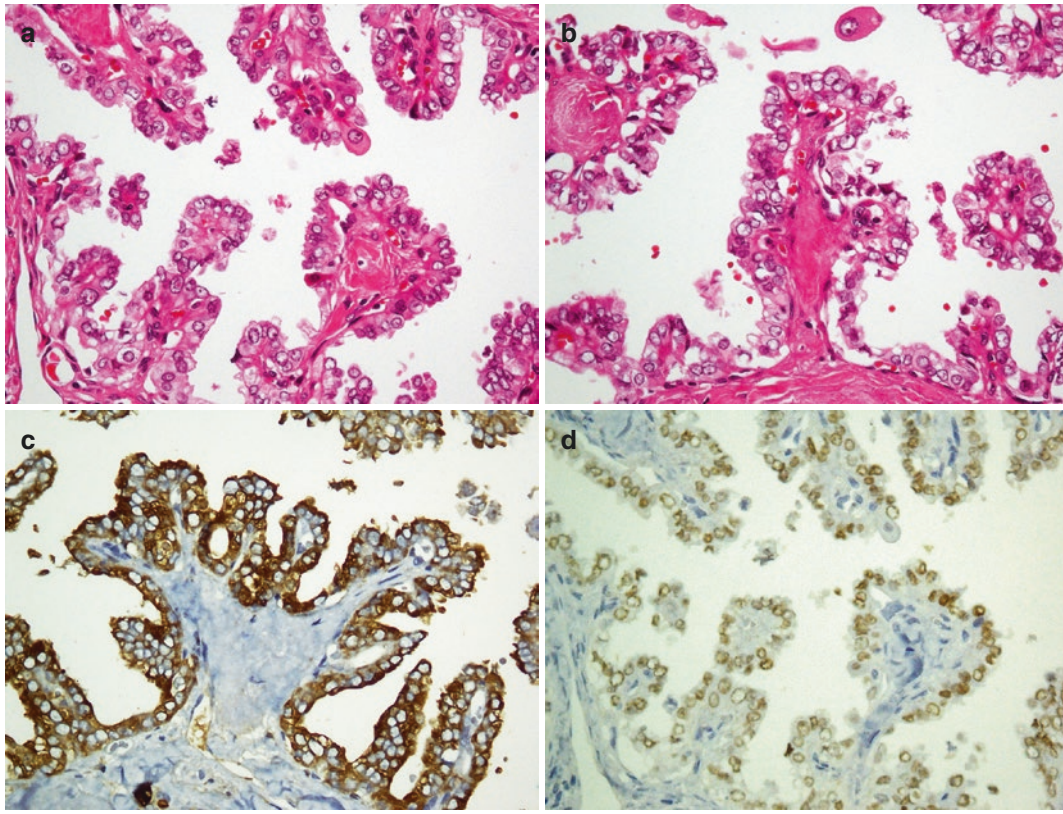

Fig. 29.16 Thyroid papillary carcinoma. Papillary projections with intranuclear inclusions (**a**) and Orphan Annie nuclei (**b**) are highlighted by thyroglobulin in cytoplasm (**c**) and TTF1 in nuclei (**d**)

Table 29.10 Immunopanel of thyroid and parathyroid tumors

First-choice antibody panel	Second-choice antibody panel	Consistent with
CK+, TTF1+, TGB+	PAX8+, CK19+	Papillary carcinoma (Fig. 29.16)
	PAX8±, VIM+	Follicular carcinoma
CK+, TTF1+, TGB−	Calcitonin+, SYN+, CGN+	Medullary carcinoma (Fig. 29.17)
CK±, TTF1+, TGB−	p53+, VIM+, PAX8±	Anaplastic carcinoma
CK+, TTF1−, TGB−	PTH+, CGN+, parafibromin±	Parathyroid tumor

Note: *CGN* chromogranin, *SYN* synptophysin, *TGB* thyroglobulin, *VIM* vimentin
References: [88–106]

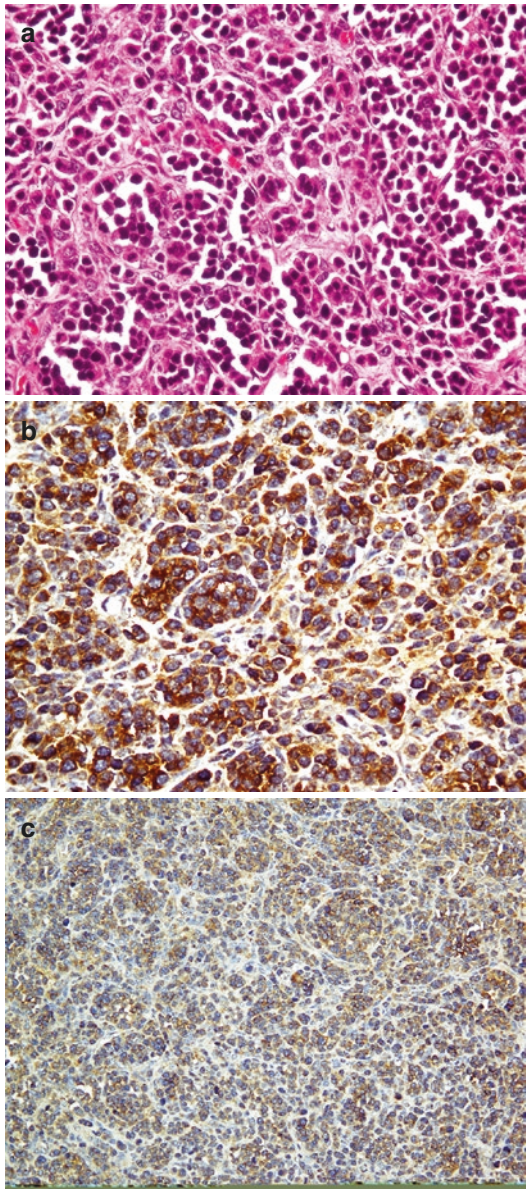

Fig. 29.17 Thyroid medullary carcinoma. Solid nests with medium size atypical cells (**a**), exhibit immunoreaction with calcitonin (**b**) and chromogranin (**c**)

as metastatic colorectal adenocarcinomas. Therefore, the importance of physical examination and imaging studies is highlighted.

29.4.2 Small-Cell Carcinoma

Small-cell carcinoma of lung usually express neuroendocrine markers and is classified as a high-grade neuroendocrine tumor. It should be noted that neuroendocrine markers including chromogranin, synaptophysin, NSE, and Leu7 (CD57) could be positive in lung non-neuroendocrine carcinomas such as adenocarcinomas and SCC. Recent studies have shown EGFR, Her2, and BRAF mutations in lung cancers, which can increase the chance for targeted therapies in these cancers [107–110]. In addition, there are growing data on novel markers such as PD-L1 and surviving to introduce them as novel prognostic markers in non-small cell lung cancers, but the results are debating and more studies are needed [111, 112].

29.4.3 Mesothelioma

Neoplasms of pleura are very rare and most tumors in this area are usually metastatic lesions. One of the most important applications of IHC is to assist pathologists in differentiating mesotheliomas from lung adenocarcinomas [113–116]. Table 29.11 shows the most frequent markers stained by IHC staining in mesothelioma compared with pulmonary adenocarcinoma (Fig. 29.18).

29.5 Immunohistochemistry of Gastrointestinal Tumors

Immunohistochemistry is used in gastrointestinal and colon cancers to particularly determine the tumor subtype and origin, especially for poorly or undifferentiated cancers for which morphology alone cannot determine the origin. Generally, it should be noted that definite tissue diagnosis clinical practice needs combination of IHC results and clinical information, including biopsy site and the patients' clinical history [117]. Previous studies show that blinded use of an IHC panel for differential diagnosis can primarily identify about 83% of tumor origins vs. 65.6% of metastasis. Several publications on IHC studies are available, and each recommends its own IHC panel for differential diagnosis. This makes it clear that there is no single IHC panel, or standard of care, for tissue determination and pathologists have long

Table 29.11 Immunohistochemistric differentiation of pulmonary adenocarcinoma (PAC) and malignant mesothelioma

Marker	Pulmonary AC	Mesothelioma	Comment
Calretinin	R	Usually +	The most specific and reproducible positive marker in mesothelioma
CDX2	R	–	About 13% positive, in pulmonary mucinous carcinomas
Cytokeratin	AE1/AE3, CK5/6 (R), CK7	CK5/6 (S), CK7 (used to differentiate mesotheliomas from sarcomas)	CK7: Most common CK in primary lung cancer (about 100% in AC, 40% in small-cell carcinoma, about 20% in carcinoid tumor and none of SCC arising from lung) CK5+ specially in lung SCC
D2–40	–	+	Usually positive specially in sarcomatoid variants of mesothelioma
EMA	S (cytoplasmic)	S (membranous)	
TTF1	+	–	
Mesothelin	–	+	
p63	–	–	Positive in pulmonary SCC
pCEA	+	–	
S100	+	–	
SMA	–	50–60%	
SP-A (Surfactant protein-A)	50%	–	
Thrombomodulin	–	+	
Vimentin	+	–	
WT1	–	60%	

Note: *pCEA* polyclonal CEA, *SMA* specific muscle antigen

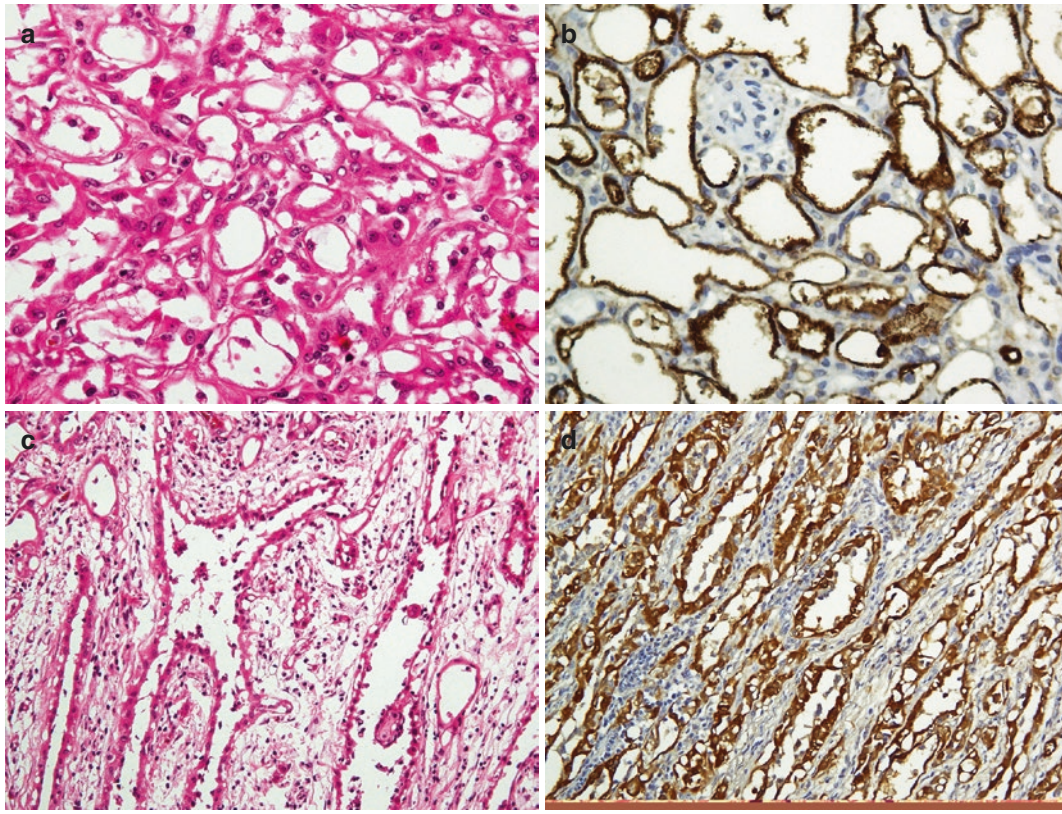

Fig. 29.18 Mesothelioma. Adenomatoid type (**a**), shows immunostaining for mesothelin (**b**) and tubular type (**c**) shows immunoreaction for calretinin (**d**)

known that tissue of origin identification is inherently a multiplex problem [118–120].

Here, the authors have briefly tried to introduce the major and common IHC markers used to differentiate frequent gastrointestinal tumors. It should be noted that the average positivity of a marker in a specific tumor differs from one study to another, as well as in different textbooks. In this chapter the most prevalent and reliable data are provided.

29.5.1 Liver

The most common primary hepatic cancer is hepatocellular carcinoma, which is well known to have a wide spectrum of histologic differentiation and a great diversity of appearances. It necessitates the application of IHC as an ancillary aid for better diagnosis of the lesion. It is important to reiterate that IHC is after all an ancillary aid. A significant clinicopathologic correlation seems mandatory for the final diagnosis. If a definitive diagnosis cannot be clinched, at the least, certain differential diagnoses can be excluded [121–125]. Immunophenotype of normal liver is summarized in Table 29.12 (Figs. 29.19 and 29.20).

Cholangiocarcinoma is a malignant tumor with characteristics mostly similar to other types of adenocarcinomas. The tumor is usually positive for CK7, CK19, CAM5.2, CK AE1/AE3, pCEA, mCEA (noncanalicular pattern), and MOC31. MUC4, MUC5AC, MUC6 can also be useful not in diagnosis, but in classification and predicting the prognosis. Additionally, CD56, which is positive in benign bile ductular proliferations and negative in cholangiocarcinomas, can be useful in differentiating malignant lesions from benign proliferation. The exception for this rule is clear cell cholangiocarcinoma, which is positive for CD56. Staining for CK7 and CK19 in

Table 29.12 Immunohistochemistry of normal liver

	Markers				
Normal tissue	Hepatocellular	Adenocarcinoma	Carcinoma	Canalicular	Others
Hepatocytes	HepPar1, TTF1 (cytoplasmic)	MOC31	CAM5.2	CD10, pCEA	B-catenin
Bile duct cells	–	CK7, CK19 (+/–), MUC6	CAM5.2, CKAE1/AE3, EMA, BerEP4	–	B-catenin

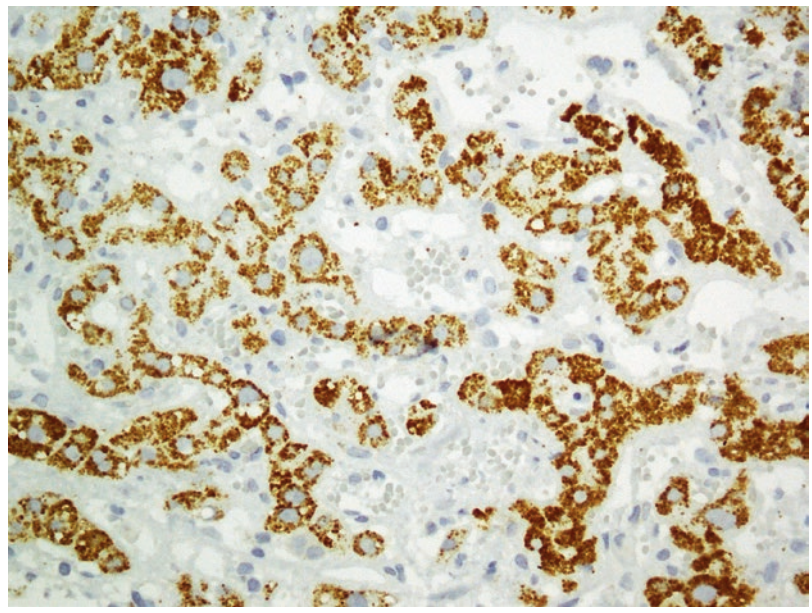

Fig. 29.19 Normal liver stains with HepPar1 showing typical cytoplasmic coarse granules of hepatocytes

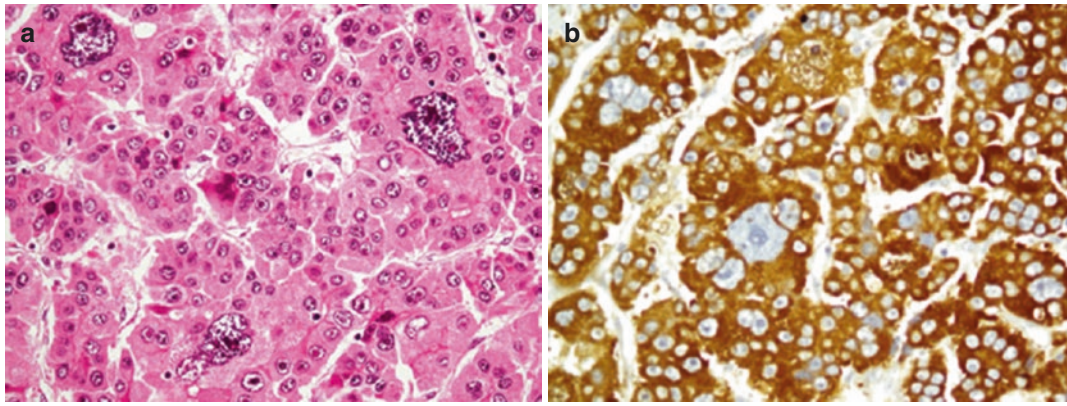

Fig. 29.20 Hepatocellular carcinoma with huge bizarre giant nuclei making diagnosis simple as malignant (**a**) exhibits reactivity with HepPar1 (**b**)

Table 29.13 Immunohistochemistry of hepatocellular carcinoma and cholangiocarcinoma

	Markers				
Tumor	Hepatocellular	Adenocarcinoma	Carcinoma	Canalicular	Sinusoidal
Hepatocellular carcinoma	HepPar1, TTF1 (cytoplasmic)	–	CAM5.2, EMA (−/+)	CD10, pCEA	CD34, FVIII
Cholangiocarcinoma	–	MOC31, CK7, CK19, MUC4, MUC5AC, MUC6	CAM5.2, CKAE1/AE3	pCEA, mCEA (noncanalicular)	–

cholangiocarcinoma can help to differentiate this tumor from HCC, which is negative for the mentioned markers [126, 127]. Table 29.13 indicates the immunophenotypes of hepatocellular carcinoma and cholangiocarcinoma.

29.5.2 Esophagus

The most common esophageal cancers are adenocarcinomas and SCC. Adenocarcinoma of the esophagus is immunophenotypically similar to gastric adenocarcinomas and there is no IHC panel to distinguish these two. Esophageal SCC is usually positive for most CK markers including CK AE1/AE3, CK 34bE12, CK5/6, CK19 (positivity increases with tumor grade whereas benign squamous lesions are negative for this marker) and p63. Additionally, most SCCs are negative for CK7 and CK20, which can be useful in distinguishing poorly differentiated SCCs from poorly differentiated adenocarcinomas positive for these two CK markers [128–130].

29.5.3 Stomach

Stomach glandular epithelium expresses CK20 and less commonly CK7 (CK7+, CK20+) and MUC5AC, distinguishing it from small intestine and colorectal epithelium. Immunoprofile of normal gastrointestinal mucosa is denoted in Table 29.14. Gastric adenocarcinoma has many histologic variants, but they have almost similar immunophenotyping. It should be mentioned that synaptophysin and chromogranin as neuroendocrine markers can be positive in gastric adenocarcinomas; therefore, positive staining with these markers is not sufficient for the diagnosis of neuroendocrine carcinoma [131–133]. Some gastric cancers may also express PD-L1, Her2/neu, EGFR, and VEGFR, which

Table 29.14 Immunoprofile of normal gastrointestinal mucosa

	Simple epithelial marker					MUC			
Normal tissue	CK7	CK20	AE1/AE3	CAM5.2	CEA	Gastric (MUC5AC)	Intestinal (MUC2, MUC4)	CDX2 (intestinal marker)	CD15
Stomach	+/−	+	+	+	+	+	−	−	+
Small intestine	−	+	+	−	−/+	−	+	+	+
Large intestine/appendix	−	+	+	+	+	−	+	+	+

Note: + (>90%), +/− (>50%), −/+ (<50%), − (<10%)

Table 29.15 Immunoprofile of gastric, small intestine, and colorectal adenocarcinoma (AC)

	Tumor associated marker						MUC			
Tumor type	CK 18/19	CK7	CK20	AE1/AE3	CAM5.2	CEA	Gastric (MUC5AC)	Intestinal (MUC2, MUC4)	CDX2 (intestinal marker)	CD15
Gastric AC	+	+/−	−/+	+	+	+	−/+	−/+	−/+	−
Small intestine AC	+	+/−	+/−	+	−/+	−/+	−/+	+/−	+/−	−
Large intestine/ appendix AC	+	−	+	+	+	+	−/+	+/−	+	−

Note: + (>90%), +/− (>50%), −/+ (<50%), − (<10%)

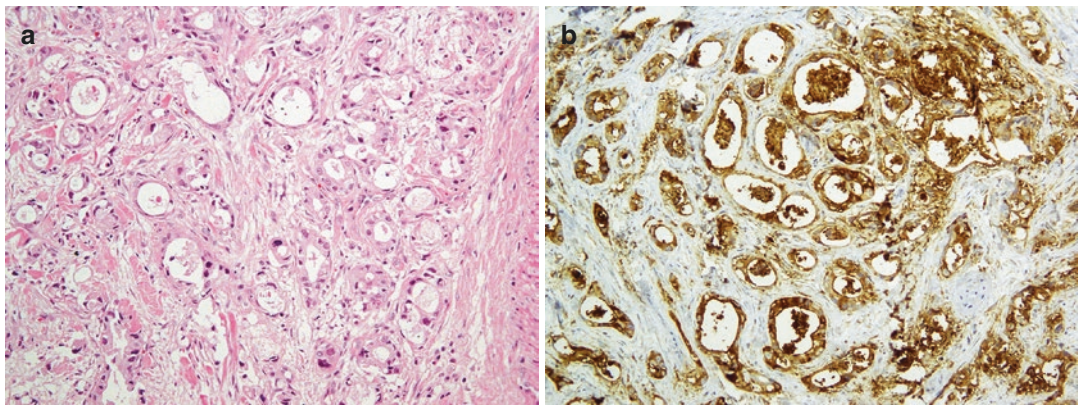

Fig. 29.21 Adenocarcinoma of stomach with atypical glands and nuclear pleomorphism (**a**) immunostained with CEA (**b**)

can be used in targeted therapies against gastric cancer [134–136]. Immunoprofile of gastric adenocarcinoma is demonstrated in Table 29.15 (Fig. 29.21).

29.5.4 Small Intestine

Immunophenotyping of adenocarcinoma is also valuable in neuroendocrine tumors (NET) [137–139]. Tables 29.14 and 29.15 summarize the immunoprofile of normal small intestine, its adenocarcinoma, as well as their comparison with stomach and colon adenocarcinoma.

29.5.5 Colon

In contrast to older studies, which have discussed colon cancers generally, recent studies reveal that colon cancers arise from two different pathways (chromosomal instability of APC gene vs. microsatellite instability (MSI) pathway with different immunophenotypic features) [123, 140–146].

Table 29.16 Immunoprofile of colon adenocarcinoma based on chromosomal instability and MSI pathways

Chromosomal instability pathway (80–85%)		MSI pathway (15–20%)	
CK20	100%	CK20	Can be negative in about 30%
MUC2	Usually positive	MLH1	Complete absence of staining with a sufficient internal control needed for a positive result
MUC5AC	Usually negative (about 30% positive, especially in mucinous carcinomas)	MSH2	
CAM5.2	Usually positive	MSH6	
MOC31	Usually positive	PMS2	
CDX2	About 90%	CDX2	Can be negative in about 20%
CK7	5–10%		
CEA	Usually positive especially monoclonal type		
CK8	Usually positive		
CK18	Usually positive		
CK19	Usually positive		
CKAE1/AE3	Usually positive		
MSI-related markers	These markers are usually positive in this subtype of colon carcinomas		

Table 29.17 Immunoprofile of normal pancreas

Marker			Normal tissue
Exocrine	Glandular/ductal	Epithelial	CAM5.2, AE1/AE3, CK7, CK8/1/8/19
		MUC	MUC1, MUC6
		ONP	–
	Acinar		Trypsin, chemotrypsin, lipase, amylase, elastase
Endocrine			CGN, SYN, NSE

Immunoprofile of normal and colon adenocarcinoma is denoted in Tables 29.14, 29.15, and 29.16.

29.5.6 Anal

The most frequent anal cancers are SCC and adenocarcinoma. Anal SCC is almost similar to SCC of other origins; nonetheless, the role of HPV is highlighted. Adenocarcinomas of anus are usually positive for CK7 and negative for CK20, CDX2, and CK5/6, which helps to differentiate them from adenocarcinomas of the colon origin [145, 147, 148].

29.5.7 Appendix

Mucinous adenocarcinomas of appendix origin can be distinguished from mucinous colorectal carcinomas with immunostaining for CK7 and MUC markers [149–151].

29.5.8 Pancreas

Pancreas is composed of glandular/ductal, acinal epithelium, and endocrine cells. Pancreatic neoplasms can be roughly divided into two categories of exocrine and endocrine system neoplasms. This part mostly discusses about the exocrine system and mostly adenocarcinomas of this area. Additionally, tumor suppressor genes including DPC4 and SMAD4 are inactivated in about 50–60% of the adenocarcinomas of this site [123, 152, 153]. Immunoprofile of normal pancreas and some pancreatic tumors are summarized in Tables 29.17 and 29.18. Figure 29.22 depicts solid-pseudopapillary neoplasm.

Table 29.18 Immunoprofile of some pancreatic tumors: pancreatic ductal adenocarcinoma (PDAC), acinar cell carcinoma (ACC), neuroendocrine carcinoma (NEC), and solid-pseudopapillary neoplasm (SPN)

Marker			PDAC	ACC	NEC	SPN (Fig. 29.22)
Exocrine	Glandular/ductal	Epithelial	CAM5.2, AE1/AE3, CK7, CK8/18/19, pCEA, PSCA	CAM5.2, AE1/AE3, CK8/18, EMA	CAM5.2, AE1/AE3, CK19	– (positive for β-Catenin, vimentin, PR, CD10)
		MUC	MUC1, 3, 4, 5AC, 6 (+/−)	–	–	–
		ONP	CA19.9, CA125, B72.3, DUPAN-2, CECAM1	–	–	–
	Acinar		–	Trypsin, chemotrypsin, lipase, amylase, elastase	–	α1-antitrypsin
Endocrine			–	CGN, SYN	CGN, SYN, NSE, CD56, CD57	CGN, SYN, NSE, CD56

Note: *CGN* chromogranin, *NSE* neuron-specific enolase, *ONP* oncoprotein, *PR* progesterone receptor, *SYN* synaptophysin

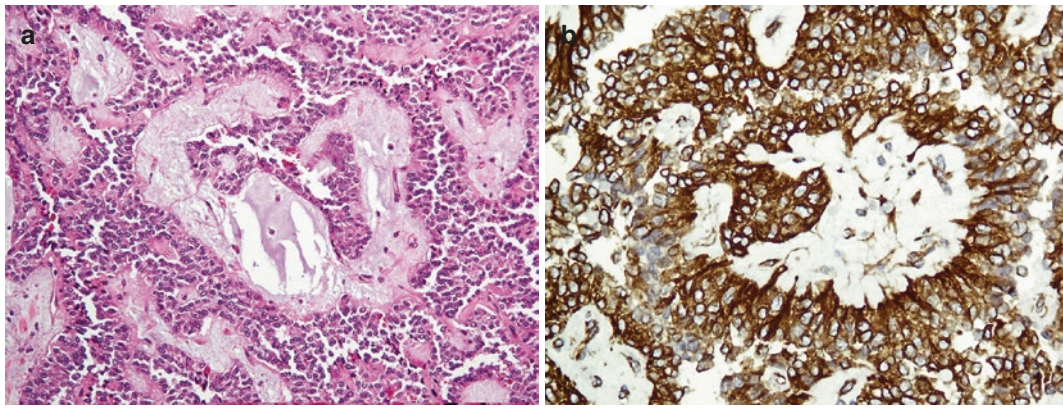

Fig. 29.22 Solid-pseudopapillary neoplasm. Papillary projection covered by relatively bland-looking cells supported by a hyalinized stroma (**a**) highlighted with vimentin (**b**)

29.5.9 Gastrointestinal Stromal Tumor

Gastrointestinal stromal tumor (GIST) is a soft tissue tumor of GI wall, which is in differential diagnosis of leiomyoma and fibromatosis. Most GISTs express C-kit (>95%), CD34, and CD99 (Fig. 29.23). Sometimes weak positivity for S100, SMA, desmin, and synaptophysin (but not chromogranin) can also be found [145, 154, 155].

29.5.10 Neuroendocrine Carcinomas

Neuroendocrine tumors arise from different organs. Most have similar morphology and tumor marker expression and the most important diagnostic clues are histologic features, as well as immunostaining for synaptophysin, chromogranin, and NSE (Fig. 29.24). In addition to the mentioned markers most of neuroendocrine tumors can express the tissue markers in which they are originated which help to diagnose the origin of metastatic neuroendocrine tumors [153, 156–158].

29.6 Immunohistochemistry of the Urinary Tract

29.6.1 Kidney

Renal cell carcinoma (RCC) is the most common tumor of the kidney with variants of clear renal cell carcinoma (CRCC), papillary renal cell carcinoma

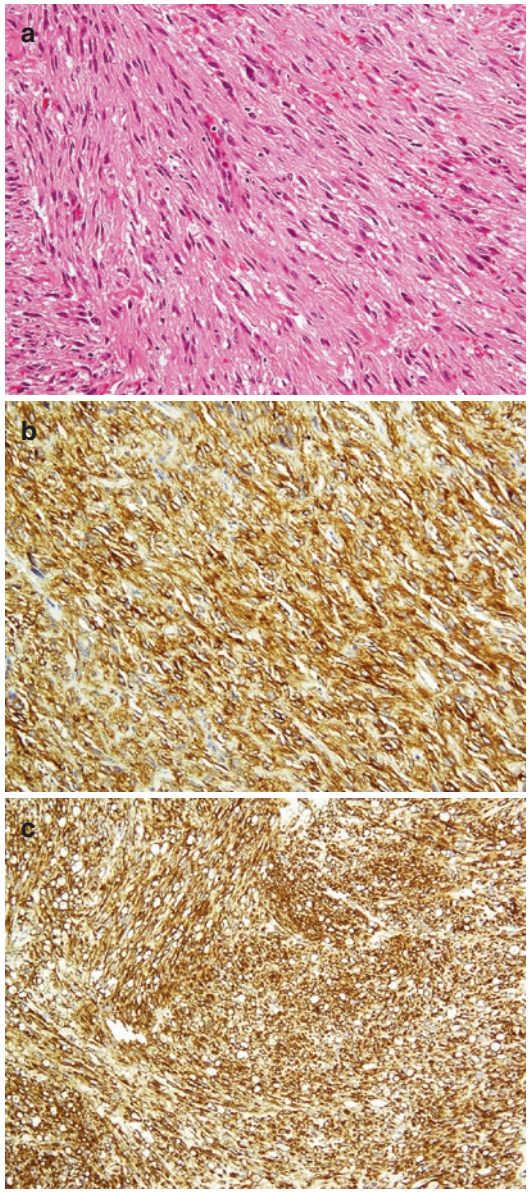

Fig. 29.23 Gastrointestinal stromal tumor. A low-grade intestinal wall tumor shows uniform spindle cells with elongated nuclei (**a**), with immunoreaction to C-kit (**b**) and CD34 (**c**)

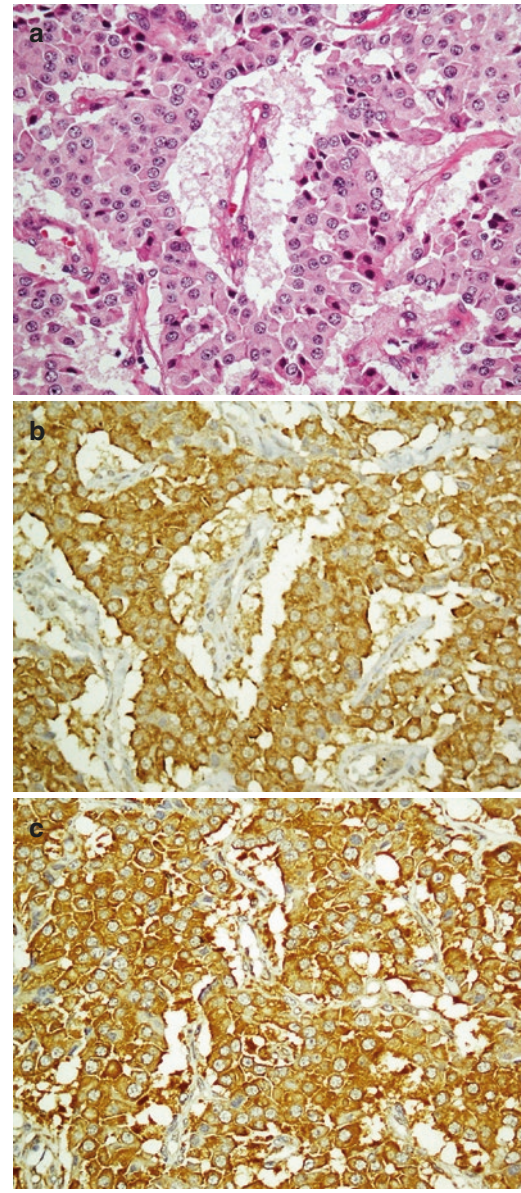

Fig. 29.24 Neuroendocrine carcinoma consists of atypical cells with round nuclei and dusty chromatin (**a**). Tumor cells are immunostained with chromogranin (**b**) and synaptophysin (**c**)

(PRCC), and chromophobe carcinoma (CC). Commonly used immunohistochemical Abs in the urinary system are summarized in Table 29.19. Immunohistochemistry is an ancillary test used to distinguish variants of RCC as well as tumors with histopathologic similarities including collecting duct carcinoma and urothelial carcinoma of the renal pelvis. Carcinomas with clear cell feature include CRCC (Fig. 29.25), papillary renal cell carcinoma, and transitional (urothelial) cell carcinoma of the renal pelvis. Differential diagnoses of carcinoma with oncocytic appearance are chromophobe

Table 29.19 Immunohistochemical markers in the urinary system tumors

Marker	Function	Immunoreaction in tumor
AE1/AE3	Pan-CK epithelial marker	RCC
CAIX	Carbonic anhydrase IX: maintenance of intracellular and extracellular pH, regulatory role in cell proliferation	PRCC
CAM5.2	Intermediate cytoskeleton filament	RCC, PRCC, CC, CDC
CD10 (CALLA)	A zinc-dependent cell membrane metalloprotein	RCC, PRCC
CD117 (C-kit)	Transmembrane glycoprotein receptor tyrosine kinase	CC, CDC, OC
CK7	LMWCK (simple epithelia)	PRCC, CC, UC, PAC (+/−)
CK20	LMWCH (simple epithelia)	UC (+/−), PAC (+/−)
34βE12	HMWCK (CK1, 5, 10, 14)	CDC, UC
EGFR	Receptor with tyrosine kinase activity	UC (+/−)
Ep-Cam	Glycosylated transmembrane cell surface epithelial protein in distal nephron	PRCC (+/−), CC, CDC
HMWCK	Intermediate cytokeratin filaments of prostate basal cell	"Negative" marker in PAC
Ki-67 (MIB1)	Nuclear protein expressed in all phases of the active cell cycle (G1, S, G2, M)	Proliferative marker
Ksp-Cadherin (kidney-specific)	Calcium-dependent cell adhesion molecule plays an important role in the maintenance of tissue integrity	CC, OC
p53	Tumor suppressor protein	UC
p63	A member of p53 family transcription factor, marker of basal cells	"Negative" marker in PAC
P501S (Prostein)	A 553-amino acid protein localized to the Golgi complex	PAC
P504S (AMACR)	Enzyme mainly localized to peroxisomal structures	PRCC, PAC
PAX2/PAX8	Members of the paired box (PAX) gene family expressed in the development of the urogenital tract	RCC, PRCC, CC, CDC, OC (+/−)
PSA	330-kD glycoprotein, prostate-specific antigen	PAC
PSAP	100-kD glycoprotein, prostate-specific antigen	PAC
PSMA	100-kD glycoprotein, prostate-specific antigen	PAC
RCC	200-kD glycoprotein expressed in epithelial cells lining normal renal proximal tubule	RCC, PRCC
Thrombomodulin	75-kD glycoprotein, to convert thrombin from a coagulant protein to an anticoagulant	UC
Uroplakin III	A transmembrane protein unique to urothelium	UC
Vimentin	Intermediate cytoskeleton filament	RCC, PRCC, CDC

Note: *CC* chromophobe carcinoma, *CDC* collecting duct carcinoma, *OC* oncocytoma, *PAC* prostatic adenocarcinoma, *PRCC* papillary renal cell carcinoma, *UC* urothelial carcinoma
References: [159–193]

carcinoma, oncocytoma, and oncocytic papillary RCC (Fig. 29.26) [163–189]. The immunophenotype of collecting duct carcinoma is 34βE12$^+$, CD10$^-$, and AMACR$^-$, in contrast to PRCC, which is 34βE12$^-$, CD10$^+$, and AMACR$^+$ [159, 164]. Considering the histopathologic pattern, the following immunopanels (Tables 29.20 and 29.21) compare the immunohistochemical Abs in these tumors.

29.6.2 Bladder

Normal urothelium exhibits a unique pattern of cytokeratin expression characterized by coexpression of simple epithelium cytokeratin (CK7, CK20, and CAM5.2) and HMWCK (CK5/6 and 34βE12). While CK20 is expressed in umbrella cells of the normal urothelium, in dysplastic urothelium and carcinoma in situ, it is expressed in all layers of the urothelium [159–163, 177, 178]. CD44 is expressed in the basal layer of normal urothelium and shows focal staining of basal layers of the dysplastic urothelium [179]. Urothelial carcinomas are divided into: (1) non-invasive papillary carcinoma and (2) invasive carcinoma which can appear as papillary or non-papillary itself (Fig. 29.27). Immunohistochemistry can be helpful to differentiate urothelial carcinoma

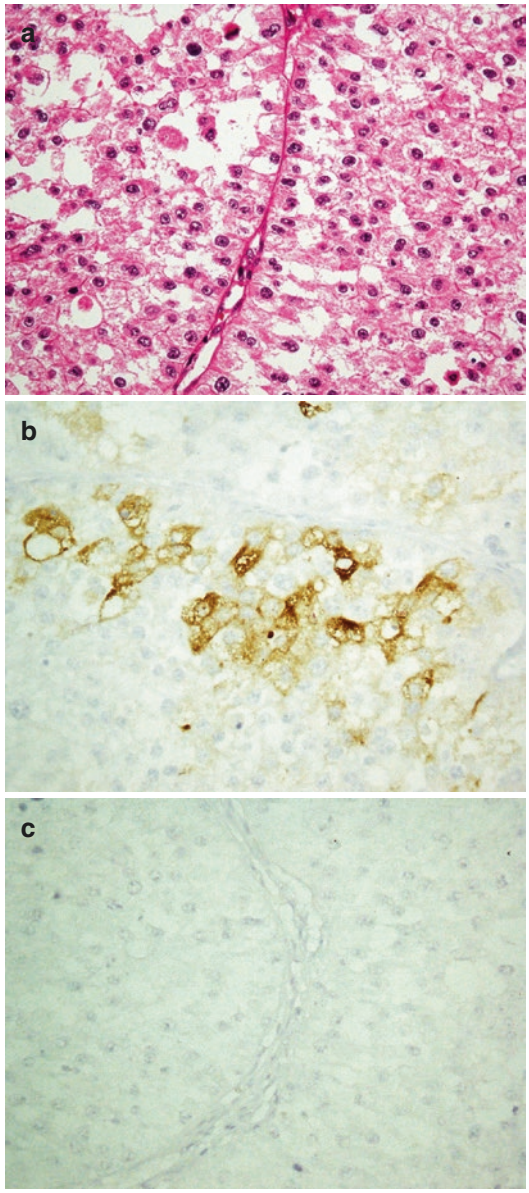

Fig. 29.25 Renal cell carcinoma with eosinophilic to clear cells (**a**) is immunostained with CD10 (**b**) but not with CK20 (**c**)

from direct extension of an adjacent primary carcinoma (prostate, colorectal, cervix, and uterine) as well as metastasis and also to distinguish variants of urothelial carcinoma. Common immunohistochemistry Abs in normal urothelium, urothelial hyperplasia, urothelial dysplasia, and urothelial carcinoma are summarized in Table 29.22.

29.7 Immunohistochemistry of Female and Male Genital Tumors

29.7.1 Uterine Cervix

The most important and also frequent cervix cancers are cervix SCCs and adenocarcinomas. Cervix SCC markers are similar to those seen in SCCs of other origins. P16 is a unique marker expressed in tumors of cervix, which can help in differentiating this lesion from same counterparts from uterine or other origins. Adenocarcinomas of cervix also express most adenocarcinoma markers. One of the advantages of IHC is to differentiate adenocarcinomas of cervix from endometrium. Cervix adenocarcinomas usually express p16 and CEA, and are negative for vimentin and ER, whereas endometrium adenocarcinomas have a reverse expression pattern [185–190].

29.7.2 Vulva and Vagina

As other organs, various malignancies can occur in these two organs but similar to cervix the most common cancer of these two sites is SCC, with IHC marker expression similar to cervix counterparts [191, 192].

29.7.3 Uterine Corpus

Uterine tumors are of myometrium or endometrium origin. The myometrial tumors are usually sarcomas and were discussed in the sarcoma section. The endometrium may develop various cancers, but the most frequent one is endometrial adenocarcinoma. Endometrial adenocarcinoma has some variants in which endometrioid adenocarcinoma is the most frequent one. Endometrioid adenocarcinoma usually expresses CK7, CA125, ER, PR, and vimentin, but is negative for CEA, CK20, and p16. Some endometrial carcinomas express Her2/neu marker, which along with ER and PR markers can be used in targeted therapies [185–190, 193–196].

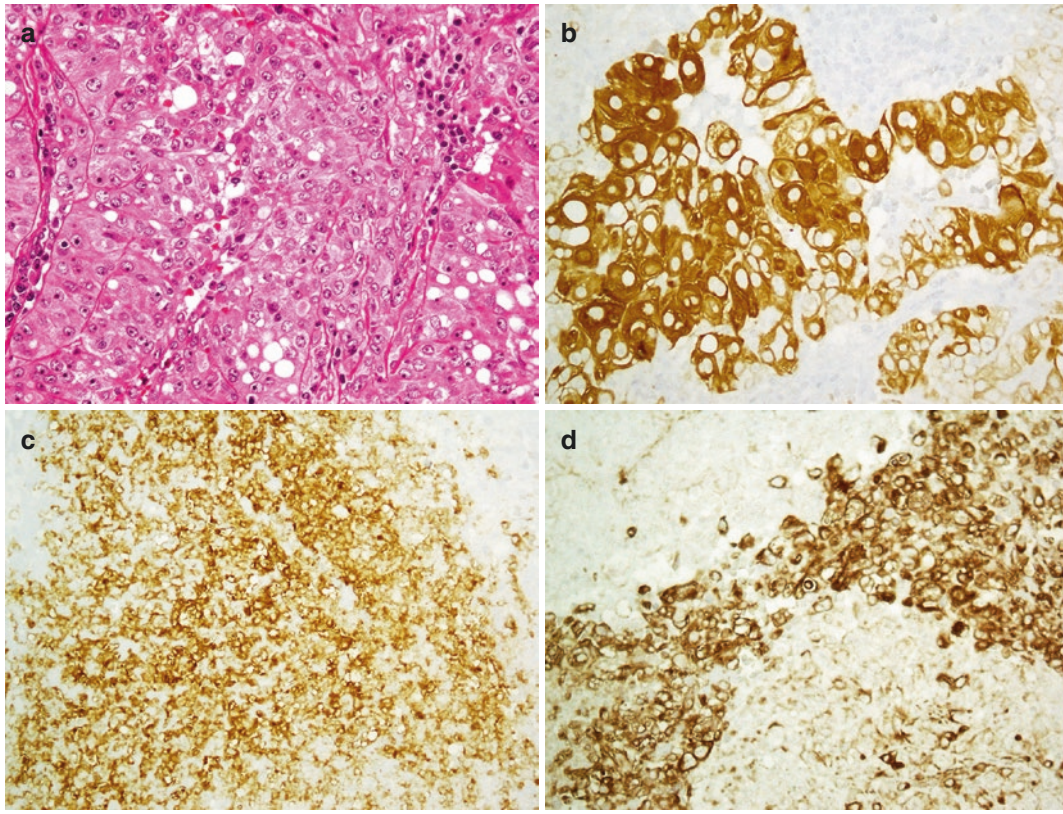

Fig. 29.26 Papillary renal cell carcinoma with oncocytic feature (**a**). Tumor cells are positive for CK7 (**b**), CD10 (**c**) and vimentin (**d**)

Table 29.20 Immunoprofile of kidney carcinoma with clear cell appearance: Clear RCC (CRCC), papillary RCC (PRCC), and urothelial carcinoma (UC)

Tumor	CK7	CK20	Vimentin	RCC	CD10	PAX2/8	AMARC	Uroplakin	p63
CRCC	–	–	+	+	+	+	–	–	–
PRCC	+	–	+	+	+	+	+	–	–
UC	+	+	–	–	–	–	–	+	+

References: [159–169]

Table 29.21 Immunoprofile of kidney carcinoma with oncocytic cell appearance: oncocytic papillary RCC (OPRCC), chromophobe carcinoma (CC), and oncocytoma (OC)

Tumor	CK7	CK20	CAM5.2, EMA, AE1/AE3	Vimentin	RCC	CAIX	CD10	CD117	Ep-Cam	Ksp-Cadherin
OPRCC	+	–	+	+	+	+	+	–	+	–
CC	+	–	+	–	–	–	–	–	+	+
OC	–	–	–	–	–	–	–	+	–	+

References: [172–176]

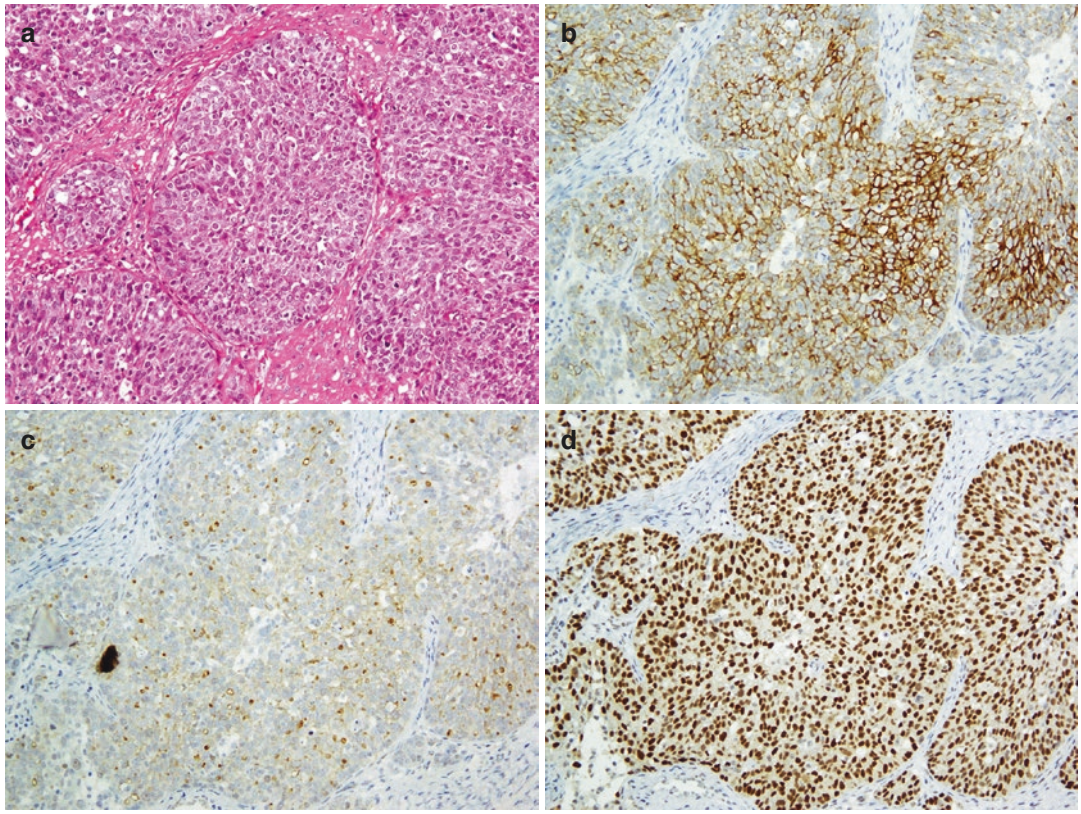

Fig. 29.27 Transitional cell carcinoma, invasive, non-papillary type (**a**). Tumor cells exhibit immunoreaction with CK7 (**b**), CK20 (**c**) and p63 (**d**)

Table 29.22 Antibody immunoprofile in normal urothelium, urothelial hyperplasia, dysplasia, and carcinoma

Marker	Normal urothelium	Urothelial hyperplasia	Urothelial dysplasia	Urothelial carcinoma
CK7	+	+	ND	+
CK20	+ U	+	+	+
34βE12	+ B	ND	ND	+
CD44	+ B	ND	−/+	ND
EGFR	−/+	+	+/−	+/−
p63	ND	ND	ND	+[a]
UPIII	+ U	ND	ND	+[a]
TM	+ U	ND	ND	+[a]
p53	−	−	+	+[a]

Note: + (>90%), +/− (>50%), −/+ (<50%), − (<10%). *B* basal layer, *TM* thrombomodulin, *U* umbrella cell, *UPIII* uroplakin III
References: [177–184]
[a]Non-invasive carcinoma > invasive carcinoma

29.7.4 Ovary

Except the intestinal type of mucinous adenocarcinoma, all primary ovarian carcinomas are CK7-positive and CK20-negative (Fig. 29.28). This can be used in differentiating primary ovarian carcinoma from metastatic tumors [149–151, 189, 197–200]. The immunophenotype of primary ovarian tumors is described in Table 29.23.

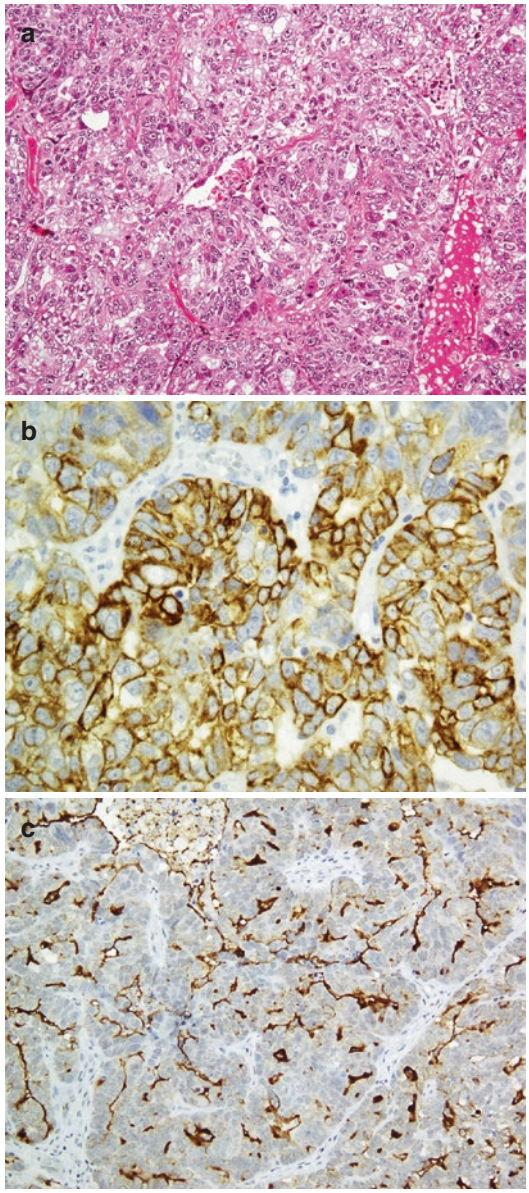

Fig. 29.28 Ovarian serous carcinoma poorly differentiated (**a**), shows immunoreaction with CK7 (**b**). CA125 is highlighted in the luminal surface (**c**)

29.7.5 Breast

Breast cancer is one of the most common malignancies with various histopathological types; however, adenocarcinomas and its two subtypes including invasive ductal (IDC) and lobular carcinomas (ILC) comprise the majority. Most breast cancers including IDC and ILC are positive for mammaglobin, GCDFP15, ER, PR and some are positive for Her2/neu markers. Additionally, epithelial tumor markers, CK (especially CK7) and EMA, are also positive in these tumors [201–206]. The lack of reaction with myoepithelial markers is in favor of an invasive carcinoma. Both normal (Fig. 29.29) and proliferative glands (Fig. 29.30) as well as ductal carcinoma in situ (Fig. 29.31) exhibit reactivity with myoepithelial markers. Application of p63 and calponin or p63 and SMA is a good way to evaluate the presence of myoepithelial cells [201, 207]. Immunoprofile of normal breast glands and breast cancers are summarized in Tables 29.24 and 29.25 (Figs. 29.32 and 29.33).

29.7.6 Prostate

Prostate gland is composed of two layers, epithelium and basal cell layer. Normal prostate epithelium exhibits immunoreactivity with prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), prostate-specific acid phosphatase (PSAP), and prostein (P501S) whereas prostate basal cells display immunostaining with HMWCK (34βE12), p63, and S100A6 (Fig. 29.34) [159–163, 210]. Immunolabeling for basal cell markers is usually used in a mode of "negative" diagnostic marker in order to show the absence of basal cells in prostate carcinoma (Fig. 29.35). Basal cell cocktail is a mixture of basal cell markers (HMWCH and p63 or CK5/6 and p63) used to highlight the presence of basal cells in normal glands, which differentiates them from prostate intraepithelial neoplasia (PIN) and prostate adenocarcinoma [211]. In addition, prostatic adenocarcinomas usually express α-Methylacyl coenzyme A racemase (AMACR) enzyme, which is negative in normal prostatic epithelium and helps to differentiate its malignant lesions from benign neoplasms. Most of metastatic carcinomas from prostate origin exhibits reactivity to CK 7 and CK20 as well as PSA (Fig. 29.36) Table 29.26 summarizes the immunoprofile of normal prostate glands as compared with PIN and adenocarcinoma.

Table 29.23 Immunophenotype of ovarian cancers

Epithelial tumors		Germ cell tumors				Stromal tumors (almost always negative for EMA)	
Serous (Fig. 29.28)	Mucinous	Dysgerminoma	Yolk sac	Embryonal carcinoma	Chori-carcinoma	Granulosa cell tumor	Sertoli-Leydig cell tumor
EMA CK7 CA125 DPC4 ER PR WT1	EMA CK7 CK20 mCEA CDX2 MUC5A	PLAP CD117 (c-kit) Oct-4 D2-40	PLAP AFP CK AE1/AE3 Glypican-3	PLAP Oct-4 CK AE1/AE3 CD30	HCG Inhibin CK	Inhibin CD99 WT1 Calretinin CD56	CK CD99 WT1

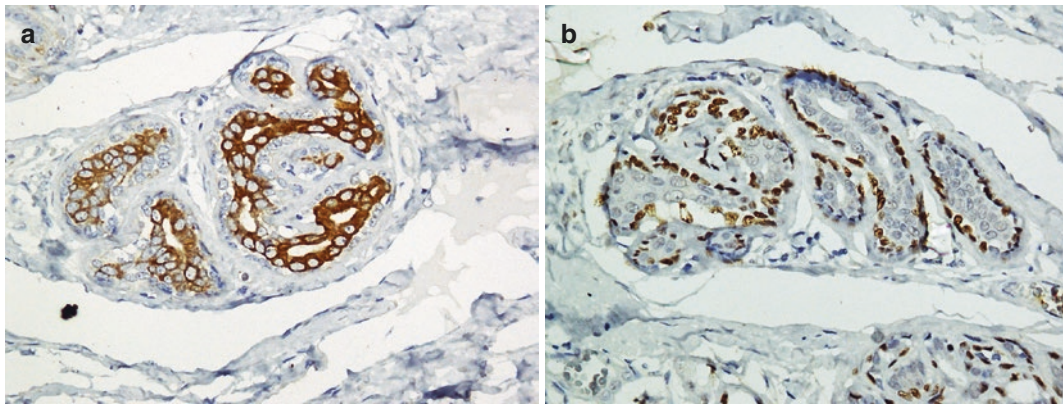

Fig. 29.29 Cytokeratin (**a**) stains epithelial cells and p63 (**b**) stains myoepithelial cells of normal breast glands

29.7.7 Testis

Tasticular tumors are classified into germ cell tumors and sex-cord stromal tumors. Germ cell tumors are the most common type with classic seminoma subtype comprising the majority. The definite diagnosis of these tumors is depended on proper application of the immunohistochemistric markers and histopathologic evaluation of the biopsy (Figs. 29.37, 29.38, and 29.39). Table 29.27 summarizes the immunophenotype of testicular tumors.

29.8 Immunohistochemistry of Lymphoma

Immunohistochemistry is an integrated part of diagnostic surgical pathology of Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL). Various Ags, mostly CD markers, are the targets of IHC. Neoplastic lymphoid cells express the same CD Ags with some aberrancy in type and amount. Several oncogene products are also expressed in some lymphomas (i.e., Follicular lymphoma). These Ags have diagnostic and probably prognostic value. Proliferative Ags like Ki67 are also of great value.

Morphology is the main stem of lymphoma diagnosis; nonetheless, IHC seems mandatory for the diagnosis and typing of malignant lymphoma. As a general rule, panels should be used for immunophenotypic evaluation and there is no single marker absolutely specific for one definite lymphoproliferative disorder. Some routinely used markers are shown in Tables 29.28, 29.29, 29.30, 29.31, and 29.32 and Figs. 29.40, 29.41, 29.42, and 29.43.

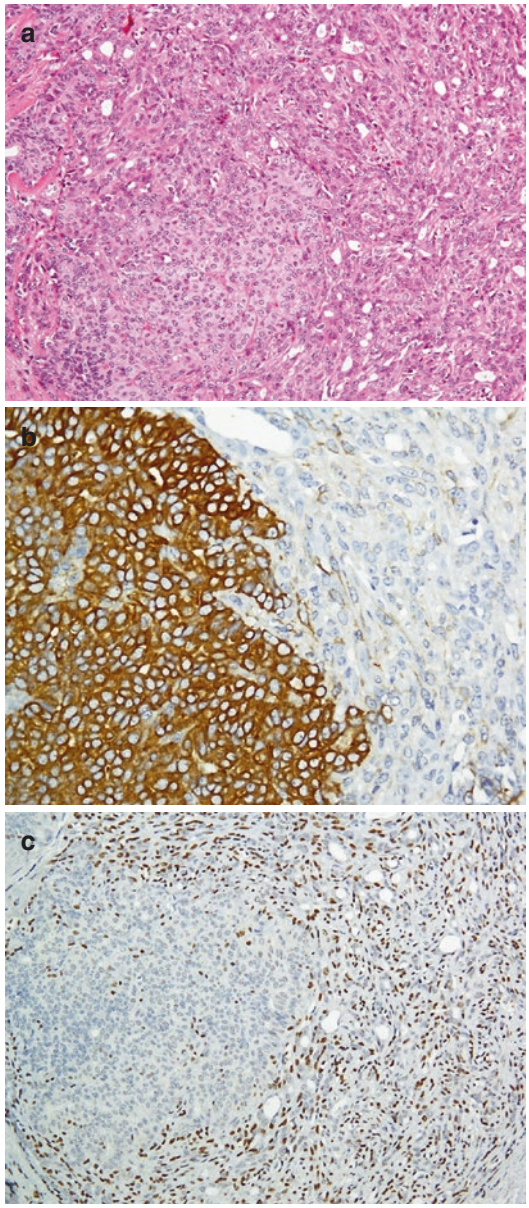

Fig. 29.30 Breast proliferative lesion (**a**). Presence of myoepithelial cells confirmed by immunoreaction to HMWCK (**b**) and p63 (**c**) which is indicative of a benign process

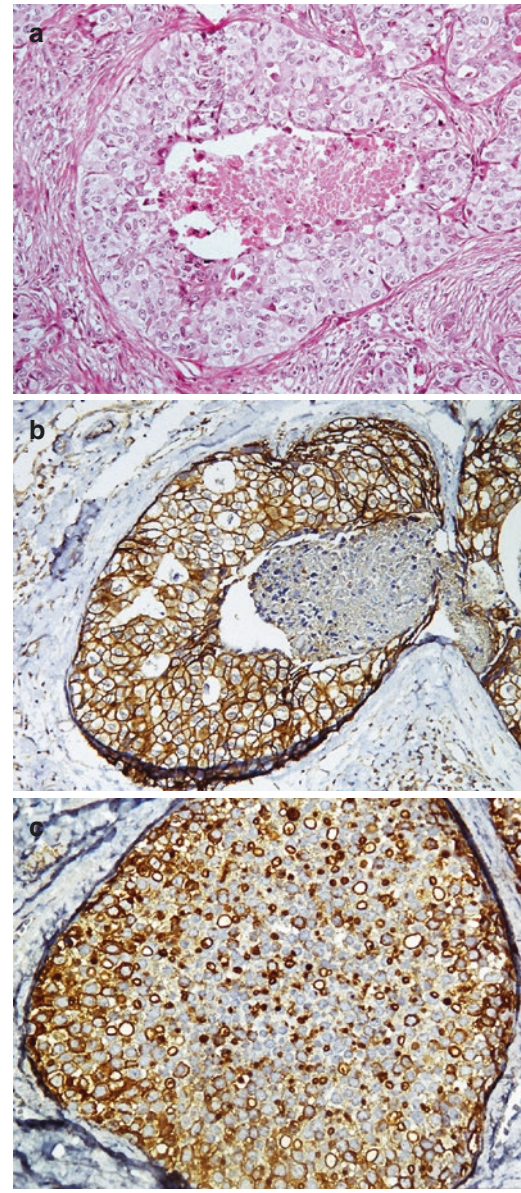

Fig. 29.31 Ductal carcinoma in situ (**a**) is immunostained with Her2neu (**b**) and CA15.3 (**c**)

29.9 Immunohistochemistry of Soft Tissue and Bone Tumors

Soft tissue sarcomas are a diverse family with different histologic origins and common histo-

Table 29.24 Immunoprofile of normal breast gland tissue

Normal epithelium	Immunoreactive antibodies
Luminal cells (LC)	CK8/18, CK19
Myoepithelial cells (MC)	CK5/6, CK14, CK17, p63, SMA, calponin, CD10
Both LC and MC	Pan-CK, AE1/AE3, CK7, S100

Table 29.25 Immunoprofile of invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) (Figs. 29.32 and 26.33)

Marker	IDC	ILC
Mamoglobin	−/+	+/−
ER	+/−	+
GCDFP15	−/+	−/+
E-cadherin	+	−
p120	+	+
34βE12	−	+

Note: + (>90%), +/− (>50%), −/+ (<50%), − (<10%)
References: [201, 206–209]

pathologic features. Given similar histopathologic features, immunohistochemistry is an ancillary method in distinguishing soft tissue tumors in order to attain a final diagnosis. As soft tissue tumor classification is based on specific line tissue origin, immunohistochemistry study by using specific Abs can be valuable in distinguishing them. Soft tissue tumors are vimentin-positive and keratin-negative tumors of a divergence family with heterogenous tissue origins. Vimentin, a nonspecific marker, appears to react with all soft tissue tumors and is considered as a control marker preserved in the tissue [256–262]. Immunohistochemistry of normal mesenchymal tissues with related tumors are summarized in Table 29.33.

29.9.1 Epithelial Markers

Recognized as an intermediate filament protein, Keratin is a sensitive and specific marker in the diagnosis of carcinomas among malignant tumors. Epithelial membrane antigen (EMA), derived from the mammary epithelium, is another epithelial marker expressed in most epithelial cells except squamous cells. Keratin and EMA are expressed exceptionally in some soft tissue tumors including synovial sarcoma, epithelioid sarcoma, chordoma, and myoepithelioma/myoepithelial carcinoma (previously known as parachordoma) [263].

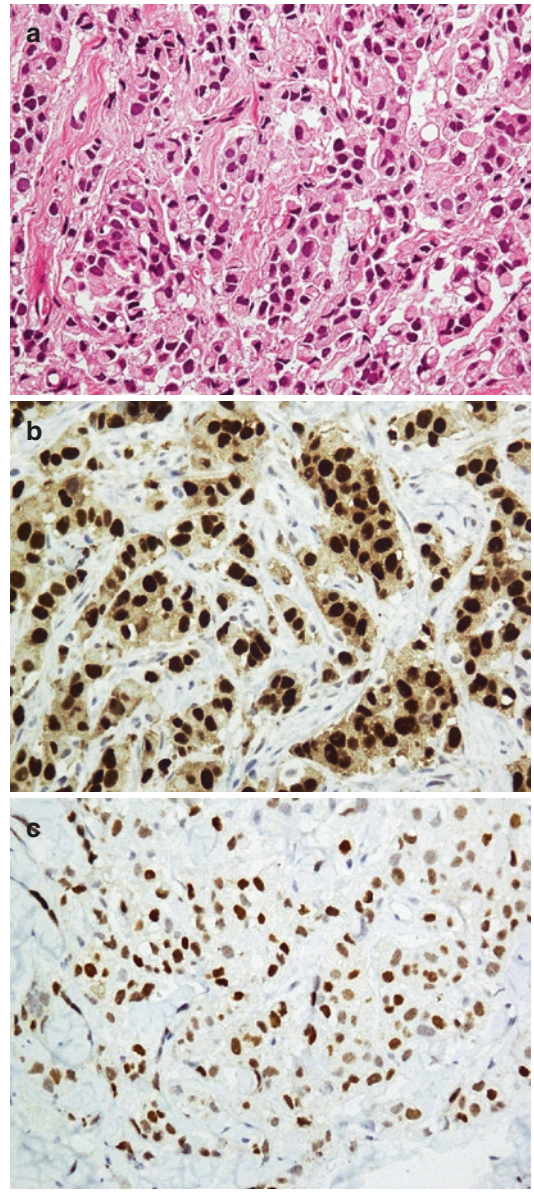

Fig. 29.32 Invasive ductal carcinoma (**a**) with ER (**b**) and PR (**c**) immunoreaction

29.9.2 Myogenic Markers

There are some Abs, which react with myogenic cells including desmin, actin, myoglobin, myoD1, myogenin, caldesmon, and calponin. Desmin

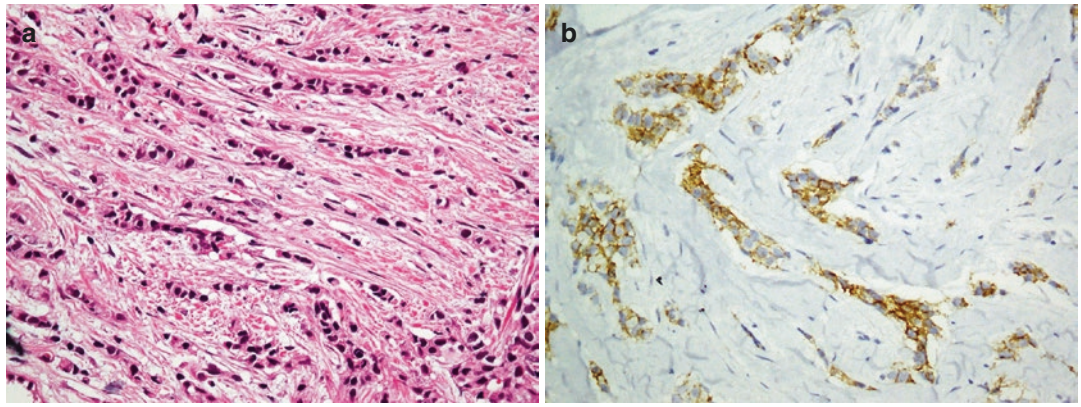

Fig. 29.33 Infiltrating carcinoma with Indian file pattern simulating lobular carcinoma (**a**), revealing immunoreaction with E-cadherin which is in favor of invasive ductal carcinoma (**b**)

is an intermediate filament protein present in the cytoplasm of smooth and skeletal muscles. The Ab against this protein reacts with myogenic tumors such as rhabdomyoma, leiomyoma, rhabdomyosarcoma, and leiomyosarcoma (Fig. 29.44) [264]. Similar to desmin, actin is another myogenic protein detected in smooth and skeletal muscles. In addition, smooth muscle actin may react with some other cells like myofibroblasts and myoepithelial cells [265–267]. Myoglobin is exclusively seen in skeletal muscle cytoplasm, whereas myo-D1 and myogenin are nuclear transcription factors, which are specifically expressed in skeletal muscle nuclei [268–270]. Myogenin has technical advantages over those of MyoD1, as the latter may cross-react with an unknown cytoplasmic Ag in non-muscle cells and tumors [271, 272]. However, Abs against these Ags are useful in determining rhabdomyosarcoma (Fig. 29.45). Calponin, a smooth muscle protein, is also expressed in myofibroblasts and myoepithelial cells and limits the usefulness of diagnostic pathology [49]. A relatively smooth muscle-specific marker being expressed in cytoplasm, Caldesmon is a useful Ab in distinguishing smooth muscle tumors from myofibroblastic tumors [273]. A novel Ag of smooth muscle differentiation, transgelin is a calponin-related protein found in smooth muscle showing higher sensitivity and specificity than other markers [274].

29.9.3 Nerve and Schwann Cell Markers

First isolated from the central nervous system (CNS), S-100 protein is known as a marker of nerve sheath tumors as well as melanocytic and chondrocytic tumors. S-100 is expressed by a wide range of cell types including glial cells, neurons, Schwann cells, melanocytes, chondrocytes, lipocytes, myoepithelial cells, sustentacular cells, Langerhans histiocytes, interdigitating reticulum cells, and various epithelia [27]. CD56 (neural cell adhesion molecule) and CD57 (myelin-associated glycoprotein) are expressed by a variety of different cell types including tissues of peripheral nervous system (PNS) and CNS, as well as natural killer (NK) cells and neuroendocrine cells [275–277].

29.9.4 Endothelial Markers

von Willebrand factor (vWF) is exclusively expressed by endothelial cells and is principally used to distinguish vascular neoplasms from their morphologic mimickers. Due to low sensitivity of vWF in detecting high grade vascular neoplasms, other endothelial markers such as CD31, CD34, and FLI -1 have limited the routine use of vWF in the context of vascular tumors. Given similar sensitivity to CD34,

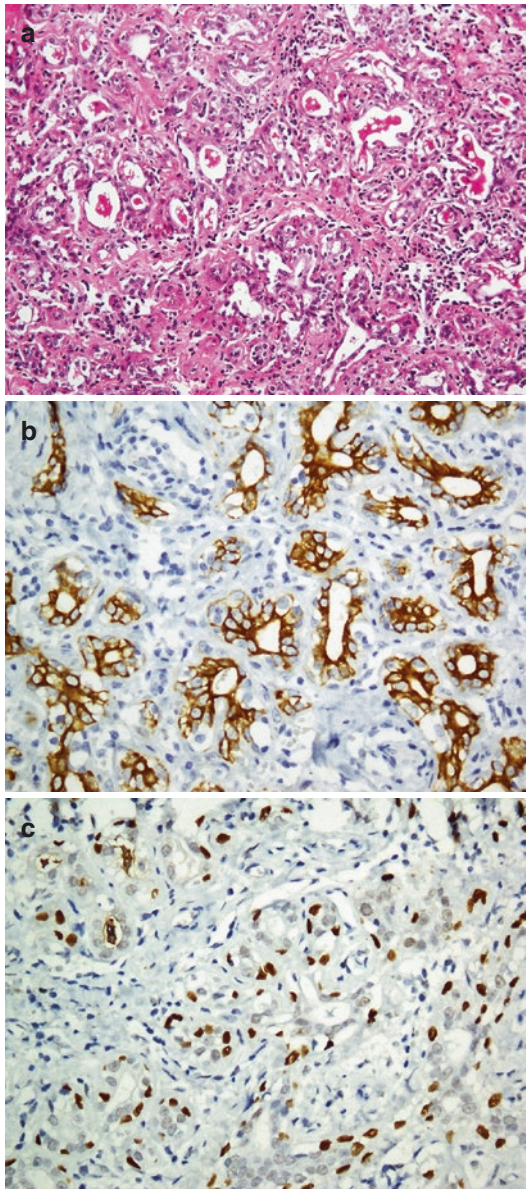

Fig. 29.34 Normal prostate epithelium (**a**) is immunostained with PSA (**b**) and basal cells are immunoreacted with p63 (**c**)

CD31 is expressed by macrophages, being a more specific vascular marker than CD34. CD34 is expressed by bone marrow hematopoietic precursor cells and dendritic interstitial cells limiting its application in vascular tumors [278–281]. As a nuclear transcription factor, FLI-1 (Freund's leukemia integration site) is an endothelial marker expressed in vascular tumors as well as ES/PNET and lymphoblastic lymphoma [56].

29.9.5 Fibrohistiocytic Markers

There are some nonspecific markers such as alpha1-antitrypsin, muramidase (lysozyme), alpha1-antichymotrypsin, cathepsin B, CD68, CD163, factor XIIIa, and the HAM 56 Ag which are expressed in melanomas, carcinomas, as well as some sarcomas like MFH [282–288]. Therefore, application of these markers is limited and should be considered after ruling out other sarcomas with specific line differentiation.

29.9.6 Lipocytic Markers

MDM2 (an inhibitor of p53 transcriptional activation) and CDK4 (a protein involved with cell cycle progression) are markers to separate dedifferentiated liposarcomas from other poorly differentiated sarcomas [289].

29.9.7 Chondrocyte Markers

Chondrocytes do not display specific markers, and show reactivity with S100 and vimentin. Chondrosarcoma also exhibits reactivity with CD57 [290]. Being as a master regulator of chondrogenesis, SOX9 is a sensitive marker for cartilogenous differentiation distinguishing mesenchymal chondrosarcoma from other small blue round cell tumors [291].

29.9.8 Osteogenic Markers

Osteocalcin (a non-collagenous intraosseous protein) with approximately 70% sensitivity is a completely specific marker for bone-forming tumors. In addition, osteonectin (a bone matrix glycoprotein participates in stromal mineralization) also a sensitivity of 90% and a specificity of

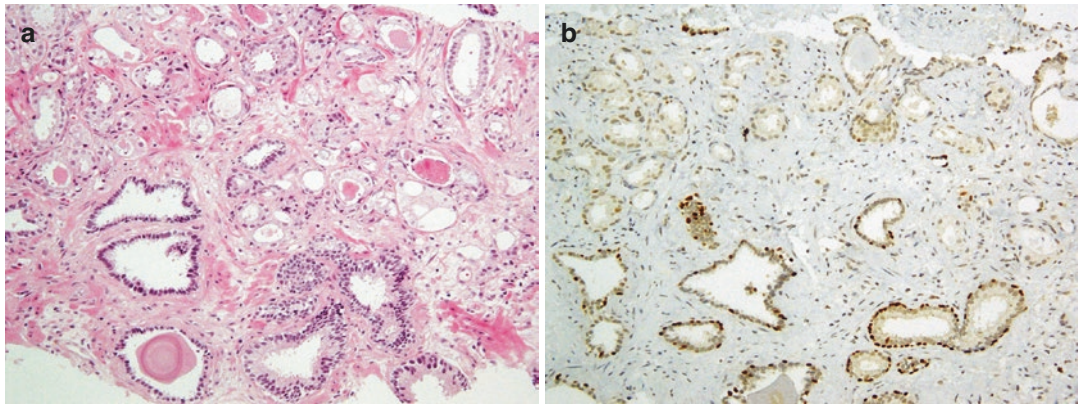

Fig. 29.35 Atypical prostate glands in the top of the picture which are highly suspicious to adenocarcinoma (**a**), show negative reaction to p63 (**b**). Some normal glands at the bottom of picture exhibit reaction with p63

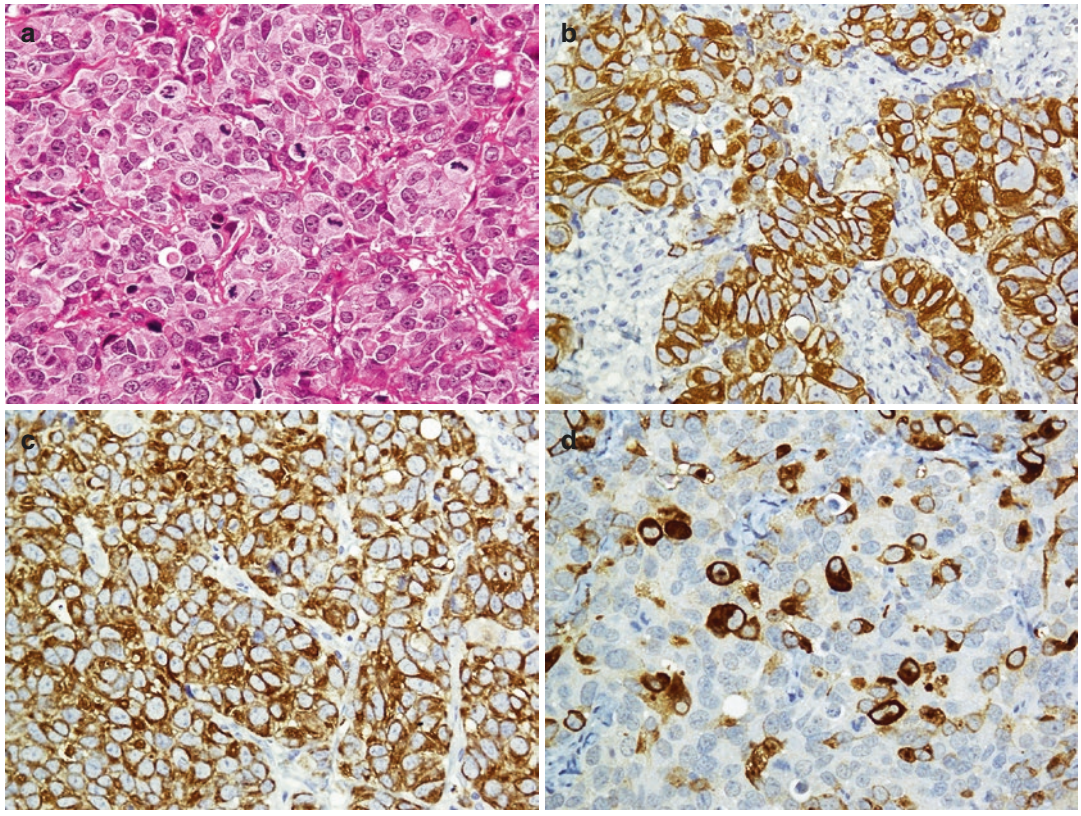

Fig. 29.36 An undifferentiated carcinoma from pelvis with high mitotic rate (**a**) demonstrates cytoplasmic reaction with CK7 (**b**), CK20 (**c**) and PSA (**d**) which support the origin of this tumor as prostate

54% in the diagnosis of osteoblastic neoplasms [292, 293]. These markers are rarely being used in routine diagnosis because the diagnosis of the osteosarcoma is based on the presence of osteoid in the H&E stained slides.

29.9.9 Unknown-Origin Soft Tissue Tumors

Ewing sarcoma/peripheral nerve sheath tumor (ES/PNET) comprises a prototype of small

Table 29.26 Immunoprofile of normal prostate (NP), high-grade prostate intraepithelial neoplasia (HGPIN), and prostate adenocarcinoma (PAC)

Marker	NP	HGPIN	PAC	Application
PSA	+E	+	+	Weak reaction in HGPAC or metastatic carcinoma, to differentiate HGPAD from other undifferentiated carcinoma (colon, urothelium)
PSAP	+E	+	+	Similar to PSA
PSMA	+E	+	++	Correlated with grade and stage, more intense in HGPAC
P501S	+E	+	+	To differentiate high grade PAC from other high-grade adenocarcinomas (colon, urothelium)
P504S (AMACR)	–	++	++	Combine with basal cell markers to differentiate HGPIN and PAC from normal prostate
HMWCK (34βE12)	+B	Partial loss	–	Complete loss in PAC ("negative" marker)
p63	+B	Partial loss	–	More sensitive than HMWCK ("negative" marker)
CK5/6	+B	Partial loss	–	More sensitive than HMWCK ("negative" marker)

Note: *B* basal cell, *E* epithelium
References: [211–219]

round cell neoplasms of bone and soft tissue exhibiting neuroectodermal features. As a product of the MIC2 gene, CD99 is a cell surface transmembrane glycoprotein diffusely present in nearly all tumors (Fig. 29.46) [294]. Clear cell sarcoma (malignant soft part melanoma) shares markers of malignant melanoma such as S-100, MART-1, HMB45, and tyrosinase [295]. Alveolar soft part sarcoma has been evaluated by the presence of Myo-D1 and myogenin [296, 297]. Desmoplastic small round cell tumor (DSRCT) is characterized by the coexpression of epithelial and mesenchymal markers [298]. The immunohistochemistry characteristics of these tumors are summarized in Table 29.34.

29.10 Immunohistochemistry of the Nervous System

The brain tumors are classified into two major groups: primary and metastatic. Primary brain tumors are further categorized into three major subtypes: neuroepithelial tumors (astrocytoma, oligodendroglioma, ependymoma, choroid plexus tumors, neuronal tumors, and pineal tumors), non-neuroepithelial tumors (meningioma, nerve sheath tumors, lymphoma, chordoma, and germ cell tumors) and primitive undifferentiated tumors (medulloblastoma, pineoblastoma, ependymoblastoma, and PNET) [299–305]. Primary origin

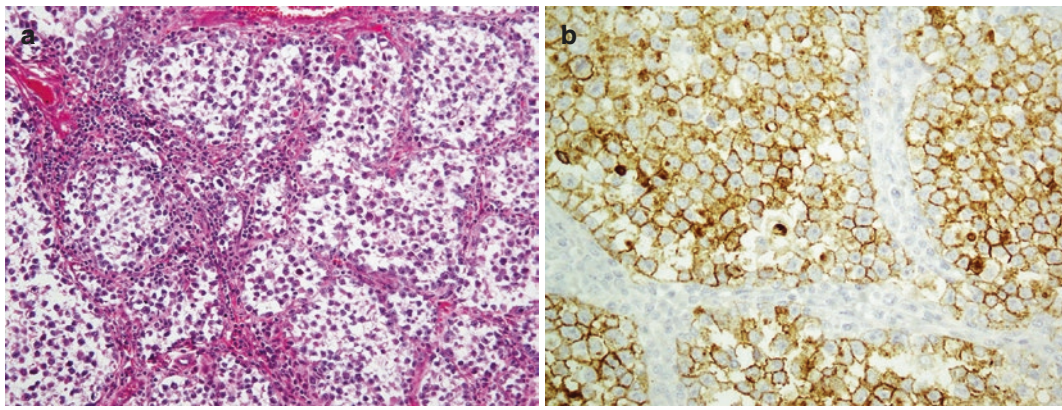

Fig. 29.37 Classic seminoma with polygonal cells and abundant watery cytoplasm (**a**) shows immunostaining with PLAP (**b**)

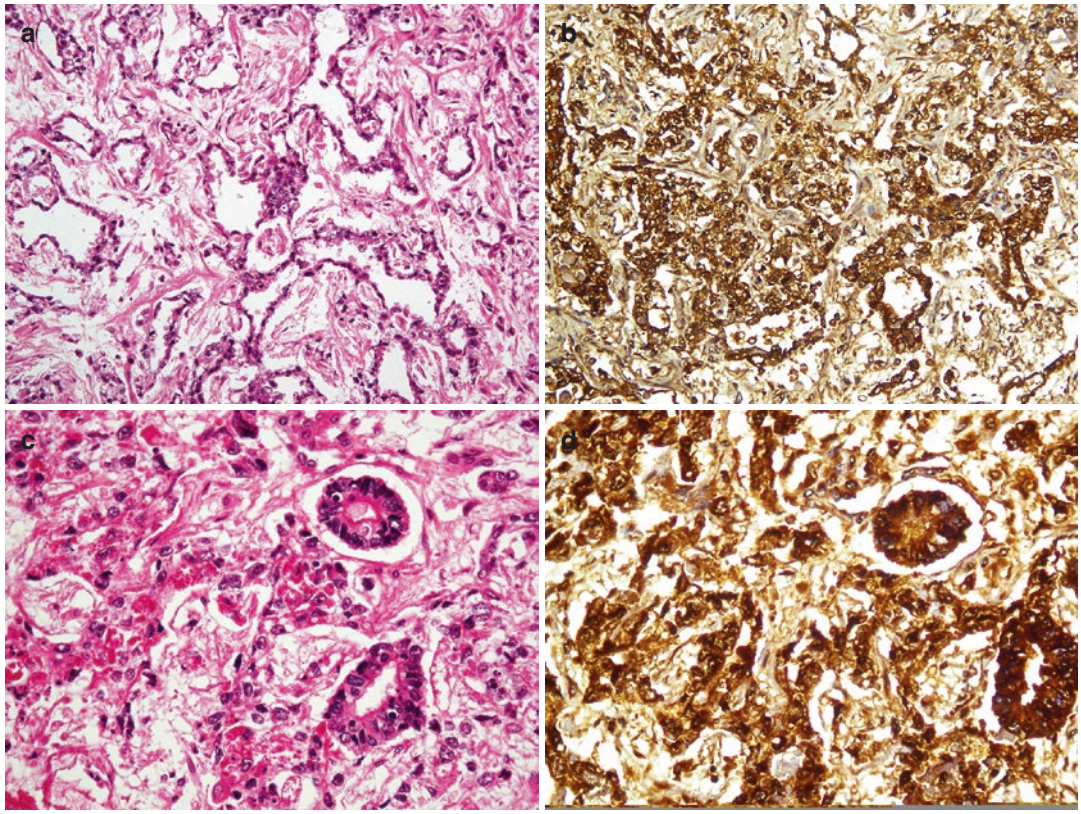

Fig. 29.38 Yolk sac tumor with tubuloglandular structures exhibits immunostaining with AFP (**a**, **b**) and glandular structures with numerous hyaline globules which are positive for AFP (**c**, **d**)

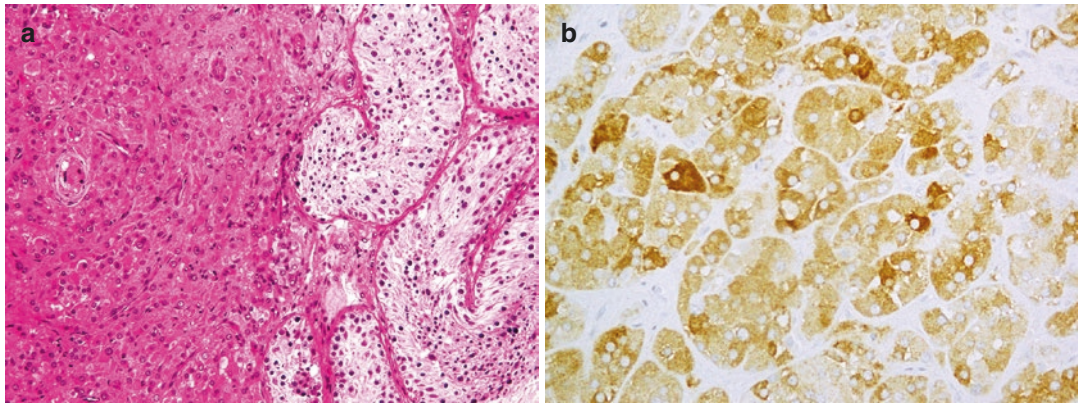

Fig. 29.39 Leydig cell tumor. Eosinophilic polygonal cells growth in the adjacent of seminiferous tubules (**a**) show immunoreaction with Inhibin-A (**b**)

of metastatic carcinoma is determined by the use of immunohistochemical panel. Commonly used IHC Abs in primary CNS tumors are demonstrated in Table 29.35.

29.10.1 Neuroepithelial Tumors

Glial tumors (astrocytoma, oligodendroglioma, and ependymoma) usually react with glial

Table 29.27 Immunophenotype of testicular tumors: classic seminoma (CS), spermatocytic seminoma (SS), embryonal carcinoma (EC), yolk sac tumor (YST), choriocarcinoma (CC), Sertoli cell tumor (SCT), and Leydig cell tumor (LCT)

Germ cell tumors (PLAP+, Inhibin−)					Sex-cord stroma tumors (PLAP−, Inhibin+)	
CS (Fig. 29.37)	SS	EC	YST (Fig. 29.38)	CC	SCT	LCT (Fig. 29.39)
C-kit+ OCT3/4+ CD117+ D2-40+	C-kit+/−	C-kit+/− OCT3/4+ AE1/AE3+ AFP+/− CD117+ CD30+	C-kit+/− AE1/AE+ AFP+ Glypican-3+ HepPar-1+	Inhibin+ AE1/AE3+ Glypican-3+ HCG+	AE1/AE−/+ CAM5.2+ Vimentin+ SMA+ SYN+ NSE+	AE1/AE−/+ GAL-3+ Vimentin+ CD99+/−

Note: + (>90%), +/− (>50%), −/+ (<50%), − (<10%)
References: [163, 220–233]

Table 29.28 Immunoprofile of precursor lymphoid neoplasms (Fig. 29.40)

Lymphoma	CD2	CD5	CD20	CD79a	PAX5	CD45	CD34	CD10	CD99	Tdt	CD43	CD56
B ALL/LBL	−	−	+/−	+	+	−/+	+	+	−	+	+	−
T ALL/LBL	+	+	−	−	−	−/+	+	+/−	+	+	+	+

Note: + (>90%), +/− (>50%), −/+ (<50%), − (<10%)
References: [234–240]

Table 29.29 Immunoprofile of small B-cell lymphomas: B-cell small lymphocytic lymphoma/chronic lymphocytic lymphoma (B SLL/CLL), mantle cell lymphoma (MCL), marginal zone lymphoma (MZL), mucosa-associated lymphoid tissue (MALT), follicular lymphoma (FL), lymphoplasmacytic lymphoma (LPL), and hairy cell leukemia (HCL)

Lymphoma	CD20	CD23	CD10	CD5	BCL6	MUM1	CD43	CyclinD1	AnnexinA1	BCL2
B SLL/CLL	+ (weak)	+	−	+	−	+/−	+	−/+	−	+
MCL	+	−/+	−	+	−	−	+	+	−	+
MZL (nodal)	+	−	−	−	−/+	+	+/−	−	−	+
MZL (MALT)	+	−	−	−	−	+/−	+/−	−	−	+
MZL (splenic)	+	−	−	−	−	+/−	−	−	−	+
FL	+	−/+	+	−	+	−	−[a]	−	−	+
LPL	+	−/+	−/+	−	−	+[b]	−/+	−	−	+
HCL	+	−/+	−/+	−	−	NT	NT	+	+	+

Note: + (>90%), +/− (>50%), −/+ (<50%), − (<10%)
References: [234–237, 241–249]
[a]Maybe positive in grade 3
[b]More intense in plasmacytoid cells

Table 29.30 Immunoprofile of some aggressive mature B-cell lymphomas: diffuse large B-cell lymphoma (DLBL), T-cell/histiocyte-rich B-cell lymphoma (TC/HRBCL), and anaplastic large-cell lymphoma kinase (ALK)

Lymphoma	CD20	CD10	MUM1	Bcl-2	Bcl-6	CD30	Ki-67	EMA	CD45	CD138
DLBCL (NOS) (Fig. 29.41)	+	+[a]	−[b]	+/−	+[a]	−[a]	<90%	−	+	−
TC/HRBCL	+	−/+	−/+	+/−	+	−	<90%	+	+	−
DLBCL Plasmablastic	−[a]	−	+	−	−	+/−	>90%	+	−[a]	+
DLBCL-ALK + (Fig. 29.42)	−	−	+/−	−	−	−	<90%	+	+ weak	+
Burkitt lymphoma	+	+	−	−[a]	+	−	>95%	−	+	−

Note: + (>90%), +/− (>50%), −/+ (<50%), − (<10%)
References: [234–237, 246, 249–252]
[a]Some cells may be weakly positive
[b]Positive in non germinal centers (35–65%)

Table 29.31 Immunoprofile of some mature T-cell/NK cell lymphomas: mycosis fungoides (MF), adult T-cell lymphoma/leukemia (ATLL), angioimmunoblastic T-cell lymphoma (AILT), anaplasticg large-cell lymphoma (ATCL), and T-cell lymphoma (TCL)

Lymphoma	CD3	CD5	CD4	CD8	CD30	ALK	TIA1	CD56
MF	+	+	+	–	+[c]	–	+[c]	–
ATLL	+	+	+[a]	–[a]	+/–	–	–	–
AILT	+	+	+	–	+[c]	–	–	–
ALCL	–/+	+	+	–	+	+ (60–80%)	+/–[d]	–
Subcutaneous Panniculitis-like TCL	+	–	–	+	–	–	+	–
Cutaneous TCL	+	–	–	–/+	–	–	+	+
Hepatosplenic TCL	+	–/+	–	–/+	–	–	+	+
Nasal or nasal-type NK/TCL	+ (cytoplasmic)	–	–	+/–	–	–	+	+
Enteropathy-type TCL	+	–	–	+	+/–	–	+	+[b]

Note: + (>90%), +/– (>50%), –/+ (<50%), – (<10%)
References: [234–237, 253–255]
[a]Most cases
[b]Subset with monomorphic small-cell morphology
[c]Some large cells
[d]More often ALK positive cases

Table 29.32 Immunophenotypic features of classic Hodgkin lymphoma (CHL) and nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) (Fig. 29.43)

Lymphoma	CD20	Pax-5	CD15	CD30	Facsin	EMA	ALK-1
CHL	+/–	+ (weak)	+	+	+	–/+	–
NLPHL	+	+	–	–/+	–	+/–	–

Note: + (>90%), +/– (>50%), –/+ (<50%), – (<10%)
References: [234–237]

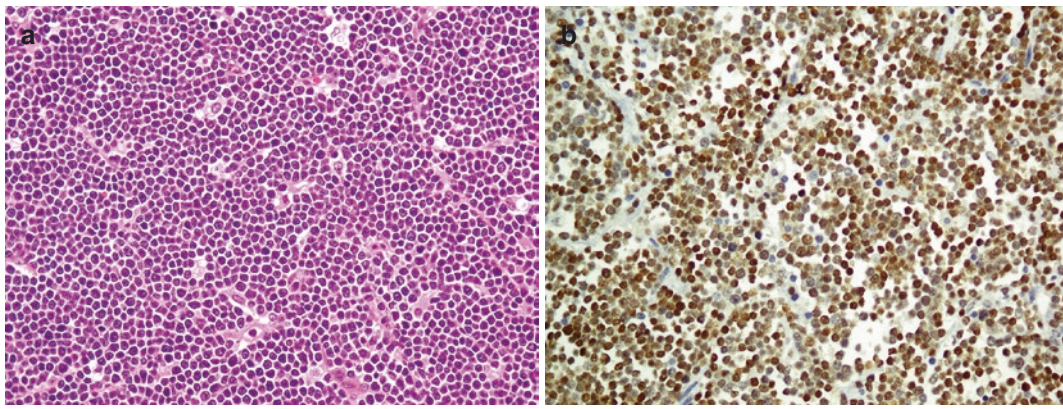

Fig. 29.40 Lymphoma with starry sky feature declares a highly proliferative phase (**a**) in which antibodies to terminal deoxynucleotidyl transfer (TdT) marks it as a precursor lymphoid neoplasm (**b**)

fibrillary acidic protein (GFAP) [160, 161, 306]. Oligodendroglioma variably expresses GFAP and commonly reacts with Leu7 and S-100 [307, 308]. Moreover, GFAP is present in other mixed glial and neuronal-glial tumors including oligoastrocytoma and ganglioglioma (Fig. 29.47) [306]. Neurocytoma and pineal tumors are GFAP-negative and synaptophysin-positive.

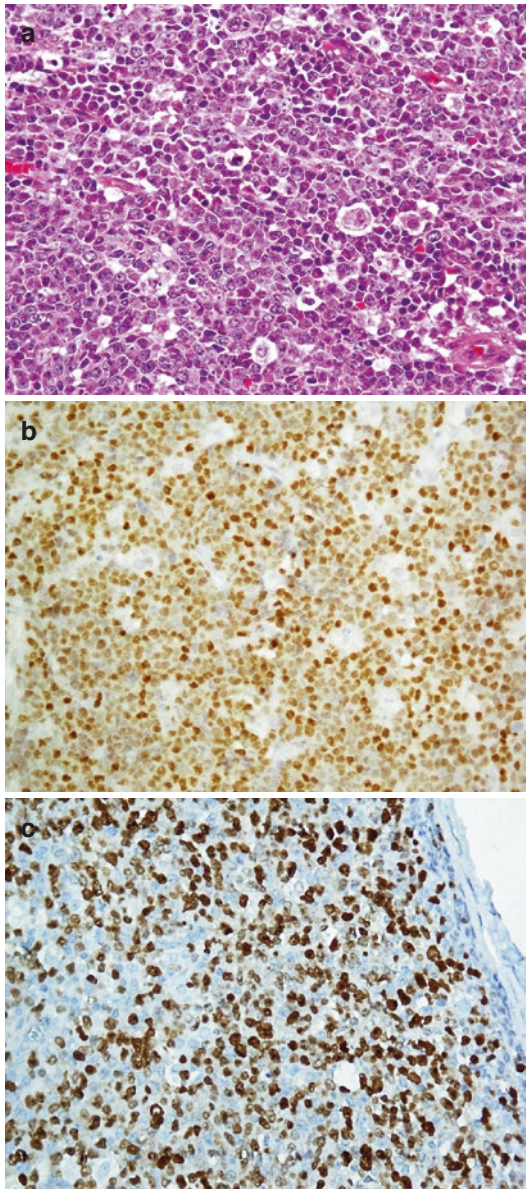

Fig. 29.41 Diffuse large B-cell lymphoma (NOS) (**a**) weakly reacts with Bcl-6 and (**b**) indicates a high proliferative index by Ki-67 (**c**)

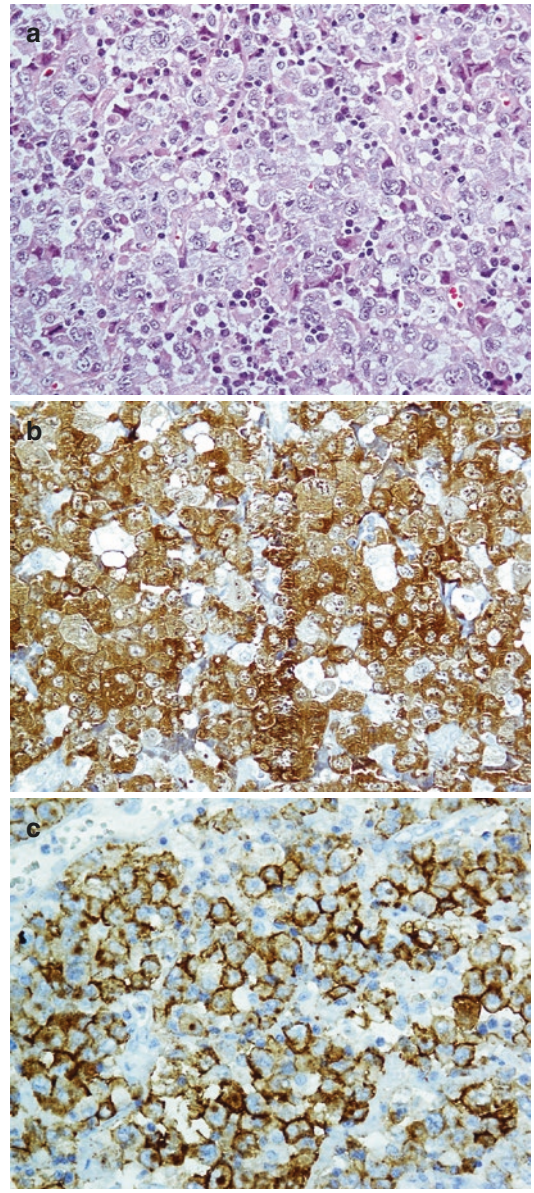

Fig. 29.42 Diffuse large B-cell lymphoma (ALK). Large anaplastic cells intermixed with lymphoplasma cells (**a**) are strongly positive for ALK (**b**) and EMA (**c**)

Among neuroepithelial tumors, choroid plexus tumors demonstrate reactivity with epithelial markers such as cytokeratin, CAM5.2, and EMA. Additionally, transthyretin, as a potential marker, and IGF-II, as a newer marker, are positive in choroid plexus tumors [309–311]. Pineal tumors are GFAP and epithelial-negative tumors, which exhibit reactivity with synaptophysin and neurofilament (Table 29.36).

29.10.2 Non-neuroepithelial Tumors

Among non-neuroepithelial tumors, meningiomas are positive for EMA, which differentiates

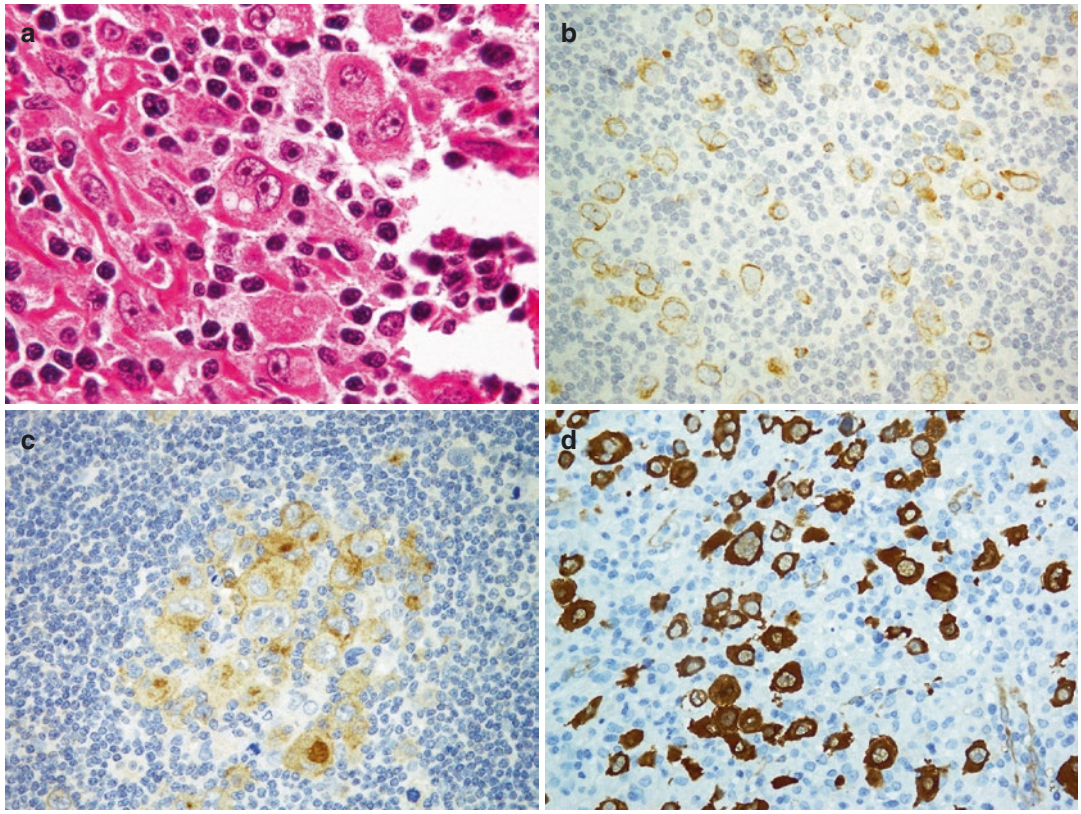

Fig. 29.43 Hodgkin lymphoma. Typical Reed–Stenberg cell with mirror binuclear feature of "Owl's eye"(**a**) weakly reacts with CD 15 (**b**) and CD30 (**c**) and strongly reacts with fascin (**d**)

Table 29.33 Immunohistochemical antibodies of normal mesenchymal tissues and related tumors

Soft tissue	Markers of soft tissue	Related tumor	Immunoreactive markers
Chondrocyte	S100, SOX9, vimentin	Chondrosarcoma	S100, vimentin, CD57, SOX9: sensitive marker for cartilaginous differentiation
Endothelial cells	Vimentin, CD-31, CD-34, FLI-1	Angiosarcoma	CD-31, CD-34, FLI-1
	D2-40 (lymphatic endothelium)	Lymphangiosarcoma	D2-40
Fibroblasts	Vimentin, CD10, CD99	Fibrosarcoma	Vimentin
Fibrohistiocyte	CD68, CD168, a1AT, cathepsin B, factor IIIA, HAM 56	Malignant fibrous histiocytoma	CD68
Lipocytes	Vimentin, S100 (variable), calretinin, MDM2, CDK4, CD-34	Liposarcoma	S100, MDM2, CDK4
Osteoblast	CD56, osteocalcin, osteonectin, vimentin	Osteosarcoma	Osteocalcin, collagen IV, CK, EMA, CD99, S100, desmin, SMA, factor 13
Nerve/Schwann cell	Vimentin, S100, CD56, CD57	MPNST	S-100
Skeletal muscle	Desmin, myoglobin, CD56, GFAP	Rhabdomyosarcoma	Myogenin, myo-D1, PLAP, WT-1
Smooth muscle	Desmin, NSE, SMA, MSA	Leiomyosarcoma	Desmin, SMA, MSA, h-caldesmon, collagen IV
Synovial cell	CD68, clusterin	Synovial sarcoma	CK, EMA, vimentin, CD68, CD-99, E-cadherin, Collagen IV

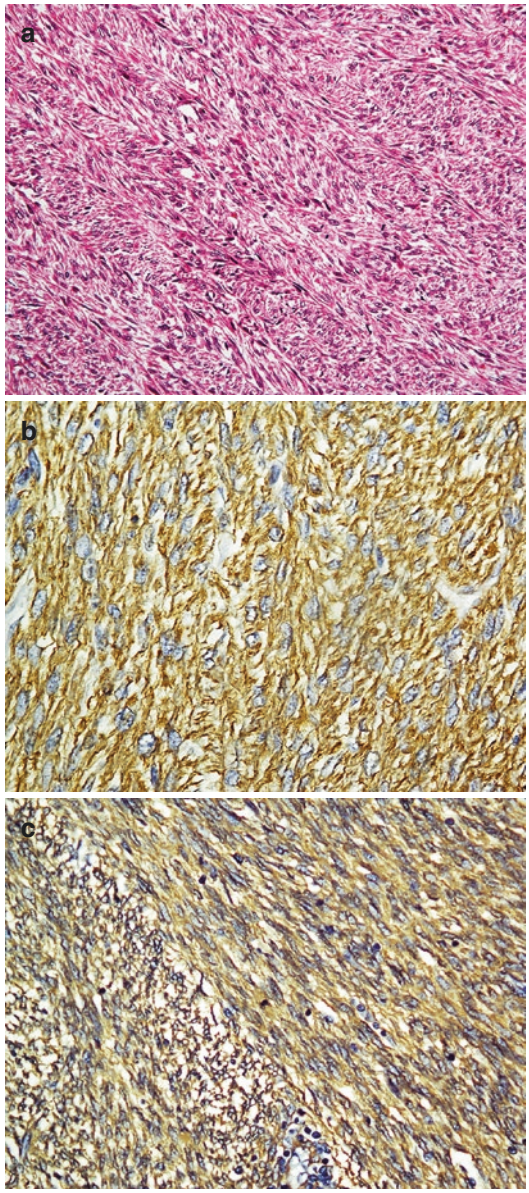

Fig. 29.44 Leiomyosarcoma. Spindle cells arranged in interlacing cross-striated fascicles (**a**) are immunostained with desmin (**b**) and H-caldesmon (**c**)

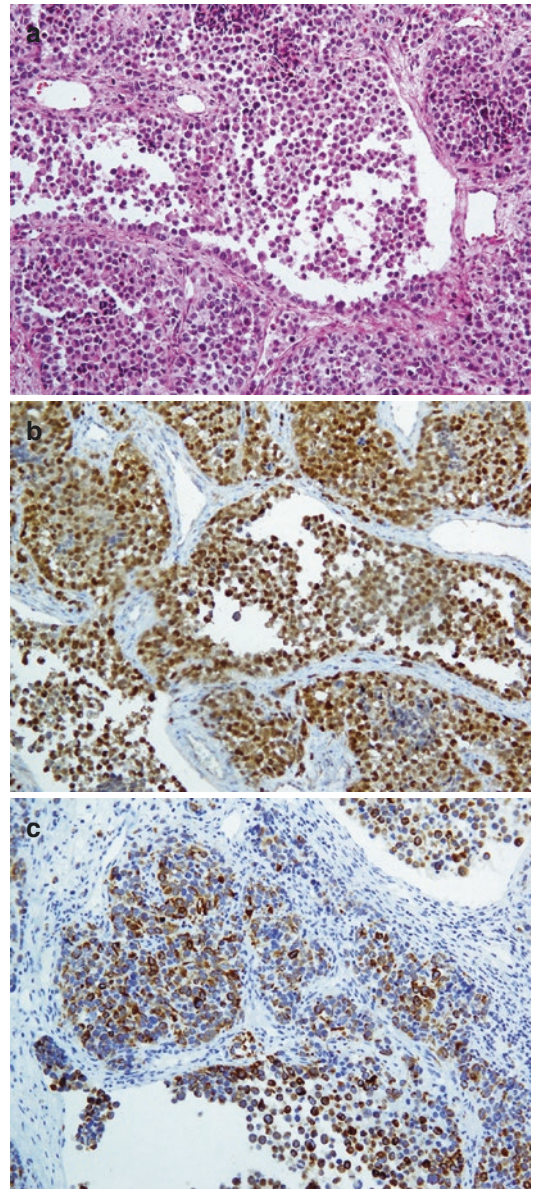

Fig. 29.45 Alveolar rhabdomyosarcoma. Large polygonal cells with alveolar pattern (**a**) are highlighted with myogenin (**b**) and desmin (**c**)

them from nerve sheath tumors, and are negative for GFAP which distinguishes meningioma from gliomas. Schwannoma is distinct from glioma, meningioma, and neurofibroma by showing reaction to collagen type IV. Neurofibroma differs from schwannoma by having neurofilament-positive axons. Primary and secondary brain lymphomas express LCA as a common marker and CD3 and CD20 as differentiating markers of T-cell and B-cell type lymphomas, respectively. Arising from notochord remnants, chordomas are malignant tumors along the axial skeleton

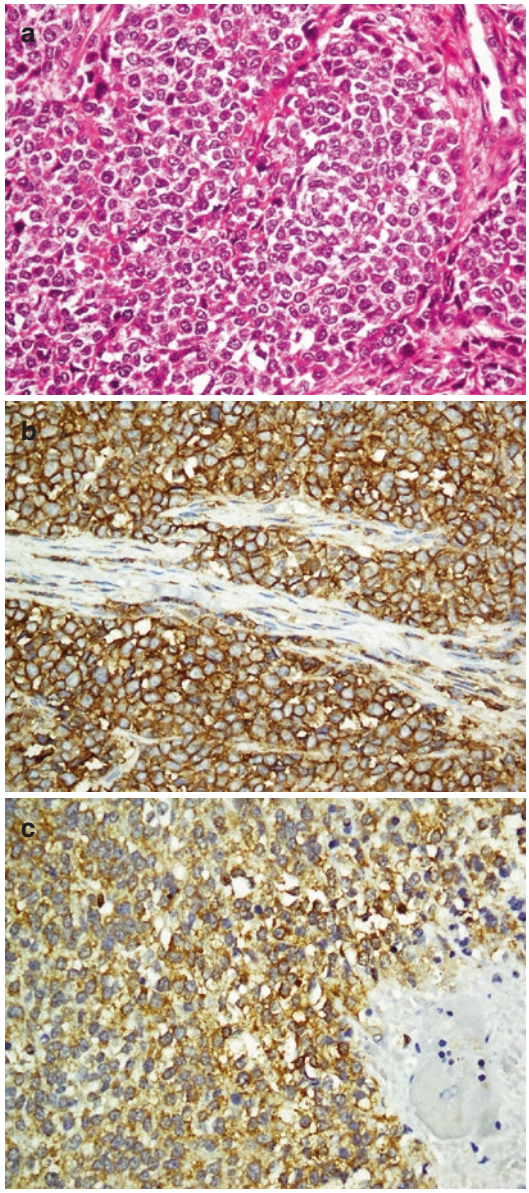

Fig. 29.46 Small round cell tumor (**a**). Immunoreaction with MIC2 (**b**) and NSE (**c**) antibodies supports the diagnosis of PNET

recognized by characteristic physaliphorous cells with large intracytoplasmic vacuoles. Chordoma exhibits reactivity for CK and EMA as well as S100, whereas chondrosarcomas lack these features (CK/EMA-negative and S100-positive). Primary germ cell tumors are found along the midline in the pineal and suprasellar regions which demonstrate immunostaining with

Table 29.34 Immunoprofile of unknown-origin soft tissue tumors: Ewing sarcoma/peripheral neuroectodermal tumor (ES/PNET), clear-cell sarcoma (CCS), alveolar soft part sarcoma (ASPS), and desmoplastic small round cell tumor (DSRCT)

Panel antibodies	ES/PNET	CCS	ASPS	DSRCT
CD99/FLI-1	+	–	–	–
S100/HMB45/ MITF/Melan-A	–	+	–	–
TFE3	–	–	+	–
NSE	+	–	–	+
Desmin	–	–	–	+
CK/EMA	–	–	–	+
WT1	–	–	–	+

References: [294–298]

placental alkaline phosphatase (PLAP), alpha fetoprotein (AFP), beta-HCG, and CEA (Fig. 29.48) (Table 29.37).

29.10.3 Undifferentiated Tumors

Medulloblastoma, pineoblastoma, ependymoblastoma, and PNET are primitive undifferentiated tumors commonly located in the posterior fossa, pineal gland, periventricular area, and anterior fossa, respectively. Medulloblastoma, pineoblastoma, and ependymoblastoma differentiate from PNET by negative reaction for CD99. Ependymoblastoma can be distinguished from meduloblastoma/pineoblastoma/PNET by the absence of reactivity to synaptophysin and neurofilament (Table 29.38).

29.10.4 Proliferative Markers

MIB1 (Ki67) is an Ab that detects proliferating cells in various phases of the cell cycle, and is important in the grading of CNS tumors. It is used to predict patient outcome and distinguishes long- and short-time survivals in patients with glial tumors (Table 29.39 and Fig. 29.49). p53 and EGFR overexpression can be defined immunohistochemically. Overexpression of p53 is associated with tumor progression in glioblastoma multiforme (GBM). EGFR overexpression correlates with poor prognosis in gliomas and is

Table 29.35 Commonly used antibodies in primary CNS tumors

Antibody	Normal brain	Tumor
EMA	Epithelial, perineural, meningothelial cells	Meningioma, chordoma, medulloblastoma
GFAP	Glial cells	Glial tumors except oligodendroglioma, medulloepithelioma, choroid plexus tumor, ganglioglioma
Leu7 (CD57)	Oligodendroglial cells, Schwann cells	Oligodendroglioma, schwannoma, Neurofibroma, oligoastrocytoma
Neurofilament	Neuropil	Ganglion cell tumors, neurocytoma, pineocytoma, neurofibroma, medulloblastoma, PNET
NSE	Neuroectodermal and neuroendocrine cells	Neuroblastoma, hemangioblastoma, PNET, oligodendroglioma
S-100	Glial cells, Schwann cells, dendritic and Langerhans cells, melanocytes, other mesenchymal cells	Gliomas, meningioma, schwannoma, neurofibroma, chordoma, craniopharyngioma, PNET, medulloblastoma, pineoblastoma, neuroblastoma, melanoma, chondroid tumors
Synaptophysin	Neuroendocrine cells, neuropil	Neurocytoma, ganglion cell tumors, pineocytoma, choroid plexus papilloma, medulloblastoma, pineoblastoma, neuroblastoma, PNET, oligodendroglioma, dysembryoblastic neuroepithelial tumor
Vimentin	Meningoendothelial cells, other mesenchymal cells	Meningioma, gliomas, chordoma, ependymoblastoma, hemangiopericytoma, ganglioglioma, embryonal tumors
Collagen IV	Ganglion cell, Schwann cell, other mesenchymal cells	Ganglion cell tumor, schwannoma, medulloblastoma/ pineoblastoma

References: [160, 161, 306]

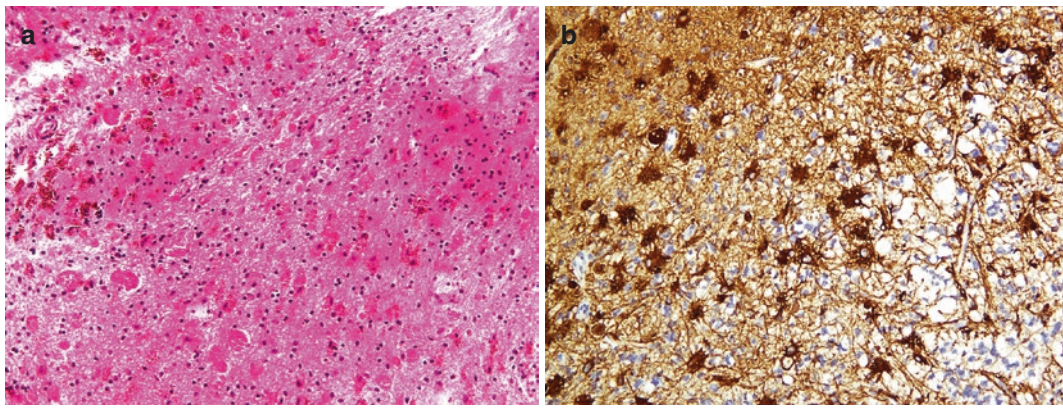

Fig. 29.47 Fibrillary astrocytoma with proliferation of atypical astrocytes (**a**), exhibit GFAP-positive cytoplasmic processes (**b**)

Table 29.36 Immunopanel of neuroepithelial tumors

First-choice antibody panel	Second-choice antibody panel	Consistent with
GFAP+, EMA−, CAM5.2−	Vim+, NF+, S100+	Astrocytoma (Fig. 29.47)
	Leu7+, NSE+, S100+	Oligodendroglioma
GFAP+, EMA (R), CAM5.2 (R)	Vim+, S100+	Ependymoma
GFAP (S), EMA+, CAM5.2+	Laminin+, SPN+, S100+, IGF-II+	Choroid plexus papilloma
GFAP−, EMA−, CAM5.2−	SPN+, NF+	Central neurocytoma
	SPN (S), NF (S), Collagen IV+	Ganglion cell tumor
	NSE+, SPN+, NF (R)	Pineal tumor

Note: *N* negative, *R* rare, *S* sometimes
References: [160, 161, 306–308, 312–320]

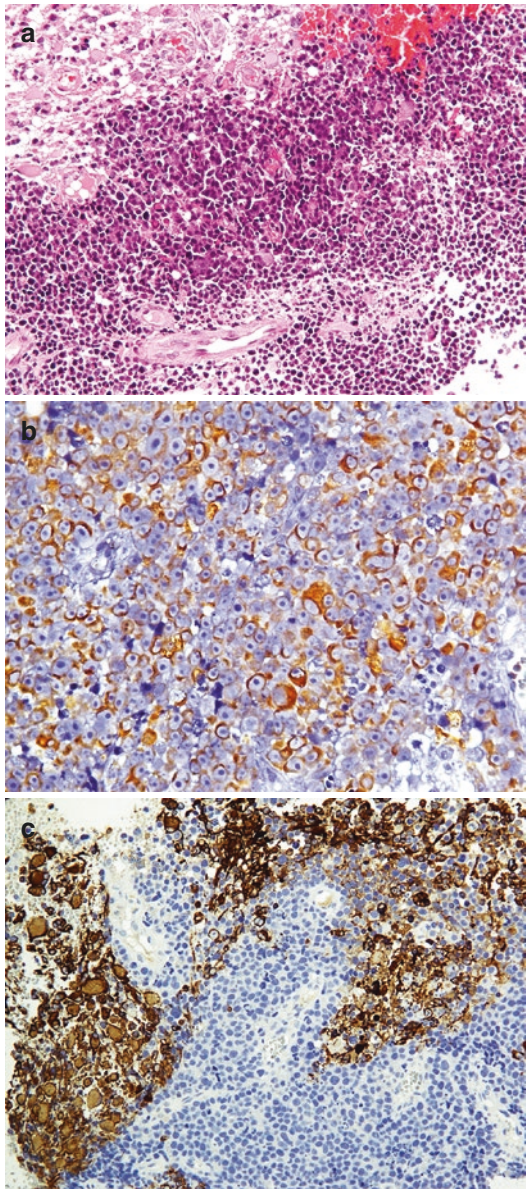

Fig. 29.48 Germinoma. (**a**) A tumor with relatively medium to large polygonal cells resembling an undifferentiated tumor surrounded by reactive astrocytes (upper right corner). Tumor cells react with PLAP (**b**) and reactive astrocytes stain by GFAP (**c**). Courtesy of Dr. Taghi Ghiasi-Moghadam, Mashad, Iran

not present in low-grade gliomas. As a new therapeutic target, EGFR tyrosine kinase inhibitors are used for the treatment of GBM.

29.11 Immunohistochemistry of Pediatric Tumors

Solid pediatric tumors comprise a heterogenic group of variable entities with morphologies including small round cells, spindle cells, and polygonal cells. Small round cell tumors include neuroblastoma, rhabdomyosarcoma, Ewing's sarcoma/PNET, desmoplastic small round cell tumor, Wilms' tumor (Fig. 29.50), small-cell osteosarcoma, lymphoma, and melanoma. Rhabdomyosarcoma, Wilms' tumor, and melanoma also display spindle cell components or present as pure spindle cell tumor. Polygonal cell tumors of childhood comprise of rhabdomyosarcoma, malignant rhabdoid tumor, osteosarcoma, and melanoma [341, 342].

Frequently confused with primitive neuroectodermal tumors (PNETs), neuroblastoma is the most common malignant tumor of the posterior mediastinum in pediatric patients with morphology of small round cell tumor. Neuroblastoma has a predilection for adrenal glands and sympathetic ganglia, whereas PNETs are cholinergic tumors [343, 344]. Expression of CD44s and c-kit receptor correlates with favorable prognosis in a subset of neuroblastoma [345, 346]. Rhabdomyosarcoma is the most common pediatric soft tissue sarcoma subclassified into embryonal, botryoid, alveolar, and spindle cell subtypes. Embryonal rhabdomyosarcoma (including botryoid), the most common type in childhood, usually displays small-cell morphology, whereas the alveolar variant usually exhibits features of polygonal cells [347–350].

Initially regarded as an undifferentiated sarcoma of the bone and soft tissue, Ewing's sarcoma/primitive neuroectodermal tumor (ES/PNET) is now being classified as a small round cell tumor with varying degrees of neuroectodermal differentiation with pseudorosette formation [351]. Desmoplastic small round cell tumor is an aggressive, malignant tumor usually involving the abdominal or pelvic cavity of children or young adults with the morphology of small round

Table 29.37 Immunopanel of non-neuroepithelial tumors

First-choice antibody panel	Second-choice antibody panel	Consistent with
Vimentin+, S100+	EMA+	Chordoma
Vimentin+, S100 (R)	EMA (S)	Meningioma
Vimentin−, S100+	Leu7+, collagen IV+, GFAP (R)	Schwannoma
	Leu7+, NF+, EMA+	Neurofibroma
Vimentin−, S100−	LCA+, L26+	Lymphoma
	PLAP+, HCG+, AFP+	Germ cell tumor (Fig. 29.48)

Note: *N* negative, *R* rare, *S* sometimes
References: [160, 161, 306–308, 321–326]

Table 29.38 Immunopanel of primitive undifferentiated tumors

First-choice antibody panel	Second-choice antibody panel	Anatomic site	Consistent with
SYNP+, S100+	NF (R), GFAP (R), Collagen IV+, Vim (S), CD99−	Posterior fossa	Meduloblastoma
		Pineal gland	Pineoblastoma
	NF (R), GFAP (R), Collagen IV−, Vim−, CD99 (S)	Anterior fossa	PNET
SYNP−, S100+	NF−, GFAP (R), Collagen IV−, Vim (S), CD99−	Cerebrum, cerebellum	Ependymoblastoma

Note: *N* negative, *R* rare, *S* sometimes
References: [160, 161, 306–308, 319, 327–332]

Table 29.39 Proliferative factor of MIB1 in some CNS tumors and correlation with survival (Fig. 29.49)

Tumor	MIB1 %		Survival
Astrocytoma	<2		80%
	>2		20%
Anaplastic astrocytomas	5–10		–
Glioblastoma multiforme	>10		–
Oligodendroglioma	<5		Longer survival
	>5		Shorter survival
Ependymal tumor	>5		Shorter survival
Choroid plexus papilloma	3.7		<6% nonaggressive
Choroid plexus carcinoma	14		>6% aggressive
Meningioma	Ozen study	Abramovich study	Lanzafame study
Benign (grade 1)	1.2	1	<1% no recurrence
Anaplastic (grade 2)	2.3	5.5	>1% recurrence
Malignant (grade 3)	6.7	12	
Medulloblastoma	50%		–

References: [160, 333–340]

cells arranged in nests and separated by a dense collagenized and desmoplastic stroma [298].

Wilms' tumor (WT) or nephroblastoma is the most common pediatric neoplasm of the kidney derived from nephrogenic rests displaying divergent differentiation. The classic histopathologic pattern of WT consists of triphasic elements of blastemal, epithelial, and stromal components. Blastemal component is composed of small round cells exhibiting reactivity with vimentin and desmin. Epithelial component shows staining with cytokeratin, whereas stromal component demonstrates variable reactivity based on its differentiation pattern [352, 353]. Lacking a characteristic immunohistochemical profile, the diagnostic feature of osteosarcoma is the presence of osteoid, which can be distinguished from other undifferentiated small round cell tumors

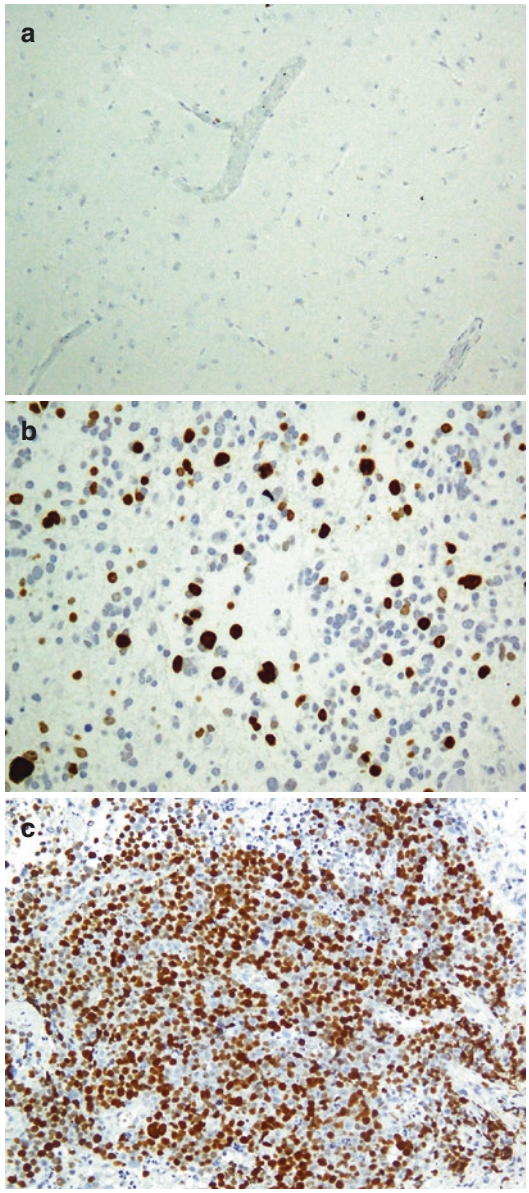

Fig. 29.49 Proliferating marker of Ki-67 is "non-reactive" in normal brain (**a**), 30% reactive in astrocytoma (**b**) and 80% reactive in germinoma (**c**)

[354, 355]. Originally described in the kidney and CNS, malignant rhabdoid tumor is a highly aggressive neoplasm of the childhood with a tendency of widespread metastases. Malignant rhabdoid tumor is a densely cellular tumor comprised of cords and sheets of polygonal cells with abundant eosinophilic cytoplasm and large eccentric nuclei containing prominent eosinophilic nucleoli [356, 357]. Table 29.40 displays an immunopanel to the diagnosis of common pediatric tumors.

29.12 Immune Surveillance, Immune Editing, Immune Constant of Rejection, Immune Contexture, and Immune Scoring of Cancers

Cancer is a complex disease involving cellular and molecular interactions between the tumor and the immune system [373]. The concept of *"Immunosurveillance,"* first described by Lewis Thomas and Macfarlane Burnet, refers to the detection and destruction of tumor cells by the immune system [374, 375]. This theory has been supported by the analysis of experimental and clinical tumor microenvironment data. The strongest argument for the existence of immunosurveillance is that immunodeficient hosts are associated with increased frequency of cancers. In addition, regression of primary and metastatic tumors has been attributed to immunologic mechanisms, but many other factors may have been responsible (e.g., hormonal, nutritional, or vascular). Tumor microenvironment is a complex milieu comprised of extracellular matrix and host cells, including mesenchymal, endothelial, and immune cells. During carcinogenesis process, the neoplastic cells constantly interact with host cells, extracellular matrix, and bioactive molecules, which constitute the tumor microenvironment [376–378].

The concept of *"cancer immunoediting,"* proposed by a series of mouse model publications that immune deficiencies are associated with tumor aggressiveness, describes how the immune system encounters with tumor cells during tumorigenesis [379–382]. Immune cells engage to combat with cancer cells in three sequential phases: cancer elimination, cancer equilibrium, and cancer escape. In the elimination phase, the immune system clears most tumor cells; a population of immune-resistant tumor cells appears in the equilibrium phase; and finally, in escape phase, the

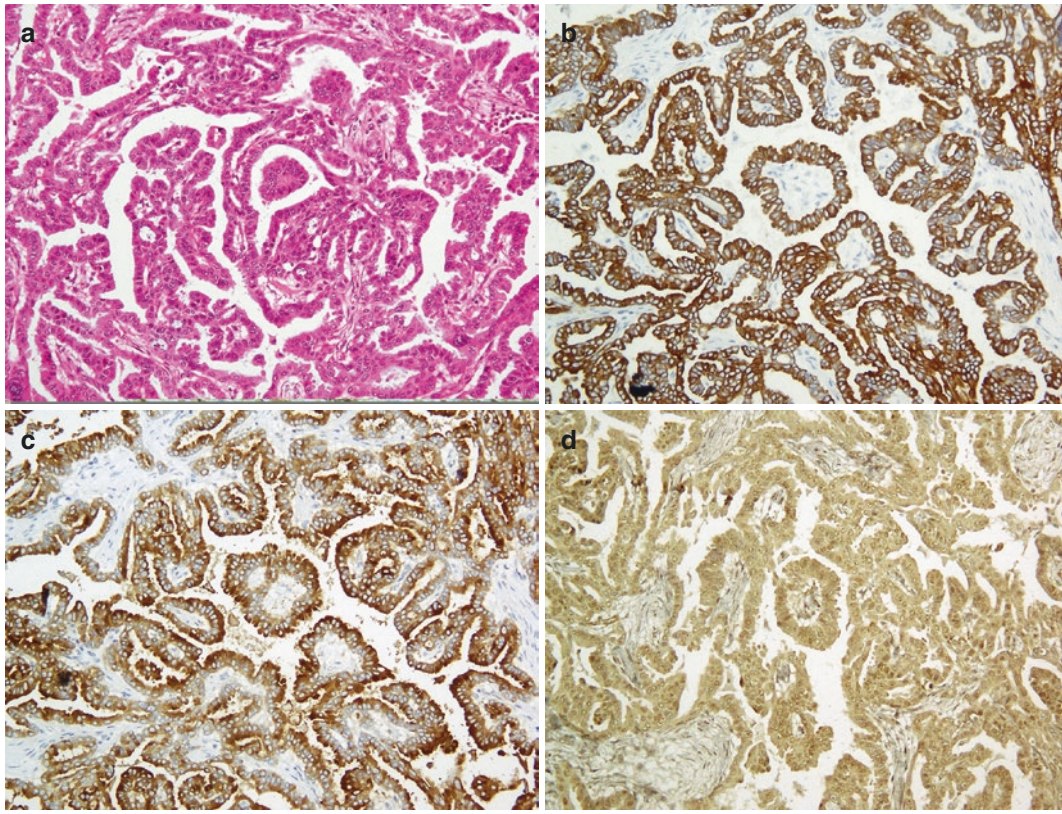

Fig. 29.50 Wilms' tumor. Epithelial component with tubuloglandular structures (**a**) showing immunoreaction with CKAE1/AE3 (**b**), EMA (**c**) and WT1 (**d**)

Table 29.40 Immunopanel of pediatric tumors

First-choice antibody panel	Second-choice antibody panel	Additional antibody/ histopathologic feature	Consistent with
AE1/AE3+, CAM5.2+, VIM+	DES+, WT1+, EMA+	SYN+, CHG+, NSE+/small round cell	Wilms tumor
		SYN+, CHG+, NSE+/ polygonal cell	Malignant rhabdoid tumor
		SYN–, CHG–, NSE+/small round cell	Desmoplastic small round cell tumor
AE1/AE3–, CAM5.2–, VIM+	DES+, MYOG+, MyoD1+	MSA+, CD99±, CK±/small round/spindle/pylogonal cell	Rhabdomyosarcoma
	DES–, MYOG–, MyoD1–	CD45+/small round cell	Lymphoma
		CD99+, S100+/small round/ polygonal cell + osteoid	Osteosarcoma
		CD99+/small round cell	ES/PNET
		S100+, SYN+, CHG+, NSE+/ small round cell	Neuroblastoma
		S100+, HMB45+, MART1+/ small round/polygonal cell	Melanoma

References: [54, 358–372]

tumor develops strategies to evade immune destruction. The last phase is a consequence of immune exhaustion and inhibition or results from the emergence of tumor cell variants (Fig. 29.51).

It is now well known that innate and adaptive immune systems can promote tumor development and progression through immunosurveillance. However, there are many interactions between the innate immune cells [macrophages, neutrophils, mast cells, NK cells, and immature dendritic cells (DC)] and the adaptive immune cells [mature DC, B-lymphocytes, T lymphocyte, and regulatory T-cells (Tregs)]. Initially mediated by innate immunity, interaction between tumor cells and immune system develops and the tumor is eliminated through adaptive immune system activation [383, 384]. The immune-mediated, tissue destruction process described by the concept of *"immunologic constant of rejection"* (ICR) which includes the coordination of interferon-stimulated genes (ISGs) pathway and immune effector functions (IEFs) pathway. This constant demonstrates the activation of ISGs, recruitment of cytotoxic immune cells (primarily through CXCR3/CCR5 ligand pathways), and activation of the IEFs pathway (IEF genes; granzymes A/B, perforin) [385, 386].

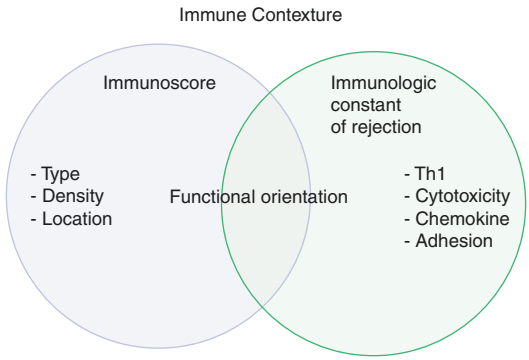

Fig. 29.51 The "Immune contexture" at the background is defined by combination of immune variables associating the nature, density, functional orientation, and distribution of immune cells within the tumor. The "Immunoscore" and the "Immunologic constant of rejection" are overlapped by functional orientation

The *"immune contexture"* is characterized as the density, type, location, and functional orientation of adaptive immune cells within the tumor, which is essential to accurately define the impact of cancer prognosis [387–389]. Parameters of the immune contexture comprise of $CD3^+$ density, cytotoxic $CD8^+$, and memory $CD45RO^+$ T-cells, their location at the tumor center (CT) and invasive margin (IM), combined with the quality of tertiary lymphoid structures (TLS) (Fig. 29.52). Evaluation of immune contexture in the clinical setting will provide prognostic and predictive benefits [387, 388].

In human, the presence of tumor infiltrating lymphocytes (TILs) has been reported as a favorable prognostic factor in many primary tumors. The high density of TILs associated with good prognosis has been well documented, not only to various organs of cancer origin (such as breast, colon, lung, head and neck, kidney, bladder, ovary, prostate), but also to various cancer cell types (adenocarcinoma, squamous cell carcinoma, large-cell cancer, melanoma, etc.) (reviewed in [389–391]) (Fig. 29.53). The quantification of TILs allowed defining a novel scoring system based on the densities of two lymphocyte populations ($CD3^+$ and $CD8^+$), both in CT and in IM of tumors. Based on the immune contexture, a standardized, simple, powerful immune scoring system ("Immunoscore") was determinate. Immune classification of cancers provides a scoring system

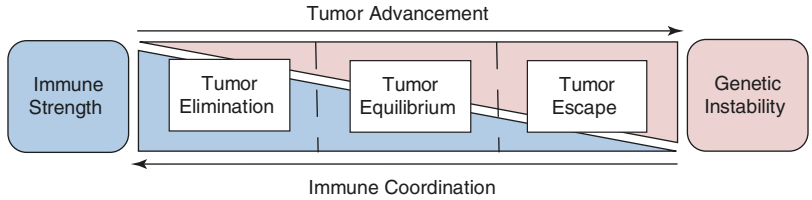

Fig. 29.52 Cancer-immune spectrum. The immunoediting theory describes how a tumor can evade from immune destruction and how immune system restraint the tumor

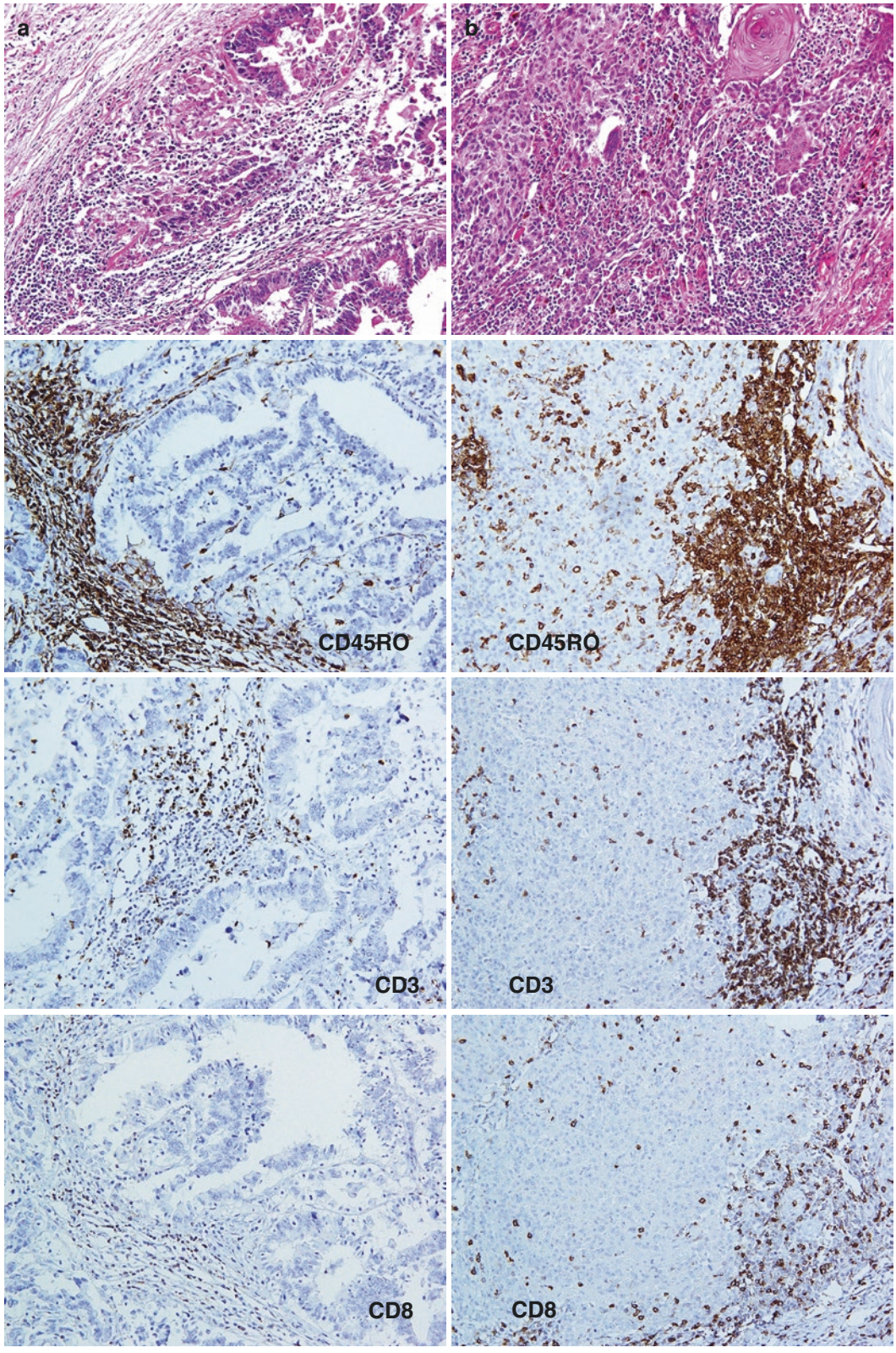

Fig. 29.53 (**a**) Colon adenocarcinoma and (**b**) skin SCC with surrounding TILs, immunostained with CD45RO, CD3, and CD8

Table 29.41 The characteristics of immune contexture, Immunoscore, and immunologic constant of rejection

Concepts	Characteristic
Immune contexture	Type, density, location, and functional orientation of adaptive immune cells (Th1 cell, cytotoxicity, chemokine, adhesion)
Immunoscore	Standardized, simple, quantitative, routine test derived from the immune contexture
Type	$CD3^+$ T-cell, $CD8^+$ T-cell
Density	Quantification (cells/mm^2)
Location	Tumor center, invasive margin, tertiary lymphoid islets
Immunologic constant of rejection	Immune-mediated, tissue destruction processes: (a) Interferon-stimulated genes pathway (b) Cytotoxic immune cells (primarily through CXCR3/CCR5 ligand pathways) (c) Immune effector functions pathway (IEF genes; granzymes A/B, perforin)

ranging from Immunoscore 0–4; and low to high densities of both lymphocyte populations in CT and IM of tumors (Table 29.41). The Immunoscore system has shown to have a prognostic significance superior to AJCC/UICC-TNM staging systems. Thus, incorporating the Immunoscore into traditional staging systems has an essential prognostic and predictive value [392, 393].

In 2012, an international task force was initiated to promote the Immunoscore in routine clinical settings as a new component of cancer classification, designated TNM-I (TNM-Immune) [394]. The purpose of the Immunoscore international task force was: (1) to validate the feasibility and reproducibility of the Immunoscore, (2) to validate the major prognostic and predictive power of the Immunoscore in colon cancer patients. In order to become globally applicable in routine clinical setting, evaluation of the Immunoscore must be pathology based, feasible in routine settings, simple, inexpensive, rapid, robust, reproducible, quantitative, standardized, and powerful [30, 394].

Multiple laboratory variables influence the validity and reliability of immunoscoring in the clinical setting, which need to coordinate with distinct criteria. They are included in the complexity of quantitative IHC assay, variable protocols across laboratories, and immune cell analysis accompanied by uneven region selection criteria and variable ways to quantify TILs. An effort for harmonization and reproducibility of IHC method recommends laboratories to test the prognostic value of Immunoscore using initial guidelines [393, 394]. It is also acknowledged that additional markers may be used to further refine the prognostic value of the Immunoscore.

Concluding Remarks Besides conventional histopathologic evaluation of various tissues, IHC has provided a significant aid in diagnosis, and its role is growing not only in arriving diagnosis but also for targeted therapies and predicting prognosis. Recently, various markers have been introduced which have therapeutic or prognostic value. Notably, it should be emphesized that IHC has some limitations and should be used in an appropriate setting by an experienced pathologist to avoid misdiagnosis. Additionally, a panel of related antibodies instead of single marker are needed to yield at a correct and precise diagnosis.

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30 Fluorescent In Situ Hybridization: Methods and Application in Cancer Diagnosis

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Contents

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30.1 Basic Principles

Fluorescent in situ hybridization (FISH) is a molecular cytogenetic technique developed in the 1980s [1] used for the identification, localization, and determination of the presence or absence of specific nucleotide sequences. The technique takes advantage of the inherent ability of complementary strands of DNA or RNA from

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different sources to hybridize. In situ hybridization is based on the principle of annealing a labeled nucleic acid probe to complementary sequences within cells or tissue mounted (in situ) on a microscope slide. "Fluorescence" refers to the ability to emit light from a reaction within the emitter that renders the visualization of the probe under the microscope possible. Among its advantages in comparison to conventional cytogenetic techniques that require live mitotic cells is its ability to be applied to all nucleated cells or tissues given that the target nucleic acid is not degraded, can be mounted on a slide, and is accessible to the probe. It is a molecular based assay, and therefore is highly sensitive and specific, with a speedy assay time. FISH on nuclear DNA is a powerful tool in the identification of chromosome aneuploidies, segmental gains or losses of chromosomes, rearrangements, gene fusions, and gene amplifications. It can also be applied for the characterization of the highly rearranged chromosomes often present in karyotypes of cancerous cells.

In all its applications, the FISH procedure involves mounting of the specimen on the slide, preparation of the sample, design and choice of probe, pre-hybridization measures, hybridization step, post-hybridization washing of the slides, and microscopic analysis. All these steps shall be reviewed with special attention to the problems that may arise during the procedure. A major limitation of FISH is the limited number of probes that can be simultaneously applied and analyzed. The evolution of other molecular cytogenetic techniques such as multiplex FISH, spectral karyotyping, or array-based comparative genomic hybridization has overcome this limitation. We shall discuss these techniques briefly and the potential that they offer for the future.

30.1.1 Materials

FISH involves the application of nucleic acid probe/probes to complementary DNA in cells and tissues. Towards this end a direct or indirect fluorescent-labeled probe and a sample of cells, tissue, or metaphase spreads fixed on microscopic slides are required. We will proceed to consider each of these requirements separately.

30.1.1.1 Target Samples

Of the major advantages of FISH is the wider range of samples that it can be applied to in comparison to cytogenetic. Theoretically, it is possible to perform DNA FISH on any nucleated cell that can be adhered or fixed onto a microscopic slide. This encompasses all cells including those suspended in fluid such as peripheral blood, those obtained by smear preparations such as buccal smear or from disassociation of fresh tissue such as lymph nodes, and even those processed from cut sections of frozen tissue and paraffin-embedded tissue.

Compared to other molecular techniques the major advantage of FISH is its inherent capacity to present visual evidence of the location of the target and its microscopic morphology. As a result, in addition to the presence/absence of the target we can establish the location of the target within the chromosomes, cells, or tissue and their identity.

Essential requirements are first the integrity of the DNA in the nuclei of the cells of interest and second the preparation of a monolayer of these cells. Both conditions are necessary to allow the probe to penetrate the cells and hybridize to the DNA and then for the visualization of the signals within the nuclei. Processing of the samples and slide making is most often specific to the nature and type of sample.

30.1.1.2 FISH Probes

FISH probes are designed for three basic groups of DNA sequences: repetitive sequences such as centromeres, telomeres, NOR regions, non-repetitive sequences such as chromosome-specific centromeric regions, whole-chromosome arms or whole chromosomes, and locus-specific sequences.

Centromere probes are either universal or chromosome specific. The universal probe is designed to hybridize with the alphoid satellite DNA of centromeric region common to all centromeres and will hybridize to all centromeres

and the long arm of chromosome Y simultaneously. A chromosome-specific centromeric probe is designed to hybridize the sequences adjacent to the alphoid DNA of the centromere and specific to the chromosome in question. Certain chromosomes have regions of homology in the sequences adjoining their centromeric DNA, for instance chromosomes 13 and 21, thereby resulting in cross-hybridization.

Telomere probes are designed to hybridize the repetitive TTAGGG sequence common to all chromosomes. Subtelomeric probes however are unique to each chromosome and designed to hybridize with the unique sequences adjacent to the telomere on the short and long arms of the chromosome. They can be used to identify rearrangements involving the most distal regions of the chromosome arms. Their only limitation in many cases is their smaller size in comparison to many other probes.

NOR probes are designed to hybridize the ribosomal DNA sequences on the short arms of acrocentric chromosomes. As there is no variation in these sequences they are not chromosome specific and cannot be used to identify the chromosomes. Whole band, arm, or chromosome FISH probes are a series of locus-specific probes designed to cover the whole length of the region to be identified.

Locus-specific, gene-specific probes are designed to hybridize the sequences within or adjacent to specific genes or regions of interest. Increased GC content of the sequence will increase the specificity of hybridization.

30.1.1.3 Probe Types

Probes used in molecular biology are invariably RNA or DNA sequences, usually 100–1000 nucleotides, complementary to a specific DNA sequence. The sequence should be long enough to ensure specific bonding without causing physical impediments. In general, the longer the probe the more specific the bonding, yet inversely the lower the intensity of the signal in certain tissues due to the physical barriers impeding penetration of the probe.

There are various options in the choice of probes, DNA or RNA, and when DNA, single or double stranded. Probe labeling with fluorescent tags can be done directly or indirectly. The requirements of the analysis will determine to a great extent the choice of probes as there are advantages and disadvantages for each probe type.

RNA Probes

RNA probes are invariably synthesized by in vitro transcription with incorporation of fluorescent-labeled nucleotides [2]. Despite the fact that RNA probes have the advantage of being single stranded with less chance of reannealing, a higher fluorophore incorporation yielding higher signal intensity per size, and a relatively higher thermodynamic stability compared to DNA probes, they are less commonly used for DNA targets as they produce high levels of background.

DNA Probes

DNA probes can be synthesized and labeled as single-stranded or double-stranded probes. Double-stranded DNA probes are more stable as they will reanneal and thus do not require freezing, but will need to be denatured before application to the target. Single-stranded DNA probes, however, are less thermodynamically stable and will require freezing; yet they are more densely labeled in comparison to the double-stranded probes. The amount of incorporated labeled nucleotides will determine the signal intensity of the probe.

Single-stranded DNA probes can be prepared by primer extension on single-stranded template [3] by PCR [4], or by chemical synthesis of oligonucleotides. Chemical synthesis of oligonucleotides leads to oligomer probes usually ranging from 18 to 50 rarely up to 100 nucleotides. To compensate for the short length of these probes and hence the low signal intensity for in situ applications they may be designed in a series of probes sequentially complementary to the target.

Double-stranded probes are synthesized by nick translation [5], random priming [6], or polymerase chain reaction (PCR) [7] in the presence of a labeled nucleotide.

Major sources of locus- or gene-specific DNA probes are plasmid, BAC, PAC, or YAC, clones from the human genome library. A challenge in

the use of these probes is the presence of repetitive DNA sequences that could lead to cross hybridization or background and need to be eliminated from the reaction by the use of Cot-1 human DNA during hybridization.

Labeling of Probes

Fluorescent labeling of probes involves a process by which the nucleotides incorporated into the probe are chemically conjugated to a fluorophore, "direct labeling," or to a molecule that can bind to a fluorophore "indirect labeling." Despite the fact that indirect labeling has the potential for producing more intense signals compared to the direct labeled probes, the added incubation steps, and the higher background produced by nonspecific binding of the antibodies to the slide and the specimen itself, direct labeling is more often the choice in medical applications.

A range of fluorophores are used for the labeling of the probes. Most commonly used probes are labeled with fluorophores emitting signals in the visual spectrum of red (TRITC/spectrum orange/Texas Red) and green (FITC/green). Many other commercially available probes may also incorporate a third color of blue (Aqua). It is highly recommended to choose microscopic filters appropriate to the optimal emission range for the probes being used as a fluorophore may not be optimally detected with another filter for the same color.

30.1.2 Methodology

30.1.2.1 Sample Preparation

A good sample preparation will ensure proper hybridization and enable accurate analysis of results. It is therefore more provident to take time to prepare well-spread specimens in the case of suspension samples, and sufficiently exposed nuclei in the case of smears and paraffin-embedded preparations. Here we present briefly the basic principles.

Suspension Cell Preparation

Cells suspended in fluid most often used for FISH include peripheral blood, bone marrow, and occasionally other fluids such as pleural effusion. To obtain proper mounting of cells to enable efficient probe hybridization and enough number of cells, there are various methods that will vary according to laboratory experience. It is important to prepare slides with sufficient number of the cells of interest while at the same time eliminating those cells that are not of interest.

For peripheral blood and bone marrow samples which are the most frequently used suspension samples in the study of hematological disorders, it is helpful to eliminate the red blood cells before fixing the sample onto the slide. This will help eliminate unnecessary background noise. One method is to prepare the sample according to routine cytogenetic harvesting procedure which includes the use of KCL hypotonic solution. The resulting swelling will eliminate the cytoplasm. The pellet is fixed with Carnoy's fixative solution which lyses the red blood cells prior to slide making.

Alternatively, one can use FICOLL to eliminate the red blood cells and prepare direct smears of the cells. The advantage of this technique is that it will maintain the structure of the cell and enable recognition of the leukemic cells. For instance in the case of multiple myeloma where the plasma cells are the cells to be studied, maintenance of the cellular structure and visual recognition of these cells by cIG FISH are essential for the efficiency of the study.

For many studies and samples as in the study of multiple myeloma, enrichment of the affected cells, i.e., plasma cells, may be necessary and should be done accordingly. For T-cell or B-cell lymphocytes a 48–72-h culture with appropriate mitogens such as phytohemagglutinin M and pokeweed, respectively, will increase the cell count. The maintenance of a backup culture of the sample regardless of the indication may prove to be beneficial.

Smear Preparation

In many cases, we are able to obtain slides from fresh tissue such as lymph nodes or tumoral tissue prior to fixing in formalin for pathological study. The advantage of these preparations is the lack of fixation and the structural changes resulting from formaldehyde treatment. However, this

is limited to use for tumors that have distinct gross appearance and do not rely on microscopic identification and separation of the malignant cells, for example in large neuroblastomas.

Solid Tumor Preparation

Most solid tumors will be referred for FISH tests following initial pathological study and in many cases after immunohistochemistry has been completed. Therefore they are commonly embedded in paraffin blocks. For appropriate FISH study it is essential that certain criteria be taken into account in the preparation of these blocks. The process of fixation can greatly influence the efficiency of hybridization of probes. The most commonly used fixative is formalin at optimal concentration of 4% formaldehyde w/v and pH of 6.8–7.2.

The maintenance of these optimal conditions will determine to a great extent the efficiency with which FISH and in general all ISH techniques can be applied.

30.1.2.2 Pre-hybridization Treatment

All samples will require various degrees of pretreatment to make the DNA in the nuclei accessible to the fluorescent probe and to eliminate the autofluorescence that could result from poor or insufficient digestion of slides. The extent and nature of pretreatment required will depend on the nature of the sample, its cellular density, protein content, and the process of slide making including the time and manner of fixation. It is always fortuitous to spend time on trying to obtain an appropriate sample prior to hybridization as this will determine to a great extent the success of the analysis following hybridization. In our experience this might require several attempts at digestion and subsequent application of DAPI and microscopic evaluation of the efficiency of digestion by determining the degree of visible background or cellular fluorescence using the various filters.

Most protocols will involve a drying process where the slides are incubated in an oven at 50–80 degree centigrade prior to pretreatment. The time of incubation will depend on the age of the slides and the ambient humidity. This helps to eliminate any excess water that may be retained on the slide which will trap the probes and result in nonspecific background signal. It also helps to maintain the morphology of the nuclei and chromatin.

Following all pretreatment protocols the slides are dehydrated by a serial ethanol wash prior to applying the probe.

We will review the basic principles and protocols for the various sample types. Although there are many commercially available kits for the preparatory steps, they all follow the same basic principles that we will address and present here. The whole procedure is highly dependent on the specimen and the sample. We find it to be beneficial to work through the procedure by a series of trial-and-error attempts to obtain a working baseline protocol for the laboratory.

Suspension Samples

The slide making process should attempt to eliminate the unwanted cells and to retain the cells of interest facilitating analysis. It is often necessary to further eliminate any cytoplasmic debris or proteinaceous material that could interfere with the penetration of the probe. Treating the slides with mild proteinase digestion enzymes such as proteinase K or trypsin or simply washing in diluted glacial acetic acid will often prove to be sufficient. The concentration of the enzyme, the time of exposure, and the temperature will determine the extent of digestion and may require successive attempts before the optimal result is obtained.

Tap/Smear Preparations

The pre-hybridization procedure for fresh sample preparations is basically the same as for suspension samples. Archival sample preparations and smears may require further processing. For example, at times, fresh blood and bone marrow specimens may not be available for FISH studies and the smears prepared for morphological study at diagnosis will provide the only material that can be used. In these cases, it is best to remove the red blood cells of the slides by immersing the slides in Carnoy's fixative prior to pretreatment. This not only removes the red blood cells but it will also ensure further fixation and hardening of the cells.

Solid Tissue Samples

Working with paraffin-embedded specimens is probably by far the most challenging of all FISH procedures. The diversity of the specimens, their cellular composition and density, their protein content, and the different fixation processes that the specimens have been exposed to will determine the extent and strength of pretreatment measures and their success.

The most commonly used fixative for tissue preservation is formaldehyde. Formaldehyde can react with groups on lysine, arginine, cysteine, tyrosine, threonine, serine, and glutamine forming reactive complexes which may combine with each other forming methylene bridges (cross-links) or with hydrogen groups [8]. Though washing the tissue after formalin fixation can reverse some of these reactions important cross-links will remain and are deterrent in the accessibility of DNA to the probe [9]. To be able to perform FISH on these specimens it is necessary to effectively remove these cross-links and to dissolve the protein structures, thus making the nuclei accessible to the probes. The efficiency with which this can be done is greatly affected by the length of time of fixation which should not optimally exceed the 24 h, temperature of fixation process, and appropriate buffering of the formalin preparation.

The first step in preparation of FFPE specimens for FISH is the deparaffinization which is usually done by immersing the slides in serial xylene solutions. The slides are dehydrated again by immersion in 100× ethanol and air-dried.

Treatment with acid will help remove the histones from the DNA and to deproteinize the chromatin. Most often 0.2 HCl is used followed by rinsing the slides in water and sodium chloride, and trisodium citrate dihydrate solution (SSC).

This step is followed by chaotrope treatment which involves compounds that will disrupt the molecular structures that stabilize the proteins, nucleic acids, and polysaccharides. This treatment is necessary for the removal of the protein cross-links that result from the fixation in formaldehyde and will deter the process of hybridization of the probe to the DNA. The most commonly used compound is preheated 1 M sodium thiocyanate and 1 M NaSCN solution. After this step we will proceed with proteinase digestion of the protein as we do with all other slides.

The time of exposure to the various steps and the concentration and the temperature of the solutions will vary according to the sample and may be achieved for various specimens through trial-and-error attempts. Again, it is worthwhile to spend time at this stage to ensure proper exposure of the nuclei and appropriate elimination of the protein in order to reduce the autofluorescence.

30.1.2.3 Hybridization

The concept of hybridization is simple and common to all samples and probes. We need to denature the DNA of the probes and the target samples, and thereafter incubate them together under appropriate conditions allowing for hybridization to take place. These conditions need to be stringent enough to prohibit nonspecific binding and yet provide enough chance for the probe to find its target.

All commercial probes provide protocols, and are common in the basic concepts. The basic steps will invariably include a denaturation step. To denature the DNA of the sample and the probe, both will have to be brought to the DNA denaturation temperature of 94–95 degree centigrade. This can be done for the specimen slide by placing the slide on a heating block and giving it enough time to ensure that denaturation of DNA has taken place without leaving the slides to dry causing damage to the nuclear framework. We can denature the probe by placing the tube containing probe suspended in hybridization buffer in a heating block at same temperature, or as is more commonly done we can co-denature by placing the probe on the slide and denaturing them together on heating block.

Once the probes have been denatured, we can proceed with the hybridization step, which will involve incubating the specimens with the probe in a humid setting at 37–40 degree centigrade for a few hours to overnight. The time should be long enough for the specific hybridization of the probe to take place. We must ensure that there is enough hybridization buffers to allow for the hybridiza-

tion to take place in a moist and humid setting such that the slides do not dry and maintain the probes in liquid suspension.

30.1.2.4 Post-hybridization

Following the hybridization procedure it is essential to remove all nonspecific probe and hybridization buffer from the slides prior to microscopic analysis. Again, most commercial probes will have suggestions and protocols for the wash procedures. These steps will invariably include an initial wash procedure that involves removal of the slide cover and the hybridization buffer from the slide. This step is usually followed by a wash procedure aimed at removing all nonspecifically bound probes by submerging the slides in a preheated solution most often in the 70 degree centigrade range.

The following steps are concentrated on the removal of all salt solutions and crystals from the slide surface. The slides are often air-dried and thereafter ready for microscopic analysis. It is highly recommended to analyze the slides immediately following the wash procedures.

30.1.2.5 Counterstaining

In most FISH studies, the nucleophore DAPI counterstain is used which will stain the nucleus uniformly enabling us to visually see the whole nucleus and to recognize the specific signals within them.

30.1.3 Microscopy/Analysis

Basis of fluorescent analysis of specimens relies on the expertise of the technologist and the appropriate equipment. It is essential to use appropriate filters for the wavelength of the fluorophores of the probes being used. We highly recommend checking and choosing the optimal filter for each probe to ensure easier analysis of the signals under the microscope. For instance the red fluorophore platinum bright 590 with excitation at 587 and emission at 612 is best seen with the Texas Red filter with excitation filter of 580/25 and emission filter of 625/30 and not with the TRITC/orange filter that has excitation at 546/22 and emission at 590/23, which would in turn be more appropriate for the red fluorophore platinum bright 550 with excitation at 550 and emission at 580.

Most laboratories involved in the handling and interpretation of FISH results will find it necessary to invest in FISH imaging cameras and software, which will facilitate the interpretation, analysis, and storage of data and results.

30.1.3.1 Interphase

In many instances, especially when dealing with archival material, we have only interphase cells for analysis and it is especially important to choose them appropriately, as results can otherwise be misleading. First, we must identify and choose the cells of interest for exclusive scoring. For suspension preparations, this may involve a pre-slide making selection method such as cell sorting or may require the identification of the cells by immunohistochemical staining. At times, the population of the malignant cells is so abundant that by scoring appropriate numbers of interphase nuclei we are able to establish with relative certainty the inclusion of the clone of interest.

For slides prepared from paraffin-embedded tissue it is customary to locate the area with the highest population of tumoral cells on H&E preparations of same specimen prior to or simultaneously with the analysis.

Second, the analyst must strive to locate intact nuclei that have the least degree of folding or overlap with other nuclei/cells, thereby decreasing the likelihood of extra or lost signals. The number of signals is scored within the selected nuclei and theoretically corresponds to copy numbers of the regions of DNA homologous to the probes (Fig. 30.1).

Another consideration in the study of nuclei in slides prepared from paraffin-embedded tissue is the fact that there is the possibility of broken nuclei. In the sectioning of cells, there is always the possibility of losing signals because of the segmentation of the nucleus. This can be compensated for most efficiently by scoring a larger number of cells and in some cases by including only cells in the study that have appropriate number of control signals.

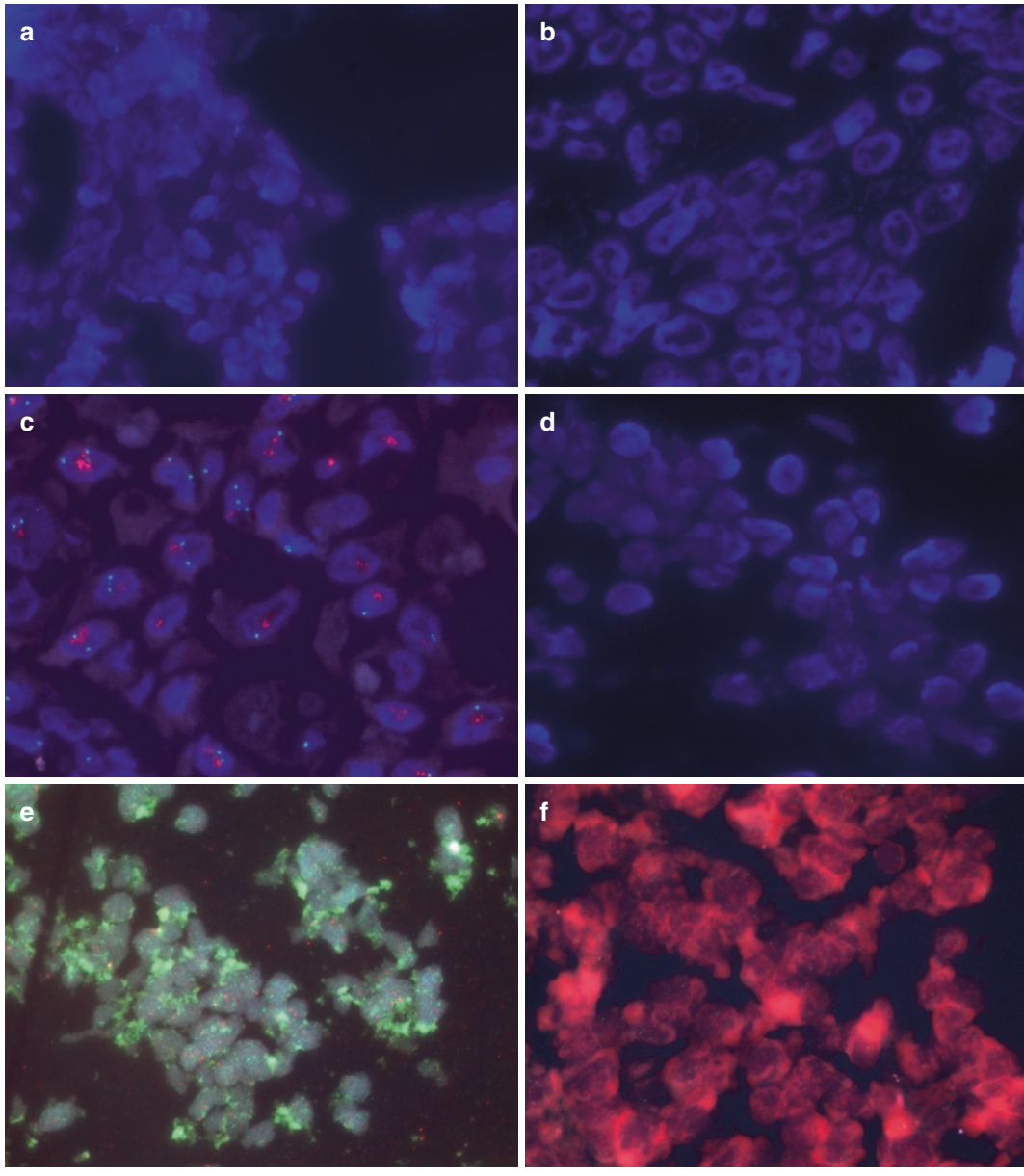

Fig. 30.1 Digestion of the preparation made from formalin-fixed paraffin-embedded tissue is extremely crucial in the success of FISH analysis as it will make the nuclei accessible to the probe. It is therefore worthwhile to invest time on proper digestion and time allowing to proceed with consecutive trial and errors. It is possible to check the success of the digestion by viewing the slide under the microscope with DAPI staining. (**a**) The tissue is not properly digested and as can be seen the nuclei are not individually visible but appear as a clump. (**b**, **c**) The tissue has been overdigested and the morphology of the nuclei has been compromised creating holes. There is the risk of loss of signals in these samples. (**d**) The tissue is properly digested and the nuclei are intact but separately visible. (**e**, **f**) As a result of underdigestion there is extreme autofluorescence. This can be viewed under the microscope prior to hybridization of the probe by checking the tissue preparation following digestion using various filters

30.1.3.2 Metaphase

The application of FISH for metaphase chromosomes is only possible on mitotic cells following culture. In the study of malignant processes this is most often used for study of leukemia and chronic proliferative processes. Obtaining metaphase from solid tumors is a more difficult process and involves the processing of fresh tissue specimens prior to any fixation.

Often, FISH study of metaphase spreads is limited to those cases where a karyotype has already been studied. FISH may be performed for clarification of findings in metaphases. It is a useful tool for the study of the difficult-to-interpret karyotypes, where a rearrangement is suspected but cannot be conclusively defined. The advantage of having a karyotype on which to do the FISH study is that a single probe may help determine the second partner chromosome on the karyotype without the necessity of going through a range of probes to identify the partner chromosome (Fig. 30.2).

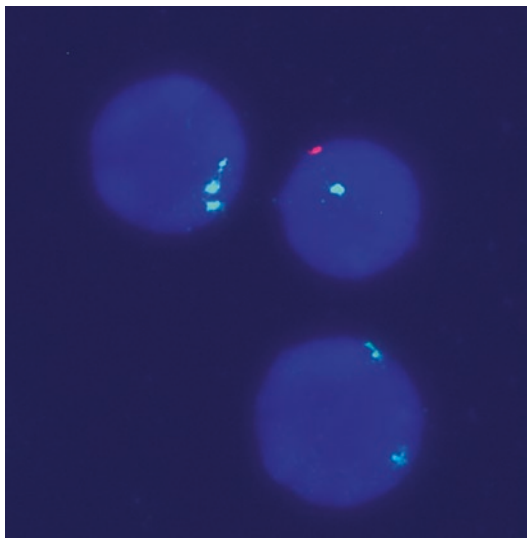

Fig. 30.2 Post-transplant chimerism was quantitated using Kreatech SE X(DXZ1)/SE Y(DYZ3) sex chromosome centromeric probes. The test is often requested for sex mismatch transplant as a designation of the success of the graft. In this case the donor is XX demonstrated by the cells with the two green signals, and the recipient is XY, those cells with one green and one red signal

At other times, it may be performed for the identification of cryptic rearrangements that are not detectable in routine cytogenetic analysis (Fig. 30.3).

30.1.3.3 Probe Design

One of the greatest achievements of molecular cytogenetics has been the advent of multicolor FISH, which allows for the use of different fluorophores simultaneously. The combination of the fluorophores has made possible variations in the design of probes specific to the needs of the case to be studied. Most FISH probes used in medical applications today are locus-specific probes and it is common practice to include a control probe labeled with different fluorophore in each FISH experiment. The control probe can be another locus-specific probe on the same or another chromosome. The variable design of the combination of these probes and their labeling will determine the application and the information that can be derived from the FISH procedure.

Enumeration Probes

These probes are designed with the intention of determining the copy number of a chromosome, region, or gene. They can therefore be centromeric probes, region-specific probes, or as is often the case locus-specific probes. The probe is often labeled with a given fluorophore emitting a red/green signal under the microscope and often there will be a second probe, which may or may not be on the same chromosome to be used simultaneously as the control, which is labeled with the other fluorophore red/green that has not been used. These probes can be used to count the copy number of a given chromosome, or a specific gene. For example, gain of chromosome 8 is a common finding in myelodysplastic syndrome, or acute myeloid leukemia. It is possible to count copy numbers of chromosome 8 by using the FISH probe that is designed for centromere of chromosome 8 labeled in one color fluorophore

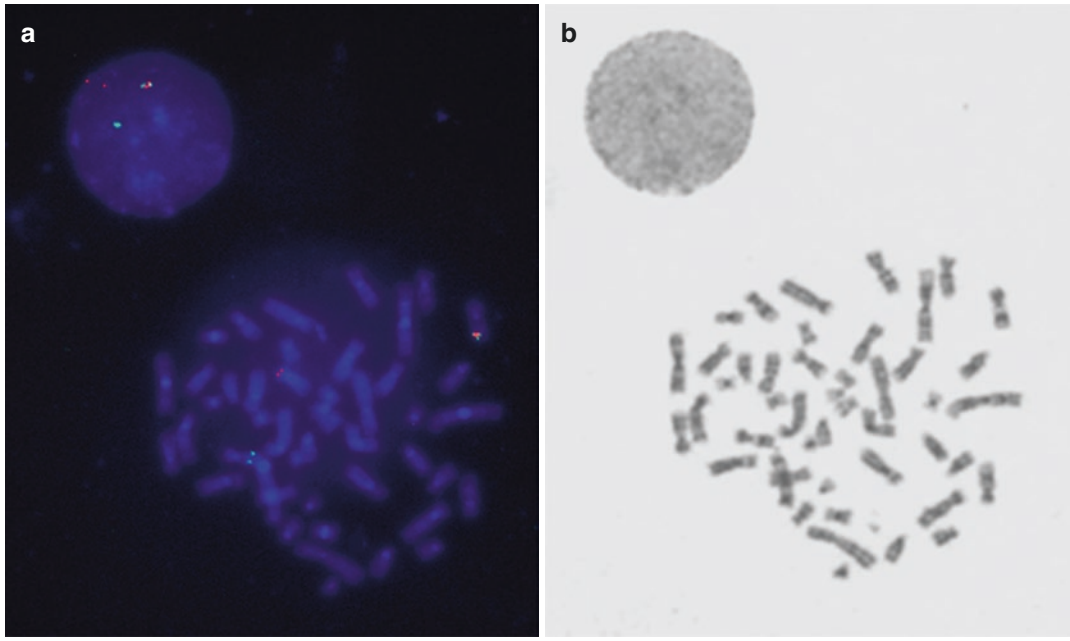

Fig. 30.3 Rearrangement of 11q23 was suspected in an otherwise apparently normal karyotype. To identify the partner chromosome FISH was performed using POSEIDON Kreatech MLL break-apart probe (**a**). The fused signal was observed on normal chromosome 11. A green signal corresponding to centromeric region of the MLL gene was present on the rearranged chromosome 11 while the distal region of MLL gene presented by the red probe was present on the short arm of chromosome 9 identified on the karyotype (**b**)

along with a locus-specific probe designed for MYC on 8q24 labeled in another color fluorophore. The presence of extra copies of both signals will suggest the presence of an extra copy of the chromosome 8. Loss of the p53 tumor-suppressor locus is often an adverse finding in many tumorigenic processes and is analyzed using enumeration probes specific for the locus along with a control probe that may or may not be on the same chromosome (Fig. 30.4).

Break-Apart Probes

Break-apart probes are most often used for the identification of rearrangement involving a specific gene of interest. Two probes are designed in close proximity to each other, most often within or flanking a given gene of interest and each probe is differentially labeled with green/red fluorophores. When there is no rearrangement we obtain a fused signal whereas in the

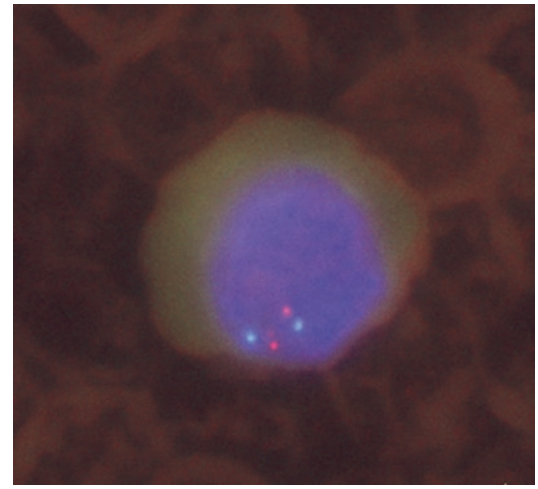

Fig. 30.4 FISH performed on plasma cells identified by cIG FISH using Poseidon Kreatech P53 enumeration probe. The gene locus is labeled in red and the centromere is labeled in green as control. As evident, there are two red and two green signals indicating that there has been no loss of the p53 locus

case of a rearrangement of the gene we would expect a visible separation of the signals in excess of the size of one signal. This is used for the identification of rearrangements of highly active and oncogenic loci such as MYC, IGH, and EWS. These loci have multiple rearrangement partners but their involvement is the driving oncogenic factor. One FISH probe will identify this involvement; further testing for the partners can be sequentially performed when and if warranted. For example, EWS–FLI1, resulting from t(11;22) (q24;q12), is seen in approximately 85% of cases of ES/PNET. Variant fusions of EWS with other ETS family genes—ERG (at 21q22), ETV1 (7p22), E1AF (17q12), and FEV (2q33)—have been found in rare cases of ES/PNET. Using a EWS break-apart probe will potentially detect both kinds of rearrangements in one assay. There does not seem to be any prognostic difference in the various rearrangement partners; therefore, in many cases, evidence of the EWS rearrangement will suffice in the confirmation of diagnosis (Fig. 30.5).

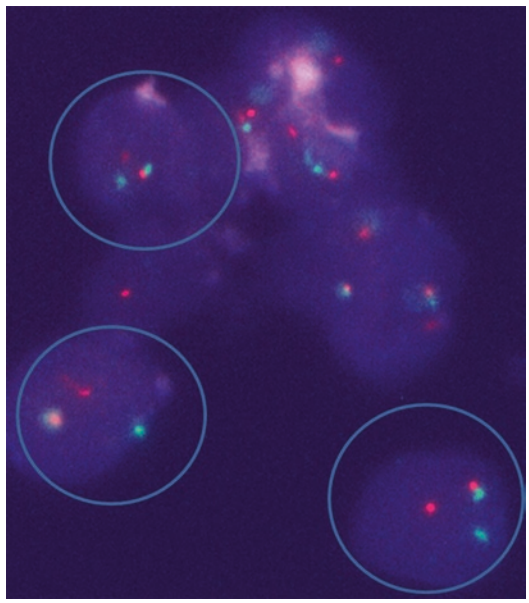

Fig. 30.5 FISH on paraffin-embedded tissue using POSEIDON Kreatech EWS 22q11.2 break-apart probe. Among the nuclei present in this setting we would choose the nuclei that have no overlap with other cells. The probe is designed such that any rearrangement of the EWS locus (fused signal) will lead to separation of the signals

Fusion Probes

Fusion probes are used for the identification of one or two fusion products resulting from a chromosomal rearrangement involving two loci from two different chromosomes. The probe/s on each chromosome will be labeled in the same color while those on the second chromosome will be of a different color. They often span or flank the breakpoint at the site of the rearrangement. These probes can be designed for the detection of the one tumorigenic fusion product and are called single fusion, or they can be designed to identify the additional second fusion on the reciprocal chromosome and are called dual-fusion probes. In the first case, the probes will target two regions flanking the gene of interest on the one chromosome and one region within or flanking the other gene of interest on the second chromosome. Most often the chromosome that is bearer of the tumorigenic fusion product will have a single probe and bear the fusion signal in the case of a rearrangement. In the case of dual-fusion probes, probes are targeted to two sequences within or adjacent to each of the two genes or breakpoints of interest one on each chromosome.

The advantage of using the dual-fusion probes for detection of fusion gene products is that it will convey additional information that may influence the interpretation. It can demonstrate the break-apart of one gene in the absence of the fusion product for which it is being tested (Fig. 30.6). For example a probe designed to detect the t(8;14) MYC/IGH rearrangement will also detect MYC rearrangement in the absence of IGH rearrangement and vice versa by showing an extra signal corresponding to the rearranged gene without a fusion signal.

It can identify amplification of the fusion gene product, for example extra copies of the BCR/ABL fusion gene product as shown in Fig. 30.7 in a case of acute-phase CML.

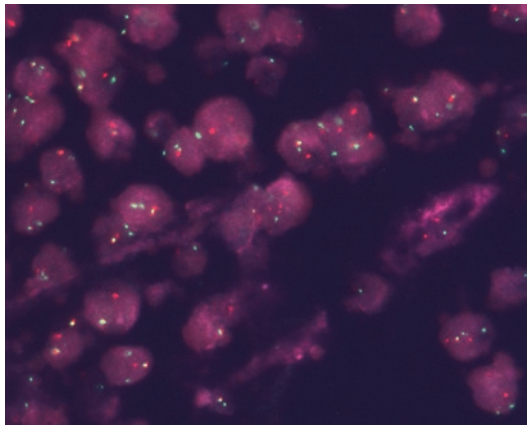

Fig. 30.6 FISH was performed using POSEIDON Kreatech t(8;14) MYC/IGH dual-fusion probes where the signals on MYC are labeled in red and the signals on IGH are labeled in green. In the reciprocal translocation of (8;14) where rearrangement occurs on both chromosomes, there are two fusion products MYC/IGH on chromosome 8 and IGH/MYC on chromosome 14. In actual fact, there may be many variations of the rearrangement, where one chromosome may be lost or deleted, or fusion may be the result of an insertional rearrangement. In this case, there is only one fusion gene product and the second fusion product has been lost

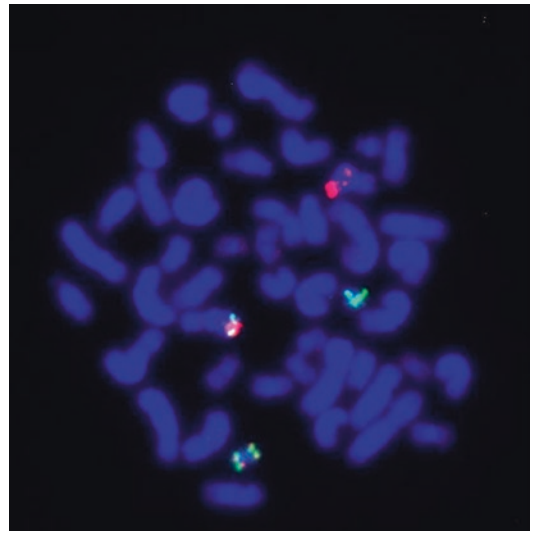

Fig. 30.7 FISH was performed using CYTOCELL 9;22 bcr/abl dual-fusion probe. In a reciprocal translocation, two fused signals corresponding to the reciprocal rearrangement are expected. In this case in addition to the two fused signals there is an additional fused signal corresponding to an extra copy of the Philadelphia chromosome which was present in the karyotype as an isochromosome

30.1.4 Other FISH/Labeling Techniques

30.1.4.1 Chromosome Painting/ Multiplex FISH

Chromosome painting refers to the hybridization of a series of probes complementary to the whole length of chromosomes allowing the visualization of all regions of that chromosome in metaphase spreads [10]. These complex DNA probes are derived from a single type of chromosome following flow-sorting or microdissection, subsequent amplification, and labeling by degenerate oligonucleotide polymerase chain reaction [11]. It enables the identification of numerical and structural aberration of whole chromosomes, but it cannot identify intrachromosomal rearrangements including deletions, duplications, and inversions. It is a strong tool for the detection of rearrangements, especially complex ones that are difficult to characterize in cytogenetic analysis.

Whole-chromosome painting is available for every human chromosome, allowing the simultaneous painting of each of the 24 chromosomes in distinguishable fluorescent colors. This capacity gave rise to two independent FISH techniques, multiplex FISH (M-FISH) [12] and spectral karyotyping (SKY) [13]. In both techniques, all 24 chromosomes are differentially labeled, and images are collected with a fluorescence microscope that has filter sets for each fluorochrome, and a combinatorial labeling algorithm allows separation and identification of all chromosomes, which are visualized in characteristic pseudo-colors [14]. M-FISH and SKY both rely on digital imaging equipment and appropriate software for discriminating the differentially labeled probes. SKY analyzes the spectral signature at each pixel of the image, while M-FISH uses specific narrow band-pass fluorescence filter sets for this distinction [15].

M-FISH and SKY have been most extensively used for the characterization of unbalanced translocations, complex chromosomal rearrangements, and marker chromosomes that are

common in solid tumors. The major limitations of these techniques are their expense and limited availability in general.

30.1.4.2 FISH/Immunohistochemistry Combined Techniques

For appropriate FISH study in many specimens, it is essential to identify the nuclei of the cells of interest to ensure the scoring of appropriate cells. Cell sorting prior to the sample preparation is an appropriate option, but may not always be possible. It is therefore advantageous to be able to identify the cells by another technique. One such technique is the use of immunohistochemistry antibodies specific for the cells of interest prior to hybridizing with FISH probes. For example, in the study of plasma cells in multiple myeloma, it is possible to identify the cells by immunohistochemical staining of the cells with cIG. This is a promising technique for samples where the cells are not morphologically recognizable and require other identifiable stains.

30.1.5 Microarray Comparative Genomic Hybridization

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique that was initially developed in 1992 and applied for the study of solid tumors [16]. It is a DNA-based test whereby DNA of the test sample and the DNA of a normal reference sample are differentially labeled and jointly hybridized to a platform. Initially, in CGH, the platform was a slide prepared from human metaphase preparations. The differential intensity of the signals from the two DNA samples hybridized to each chromosome was measured and compared using a fluorescent microscope. The ratio of test DNA sample signal intensity to that of the reference was measured by software. This ratio was used to determine the copy number of the test genomic DNA for each chromosome segment. The resolution of the technique was limited to the resolution of the metaphase chromosomes which is around 5 Mbs.

Following the human genome project and the availability of the human genome library, the metaphase platform was replaced by a DNA-based platform [17]. In microarray-based comparative genomic hybridization, the slides are spotted with a microarray of genomic human DNA, supplied from BAC clones or synthesized oligonucleotides. The test and reference samples are differentially labeled as before, combined with Cot-1 human DNA, and hybridized onto the microarray slide. The microarray slide is scanned using a laser scanner and a ratio of the signal intensity for each spot determined by a software that recognizes the spots and their corresponding chromosomal and nucleotide location. The ratio of label signal intensity of test to reference sample will determine the copy number of the test sample for the genomic content represented by the spot. A ratio of 2:2 represents a diploid situation, a ratio of 3:2 represents a copy number gain, and a ratio of 1:2 represents a copy number loss.

The advantage of the microarray is the increased resolution and the limitless variation of the genomic content that could be spotted on the slide, whole genome, or targeted regions of the genome.

In recent years, array-based genomic comparative hybridization has become a routine part of the analysis of the genomic content of many tumors. It is an objective reproducible assay with the comprehensive analysis of a karyotype at a much higher resolution and the sensitivity of molecular techniques. It is DNA based and can therefore be applied to any archival material as long as good-quality DNA can be extracted. It has the advantage of being able to explore various markers simultaneously in one assay. However, it is limited in that it will not detect balanced rearrangements, ploidy changes, and low mosaicisms.

30.1.6 Clinical Application in Cancer Setting

FISH is a powerful and versatile diagnostic tool and its application in cancer setting has high clinical significance for tumor diagnosis and prognosis [14]. FISH is a highly sensitive, specific, and rapid turnover method with a high efficiency of

hybridization and determination of translocations, deletions, inversions, and amplification of target genes [18]. FISH is usually applied in metaphase and interphase chromosomes for the study of hematologic malignancies and solid tumors, respectively.

Hematologic malignancies including leukemia and lymphoma are frequently characterized by recurrent chromosome breakpoints that produce chromosomal rearrangement and translocation. The identification of specific translocations in many of these malignancies is possible with FISH method by finding fusion genes. In contrast, the vast majority of solid tumors are defined by a specific pattern of chromosome gains and losses that are tumor type specific. Here, we introduce most important chromosomal changes in hematologic malignancies and solid tumors diagnosed by FISH.

30.1.6.1 Hematologic Malignancies

FISH analysis in hematologic malignancies is a rapid and reliable complementary method to identify the specific chromosomal rearrangements in both mitotic and interphase cells, predict poor prognostic outcome, and determine the best therapeutic approach. FISH is regularly utilized as an initial assessment in conditions with normal karyotype, poor chromosome morphology, low mitotic activity of leukemic cells, and considerable karyotypic variability and complexity [19]. A more representative assessment of abnormal cells is provided when the proliferative activity is low or dividing cells do not represent the neoplastic clone [10, 20]. Monitoring of evolving cytogenetic abnormalities throughout the course of the disease allows clinicians to treat patients more effectively and assess their responses more efficiently. They are also used to differentiate the heterogeneous nature of the leukemias, manifested by the different genetic subtypes. For example, some gains and deletions have prognostic and predictive value in hematologic malignancies including gain of 1q/1p in multiple myeloma (Fig. 30.8). For the detection of residual disease in patients with hematologic malignancies or after allogeneic bone marrow transplantation, interphase FISH is the method of choice [20].

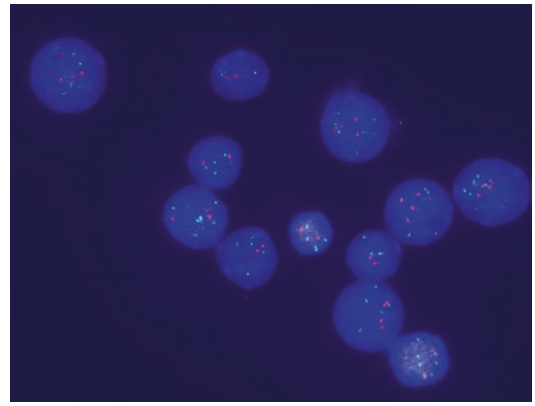

Fig. 30.8 FISH was performed using Kreatech 1p36/1q21 probes. There are multiple copies of both green and red signals compatible with additional copies of both arms of chromosome 1. The gain of 1p/1q is a prognostic factor in multiple myeloma

Here we represent some FISH probes usually applied in the hematologic malignancies with corresponding genes, functional sequence, and clinical outcome (Table 30.1).

30.1.6.2 Solid Tumors

In solid tumors, FISH is usually applied in the interphase cell nucleus for the simultaneous assessment of chromosomal aberrations, cellular phenotype, and tumor morphology [21]. Interphase FISH is analyzed on tumor cell smear, touch preparations, or formalin-fixed, paraffin-embedded tissue sections, thus enabling retrospective analyses and correlation of chromosome alterations with biological and clinical end points. Interphase FISH can screen large numbers of cells and identify chromosomal aberrations in a small subpopulation, thus providing the opportunity to identify early lesions and determine poor prognostic outcome and the best therapeutic approach [19]. For example, assessment of HER2 by FISH method can be performed on paraffin-embedded tissue sections to determine gene amplification. HER2 status is primarily evaluated to determine patient eligibility for anti-HER2 therapy. It may identify patients who have a greater benefit from anthracycline-based adjuvant therapy. Some assays use a single probe to determine the number of HER2 gene copies present, but most assays include a chromosome enumeration probe

Table 30.1 FISH analysis in hematologic malignancies with corresponding gene and clinical outcome

Disease	Chromosomal abnormality	Fusion gene/function	Clinical outcome
Myelodysplastic syndrome	Deletion of 5q, 20q, loss of chromosome Y		Low-risk disease
	Trisomy 8		Intermediate risk
	Complex karyotype (abnormality of chromosome 5, 7), del (7q)		Poor prognosis
ALL, B-cell type	t(12;21)	CBFα-ETV6	
ALL, T-cell type	Diverse chromosomal translocation	NOTCH1	
Chronic lymphocytic leukemia (CLL)	del (13q)		Low-risk disease
	Trisomy 12, del (6q)		Intermediate risk
	del (11q), del (17q), Rearrangements of 14q32		Poor prognosis
CML	t(9;22) (q34;q11)	BCR-ABL rearrangement	
AML	t(8;21), t(15; 17), inv.(16), t(16; 16)		Favorable group
	Gains of chromosomes 6, 8, 11, 13, 21, and 22; loss of the Y chromosome; del (7q, 9q, 12p, and 20q)		Intermediate group
	−5, −7, del (5q), inv. (3), t(3; 3), t(9; 11), t(11; 19), 20q, 21q, del (9q), t(9;9) and t(9; 22)		Poor prognosis
Therapy-related AML	t(3;5)	AML1-ETO rearrangement	
	t(8;21)(q22;q22)		
Multiple myeloma	del (13q), and 17p, and 11q rearrangements		Unfavorable cytogenetic abnormalities
	t(11;14)	CCND1-IGH rearrangement Cyclin D1 overexpression	Good prognosis in patients receiving high-dose chemotherapy and stem cell transplant
	t(4; 14)	MMSET-FGFR3	Poor prognosis after high-dose therapy
Burkitt's lymphoma	t(8;14)(q24;q32)	MYC-IGH	
	t(8;22)		
	t(2;8)		
Mantle cell lymphoma	t(11;14)	CCND1-IGH Cyclin D1 overexpression	
Extranodal marginal zone lymphoma	t(11;18)	MALT1-IAP2	
	t(1;14)	BCLW-IgH	
	t(14;18)	MALTl-IgH	
Anaplastic large-cell lymphoma	t(2;5)	NPM-ALK Constitutively activation of various signaling pathways	
Follicular lymphoma and B-cell lymphoma	t(14;18)(q32;q21)	IGH-BCL2 BCL2 overexpression	

(CEP17) to determine the ratio of HER2 signals to copies of chromosome 17 (Fig. 30.9). Overexpression of HER2 is both a prognostic and predictive factor but it should be underlined that in situ component of breast carcinoma has no predictive value and FISH analysis should be performed on the invasive carcinoma (Fig. 30.10). Prolonged fixation in formalin, decalcification, and insufficient protease treatment of tissue are factors to obtain negative result.

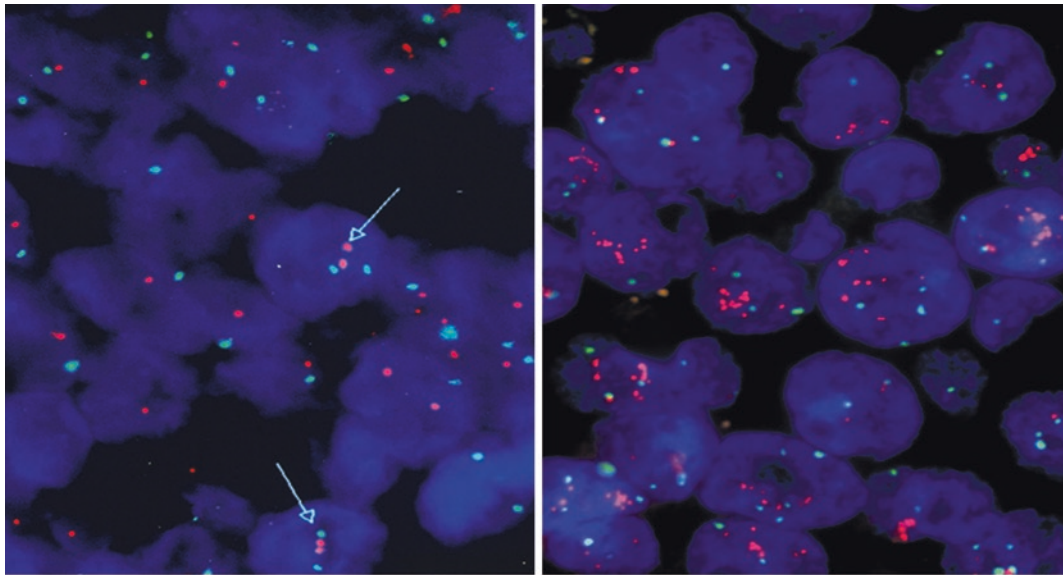

Fig. 30.9 Identification of HER2 with FISH. Her2/Neu amplification is measured by calculating the ratio of locus-specific signals to the centromere-specific probes. The latest CAP/ASCO guidelines specify positive HER2 amplification as FISH ratio higher than 2.2 or HER2 gene copy greater than 6.0 and negative HER2 amplification as FISH ratio lower than 1.8 or HER2 gene copy less than 4.0. FISH ratios of 1.8–2.2 or HER2 gene copy of 4.0–6.0 are considered as equivocal. In these images, Her2/Neu locus is labeled in red and the centromere is labeled in green. In the first image the ratio is less than 1.8 compatible with negative amplification and in the second image ratio is greater than 2.2 and compatible with amplification

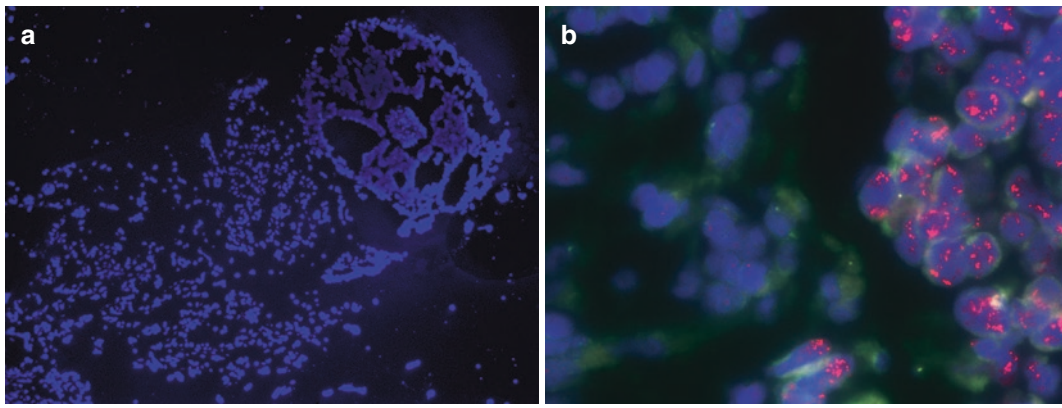

Fig. 30.10 The overview of the tissue and the selection of regions for performing FISH counts are essential in the interpretation of tumoral variations. It is important to analyze only those tumoral cells that are of value in the proliferation of the line. The above left images are overviews of tissue prepared from an invasive breast adenocarcinoma. As seen in the DAPI images there are multiple in situ foci. The cells within those in situ formations are shown on the right after application of Her2/Neu probe. As can be seen, there is amplification of the signals in these cells whereas the surrounding tissue is negative for amplification. The inclusion of these cells in the final count will affect the interpretation resulting in a false positive, as in situ formation is not representative of the tumoral population and should not be included

Genomic comparative hybridization offers an elegant, simple, and fast procedure and has become a routine part of the analysis of the genomic content of many tumors. The greatest impact of CGH is on the analysis of solid tumors and lymphomas whose study by conventional cytogenetics has been limited. Genomic analysis of DNA extracted from formalin-fixed, paraffin-embedded tissue allows the retrospective identification of chromosomal aberrations, and thus facilitates the correlation of cytogenetic findings with tumor phenotype, clinical course, and prognosis [22]. As with any technique, CGH has its limitations: it cannot detect balanced chromosomal translocations, inversions, point mutations, and aberrations.

The applications of FISH in solid tumor including sarcomas, carcinomas, and neuroepithelial tumors with corresponding gene and functional sequence are denoted in Tables 30.2, 30.3, and 30.4.

Table 30.2 FISH analysis in some sarcomas with corresponding gene and functional sequence

Tumor	Chromosomal abnormality	Involved gene/function
Alveolar soft-part sarcoma	t(X;17)	ASPSCR1-TFE3
Clear-cell sarcoma	t(2;22)(q34;q12)	EWSR1-CREB1
Dermatofibrosarcoma protuberans	Ring chromosome, t(17;22)	PDGFRB
Desmoplastic small round-cell tumor	t(11:22)(p13;q12)	EWSR1-WT1
Endometrial stromal tumor	t(7;17)	JAZF1-JJAZ1
Ewing sarcoma/PNET	t(11;22) t(21;22), t(7;22), t(17;22)	EWS-FLI1/activates transcription factor FLI1
Fibromatosis	del 5q	APC inactivation
Inflammatory myofibroblastic tumor	Translocation of 2p23	ALK overexpression
Liposarcoma		
Well differentiated/ dedifferentiated	Ring chromosome 12, amplification (12q15)	MDM2 amplification
Myxoid-round cell type	t(12;16)	FUS-DDIT3
	t(12;22)	DDIT3-EWSR
Solitary fibrous tumor	Inversion	NAB2-mediated constitutional activation of ERG transcription factors
Synovial sarcoma	t(X;18)(p11.2;q11.2)	SYT-SSX1/SYT-SSX2
	t(X;20)(p11.2;q13)	SS18L1-SSX1

Table 30.3 FISH analysis in some carcinoma with corresponding gene and functional sequence

Tumor	Chromosomal abnormality	Involved gene/function
Breast carcinoma	17q amplification	HER2
Colorectal carcinoma	17p12	EGFR
Gastric carcinoma	t(1;9), −8p, polysomy of chromosome 20	
Lung carcinoma		
NSCLC	t(2;2)(p21;p23)	EML4-ALK
SCLC	10q, 8p gene mutations	PTEN, FGFR1
	3q amplification	SOX2
NUT midline carcinoma	t(15;19)	NUT-BRD4
Prostate carcinoma		ERG-TMPRSS2 and ETV1-ETS
Renal cell carcinoma		
Clear-cell RCC	del 3p	VHL inactivation with CAIX overexpression
Papillary RCC	Trisomy 7 and 17, deletion X	MET activation
Translocation RCC	t(X;17), t(X;1), t(6;11)	TFE3, TFEB
Seminoma	Isochromosome (12p)	Gain of chromosomal material

Table 30.4 FISH analysis in neuroepithelial tumors with corresponding gene and functional sequence

Tumor	Chromosomal abnormality	Involved gene/function
Astrocytoma	BRAF duplication/fusion	BRAF
Ependymoma	+19	Unknown
Glioblastoma	7p12 amplification	EGFR
	10q23	PTEN, poor prognosis
Medulloblastoma	i(17q10) [17p/17q]	TP53
Meningioma	Monosomy 22	NF2 mutation, MN1?
Neuroblastoma	2p24 amplification	N-MYCgeh
Oligodendroglioma	del 1p/19q	Unknown
Schwannoma	Monosomy 22	NF2 mutation

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Cancer Molecular and Functional Imaging

31

Farnaz Najmi Varzaneh and
Behnoud Baradaran Noveiry

Contents

31.1 Introduction

When cancer lesion is diagnosed, it is usually well far beyond the initial phase of the conversion of normal cells to abnormal [1–3]. Opportunity and challenges of tumor's early diagnosis have led the scientists to move forward to molecular imaging [4–7]. The ability to see inside the tumor cells and its environment, at a molecular level, is challenging. Study in this era allows researchers to completely understand how cancer cells initiate, grow, and spread [8–11].

Molecular and functional imaging techniques for detecting major characteristics of the tumor microenvironment such as angiogenesis and metastasis and evaluating treatment response have been developed recently [12–15]. The

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Cancer Molecular and Functional Imaging Program describes the potential use of CT and MR imaging biomarkers to differentiate cancerous cell from normal cells and also aggressive cancer from low-grade or benign tumors [16–19].

Many of these MRI biomarkers are adaptable into clinic, and well suited for "bench-to-bedside" approaches to develop the essential techniques for targeted treatments and attain the expected response against cancer [19–23]. Following the detection of cancerous cells, finding the most effective therapy based on biologic and genetic characteristics of tumor cells and interfering immune pathways to improve outcome would be the proceeding steps [24–26].

This chapter discusses the role of molecular and functional imaging in cancer immunotherapy.

31.2 Early Diagnosis of Cancer: Imaging at the Molecular Level

31.2.1 General Consideration

Molecular imaging, originated from the field of radio-pharmacology, is a kind of diagnostic imaging which provides detailed pictures of organisms inside at a molecular and cellular level [27].

While other diagnostic imaging techniques such as X-rays and computed tomography (CT) mainly provide structural pictures, molecular imaging allows clinicians to find the tumor function and to measure its chemical and biological pathways [22, 28].

Molecular imaging capabilities are listed as follows: providing functional and biochemical information which is unattainable via other imaging technologies, detecting important changes in cells and tissues at earlier stages compared with CT and MRI, and assisting in treatment planning by demonstrating specific molecular features [29, 30].

Molecular imaging routinely uses labeled probes that are injected into the patient's body. Once the imaging agent is administered, it accumulates in a target organ or attaches to cell surface receptors [31]. The imaging agents are detectable by imaging devices which generate body or tissue pictures based on agent distribution [32]. The pattern of agent distribution allows physicians to understand the function of organs and tissues within the body.

As a valuable tool for managing the patients' care, the growing field of molecular imaging helps clinicians to characterize the extent or spread of the disease in the body [13], offer personalize healthcare medicine, and choose the most effective therapeutic method, which are matched with the tumor type and tailored to fit individual patient's requirements based on unique biologic characteristics [33], ascertain patient's response to specific drugs [34], adjust treatment plans according to cellular activity changes, and manage ongoing care by evaluating tumor progression and detecting tumor recurrence [35, 36].

Molecular imaging with incorporating elements and development of pertinent materials such as imaging agents, reporter constructs, ligands, and probes is a rapidly evolving field. Various molecular imaging techniques such as PET/CT, magnetic resonance imaging (MRI), bioluminescent imaging (BLI), and fluorescence imaging (FLI) are applied for tracking immune and stem cell. We will discuss development and utilization of each modality separately [37–39].

31.2.2 Molecular PET and PET/CT Imaging in Oncology and Immunology

Positron-emission tomography (PET) is one of the most promising new techniques in nuclear medicine which provides vision into the biological behavior of tumors rather than their morphological features and allows physician to observe numerous physiological and biochemical processes in vivo [40]. PET scans use a radioactive tracer on a biologically active molecule that is presented into the patient's body to measure the cellular activity of the cell or the part of the body being examined [41, 42].

Fludeoxyglucose (FDG), an analogue of glucose, is the most common biologically active molecule used in PET which provides real-time and valuable information regarding the metabolic behavior of tracer based on the increased glucose uptake and glycolysis of cancer cells and shows metabolic abnormalities before alterations in morphology occur [43, 44].

A whole-body FDG-PET scan to explore the possibility of cancer metastasis is the most common type of PET scan in standard healthcare system [45]. The most limiting factor in PET scan is the poor structural landmarks that functional PET imaging generally provides [46, 47].

Integrated PET/CT is a new and powerful imaging modality that allows the acquisition of co-registered PET and CT data in one fused image and has synergistic benefits over each modality alone [48–50]. Therefore, the new modality PET/CT provides combined anatomical and functional imaging information and allows the physician to determine the exact location, extent of the tumor, and biological characterization of morphological abnormalities [51, 52].

Currently, most of the PET/CT studies in oncology use FDG as a tracer; however, the evolving demand to assess tumor angiogenesis, tumor hypoxia, and tumor cell proliferation has led to the development of other particular labeled tracers including amino acid F-fluoroethyl-L-tyrosine (FET) and thymidine analogue 3′-deoxy-3′-(18) F-fluorothymidine (FLT) [53, 54].

Tumor angiogenesis is a complicated biological process which presents as a central mechanism in tumor growth and metastasis [55, 56]. Vascular endothelial growth factor (VEGF) is a potent proangiogenic factor that is secreted by growing stromal or cancerous cells [57, 58].

The VEGF secreted from cancerous cells stimulates tumor survival, cell proliferation, endothelial migration, and invasion and is therefore an important target for cancer therapy [59]. VEGF mediates its effects through numerous tyrosine kinase receptors [60]. Several VEGF targeted agents, including antibodies and soluble decoy receptor, have been developed recently [61]. As these targeted therapies undergo clinical evaluation, molecular imaging techniques such as dynamic contrast-enhanced-MRI (DCE-MRI), diffusion-weighted MRI (DW-MRI), and nuclear imaging modalities have been used to evaluate treatment response to anti-VEGF antibody [62, 63].

Hypoxia is another pathological condition which occurs when the system fails to supply oxygen to meet the main tissue demand [64]. The hypoxia phenomenon is present in most solid tumors and has been linked with a trend toward poor prognosis and ultimate poor clinical outcome [65].

Detecting and characterizing of hypoxia within tumors are of the highest clinical importance because tumor-cell aggressiveness, metastatic extent, and increased rate of recurrence are all associated with hypoxia [66, 67].

During the last decades, there has been growing appeal toward assessment of tumor hypoxia at sites inaccessible to invasive procedures [68]. As such, considerable effort has been put toward development of imaging modalities that can directly measure oxygen distribution and therefore hypoxia within tumor [65].

PET imaging, based on retention and uptake mechanism and through great number of tracers, is one of the most extensively investigated imaging modalities with the highest specificity for identification of hypoxia in solid tumors [69, 70].

In addition to the role of PET and PET/CT in oncology and cancerous cells, the use of nuclear medicine in characterization and diagnosis of infectious and inflammatory diseases and its role in therapeutic strategy have been rapidly evolving [71, 72]. In this regard, the role of PET/CT in detecting subclinical arthritis in preclinical RA [73], bone infection [74], vasculitis [75], and chest and abdominal inflammation [76] and its potential novel application for diagnosis and treatment evaluation have been discussed. However, since MRI is a better tool to detect soft tissue, PET/CT scan has lack of strong evidence in autoimmune pancreatitis [77, 78], inflammatory bowel disease [79], autoimmune thyroiditis [80], etc.

31.2.3 Molecular PET/MR Imaging and Functional MRI in Cancer

PET/MRI is a hybrid imaging modality, which provides MRI soft-tissue structural data incorporated with PET functional imaging information [81].

However, PET/MRI has convincing inherent advantages compared to PET/CT in terms of safety, lack of ionizing radiation, excellent soft-tissue contrast, and being a practical tool for staging and restaging of tumors; some recent studies report that these two modalities perform equally in most types of cancers [82]. In addition to FDG probes which is the most common probe used in PET, alternative probes have been used recently [83].

Fluorothymidine (FLT), an analogue of thymidine, is taken up by nucleoside transporters which are expressed on the cell surface and accumulated in highly proliferative tissues since it is incorporated into the nucleus during DNA synthesis, and is being used clinically for PET imaging of tumor proliferation [84]. In some studies, measuring cell proliferation with FLT provides better tumor specificity compared to measuring glycolytic activity with FDG which is due to elevated glycolysis and not limited to cancer cells [85].

FLT-PET could be a promising technique in diagnosing extramedullary sites of leukemia, particularly in brain in which there is a high level of physiologic FDG accumulation [86]. FLT-PET may also allow the detection of therapies planned to boost immune cell proliferation during cancer immunotherapy [87]. Functional MRI, including diffusion-weighted MRI (DWI), dynamic contrast-enhanced (DCE)-MRI, and dynamic susceptibility contrast (DSC) MRI, is also gaining increasing interest for a range of oncological applications from cancer detection to therapy response [88, 89].

DCE and DWI MRI sequences reflect changes in oxygenation, perfusion, vascularity, tumor microenvironment, and tissue physiology of the tumor and could be used as a surrogate biomarker for identifying early tumor response and treatment outcome which cannot be detected by conventional techniques [90]. Therefore, with the help of molecular imaging, treatment regimes in oncologic patients would be tailored according to tumor response which will have a positive effect on patient's quality of life and survival rate [91].

31.2.4 Bioluminescent Imaging (BLI) and Fluorescence Imaging (FLI) in Cancer

Bioluminescence imaging (BLI) is an imaging technology which is based on detecting visible light emitted from light-generating enzymes to report organism activity at a molecular level and to monitor transgene expression, progression of infection, tumor growth and micrometastasis, cell viability, cellular trafficking, protein-protein interactions, and gene therapy [92]. BLI is most commonly accomplished with luciferase in 2D/3D mode and the imaging technique is cost effective and very sensitive in animal models [93].

In vivo noninvasive BLI interrogation is widely becoming a method for modern biological research and is considered as a pivotal tool for tracking immune cells and optimization of cell-based therapy [94].

In this regard, the role of bioluminescent signals in localizing ER transcriptional activity in breast cancer [95], monitoring AFP-producing HCC by a chemical carcinogen in live animals [96], and androgen-independent prostate tumors in transgenic mice [97, 98] has been studied previously.

In vivo fluorescence imaging (FI) detects fluorescence release from fluorophores of small animal models by using sensitive cameras; provides a wide range of information including the location and dynamics of gene expression, tracking information of dendritic cell (DC) migration into lymph nodes and primary macrophage migration toward induced inflammatory lesions, protein expression, and molecular interactions in cells and tissues; and improves detection of malignant lesions at earlier stages [99, 100].

FI showed promising results in early detection and reduction of invasive procedures in cervical cancer [101], esophageal carcinoma [102], colorectal cancer [103], and bladder cancer [104].

31.3 Targeted Immunotherapy Based on Molecular Imaging

Immunotherapy is developed as a promising therapeutic approach for cancer treatment by stimulating the immune system against cancerous cells. Dendritic cells, T-cells, B-cells, and natural killer cells are among the immune cells which have important roles in cancer treatment [105–107].

While initial immunotherapies focused on stimulating T-cell activity, current immune-checkpoint inhibitors are being developed as antitumor immune responses.

The exclusive features of molecular imaging allow us to develop our knowledge of the role of immune cells against cancers in research era and clinical settings [108]. Recently several imaging strategies have been used to detect the distribution of immune-checkpoint molecules and identify patients who would probably take advantage from immunotherapies [87].

Up to now, three primary targets of checkpoint inhibition including the programmed death protein-1 receptor (PD-1), its ligand (programmed death ligand-1 [PD-L1]), and the cytotoxic T-lymphocyte-associated antigen-4 receptor (CTLA-4) have been offered [109].

PD-1 is expressed on the surface of activated T-cells, B-cells, and macrophages and is a negative regulator of T-cell activity. Several studies suggest that PD-1 and its ligands (PD-L1 and PD-L2) negatively regulate immune responses [110].

Interaction of PD-1 and PD-L results in inhibition of T-cell activation; thus, immunotherapy approaches that interfere with the PD-1 checkpoint would boost up anticancer activity [111]. Imaging techniques targeting PD-1 are distinctive since PD-1-targeted probes allow for imaging PD-1 expressed on the immune cell surface [112].

Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is a transmembrane inhibitory glycoprotein receptor expressed on activated T-lymphocytes; and it was recently discovered that CTLA-4 expression may be found by many tumor types [113, 114]. CTLA 4-targeted antibodies provide therapeutic efficacy in several cancers and molecular imaging such as PET helps screen patients by measuring levels of CTLA-4-positive T-cells and identifies individuals that are more likely to respond to anti-CTLA-4 immunotherapy [115]. Since molecular imaging of immunotherapy targets such as PD-1, PD-L1, and CTLA-4 has shown potential in preclinical settings as an effective tool to fight against cancer, we review molecular imaging in PD-1, PD-L, and CTLA-4 immune-checkpoint antibodies in details.

31.3.1 PD-1/PD-L

Immunotherapy blockade approaches are becoming more common in the management of numerous cancers; thus, the field is expected to keep up their rapid growth [116]. PD-1 and its ligand, PD-L1, play an important role in tumor immune evasion and the creating of tumor microenvironment suitable for tumor growth and development [109]. PD-L1 is expressed in different types of cancers, including melanoma [117], renal cell carcinoma [118], non-small cell lung cancer [119], and hepatocellular carcinoma [119].

Hindering the PD-1/PD-L1 pathway could reverse the tumor signals and develop the endogenous antitumor immune cytokines [120]. Therapeutic blockage of PD-1 and PD-L1 signaling axis with monoclonal antibodies has shown notable achievements in cancer therapy [121]. Visualizing the complex interactions between the immune system and tumor cells can offer important information regarding biomarkers that may be potential candidates for future immunotherapies [122].

Since PD-L1 is often expressed on the actual tumor cells, imaging modalities to detect this target have proven to be more reasonable than those for targets that are expressed only on immune cells [123]. Therefore, several anti-PD-L1 imaging agents have been developed to measure PD-L1 expression in preclinical settings [124].

"Immuno-PET" as a noninvasive tool measures the expression of PD-L1 throughout an entire tumor simultaneously, without the need of invasive procedures like biopsy [124].

Due to high affinity and specificity for PD-L1, a radiolabeled trace PD-1 could thus serve as an effective PET probe to assess tumor PD-L1 expression [125]. Therefore, by visualizing the PD-1/PD-L1 interaction, quantifying its expression, and mapping the bio-distribution of tracers, development of checkpoint-blocking drugs and their efficacy can be monitored.

31.3.2 CTLA-4

CTLA-4 is an inhibitory T-cell receptor that acts as a negative regulator of peripheral T-cell function. CTLA-4 is closely related to CD28 and they both bind with B7-1 (CD80) and B7-2 (CD86); however, CTLA-4 has >10-fold greater affinity in binding B7-1/B7-2 to transmit inhibitory signals [126].

CTLA-4 role is to induce peripheral immune tolerance by suppressing T-cells that are no longer needed. Both PD-1 and CTLA-4 signal inhibit T-cell activation; however, PD-1 ligation inhibits a more upstream membrane proximal step. The efficacy and safety of anti-CTLA-4 antibodies (tremelimumab) for treatment of HCC [127], melanoma [128], and non-small cell lung cancer [129] have been studied before.

PET-based whole-body molecular imaging as a noninvasive imaging has the ability to detect changes induced by treatment with anti-CTLA-4 antibodies in melanoma [130], NSCLC [131], and HCC [132].

31.4 Concluding Remarks

Immuno-oncology is a thrilling field in cancer therapy with the potential to control the growth of numerous malignancies by stimulating the body's immune system to target and fight against cancer [133]. Imaging modalities are needed to assess tumor response post-immunotherapy and further managements. Anatomic features are not sufficient biomarkers to predict response to immunotherapy; therefore functional and molecular imaging modalities are also needed to provide supplement information and monitor immune-based response. Visualization and tracking of immune cells and immunotherapy targets such as PD-1, PD-L1, and CTLA-4 would be extremely helpful in preclinical and clinical studies. In the future, molecular imaging of immunotherapy targets may improve patient classification based on tumor response and provide insight for development of novel immunotherapy targets.

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Cancer Imaging with Radiolabeled Monoclonal Antibodies

32

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32.1 Introduction

Cancer is a major public health issue and one of the leading causes of mortality, morbidity, and decreased quality of life worldwide. According to WHO estimates for 2011, cancer causes more deaths than all coronary heart disease or all stroke [1]. The ongoing global demographic and epidemiologic transitions signal an ever-increasing cancer burden over the next decades, specifically in low- and middle-resource countries, with over 20 million new cancer cases expected annually as early as 2025 [2, 3]. It is widely acknowledged that early and accurate detection of cancer could set the stage for successful treatment. Greater public awareness and increased use of screening tests have played a significant role in the detection of cancer in early stages. There is a need to reach precise answers to questions regarding the tumor location, size, spread to lymph nodes, and involvement of critical anatomical structures, so as to combat cancerous cells through modern clinical cancer therapeutic approaches. Such questions are being answered, at ever-increasing spatial resolution, through the application of traditional anatomical imaging methods such as computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound (US). Although these methods still represent the mainstay of clinical imaging, it has become clear that the acquisition of molecular and physiological information by nuclear magnetic resonance and optical imaging technologies could vastly enhance our ability to fight cancer [4–6]. As molecular imaging allows the integration of the molecular and physiological information specific to each patient with anatomical information obtained by conventional imaging methods, it is acknowledged to play a central role in the transformation of the way in which cancer is clinically managed, with the hope to detect molecular or physiological alterations that signal the presence of cancer at a curable stage and to evaluate and adjust treatment protocols in real time in the forthcoming years. Among the aforementioned molecular imaging techniques, radioimmunodetection (RID), also known as radioimmunoscintigraphy, a diagnostic procedure allowing in vivo imaging of tumors using radiolabeled antibodies and standard gamma scintillation cameras, has been a topic of intensive research during the past five decades. A large array of antibodies directed against many human tumor antigens have been developed and labeled with a variety of radioisotopes in the abovementioned era and these investigations have led to many problems in the early years, most of which were related to the unpredictable nature of polyclonal antibodies as well as the variations in different serum preparations [7, 8]. However, the introduction of well-characterized tumoral antigens and the development of hybridoma technology for the production of specific monoclonal antibodies (mAbs) renewed interest and expectations in the field of tumor imaging. And all these resulted in the capability of most nuclear medicine departments to image cancer patients with radiolabeled monoclonal antibodies so as to identify the metastatic spread of specific cancers, staging of cancer in patients before surgery, and follow-up of patients at high risk for recurrence [9].

In this chapter, a glance at the basic components of RID systems as well as some of the clinical experience and future directions of tumor imaging with radiolabeled monoclonal antibodies in cancer patients has been made. Then the past and current limitations of RID are discussed. At the end, the clinical utility of RID has been discussed. Much has also been written about the role of monoclonal antibodies as radiotherapeutic

agents in the treatment of cancer. However, this area is beyond the scope of this chapter.

32.2 Historical Perspective

In the development of a RID system, following the selection of an appropriate antigen, an antibody against the antigen is prepared and labeled with an isotope, and eventually injected into patients with the hope of targeting tumors with high specificity. The beginning of cancer RID dates back to 1948, when Pressman et al. labeled antibodies against normal rat organs [10] and then rat tumors in 1953 [11] for localization after intravenous injection. This tumor targeting in rat models with radiolabeled polyclonal antibodies was later established by Bale et al. [12]. Later, radiolabeled antibodies targeting fibrin or fibrinogen were evaluated for tumor targeting [13, 14], but showed unpromising results to localize various tumors studied in animals or humans [13, 15–17]. However, administering large doses of radio-iodinated antibodies to human fibrinogen, short-term remissions were obtained in a few selected patients with cancer [15, 17]. Thereafter, Belitsky et al. [18] reported tumor imaging using radiolabeled antibodies to an undefined renal cancer antigen. Investigations performed during the abovementioned 20-year time span resulted in an important development, which was the use of paired radioiodine labeling of different immunoglobulin preparations in order to show specific localization of the antibody of interest [19].

The next era in RID began following two major accomplishments, including the development of specific antibodies against defined cancer-associated antigens such as human chorionic gonadotropin (hCG) and carcinoembryonic antigen (CEA), and the use of human tumor xenograft models to evaluate the targeting of these antibodies [20–23], which finally led to the improved targeting of the polyclonal antibodies by affinity purification, so as to increase their immunoreactivity [23]. Iodine-125 (^{125}I) and ^{131}I were the exclusive radiotracers, used for antibody labeling in those ages.

The transition from polyclonal antibody preparations labeled predominantly with ^{131}I to "tailored designed" monoclonal antibodies labeled with diverse radionuclides, including ^{131}I, ^{123}I, indium-111 (^{111}In), and technetium-99 m (^{99m}Tc), has revolutionized the radiolabeled antibody preparations during the past 40 years. This revolution, in turn, owes to the discovery and eventual purification of tumor-associated antigens, such as CEA, alpha-fetoprotein (AFP), hCG, and prostate-specific membrane-bound antigen (PSMA), which culminated in the improvement in antibody production, in a way that either animals or in vitro cell preparations could be immunized with purified and concentrated tumor-associated antigen preparations rather than with tumor extracts. Several studies have demonstrated the value of monoclonal antibodies as targeting agents over the polyclonal antibody preparations. Some prominent studies in this line have been conducted by Mach et al. [24], who firstly published an important paper using ^{131}I-labeled anti-CEA polyclonal antibody with the resultant imaging of 40% of known antigen-positive lesion in patients with colon cancer. However, the same group [25] later reported improved results (sensitivity of 73%), using an anti-CEA monoclonal antibody preparation, and concluded the clinical promise of RID with specific monoclonal antibodies. Therefore, this improvement in antibody production dramatically enhanced the specificity of antibodies and the outcome in clinical trials.

Much of the improvement in the quality of RID studies during the past 40 years can be attributed to the progress in our understanding and choice of tumor-associated antigens, the manufacture of more specific monoclonal antibodies, the selection of radionuclides, the changes in radiolabeling chemistry, as well as the advances in imaging techniques, such as the addition of single-photon emission computerized tomography (SPECT), positron-emission tomography (PET), use of dual-isotope technology, and image fusion. A brief discussion of these factors, contributing to RID improvement, follows.

32.3 Radioimmunodetection in the New Era of Personalized and Precision Medicine

Discovery of genomic alterations driving cancer cell proliferation and survival has been a major actor in increasing our understanding of cancer cell biology and in significant improvement in the development of diagnostic techniques for early tumor detection and treatment response evaluation in recent years. One of the major challenges in anticancer drug development is the identification of molecular predictive biomarkers to guide patient selection. Therefore, in spite of the prominent progress achieved to date, continued research is warranted to improve patient outcomes through establishment of novel strategies to combine molecular diagnostic and therapeutic components while reducing toxicity.

Molecular imaging, a discipline allowing characterization of cellular process directly in living subjects and merging knowledge from various sources, including imaging, cell biology, and pharmacology, to improve cancer diagnosis and treatment, has become a powerful tool in the development of personalized cancer detection and treatment over the last decade [26]. Using noninvasive techniques, molecular imaging enables in vivo definition of the molecular features of cancer cells and allows for the characterization and measurement of cell functions [27]. Depending on the clinical application, contrast agent, and tumor location, different molecular imaging modalities may be used [28], comprising positron-emission tomography (PET), utilizing positron-emitting radioactive tracers and a tomographic scanner creating a 3D image, following the detection of the radiotracer signal [29], as well as the single-photon emission computerized tomography (SPECT), which requires a gamma-emitting radioisotope and a tomographic imaging system for signal detection, both of which provide metabolic and functional information. Identification of the molecular features of cancer in vivo allows clinical application of molecular imaging in the early cancer detection, staging, noninvasive evaluation of the tumor phenotype, patient stratification, follow-up, treatment guidance, response evaluation, early detection of resistance to therapeutic agents, surgery guidance, and drug delivery monitoring [29–33]. Furthermore, delivery of a therapeutic radionuclide carried by a molecular imaging tracer directly to tumor cells enables the extension to targeted radiotherapy in the case of SPECT and PET [34, 35]. The relatively low spatial resolution of these molecular imaging modalities is often compensated by CT, or more recently MRI co-registration for precise anatomical localization with improved resolution [36, 37].

The increased understanding of molecular alterations driving tumor progression has led to the development of novel theranostic probes characterized by two components, a targeting moiety (such as antibody), identifying a specific cancer cell or the tumor microenvironment target, and a signaling reporter (such as a radionuclide) that could be detected by SPECT or PET, resulting in the integration of imaging and therapeutic functionalities [30]. Despite the promising advantages of molecular imaging probes, there are still several unmet bench-to-bedside challenges, which need to be resolved [38, 39].

32.4 The Antibody Revolution: Story of the Magic Bullet

Radiolabeled antibodies have been developed for imaging and therapeutic purposes for more than 30 years. Following the Ehrlich description of specific cytotoxic agents against cancer tissue [40], the feasibility of this approach has been a topic of intensive research. However, the real starting point was the introduction of the hybridoma technique [41], which made isolation of large quantities of antibodies with predefined specificity possible. This procedure was first described by Kohler and Milstein in 1975, who developed a laboratory technology to produce an in vitro immortal cell line of hybridoma cells capable of producing antibodies of predetermined specificity in large quantities [41]. The process is as follows: After the injection of a

carefully selected tumor-associated antigen into a mouse, the mouse responds through the production of abundant antibodies against the antigenic material in spleen B lymphocytes. Afterwards, B lymphocytes are extracted and positioned in separate culture media, after the splenectomy. As the myeloma cell genes could make the hybridoma cells capable of growing indefinitely in vitro, B lymphocytes are then mixed with mutated non-immunoglobulin-secreting myeloma cells and chemically merged, forming hybridoma cells, so as to evade their short-term survival in tissue culture medium. As each lymphocyte contains certain genes coding for specific monoclonal antibodies, they could produce a single type of antibody. However, these genes can be isolated, purified, and tested for desired immunoreactivity and specificity. Consequently, the hybridoma cell secretes a single antibody and stays in culture systems for a long period. Additionally, multiple clones of cells can be produced from single antigen injection and those cells making the desired antibody are cultured [42]. Developments in recombinant DNA technology circumvented the limitations of the first generations of mAbs of murine origin for clinical use due to their immunogenicity, through the production of chimeric (c-mAb), humanized (h-mAb), and complete human mAbs [43].

Real progress on developing diagnostic strategies using monoclonal antibodies has been made with the identification of the tumor-associated antigens (TAA) identified for a series of human tumor types, which could be either aberrantly expressed antigens, differentiation antigens expressed during organogenesis, or expressed elsewhere in nonrelated normal tissues [44–47]. Identification of different genomic mutations in oncogenes or tumor-suppressor genes responsible for cancer growth, proliferation, and metastasis has been a milestone in molecular characterization of tumors and subsequent development of agents targeting molecular alterations in cancer pathways in the last decade [48].

The use of anticancer mAbs that can be linked to radionuclides to allow in vivo imaging of the target enabling diagnosis, staging, and molecular characterization of tumors is known as a promising approach and has been examined in several preclinical studies and clinical trials [49]. Molecular imaging techniques offer the opportunity to reveal target expression on tumoral lesions throughout the body and to depict the temporal alterations in target expression through imaging at different times, and in this way to overcome certain limitations of other conventional in vitro techniques commonly used to assess receptor/antigen expression on tumor tissue, including enzyme-linked immunosorbent assay (ELISA) and flow cytometry on blood cells for hematological malignancies or immunohistochemistry (IHC) for solid tumors, as these methods require invasive biopsy and are unable to evaluate the changes in target expression between a primary lesion and sites of metastasis in an individual or in the same lesion over time, or to assess inter-tumor heterogeneity as well as intra-tumor heterogeneity in a lesion due to the sampling approach.

It is well known that the monoclonal antibody selected for RID should yield the highest tumor-to-background ratio at the earliest time after injection. However, the heterogeneous expression of antigens between different tumor sites, varying degrees of TAA expression in the cells of the same tumor, and temporal modulation of their expression lead to suboptimal tumor targeting with monoclonal antibodies through incomplete cell targeting. In addition, other parameters such as the size of the tumor mass, antigen density, tumor physiology, fate of antigen-antibody immune complex, presence of circulating antigen, monoclonal antibody format, monoclonal antibody dose, route of administration, and monoclonal antibody circulatory half-life have been postulated to affect tumor targeting with monoclonal antibodies [50].

Using the previously mentioned hybridoma technology, a wide spectrum of monoclonal antibodies against TAAs have been developed, and are being currently investigated in various clinical trials so as to have an estimate of the safety and efficacy of the recently produced radionuclide-labeled monoclonal antibodies. Alteration of the basic structure of these monoclonal antibodies culminates in the modification

of the behavior and imaging characteristics of the complex. Together with intact mAb molecules (with the molecular weight of 150 kDa), mAb fragments and engineered variants, including fragment for antigen-binding unit (Fab), $F(ab')_2$, F(ab'), single chain Fv (scFv), and covalent dimers $scFv_2$, diabodies, and minibodies (with molecular weights ranging from 25 to 100 kDa), are being clinically implicated [43]. Radionuclide labeling of such small variable fragment of the monoclonal antibody molecule, which is the product of intact immunoglobulin G molecule chemical dissection with proteolytic enzymes such as pepsin or papain, results in brisk penetration to the target by quick leave of the vascular space and entrance to the tumor, less immunogenic properties than the intact antibody due to the lack of the Fc portion of the parent molecule, and rapid visualization of the tumor. However, the short residence time of this fragment in tissues makes it suboptimal for the detection of certain solid tumors. On the other hand, certain features of the intact immunoglobulin G, including slower clearance from vascular space due to the large size, less tumoral penetration, long serum and tissue residence time, and long time required between injection and imaging for an optimal target-to-background ratio, make them different from Fabs. The long-lasting binding of the whole immunoglobulins to tumor cells renders these agents ideal for the detection of various tumors. The aforementioned characteristics of the whole immunoglobulins, such as the long-lasting binding of the whole immunoglobulins to tumor cells and the resultant optimal tumor-to-nontumor ratios, in general, render these agents ideal for the therapeutic purpose, while the optimal format for diagnosis is still under investigation. New strategies, such as the use of pretargeting approaches, which separates the targeting antibody from the subsequent delivery of an imaging or therapeutic agent that binds to the tumor-localized antibody, have evolved to overcome some discussed obstacles [51].

Labeling of monoclonal antibodies with γ-emitting radionuclides and subsequent imaging with a single-photon emission computerized tomography (SPECT) camera have been carried out for diagnostic purposes thus far. Currently, four technetium-99 m (^{99m}Tc)- or indium-111 (^{111}In)-labeled murine mAbs (m-mAbs) have the FDA approval for cancer imaging [52], all of which had been mainly applicable in the staging of suspected recurrent or metastatic disease. The list of these radiolabeled mAbs is as follows: arcitumomab for colorectal cancer imaging (CEAScan™; Immunomedics, Morris Plains, NJ; ^{99m}Tc-labeled F(ab') to carcinoembryonic antigen [CEA]), capromab pendetide for prostate cancer imaging (ProstaScint™; Cytogen; ^{111}In-labeled IgG to prostate-specific membrane antigen), satumomab pendetide for imaging ovarian and colorectal cancer (OncoScint™; Cytogen, Princeton, NJ; ^{111}In-labeled IgG binding to the tumor-associated glycoprotein 72 antigen), and nofetumomab merpentan for small-cell lung cancer imaging (Verluma™; Boehringer Ingelheim, Ingelheim, Germany; ^{99m}Tc-labeled Fab to epithelial cell adhesion molecule). The clinical impact of these agents has not been impressive thus far. Among the four aforementioned agents, only ProstaScint remains commercially available, while the others are obsolete. This perspective might change with the use of some novel mAb formats directed against better targets, which are also suitable for therapy, in combination with cameras with improved characteristics.

To sum up, following Eisen observation of proteins being labeled with ^{131}I without alteration in their immunological specificity in 1950 [53], radiolabeling of antibodies was pioneered. Several other radionuclides (discussed under the next subheadings in detail), useful in both tumor imaging and therapeutic approaches, have been investigated since then. A multitude of radionuclides has been vectorized by monoclonal antibody derivatives to diagnose cancer since the initial establishment of radioimmunoconjugates and their subsequent use in clinical practice. Recent improvements in the production of monoclonal antibodies with higher specificity and greater binding affinity have been the consequence of several recent trials. Notwithstanding the fact that radioimmunoconjugates have exhibited promising efficacy, there is no doubt that these compounds still have limitations. However,

these stumbling blocks can partially be overcome by advancement in their usage optimization, highlighting future opportunities in radioimmunoconjugate imaging.

32.5 Theranostics

A treatment approach, based on radiolabeling compounds of interest, in which a single agent is used for both diagnostic and therapeutic purposes, is called theranostics. Using imaging methods such as SPECT or PET, this strategy is capable of assessing drug target expression and the actual presence of the drug at the tumor site in vivo in cancer patients. Among theranostic strategies, those using monoclonal antibodies (mAbs) and antibody-related therapeutic, including antibody-drug conjugates (ADCs), engineered antibody structures (minibodies, diabodies, and nanobodies), bispecific antibodies (bispecific T-cell engagers [BiTEs]), and radiolabeled antibodies for radioimmunotherapy, belonging to ever-expanding effective anticancer therapeutic agents, are of great interest. These agents, specifically designed against targets on the tumor cell membrane and immune cells together with targets in the microenvironment, and being easily radiolabeled, show promising characteristics for theranostic approaches. The mAbs could be administered either as noncurative agents, capable of lengthening disease-free survival [54–56], or as curative factors, increasing overall survival in cancer patients. The examples of the latter group consist of trastuzumab (antihuman epidermal growth factor receptor 2 (HER2) antibody) and ipilimumab (anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibody), which enhance the overall survival breast cancer and melanoma patients, respectively [57, 58].

The other focus of interest regarding theranostic approach can be its ability to provide information on the tumor target heterogeneity and successful drug delivery to tumor lesions, both of which could not be fully evaluated before the introduction of these novel approaches. It is widely accepted that not all individuals in a certain patient population using a drug with proven clinical benefit will have the same outcome; this variability is partly related to the heterogeneity in tumor target expression, tumor vascularization, or presence of an immunosuppressive tumor microenvironment. Furthermore, biopsy of a single tumor lesion was frequently required to make treatment decisions in both routine practice and drug development and the blood-based pharmacokinetic analyses were the only available method to determine dosing schedules, prior to the advent of theranostic approaches. As a result, molecular antibody imaging could set the stage for patient enrichment, drug development, and clinical decision-making. However, such therapeutic approaches are beyond the scope of this chapter.

32.6 Diagnostic Radioisotopes

Monoclonal antibodies and antibody-related therapeutics can be efficiently labeled with a wide spectrum of radionuclides, including indium-111 (^{111}In), iodine-123 (^{123}I), iodine-124 (^{124}I), iodine-131 (^{131}I), lutetium-177 (^{177}Lu), technetium-99 m (^{99m}Tc), copper-64 (^{64}Cu), gallium-68 (^{68}Ga), yttrium-86 (^{86}Y), and zirconium-89 (^{89}Zr), which are those most commonly used for cancer molecular imaging with mAbs and antibody-related therapeutics (Table 32.1). Using various labeling methods, these radiolabeled agents can therefore be administered in both murine and humans experiments [59].

As the radioactivity needs to be detected for a proper period of time for the mAbs or the antibody-related drugs to get to the specific cellular target while minimizing the duration of exposure to harmful radiation, an essential step to choose an appropriate radionuclide to label monoclonal antibodies with is matching of the physical half-life of the radionuclide and the serum half-life of the mAb, ranging from 30 min to 30 days (depending on the size and structure of mAb, the IgG subtype from which the mAb is derived, and whether the mAb is fully human, humanized murine, or chimeric). As stated in the preceding sections, the serum half-life is shorter for mAb fragments than for an intact mAb,

Table 32.1 Characteristics of radionuclide used in radioimmunoconjugate-associated cancer imaging and therapy

Technique	Radio-isotope	Half-life	Application
SPECT	^{99m}Tc	6.0 h	Diagnostic
	^{123}I	13.2 h	Diagnostic
	^{131}I	192.5 h	Diagnostic/therapeutic
	^{111}In	67.3 h	Diagnostic
	^{177}Lu	159.5 h	Therapeutic
PET	^{64}Cu	12.7 h	Diagnostic
	^{68}Ga	67.7 min	Diagnostic
	^{86}Y	14.7 h	Therapeutic
	^{89}Zr	78.4 h	Diagnostic
	^{124}I	100.3 h	Diagnostic
	^{18}F	109.7 min	Diagnostic

because of the molecular weight, which is often below the renal clearance threshold of approximately 70 kDa in mAb fragments [60].

Linking mAbs to metal-based radionuclides, such as ^{68}Ga, ^{86}Y, ^{64}Cu, ^{89}Zr, ^{111}In, and ^{177}Lu, warrants the use of a chelator, depending on the clinical applicability, the most stable chemical link, and the radionuclide itself.

The other significant factor to note while selecting a radionuclide is whether the mAb internalizes following binding to the target (such as what happens to radiometal-labeled drugs in the metabolizing process, during which the metal-based radionuclide is trapped intracellularly in lysosomes), culminating in the higher absolute uptake of the radiotracer and resultant higher tumor-to-blood ratios [61]. ^{89}Zr is an example of positron-emitter radionuclide with competent characteristics for stable antibody labeling, which has been widely used in recent years. These characteristics include a physical half-life of 78.4 h generally matching the serum half-life of most mAbs in vivo. Its physical half-life is also compatible with the residualization time, resulting in increased tumor-to-background ratios [62].

Most radiolabeled intact antibodies have a relatively long effective half-life of between 14 and 21 days, as stated above. These mAbs are distributed throughout the body and accumulate in both the tumor and other normal tissues expressing the target, with subsequent increasing tumor-to-background ratios due to the binding of the radiotracer to tumoral targets, residualization, and clearance of the nonbound tracer from the circulation over time. Target location, target expression levels, target saturation, internalization of the mAb, perfusion, and vascularization are among the factors influencing tumor accumulation of the radiolabeled mAb [60].

It is required to specify the optimal protein dose and time point to reach the proper tumor-to-background ratio prior to imaging study. Specific activity, expressed in MBq/mg, is a certain amount of radioactivity per milligram of the linked mAb, which is generally limited to 750–1000 MBq/mg for most mAbs. When the allowed safely administered protein dose is relatively low, reaching a sufficient radioactive dose for successful imaging is challenging, and this makes both the imaging and theranostic approaches arduous [63].

32.7 Limitations of Radioimmunoconjugate Compounds

The need for a reliable supply chain of radionuclide is a significant limitation in the administration of radioimmunoconjugates, which itself has multiple obstacles comprising the cost and availability of isotope production, specific activity, radionuclide purity, and chemical yield of the chemical purification process, and the radiolabeling yield, radiochemical purity, and final activity of the immunoconjugate radiolabeling process. All these points warrant specific verifications within the supply chain to obtain a reliable source to globally supply clinical facilities on a global scale.

Notwithstanding the fact that various techniques have been evolved in order to enhance the affinity and specificity of monoclonal antibody derivatives, limited tissue accessibility to the radioimmunoconjugate compound could, in turn, restrict the antibody-antigen binding. This limitation arises from both the morphologically abnormal and a highly structurally disorganized tumor neovasculature, which could exhibit anomalies such as blood flow inversion or arteriovenous shunting [64, 65], as well as the high interstitial pressure with a low passive diffusion rate, which causes large-sized macromolecules, such as monoclonal antibodies, to take longer to diffuse into the surrounding tissues [66]. The former phenomenon results from the appearance of morphologically abnormal tumor neovasculature as a result of the quick formation of new blood vessel networks during tumorigenesis so as to maintain the metabolic demands of the tumor. And the latter could be due to the slower pace of neolymphogenesis than neoangiogenesis during tumor growth. Both these phenomena are estimated to hinder monoclonal antibody penetration in solid tumors. A multitude of methods, comprising the alteration of monoclonal antibodies' global electrostatic charge through chemical modification [67–69], along with the prior use of vasoactive compounds, such as interleukin-2, to improve tumors targeting via opening of the vascular barriers [70, 71], have been examined previously, in order to optimize tumor penetration. However, as the complete coverage of tumors by full-length antibodies and their binding to antigen-positive regions while clearing from antigen-negative regions in tumor-bearing experimental models have been demonstrated by the autoradiography studies, it has been postulated that the poor penetration of monoclonal antibodies in tumors has been partly overestimated [72–74].

Another limitation in the use of diagnostic radioimmunoconjugates to be acknowledged is the reduced contrast and the efficacy of the technique, which is partly affected by the choice of the radioisotope. The main purpose is to obtain a high contrast between tumor and the surrounding healthy tissues. In addition, certain healthy tissues, such as liver, spleen, and lung, all of which are classically metastatic loci, are known to be sites for immunoconjugate metabolism and thus resulting in the reduced contrast.

32.8 Adverse Reactions

Interestingly, tracer uptake in normal tissues can partly describe observed side effects. Depending on the amount of the administered antibody or the immune phenotype of the patient, rare adverse reactions, encompassing a group of minor side effects, including rashes, hypotension, fever, and dyspnea, may appear in less than 4% of circumstances [75]. The development of immunity, caused by the formation of human antimurine antibodies (HAMA), human anti-chimeric antibodies (HACA), or human antihuman antibodies (HAHA), is variable, as the greater the amount of protein injected, the higher the frequency of HAMA, HACA, and HAHA development, increasing the risk of possible allergic reactions in repeated RID studies. This relationship is reported to be the same for monovalent antibody fragments in patients [76]. Furthermore, the possibility of the interference of the presence of circulating HAMA, HACA, or HAHA with the immunoassay results of certain tumor markers in the blood specimens of cancer patients should be kept in mind, as these antibodies may contribute to falsely elevated laboratory values in cancer patients for up to 6 months after the injection of labeled monoclonal antibodies.

32.9 Imaging Techniques

32.9.1 Revolutionary Road: From SPECT to PET

Since the 1990s, before PET technology became broadly available, mAbs have been coupled with gamma-emitting radionuclides, such as ^{99m}Tc, ^{111}In, or ^{131}I, and imaged with planar or single-photon emission computerized tomography (SPECT) cameras. SPECT images, obtained in regions of known or suspected lesions, have offered an improved contrast of the lesion in the

section of interest, differentiating it from the overlapping structures lying near the target, visualized in a planar view. At the same time, other anatomical imaging techniques, such as computed tomography (CT) and magnetic resonance imaging (MRI), could be applied to the same body region and eventually superimposed to the SPECT images of the same region by means of an appropriate computer software program, and finally result in the generation of fused images, containing both the anatomical and functional details of the region of interest.

Although informative, SPECT camera images suffered from limited sensitivity and low spatial resolution. More importantly, a need for reliable quantitative measurements was a rationale for the implication of PET as a powerful method for mAb imaging, with greater sensitivity, improved spatial resolution, and signal-to-noise ratios, as well as the capability to perform accurate quantification [77].

32.9.2 Immuno-PET

Immuno-PET is based on the coincidental recognition of a mAb labeled with a positron-emitting radionuclide. The basic description of the underlying mechanism of such technology is as follows: Based on the initial positron energy and the density of the surrounding matters, the emitted positron travels a distance of a few millimeters, loses its kinetic energy, and finally combines with an electron, leading to the so-called annihilation process, and yields two photons, each with an energy of 511 keV emitted simultaneously in opposite directions. Provided that these two emitted photons are registered by detectors, which are placed around the body of the patient, on opposite sides of the body within a 5–15 ns time interval, it is then assumed that an annihilation event has taken place somewhere along the line between the two detectors. Thus, the location of the radiolabeled mAb can be identified by means of the calculation of the crossing of all lines. In this way, detection of the annihilation photon pairs with a PET camera results in the identification of PET conjugate distribution in a patient [78].

Immuno-PET has been sentenced to several technical advances to further improve its sensitivity and resolution through accomplishments achieved by recent investigations. Additionally, combination of PET with computed tomography (CT) or magnetic resonance imaging (MRI) makes simultaneous registration of both biologic function and anatomy possible and facilitates accurate interpretation of PET images and quantification.

Positron emitters have to fulfill several requirements, such as appropriate decay characteristics for optimal resolution and quantitative accuracy, easy and cost-effective production, capability of efficient and stable coupling to mAbs, and compatibility of their physical half-life ($t_{1/2}$) with the time required for a mAb or mAb fragment to achieve optimal tumor-to-nontumor ratios, to be appropriate for immuno-PET. At the same time, maintenance of the antibody's in vivo binding and biodistribution characteristics is mandatory.

Keeping such considerations in mind, certain positron emitters, including gallium-68 (^{68}Ga; $t_{1/2}$, 67.7 min), fluorine-18 (^{18}F; $t_{1/2}$, 109.7 min), copper-64 (^{64}Cu; $t_{1/2}$, 12.7 h), yttrium-86 (^{86}Y; $t_{1/2}$, 14.7 h), bromine-76 (^{76}Br; $t_{1/2}$, 16.2 h), zirconium-89 (^{89}Zr; $t_{1/2}$, 78.4 h), and iodine-124 (^{124}I; $t_{1/2}$, 100.3 h), have been the focus of intensive research recently; and among them, ^{89}Zr and ^{124}I, having long half-lives which allow both easier transportation and imaging at late time points for obtaining maximum information, have been shown to be suitable in combination with intact mAbs, while very short-lived ^{68}Ga and ^{18}F can only be administered in combination with mAb fragments or in pretargeting technique, the approach in which antibodies capable of both binding antigens and radiolabeled small molecular weight ligands are administered and injection of the radioactive ligand takes place following the binding and clearance of the antibody, in order to bind the pre-localized antibody. Such techniques provide enhanced tumor-to-background ratios [51, 79]. Besides, it should be noted that long half-life of radiotracers causes greater radiation burden to patients. Radionuclides such as ^{76}Br and ^{124}I positron emitters can either be directly coupled to a

mAb or conjugated indirectly through a linker, using radiohalogens and radiometals [61].

Another important consideration to be taken into account while selecting a positron emitter for immuno-PET applications is whether the mAb becomes internalized following attachment to the target antigen, which results in rapid clearance of radioimmunoconjugates such as ^{76}Br- and ^{124}I-labeled mAbs from the target cells, and therefore less tumor contrast on PET images and the resultant inability to reflect the actual mAb distribution [61]. Our understanding of the abovementioned issues will open avenues to the routine clinical application of immuno-PET.

The field of PET molecular imaging, providing reproducible noninvasive whole-body biomarkers mapping, is rapidly progressing toward clinical use today. Moreover, this modality characterized by the improved image quality, the safety, as well as the potential for proper estimation of the antigenic expression level represents a promising tool for personalized medicine. Some of the recent advances in immuno-PET come next.

32.10 Monoclonal Antibodies: Clinical Utility

As stated in previous sections, among the most relevant applications of radiolabeled mAbs [80], noninvasive in vivo detection of molecular alterations and target expression also known as in vivo immunohistochemistry [81–83], evaluation of intra- and interindividual variability in tumor uptake and normal tissue accumulation and elimination, analysis of tumor heterogeneity in mAb uptake by tomographic imaging of the lesion that may be caused by either the expression of the target receptor/antigen or the variability in blood flow, delineation of molecular response to a specific targeted therapy [84], identification of resistance to targeted therapies due to changes in target expression [85], and assessment of therapeutic index via estimating the uptake of the mAbs in tumor and normal tissues could be named [86].

Recombinant engineering techniques have resulted in further accomplishments in the diagnostic application of radiolabeled mAbs through both the better recognition of cancer biology, which leads to appropriate target selection, and the improvement of the tumor:blood and tumor:normal tissue ratios [87]. Certain radiolabeled mAbs targeting tumor antigens have been investigated in both preclinical models and clinical studies and their specificity for imaging has been clearly delineated; however, further clinical trials are required to understand the exact diagnostic, prognostic, and therapeutic roles of these mAbs. A wide spectrum of promising targets are currently being assessed in clinical trials at various stages with the hope of finding ideal antigens which are intended to be readily accessible, highly overexpressed, and expressed only within the desired target tissue, and with minimal shedding or secretion from the cell surface, circulation in the blood, and residence in the interstitial compartment. Among the mAbs, those targeting well-studied molecular patterns, including program death-1 (PD-1), program death ligand-1 (PD-L1), prostate-specific membrane antigen (PSMA), CD20, epidermal growth factor receptor 2 (HER2), and epidermal growth factor receptor (EGFR), are discussed below (Table 32.2).

32.10.1 Program Death-1 (PD-1) and Program Death Ligand-1 (PD-L1)

T-cells are pivotal actors in the anticancer immune response. These immune cells express coinhibitory receptors capable of downregulating the immune response [109], one of which is programmed death 1 (PD-1) having two ligands, namely, programmed death ligand-1 (PD-L1) and PD-L2, of which PD-L1 is expressed to a greater extent. Interaction of PD-1 and PD-L1 transduces an inhibitory signal to the T-cell, leading to the impediment of T-cell proliferation, decreased levels of effector cytokines, and potentially exhaustion, making tumor cells capable of escaping immune recognition and attack [110–112], and in this way favors cancer growth and progression [113]. PD-L1 is overexpressed in different tumors, comprising ovarian cancer, non-small lung cancer, melanoma, breast cancer,

Table 32.2 Prominent radiolabeled monoclonal antibodies used for cancer molecular imaging

Target	Radiolabeled monoclonal antibody	Malignancy	Activity	Level of study	References
PD-L1	^{111}In-PD-L1	Breast	Evaluation of target expression	Preclinical	[88, 89]
PSMA	^{111}In-J591	Prostate	Estimation of ^{177}Lu-J591 activity	Clinical	[90]
VEGF	^{89}Zr-bevacizumab	Renal cell carcinoma	Identification of everolimus activity	Clinical	[91]
		Breast	Evaluation of target expression	Clinical	[92]
	^{111}In-bevacizumab	Melanoma	Evaluation of target expression and treatment response	Clinical	[93]
		Ovary	Evaluation of target expression	Preclinical	[94]
CD20	^{131}I-tositumomab (Bexxar)	Non-Hodgkin's lymphoma	Evaluation of target expression, toxicity assessment	FDA approved	[95]
	^{111}In/^{90}Y-ibritumomab tiuxetan (Zevalin)	Non-Hodgkin's lymphoma	Evaluation of target expression, toxicity assessment	FDA approved	[96]
HER2	^{111}DTPA-pertuzumab	Breast	Identification of trastuzumab activity	Preclinical	[84]
	^{89}Zr-trastuzumab	Gastric	Evaluation of target expression and afatinib activity	Preclinical	[97]
		Breast	Evaluation of target expression and drug activity	Clinical	[98–100]
	^{64}Cu-trastuzumab	Breast	Assessment of target expression and HSP90 inhibitor activity	Clinical	[101, 102]
	^{111}In-trastuzumab	Breast	Assessment of target expression and trastuzumab activity	Clinical	[103, 104]
EGFR	^{89}Zr-panitumumab	Colon	Evaluation of target expression	Preclinical	[105]
	^{89}Zr-cetuximab	Colon	Assessment of cetuximab activity	Clinical	[106]
		Lung, head and neck	Estimation of target expression and cetuximab activity	Clinical	[107, 108]

PSMA prostate-specific membrane antigen, *HSP90* heat-shock protein 90, *DTPA* diethylenetriamine pentaacetic acid, *VEGF* vascular endothelial growth factor, *HER2* epidermal growth factor receptor 2, *EGFR* epidermal growth factor receptor

gastric cancer, renal cell cancer, and hematologic malignancy, and both PD-1 and its ligand, PD-L1, have been considered to be associated with poor outcome of cancer patients [114].

Despite the promising results of anti-PD-1 or anti-PD-L1 mAb application in cancer therapy [115–119], not all the patients respond to these immune-checkpoint inhibitors. PD-L1 expression, routinely assessed with IHC on archival tissue, has been investigated as a potential biomarker of response but divulged no definitive correlation between PD-L1 expression and response. IHC method warrants a new tumor biopsy at the time of progression [120, 121]. Certain obstacles caused by immunohistochemical analysis of PD-L1 expression in tumor biopsies, which include sampling errors and, thus, misinterpretation due to intratumoral and interlesional heterogeneity, could be successfully handled using molecular in vivo imaging with radiolabeled anti-PD-L1 antibodies, which makes the measurement of PD-L1 expression prior to the commencement of therapy, of both the whole tumor lesions and their metastases, possible. As a consequence, molecular imaging could help us with more accurate detection of PD-L1 expression and accessibility and longitudinal monitoring of PD-L1 expression during disease progression and treatment, and in this way PD-1 and PD-L1 can potentially be used as a biomarker to select patients for PD-1/PDL1-targeted therapy.

SPECT imaging with ^{111}In-labeled PD-L1.3.1 mAb in mice bearing human breast cancer xenografts was found to recognize PD-L1 overexpression levels [88]. Additionally, investigation of PD-L1 expression in cell lines and in mice bear-

ing triple-negative breast cancer and non-small cell lung cancer xenografts [89], using an analog of anti PD-L1 mAb, atezolizumab, conjugated with ^{111}In together with a near-infrared dye, found ^{111}In-PD-L1-mAb and NIR-PD-L1-mAb to be capable of deciphering different levels of PD-L1 expression in tumor xenografts, and justified the feasibility of in vivo PD-L1 evaluation by imaging.

32.10.2 Prostate-Specific Membrane Antigen (PSMA)

Prostate-specific membrane antigen (PSMA), a transmembrane protein widely present in prostate cancer cells and specifically in castration-resistant tumors [122], is a cell membrane protein expressed in all stages of prostate cancer and is known to be correlated with higher tumor stages, preoperative prostate-specific antigen (PSA) levels, Gleason scores, and a higher risk of biochemical recurrence [123–125]. This marker has been characterized in other solid tumors such as colorectal cancer, renal cancer, and glioblastoma [126, 127]. PSMA has been established as a unique biomarker particularly expressed by tumor-associated neovasculature but not produced by normal vessels [128].

Monoclonal antibodies have been evolved against PSMA [122, 129]. ^{111}In-labeled capromab pendetide, marketed as ProstaScint, is an FDA-approved antibody directed against an intracellular epitope of PSMA for the detection of nodal metastases in patients with prostate cancer; however, this epitope is regarded as a suboptimal target for antibody imaging [130]. J591 is a mAb binding an epitope on the extracellular domain of PSMA [131], which has been administered in the form of ^{111}In-J591 for in vivo assessment of PSMA expression via SPECT imaging, and has been proven as a predictive biomarker of PSA response following radioimmunotherapy with ^{177}Lu-J591 [90]. This mAB has been applied for PET imaging following being labeled with ^{89}Zr or ^{124}I and for radioimmunotherapeutic approaches after being ^{90}Y and ^{177}Lu labeled [132, 133].

32.10.3 Vascular Endothelial Growth Factor (VEGF)

VEGF-A, usually referred to as VEGF, is the most prominent tumor angiogenesis mediator, with the gene comprising nine exons and eight introns mapped to chromosome 6p21.3 [134, 135]. Signal-sequence cleavage culminates in the development of six isoforms of 121, 145, 165, 183, 189, and 206 amino acid length, respectively, among which the VEGF165 is the most frequent isoform that has a significant portion bound to heparin sulfate proteoglycans on the cell surface and in the extracellular matrix after being secreted [136]. VEGF signals after binding to the VEGF receptor 2 (VEGFR-2) [137].

A humanized type of the anti-VEGF-A monoclonal antibody (mAb) A.4.6.1., bevacizumab, directed against a common epitope which is encoded by exon 4, that is commonly present in all VEGF-isoforms, is capable of the impediment of the interaction with VEGFR-1 and VEGFR-2 [138]. Noninvasive VEGF imaging using radiolabeled bevacizumab (^{89}Zr-bevacizumab and ^{111}In-bevacizumab) was first described by Nagengast et al. in nude mice with human ovarian tumor xenografts [94]. Meanwhile, an investigation carried out by Stollman et al. [139] in order to discover any possible association between VEGF-A expression in patients with colorectal liver metastases and level of ^{111}In-bevacizumab tumor accumulation did not reveal any clear-cut correlation between the VEGF-A expression and the level of antibody accumulation. Such finding could be partly attributed to the inability to visualize the soluble VEGF121 isoform and the elevated vascular permeability in tumors [140].

32.10.4 Cluster of Differentiation 20 (CD20)

CD20, a surface antigen expressed by B-cell hematological malignancies, has been the target of a chimeric mAb, rituximab, which has shown to be highly active in non-Hodgkin's lymphoma [141]. Keeping in mind the significant radiosen-

sitivity of non-Hodgkin's lymphoma, two radiolabeled murine mAbs against CD20, namely, ^{90}Y-ibritumomab tiuxetan (Zevalin) and ^{131}I-tositumomab (Bexxar), have been developed alongside rituximab and have been evaluated for cancer radioimmunotherapy (RIT) since then. Using ^{111}In-labeled mAbs, tumors can be imaged and according to the imaging results, which demonstrate the tumor uptake and the extent of normal organ localization such as spleen uptake, patients can be selected for RIT; such an approach is regarded as radiotheranostics and was applied early in the introduction of ^{90}Y-ibritumomab tiuxetan (Zevalin), although imaging is no longer routinely performed due to the safety and dosing issues [86].

32.10.5 Epidermal Growth Factor Receptor 2 (HER2)

Human epidermal growth factor receptor 2 (HER2) is overexpressed in a multitude of malignancies, comprising breast, ovary, prostate, bladder, gastric, and lung cancers, among which its diagnostic role is most vastly studied in breast cancer. The role of HER2 as a therapeutic target has been proven in patients with breast and gastric cancers [142, 143]. HER2 overexpression is found in 15–25% of patients with breast cancer and is known to be associated with the more aggressive clinical course. A spectrum of anti-HER2 drugs has shown encouraging results in patient outcomes in both advanced and early disease settings [144]. Hence, the assessment of HER2 as an important target in the management of breast cancer is a crucial step in the diagnostic workup and the selection of optimal treatments in both early-stage and metastatic settings. Immunohistochemistry (IHC) (usually with an antibody recognizing the intracellular domain of the receptor [145]) or fluorescence in situ hybridization (FISH) is the method commonly used in the clinical practice to identify HER2 status mostly from the primary tumor and when feasible [146] repeated at the time of progression to recognize any possible discordance between the primary tumor and metastasis [147]. However, there are some issues of concern with regard to these clinically available methods that need to be resolved; such issues include the limited number of metastases that can be easily accessed for a biopsy and the heterogeneity of the disease, particularly in the metastatic setting. Table 32.3 depicts multiple anti-HER2 probes developed for both SPECT and PET as noninvasive approaches for evaluating whole-body HER2 expression patterns, among which some have encouraging results in the clinical practice.

Several studies have assessed the applicability of trastuzumab, a humanized IgG1 mAb targeting epidermal growth factor receptor 2 (HER2), radiolabeled with ^{89}Zr, ^{111}In, or ^{64}Cu as a diagnostic tool to define in vivo HER2 expression in primary and metastatic breast cancer [101, 151], and has shown significant activity in patients with HER2-positive breast cancer [153]. SPECT imaging with ^{111}In-trastuzumab has shown promising results in tumor HER2 expression detection in mouse tumor xenograft studies as well as HER2-positive breast cancer human studies [103, 104]. PET imaging with positron-emitter ^{89}Zr-labeled trastuzumab has shown good spatial resolution and high and specific tumor uptake in animal models [98]. It has been postulated that reduction of downstream pathway activation implicated in cancer cell growth and proliferation by means of HER2 downregulation could be the possible mechanisms through which trastuzumab acts as a therapeutic agent [154].

Pertuzumab, a mAb that binds HER2 at an epitope other than the one trastuzumab attaches to, inhibits dimerization between HER2 and other epidermal growth factor family receptors, and can be used in its radiolabeled form to image tumoral HER2 expression alterations following treatment with trastuzumab, since these two affect different binding sites of HER2; such studies have revealed HER2 downregulation after continued trastuzumab treatment. Indeed, ^{111}In-labeled pertuzumab SPECT imaging has demonstrated promising results in the assessment of HER2 expression in mural breast cancer xenografts [84]. Uptake of ^{111}In-labeled pertuzumab has been indi-

Table 32.3 Monoclonal antibody-derived probes to assess HER2 overexpression in patients with breast cancer

Probe	Dose	Study population	Findings	References
^{111}In- or ^{68}Ga-labeled ABY-002	80–90 mg, activity ranging from 110 to 267 MBq	*N* = 3 (advanced stage)	High rate of detection of known lesion on ^{18}F-FDG PET	[148]
^{111}In-ABY-025	100 mg, mean activity of 142.6 MBq; range, 131–154 MBq	*N* = 7 (advanced stage, including 5 HER2-positive and 2 HER2-negative tumors)	Visualization of HER2-positive metastases, comprising both the liver and brain metastases	[149]
^{111}In-trastuzumab with variable amounts of trastuzumab	185 MBq	*N* = 10 (advanced stage)	Anticipation of cardiotoxicity and response to trastuzumab	[150]
^{111}In-trastuzumab	100–150 MBq	*N* = 15 (advanced stage)	Low tumor detection rate	[103]
^{89}Zr-trastuzumab	37 MBq + either 10 or 50 mg of trastuzumab	*N* = 14 (advanced stage)	Showing metastasis in liver, bone, lungs, and brain; excellent tumor uptake	[151]
^{64}Cu-trastuzumab	130 MBq	*N* = 6 (early and advanced stages)	Showing primary tumors and brain metastasis; suboptimal visualization of liver lesions	[101]
^{64}Cu-trastuzumab	364–512 MBq, 5 mg of trastuzumab preceded by 45 mg trastuzumab infusion	*N* = 8 (advanced stage)	Identification of lesions similar to ^{18}F-FDG PET; however, some lesions were only visualized on ^{64}Cu-trastuzumab PET	[152]
^{89}Zr-trastuzumab	185 MB ± 10%	*N* = 9 (advanced stage, pathologically confirmed HER2 negative)	Detection of unsuspected HER2-positive metastases in patients with HER2-negative primary breast cancer	[98]
^{89}Zr-trastuzumab	37 MBq (±10%) ^{89}Zr-trastuzumab and 50 mg cold trastuzumab	*N* = 56 (advanced stage)	Prediction of response to the antibody-drug immunoconjugate, trastuzumab-emtansine (TDM1) in patients with HER2-positive metastatic breast cancer	[99]

cated in HER2-positive breast cancer liver metastases, as well. As depicted in Table 32.3, the study performed by Ulaner et al. [98] has suggested ^{89}Zr-labeled trastuzumab to be able to detect HER2-positive metastatic lesions and select patients who may benefit from trastuzumab treatment in whom the primary tumor was found to be negative for overexpression of HER2 following conventional investigations, such as IHC and FISH; and these are all first steps to confirm the role of imaging with radiolabeled trastuzumab in defining spatial and temporal tumor heterogeneity in HER2 expression in breast cancer patients and to help us identify more patients who would benefit from HER2-targeted therapy providing an opportunity to personalize cancer treatment.

32.10.6 Epidermal Growth Factor Receptor (EGFR)

Epidermal growth factor receptor (EGFR), a member of the erbB family of tyrosine kinase receptors [155], is a 170 kDa cell surface protein composed of an intracellular domain with adenosine triphosphatase-dependent tyrosine kinase activity, a hydrophobic transmembrane domain, and an extracellular ligand-binding domain [156], which is overexpressed in different tumors, comprising lung, colon, breast, head and neck, pancreatic, and brain, and is associated with a multitude of mechanisms responsible for tumor growth and progression, such as autonomous cell growth, inhibition of apoptosis, angiogenic

potential, invasion, and metastases [157, 158]. The binding of EGF to its ligand results in the downstream activation of RAS/RAF/MAPK and PI3K/AKT cascades [159].

Different anti-EGFR mAbs, including cetuximab or panitumumab, have shown activity in different types of solid tumors and have been approved for clinical use, while some are currently under clinical evaluation. Cetuximab is an immunoglobulin G1 mouse–human chimeric monoclonal antibody that binds with high affinity to EGFR [160], inhibits the EGFR signal transduction pathway, and causes disruption of cell cycle progression and G1-phase cell cycle arrest through blockade of DNA repair mechanisms and survival pathways, decrease in matrix metalloproteinases required for metastatic invasion, and downregulation of angiogenesis and cellular adhesion [161].

It has been recently indicated that in spite of selection based on KRAS and NRAS mutational status, which is known to be predictive of resistance to anti-EGFR therapies [162], only 50% of patients with colorectal cancer treated with cetuximab achieved a benefit [163], the finding which leads to the hypothesis that the uptake of such anti-EGFR mAbs in cancer cells may anticipate its activity. Demonstration of tumor uptake of the radiolabeled mAb by PET imaging of advanced colorectal cancer patients with ^{89}Zr-labeled cetuximab following cetuximab infusion has been shown to be able to predict cetuximab activity [106]. Furthermore, another study has proposed the overexpression of EGFR as an actor in trastuzumab resistance in HER2-positive breast cancer patients [164]. The overexpression of this marker in triple-negative breast cancer cells (estrogen and progesterone receptor negative and HER2 negative) has also been associated with poor prognosis [165].

32.11 Future Perspectives and Concluding Remarks

Radiolabeled mAbs have successfully been used in the timely diagnosis of malignancies for many years, with encouraging clinical results. The current advances in the utilization of novel radionuclides for both diagnostic and therapeutic purposes have prompted the optimization of the radioimmunoconjugates and make this an appealing method for early detection and management of different cancers. Radiolabeled mAbs, as novel molecular imaging probes, have shown to be promising agents in preclinical models to enable in vivo molecular characterization of tumors to guide diagnosis and to favor response monitoring and early resistance detection. Furthermore, a shift in the paradigm from classical tumoral antigen targeting toward tumor microenvironment targeting has been achieved through the improvement in radioimmunoconjugate methodology. In vivo visualization of specific targets and pathways using molecular imaging techniques at the preclinical stage and in clinical trials has accelerated the development of new personalized cancer therapies. Hence, by means of the current advances in the theranostic application, using radiolabeled mAbs, personalized medicine, which has long been one of the main goals of cancer research thus far, has become a reality in the arsenal of cancer treatment and this results in the effectiveness of treatment and minimizes normal tissue toxicity.

Future work should focus on the integration of molecular imaging techniques using radiolabeled mAbs to address key questions in the preclinical and clinical evaluation of novel targeted agents with special regard to the imaging of expression and inhibition of drug targets and early assessment of the tumor response to treatment. Agents allowing precise measurement of tumor targets on a whole-body image upon administration of a functional agent, which are therefore expected to provide image-guided therapy, are required to adequately assess clinical endpoints, and in this way guidance on more efficient alternative treatment strategies could be possible.

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33 Flow Cytometry in Cancer Immunotherapy: Applications, Quality Assurance, and Future

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33.1 Introduction

Cancer immunotherapy seeks to elicit or augment the antitumor immune response in a cancer patient in order to enlist the help of the patient's own immune system for long-lasting tumor control. In this context, active cancer immunotherapy refers to the use of cytokines, immunomodulatory monoclonal antibodies (e.g., blocking antibodies (Abs) for CTLA-4, PD-L1, and PD-1, but also agonistic Abs for CD40, CD137, OX40, …), cell-based products (e.g., adoptive transfer of tumor-infiltrating lymphocytes-TILs-or of engineered T-cells such as CAR-T-cells), or experimental vaccines based on various antigen (Ag) formats. When evaluating immunotherapies, particularly in the experimental setting, it is essential to monitor the immune response elicited by the treatment. Immunomonitoring delivers evidence of immunogenicity; guides the choice and dose of antigens; assesses the effects of adjuvants, immune modulators, and therapy combinations; and has the potential to reveal early biomarkers of clinical efficacy. In this respect, immunomonitoring is helpful for rational clinical development and supplements clinical efficacy parameters such as disease-free period or survival, which are often available only at later clinical trial stages.

In view of their role in the anticancer immune response, the quantity and quality of tumor-antigen-specific effector $CD4^+$ and $CD8^+$ T-cells are of particular interest. In addition, the role of immune-regulatory cells, e.g., regulatory T-cells (Tregs), myeloid-derived suppressor cells (MDSCs), or certain subsets of monocytes/macrophages that can suppress effector immune responses, is increasingly recognized, not only within the tumor microenvironment but also in its macro-environment, the lymph nodes, spleen, and blood [1–4]. Informative analysis requires multiple markers for the accurate identification and quantification of phenotypic and functional cell subsets that are typically found at relatively low frequencies in the peripheral blood. These characteristics call for an assay that is multiparametric, robust, and sensitive enough to characterize rare individual cells.

The canonical multiparameter assay for the characterization of single cells in solution is polychromatic flow cytometry; it is ubiquitously used for immune monitoring, both in preclinical tumor immunology and in cancer immunotherapy trials. While the first fluorescence-based flow cytometer dates to 1968, the past several years have brought major advances in cytometer technology, reagents, and range of applications. Minimal standards for assays and cytometer quality assurance, as well as for data reporting, are being adopted, along with automated analysis techniques, which are becoming essential for the analysis and visualization of high-throughput multiparameter flow data. Much has also been learned about the challenges facing the use of increasingly complex flow cytometry assays in clinical trials, and what needs to be done to harmonize the assays across laboratories. This chapter describes the main flow cytometry methods being applied in cancer immunotherapy, with an emphasis on recent progress in the field, challenges associated with quality control, its promise to reveal biomarkers of clinical efficacy, and further developments that are likely to be rapidly implemented in routine cancer immunology.

33.2 Main Flow Cytometry Assays in Cancer Immunotherapy

Together with immunohistochemistry, immunophenotyping by flow cytometry is probably the most commonly used assay to investigate cells in cancer immunology. Flow cytometry distinguishes human immune cells via a combination of physical properties and fluorescent markers such as labeled monoclonal antibodies (mAbs) against cell-associated molecules that are expressed at the cell membrane or intracellularly. Physical properties measured by the cytometer are forward-scattered light (FSC) which is roughly proportional to the cell size, and side-scattered light (SSC) which reflects the granularity of the cells. Markers recognized by fluorescent mAbs are mostly categorized in clusters of differentiation (CD). To date, the human cell differentiation molecule organization [5] has indexed

more than 370 CD markers [6]. "Basic" CD markers are CD3, CD4, and CD8 for T-cell subsets; CD19 or CD20 for B-cells, CD14 for monocytes; CD11c for subsets of dendritic cells; CD56 for natural killer (NK) cells; and CD15 for granulocytes. In most cases, and for obvious reasons, enumeration of the number and frequencies of immune cell types is performed on blood samples (either whole blood or PBMCs), which can be easily obtained from patients at regular intervals. More difficult to obtain but very informative is the tumor tissue, of which single-cell suspensions can be prepared for analysis of infiltrated immune cells. Indeed, the phenotype of TILs is often very different from that of autologous PBMCs, and many cell subsets can only be identified and characterized within the tumor microenvironment [7, 8]. Further markers can be added to identify endothelial cells (CD31), fibroblasts (ER-TR7, vimentin), epithelial cells (EpCAM, i.e., CD326), and particular tumor cells (e.g., CAIX for renal cell carcinoma).

Altogether, cell subpopulations are defined by combination, rather than single, markers, which is not surprising considering the complexity and plasticity of human immune cell subsets. As an example, a consensus was recently reached by a group of international experts for characterizing Tregs with a minimal set of seven nonoverlapping markers that can be used as a basic Ab panel for Treg monitoring [9]. Polychromatic cell surface flow cytometry is also useful to characterize the activation status, differentiation status, and clonality of T lymphocytes. Commonly used markers for this purpose include CD25, CD69, CD137, and CD154, CD27, CD28, CD45RA/RO. Currently, and along the clinical success of checkpoint blockade Abs, the characterization of T-cell co-receptors (e.g., PD-1, Tim-3, VISTA, LAG3, and CD40, CD137, OX40 for inhibiting and activating receptors, respectively) has become a major focus of clinical research [10, 11]. For checking the clonality of the T-cell receptors (TCR), Vβ usage can be determined with specific mAbs. Note that flow cytometry can only deliver limited information on TCR usage; hence sequencing is needed for a more detailed picture on CDR3 regions. A combination of mAbs against activation markers and chemokine receptors (i.e., CCR7 = CD197) can be used to identify naïve effector memory, central memory, terminally differentiated effector memory (TEMRA), and memory T-cells with stem cell-like features [12–16]. These differentiation stages are associated with changes in functional and proliferative properties [17] and their distribution is altered in the elderly [18, 19]; hence, this information is relevant for adoptive transfer therapy, checkpoint blockade, or vaccination in aging cancer patients.

A major interest in immunotherapy is to characterize tumor-antigen-specific T-cells. Accumulating data indicates that T-cells from patients responding to checkpoint blockade recognize tumor-specific neoantigens; as these neoantigens are generally derived from patient-individual mutations, analysis of T-cell specificities in treated patients currently requires a complex and work-intensive workflow [20–22]. In anticancer vaccination with defined Ags, monitoring is easier since the antigens are known.

The most direct characterization of antigen specificity is via the use of HLA-peptide multimers, which bind directly to the cognate TCRs. First described more than 20 years ago [23], the HLA-class I multimer assay currently serves as a versatile tool for enumerating and characterizing $CD8^+$ T-cell responses, and staining protocols are broadly available [24–26]. The detection limit of a conventional HLA-multimer test reaches approximately 0.02% of the $CD8^+$ repertoire; combinatorial staining which uses two fluorochromes per specificity allows increasing the number of T-cell specificities investigated in one sample (e.g., up to 27 specificities can be monitored simultaneously with only 8 fluorochromes) [27, 28]; this is advantageous when a limited number of cells is available, for example in the case of precious patient's material. Moreover, the detection threshold has been reported to be increased of approx. ten-fold as compared to single-color multimer staining. Coupled to the production of HLA-monomers by the UV exchange technology, this high-throughput method represents an important technical achievement for the T-cell immunology field, and

has started to deliver precious information by dissecting the antitumor T-cell repertoire in patients, including that directed at neoantigens [21, 29, 30]. Recently, the number of specificities that can be tested in one sample was enlarged to >1000 by DNA barcoding of the peptide-MHC multimers [31].

HLA-multimers are widely used to monitor T-cell responses, especially in the context of peptide-based vaccination approaches [29, 32–34]. They can easily be combined with mAb panels to assess the phenotype and differentiation status of antigen-specific $CD8^+$ T-cells [11, 35]. Also, the simple combinatorial staining approach could easily be implemented for monitoring vaccination trials, for example when applying cocktails of antigenic peptides for which many specificities need to be tested in a single PBMC sample. Limitations of HLA-multimers are that (1) both the precise T-cell epitope (i.e., the exact amino acid sequence of the peptide recognized by the TCR) and its HLA-restriction (i.e., the HLA-allelic product which binds and presents the peptide to the TCR) must be predicted in advance and (2) the assay does not deliver functional information. To date, there also remains a lack of general availability of class II multimers for $CD4^+$ T-cell detection [36].

Intracellular cytokine staining (ICS) is the most common functional assay used for monitoring antigen-specific T-cells. It is the flow cytometric method of choice when HLA-multimers are not available, if the exact T-cell epitope and/or HLA restriction is unknown, and for routine assessment of $CD4^+$ T-cell responses. ICS enables detection of multiple effector functions of both $CD4^+$ and $CD8^+$ T-cell subsets [37–39], including polyfunctional T-cells that have been associated with protection against pathogens [40, 41]. A few groups have described polyfunctional T-cells after cancer vaccination in patients, but whether these cells are associated with beneficial and long-lasting antitumor T-cell responses remains an open question [7, 42]. Intracellular cytokine staining is an intrinsically complex assay, which relies on optimal conditions during cell thawing, culture, antigenic stimulation, and of course cell staining. Optimized mAb combinations, protocols, and standardization approaches have been published [43–45].

With the development of new tools, reagents, and fluorochromes, many aspects that used to be studied with conventional methods can now be addressed with flow cytometry. This is the case for cytotoxicity, proliferation, or cell signaling (traditionally detected with radioactive substances). For assessment of killing activity, target cells (including controls) are differentially labeled using fluorescent dyes (e.g., Paul Karl Horan-PKH-or 6-carboxyfluorescein diacetate succinimidyl ester-CFSE) and incubated with the effector T-cells to be tested; killing is then measured by counting the remaining target cells. Apart from the obvious safety aspects over radioactivity-based assays advantages of addressing cytotoxicity by flow cytometry-based methods are that (1) the effector cells can be phenotyped, (2) several targets can be tested in the same tube, (3) the effector-target incubation time can be significantly prolonged (up to 24 h) compared to a classical ^{51}Cr release assay, and (4) the assay is sensitive and effective, even when low numbers of effectors are available [46–48]. Other approaches to indirectly determine the cytotoxic capacity of T (or NK) cells are the use of a mAb directed against CD107a (LAMP-1), which becomes extracellularly detectable after cytotoxic granules have fused with the cellular membrane (degranulation), and the measurement of granzyme B and/or perforin loss, or the caspase activity in the target cells [49, 50].

For measuring proliferation by flow cytometry, effector cells can be first labeled with fluorescent dyes (CFSE or other tracking dyes such as CellTrace™ reagents) and cultured for several days in the presence of relevant stimuli. Since the dyes are diluted from the mother to the daughter cells, the number of cell divisions is visible in the number of fluorescent peaks detected [51]. The frequency of proliferating cells can also be assessed directly by Ab staining of the proliferation-associated nucleus protein Ki-67, expressed at all phases of the cell cycle except the resting G_0 stage [52, 53]. Early signaling which takes place in effector cells upon activation can be detected by measuring Ca influx or

phosphorylation of components of the signal transduction cascade [54, 55].

These measurements have not been used in large-scale vaccine studies so far, probably because they are time consuming and require careful optimization and technical expertise to achieve reproducible results.

Finally, cell-free cytokine analysis can also be performed by flow cytometry with multiplex beads, a method that has been recently adapted to meet GCLP standards [56–59]. The method is based on the use of beads of known sizes that have been pre-labeled with different fluorescent intensities and coated with Abs against the different cytokines of interest. Simultaneous quantification of several soluble factors in one sample (i.e., culture supernatant, serum, or plasma) can be done by comparison to standard curves provided by the manufacturer, for example, to evaluate Th1/Th2 profiles [38]. The assay is as sensitive as ELISA, with detection limits in the range of 20 pg/ml for most cytokines and can be even more sensitive when an enhanced sensitivity system is used (below 1 pg/ml).

All these examples clearly show that flow cytometry is a versatile tool for investigations of the phenotype, frequency, and functional properties of immune cell subsets. Furthermore, assays can often be combined for multi-parametric probing of cell properties, which is advantageous as precious patient samples are spared. However, the need for both robustness and sensitivity to detect tumor-antigen-specific T-cells and/or rare cell subsets poses specific challenges for the use of these complex tools in clinical research applications. These are addressed in the following sections.

33.3 Ab Panel Development and Quality Assurance

Current state-of-the-art polychromatic flow cytometry involves multistep, multi-reagent assays followed by sample acquisition on sophisticated instruments that are able to capture up to 20 parameters per cell at a rate of tens of thousands of cells per second. Obtaining reproducible results from such a complex procedure requires well-trained staff, stringent quality management, and detailed standard protocols and operating procedures (SOPs) for panel development, cytometer calibration, reagent qualification, sample preparation, use of appropriate technical and biological controls, and careful data analysis.

We start with the factors important to consider when developing a mAb staining panel. Target molecules can have vastly different expression levels. While lineage markers such as CD45, CD3, or CD8 are expressed at very high copy numbers per cell, some important markers such as transcription factors (e.g., FOXP3 for $CD4^+$ Tregs) or chemokine receptors (e.g., CCR5 on $CD4^+$ Th1 cells) are often present at much lower levels. In addition, the available probes (such as mAb clones or HLA-peptide multimers) can have variable avidities for their respective targets. Probes are labeled with different chemical classes of fluorescent dyes that must be matched to the instrument—considering factors such as the availability of a high-power laser line with a wavelength close to the maximum absorption of the fluorescent dye—and with a detector (photomultiplier plus filters/mirrors) that has a high sensitivity in the spectral emission range of the given dye. Complicating matters, cellular autofluorescence (i.e., fluorescence due to cellular molecules such as NADPH even in the absence of all dyes) further limits the sensitivity that can be achieved with a given fluorescent probe, laser, and detector. In practical terms, autofluorescence of lymphocytes is usually limited to a distinct range of emission and absorption wavelengths [60, 61]. In general, the degree of autofluorescence determines the limit of detection of direct staining, which in earlier reports was of 3000 molecules for a standard flow cytometer [62]. Consideration of all these factors leads to the following recommendation for detecting cellular markers expressed at very low levels: use a high-affinity Ab conjugated to a fluorescent dye with high quantum yield with emission spectral range far away from cellular autofluorescence, and for which the cytometer has an appropriately matched high-power laser line and detector.

For polychromatic flow cytometry, additional constraints are set by the phenomena of optical spillover and spreading. In flow cytometry, cells are analyzed in a near-physiological aqueous solution to preserve the structural properties of biomolecules. Due to the spectral absorption of water and air, the useful spectral space is limited to the range from near UV (ca. 200 nm) to near IR (ca. 1000 nm). In addition, in aqueous solutions, both the absorption and emission of fluorochromes show relatively broad spectral lines. Together, this means that the number of fluorochromes that can be analyzed at the same time is ultimately limited.

As a further consequence, spectra of fluorescent dyes routinely overlap ("spillover") [63], requiring software deconvolution of true and observed signals (i.e., compensation). However, compensation cannot correct other errors caused by measurement, binning, and photon noise, and these errors accumulate to give an irreversible effect termed as "spreading error" or "spillover spreading" [64]. Spreading error will cause the presence of one bright fluorochrome to reduce sensitivity for spectrally-close fluorochromes present on the same cell. Use of a high-power laser close to the absorption maximum can reduce errors in photon counting, and narrow band-pass filters can reduce spillover; both these measures will reduce spreading error. Finally, probe combinations should be designed so that overlapping fluorochromes are chosen for labeling markers, which are expected to be expressed on different cells.

In practice, panel development usually starts with the definition of a "wish list" of cellular targets, followed by the prioritization of these cellular targets, characterization of their expression levels, and checking for the availability of probes and conjugated dyes appropriate for the cytometer to be used. Guidance documents [65], free tools from Ab manufacturers (spectraviewers or Guide Panel Solution from BD), and helpful software (Chromocyte [66], FluoroFinder [67], or FlowJo Panel Wizard [68]) are available. A practical limitation can be the lack of commercially available fluorochrome conjugates for individual antibody clones. Indirect staining with secondary reagents (such as the biotin-streptavidin system) is possible, but not convenient for routine multicolor applications. A better alternative is the use of new methods and kits commercially available for the self-conjugation of small amounts of Ab to fluorescent dyes [69, 70].

Based on the discussion above, the cornerstones of Ab panel development guidance are the assignment of "bright" probes for "dim" targets and strategies to avoid spreading error and autofluorescence in channels relevant for "dim" targets. It is also possible to change the optical pathway of the flow cytometer to optimize the instrument (e.g., choice of filters) according to the requirements of individual mAb panels. As the number of potential artefact interactions between dyes and/or Ab clones rapidly increases with the number of parameters in the panel and as a large number of critical parameters should be optimized (e.g., Ab concentration), the development of large (≥8 colors) panels and especially those involving separate staining steps for intracellular and extracellular targets can be an expensive iterative process requiring several man-months of dedicated work. Hence, the flow community is encouraged to share rigorously calibrated and optimized polychromatic panels via the "Optimized Multicolor Immunofluorescence Panels" (OMIPs) project [71]. For phenotyping of (malignant) leukocytes in fresh whole blood, the EuroFlow consortium has also developed polychromatic Ab panels and procedures for 8–12-color staining whereby the T-, B-, and myeloid cell subsets can be defined [72, 73].

Quality assurance (QA) of a flow cytometry assay starts with the optimization, calibration, and standardization of the cytometer itself, and we refer the reader to the technical report by the Roederer group for details [74] or to specialized books. These optimization steps must not be neglected, especially with new instruments, as they may identify faulty parts that need replacement, such as a photomultiplier tube (PMT) with reduced sensitivity or suboptimal filters, and are important to optimize general instrument parameters. Conveniently, some (but not all) of these steps have been incorporated in vendor software

packages, such as the cytometer setup and tracking (CS&T) application within BD FACSDiva that uses a proprietary mixture of calibration beads. In addition, unstained and single-stained beads are used to determine the spillover matrix for compensation. For long-term immunomonitoring, it is essential to maintain accurate records of daily monitoring checks to track reproducibility and stability.

For cell staining, reagent (e.g., mAbs) quality can be an issue, especially if the assay is performed repeatedly over time. Often, reagents used are classified as "research use only" (RUO) and can show considerable batch-to-batch variation in important properties, such as concentration of antibody-dye conjugate, concentration of free dye, and even in the spectral properties of the dye (as in the case of tandem dyes) [75]. In addition, the shelf life designated by vendors is not always based on quantitative specifications. As a result, individual reagent batches have to be pretested and pre-titrated, and optimally tests should be repeated even during the designated shelf life of a reagent. As batch sizes available from vendors are often limited, this can result in the requirement of reagent bridging (demonstration of the comparability of reagent batches) during the course of a study, leading to complex logistic and tracking processes. Reagent quality control (QC) may be facilitated by the preparation of mixtures of lyophilized reagents ("lyoplates") [76–78] that can increase reagent stability and reduce pipetting error.

Appropriate use of technical and biological controls is also vital for assay interpretation. Isotype and "fluorescence minus one" (FMO) controls can help with setting gate boundaries at the analysis stage, by defining the "negative" region. However, isotypes are not always optimal controls, even if provided from the same manufacturers; moreover, for some reagents like HLA-multimers, no perfect control exists, since each single peptide-HLA multimer is a unique reagent. In addition, Ab panels must be established on cells treated similarly to those which will be monitored afterwards; for example, activated cells not only are bigger than nonactivated cells, but also generally express different amounts of a variety of molecules. When working with TILs, it is important to verify that isolation protocols, which often include enzymatic digestion, do not modify epitope exposure [79]. Pretested, aliquoted, cryopreserved samples with prescreened, predictable properties (such as being positive or negative for individual markers in the mAb panel) can serve as valuable biological controls, which can be added regularly to assay runs in order to track the variations between reagent batches or in assay performance between operators and over time.

As flow cytometry-based methods become incorporated into clinical trials, the need for a stable and unlimited source of control cell specimens that contain a defined number of functional antigen-specific T-cells as a control becomes paramount. Cell samples containing a known number of T-cells specific for a defined Ag would allow easy assessment of the quality and accuracy of reagents and assays, and provide standard controls for comparison of results across laboratories or time. Conventional sources for reference cell samples are either (1) based on leukapheresis or buffy-coat material from healthy donors-which are restricted to reactivity against immunogenic viral Ags, expensive, and available in limited amount-or (2) dependent on the ability to generate and propagate T-cell lines/clones on a repetitive basis, which is a burdensome task. The Cancer Immunotherapy (CIMT) Immunoguiding Program (CIP) group has recently established a process for the generation of TCR-engineered reference samples (TERS) that can be used in T-cell assays. In a first proof-of-principle study, we showed that retrovirally TCR-transduced T-cells spiked at defined numbers in autologous PBMC could be used as standard samples. The T-cells could be accurately detected at all dilutions in a linear fashion, down to frequencies of at least 0.02%, and the feasibility of TERS was confirmed in a small-scale interlaboratory testing [80]. Subsequently, we established, optimized, and standardized the production of TERS obtained by transfection of modified and stabilized TCR-RNA. Such a platform offers a simple, virus-free, and scalable process for the personalized manufacturing of TERS that are stable over

time. Moreover, the analysis of the TERS is similar to that of the tested cell samples in that the same gating strategy (and even the same gates) could be used. TERS can be tested across multiple assay platforms and can sensitively detect assay variation resulting from common sources of error [81]. The kit-based production of TERS has been established [82] and the first kits are commercially available (www.jpt.com).

A final, critical aspect of quality management is the careful documentation of each procedure performed, as well as provision of detailed protocols and/or standard operating procedures (SOPs) for each stage including data analysis. Technical staff needs to be well trained and need to perform the analyses on a regular basis to keep up the performance. Participation in proficiency panels will also help improve and control laboratory standards over time.

33.4 Standardization, Validation, and Harmonization via Proficiency Programs

While HLA-multimers and ICS are commonly used for monitoring experimental immunotherapies, there are still notable obstacles to the advancement of these assays as robust biomarkers for clinical trials [83, 84]. First, there is no gold standard protocol for any of these assays. Second, correlations between in vitro immunomonitoring results and patient clinical benefits are increasingly reported [85–93], but not systematically observed. The reality is that assays performed at different institutions are not equal; this results in difficulties in comparing the efficacy of various immunotherapy approaches for the same disease type, let alone between different diseases, and this in turn hampers progress in the field.

The first approach for addressing these problems in individual laboratories is to implement a strict step-by-step assay establishment, optimization, standardization, and validation process. The use of validated assays to monitor clinical trials is now mandatory in the USA and in Europe, in line with Good Clinical Laboratory Practice. Validation does not necessarily improve performance, but indicates the strengths, weaknesses, operational range, and repeatability of assays. Validation guidelines have been published for ICS and HLA-multimer staining [94–96].

Once assays are validated in expert labs, immune monitoring may be centralized at a dedicated core facility, including for multicentric studies (for specific challenges, see Sect. 33.6). An attractive alternative to this strategy, especially at the early clinical development stage, is assay harmonization. The pros and cons of assay harmonization vs. inter-center standardization have been discussed in detail elsewhere [97, 98].

Assay harmonization is based on the participation of single laboratories in iterative testing exercises called proficiency panels. For example, pretested PBMC samples, synthetic peptides, and/or HLA-peptide multimers are shipped from a central lab to all panel participants who then use their own reagents, protocols, and analysis strategies for detecting antigen-specific T-cells. Participants then report their data, which are centrally analyzed, allowing comparison of individual assay variables and performance to detect T-cells. Thus, parameters involved in assay performance may be successively identified, corrected, and confirmed to exert an impact on subsequent panels (i.e., multistep approach). Finally, benchmarks and guidelines are formulated and disseminated to the community. Participating laboratories benefit by being able to measure their own performance in comparison to peer laboratories, and regularly taking part in proficiency panels over time can also be seen as a quality control of assay performance for individual labs. Additionally, the working group can guide laboratories to improve performance if needed while providing an exchange platform for assays and their application.

Proficiency panels can in principle be applied for any T-cell assay, including those based on flow cytometry [99–101]. In 2005, two consortia, the European Cancer Immunotherapy (CIMT) Immunoguiding Program (CIP) and the Cancer Immunotherapy Consortium of the Cancer Research Institute in the USA (CIC/CRI), launched a large program of proficiency panels,

and synergistically pioneered the concept of assay harmonization [98, 102]. From 2005 to 2017, the CIP [103] has organized 21 small- to large-scale proficiency panels, dedicated to the measurement of antigen-specific $CD8^+$ T-cells (HLA-multimers, ELISPOT, and ICS) and of other immune cell subsets relevant for immunotherapy (NK cells, MDSCs). Of note, with the increasing data on circulating myeloid subsets associated with the efficacy of immunotherapies [1–3], it will become important to rely on not only phenotypic markers but also functional measurement, similar to what is done for T-cells.

Proficiency panels have taught us that there are large variations in the performance of cell assays among the flow community. While the majority of labs do detect antigen-specific T-cells present at quite high frequencies in PBMC samples (approx. >0.2% of $CD8^+$ cells), the detection rate drastically decreases for low-frequency effectors (<0.05% of $CD8^+$ cells). This is very relevant for cancer immunotherapy, as tumor-specific T-cells are expected to be present at low frequencies in the blood, even after patient vaccination. Another lesson is that comparable performance is achievable with different laboratory-specific protocols and reagents, and that full interlaboratory standardization is not necessary for good results. Surprisingly, we also found that operator experience in a method does not necessarily predict performance, underlining the utility of a regular quality control of established methods. Finally, adoption of simple measures can lead to significant improvement in assay performance. For example, staining and acquiring larger numbers of $CD8^+$ cells increase the ability to detect low-frequency HLA-multimer-positive cells, and inclusion of a cell-resting phase improves sensitivity in the IFN-γ ELISPOT assay. In contrast, a high background production of the cytokine IFN-γ both in ICS and ELISPOT is clearly associated with decreased performance [104, 105].

Over several proficiency panel iterations, it also became clear that all steps of the assays, starting from cell handling (freezing/thawing/resting), assay conditions (reagents and protocols for mAb and HLA-multimer stainings, conditions of antigenic stimulation in ICS), and acquisition of the cells including instrument settings down to the data analysis, can benefit from harmonization for achieving comparable results between laboratories. Both CIC and CIP have observed in independent panels conducted for ICS [105, 106], HLA-multimer staining [97, 107], or MDSC detection [108] that suboptimal gating strategies strongly influence the ultimate results, i.e., the detection and deduced frequencies of the cells of interest. We also showed that analysis (gating) performed by a unique user substantially decreased the variation in the frequencies of specific cells as compared to those reported by single labs analyzing their own data or the same data [106, 109]. This is not a surprise, since manual gating is subjective and highly dependent on the experience of the experimenter and tradition in the lab. Further work is therefore needed with a focus on both data acquisition and analysis, including on automated analysis strategies that can reduce the subjectivity inherent in gating as described in Sect. 33.7.

33.5 Structured Reporting of Immune Assay Experiments

An increasing number of minimal information projects have emerged in the last years to provide guidance for structured reporting of biological assays. The first minimal information (MI) project that set the scene was the Minimal Information About Microarray Experiments (MIAME) published in 2001 [110]. It is now an established and mandatory standard for publishing microarray data for a growing list of highly recognized journals [111]. More than 40 such guidelines have emerged, asking for minimal information on reported results for next-generation sequencing (MIRING) [112], in situ hybridization, immunohistochemistry experiments (MISFISHIE) [113], cellular assays (MIACA) [114], and flow cytometry experiments (MIFlowCyt) [115]. Information on the majority of these MI projects can be found in a central portal for minimal information on biological and biomedical investiga-

tions (MIBBI) [116]. These guidelines aim at achieving two major goals: first, to annotate data to such extent that they give transparent evidence on the quality, reliability, and possible error sources of reported results, and second to use the reporting standard to systematically feed public databases [117].

Structured reporting guidelines have also been provided for immune assay experiments. As outlined before, the continuous conduct of proficiency panels over several years led to the identification of steps in the assay that critically impact the results, namely (1) the sample, (2) the assay, (3) the data acquisition, (4) the data analysis, and (5) certain characteristics of the lab environment. In concordance with these findings, a flowchart of decisions that can affect the quality of data produced in clinical trials in which immunological parameters are monitored by flow cytometry was given in a landmark publication [118]. Although the variables critically affecting the quality of the results are—for most of them—well known, only very few scientific publications provide sufficient information on these aspects in their materials and methods sections. This lack of transparency is one of the major reasons preventing meaningful comparison of published results generated across institutions. In contrast, study results reported with transparent information on the essential variables of assay conduct explicitly indicate awareness of the investigator to control critical variables and can be much better interpreted and reproduced.

To reduce the discrepancy between available knowledge on immune assay conduct and lack of critical information in scientific publications, a group of T-cell immunologists from the cancer immunology, infectious diseases, autoimmunity, and transplantation fields initiated the Minimal Information About T-cell Assays (MIATA) project [119]. The group conducted an intensive vetting process with two public-consultation periods, two open consensus workshops, and several webinars [120]. The process towards reaching a broadly acceptable guideline on the minimum information that should be provided for T-cell assays [121] can be found at the project's web page [122]. With the MIATA consensus guidelines becoming available, the implementation of more structured reporting for T-cell (and more recently for NK-cell) immune monitoring has begun. So far, eight peer-reviewed journals endorse the MIATA guidelines and assign the "MIATA label." The label indicates that authors of accepted manuscripts take great care about reporting on and control of variables that matter for T-cell assays. MIATA-compliant manuscripts are listed on the MIATA homepage leading to greater exposure of the published work, which may increase interest and citations over time. The authors therefore recommend considering structured reporting of results from T-cell assays whenever possible, especially in the context of clinical trials [123].

33.6 Organization of Immune Monitoring in Multicenter Trials

Clinical trials will often require the recruitment of patients at multiple sites in order to reduce the overall duration and costs. Two general strategies emerge on how analytical assays can be performed across different sites [124]: in the distributed analysis paradigm, each site analyzes its locally derived samples. On the contrast, in the central lab paradigm, all samples are transported to a single site for analysis. In either case, flow cytometry poses additional challenges due to the fragility of the sample and the complexity of the assay.

For distributed analysis, the assay and instrumentation at different sites must be comparable. This can be achieved via full interlaboratory standardization, as is already routinely performed in clinical flow cytometry with in vitro diagnostic (IVD)-certified reagents and instruments [125]. Due to the high development costs, the number of clinical flow cytometry products for IVD on the market is limited and focuses on the clinically most relevant tasks as, e.g., the quantification of $CD4^+$ T-cells in blood. In many cases, these applications lack the technical capabilities of modern polychromatic flow cytometry. Full-scale interlaboratory standardization (with demonstrated low interlaboratory variation) of research

assays with RUO-grade reagents and customized flow cytometric instrumentation such as the Euroflow initiative for monitoring hematological malignancies is feasible, but requires great efforts [72]. An alternative to full interlaboratory standardization discussed in Sect. 33.4 is harmonization, which can be achieved via regular participation in proficiency panels and/or testing exercises involving all labs from a certain consortium.

For highly complex flow cytometric assays within clinical trials, having all samples analyzed by the same central laboratory eliminates the need for full-scale interlaboratory standardization of participating institutes, and may be less demanding. However, maintaining sample quality becomes a critical issue with this strategy. The initial sample material for flow cytometry contains living cells (in most cases derived from blood with the addition of anticoagulants). In most cases, cells (PBMCs) have to be isolated from this sample material before the start of the flow cytometric assay. Cells are usually more fragile compared to biomolecules or small molecules. Several studies have been performed to determine how long blood can be stored or transported before peripheral blood mononuclear cell (PBMC) isolation (mostly using density gradient centrifugation), and how stable isolated cells are before the assay is started [43, 126, 127]. For simple phenotyping (e.g., $CD4^+$ T-cell counting), a 48-h delay before centralized analysis is acceptable, while the most demanding applications (such as some functional T-cell assays) require isolation of the cells within 8 h of venipuncture, followed by immediate analysis or cryopreservation of the cells [128]. Shipment to a central lab followed by processing of blood samples within 8 h is not feasible in international multicenter trials. Therefore, a mixed model may be chosen [53], whereby cells are isolated and cryopreserved from peripheral blood at individual labs close to the patient, and then shipped in the frozen state to the central lab where it is stored frozen before analysis. All stages of isolation, cryopreservation, and transport conditions should be fully standardized in this model. Standardized labeling of samples that allow the unambiguous assignment of a sample to a trial, site, patient, and visit is also critical. These procedures have to be clearly defined in the clinical trial protocol and are usually further detailed in the clinical trial laboratory manual.

As an example, demonstrating feasibility of this approach, an international, multicentric immunotherapy trial was conducted, including T-cell immunomonitoring, in which more than 40 clinical sites were trained in blood sampling, labeling, and shipping, with labels and collection tubes provided by a central laboratory. Local PBMC isolation laboratories were centrally supplied with pretested kits containing all critical reagents required for isolation and cryopreservation of PBMCs. All laboratory technicians were trained and qualified on central SOPs describing in detail the PBMC isolation and cryoconservation processes. Where required, the fresh blood was transported from the clinical sites to the PBMC isolating labs using temperature-controlled shipments. The isolated frozen PBMCs were shipped to the central lab in validated dry ice containers. Patient visits involving a PBMC sampling were carefully coordinated in advance among the clinical sites, the PBMC isolating laboratories, and the logistic service providers to ensure that the blood could be processed within 8 h after venipuncture of a patient. This process led to a successful logistic chain for 361/362 (99.7%) PBMC samples and an overall evaluability rate of 64/68 (94%) patients for T-cell immunomonitoring [53], and has been adopted for further studies [129].

33.7 Automated Analysis of Flow Data

As discussed in Sects. 33.4 and 33.5, the standard approach for analyzing flow cytometry data is by the visual identification of cell subsets of interest on histograms or two-dimensional scatter plots. With multiparameter data, gating consists of first choosing a gating strategy, i.e., a sequence of 2D dot plots that is designed to allow identification of the cells of interest. For example, a possible gating strategy for identifying HLA-multimer-

positive CD8+ T-cells might be FSC-H/FSC-W (singlets), FSC-A/SSC-A (lymphocytes), CD3/viability dye (viable T lymphocytes), CD4/CD8 (basic T lymphocyte subsets), and CD8/multimer. In each dot plot, cells of interest are included, and other events excluded by the use of elliptical or polygonal gates, or sometimes by splitting the dot plot into quadrants. The exact location and shape of these gates may be based on experience, or by comparison with negative (e.g., isotype, FMO, or unstimulated control in ICS) and positive (reference sample or T-cell clone, or superantigen stimulation) controls. After a gating strategy has been set, it is typically applied in common to all flow cytometry samples in the batch being analyzed. Some researchers will also adjust gates for individual samples to take individual variability into account. In general, there is no consensus or accepted standard gating strategy, and individual laboratories may apply different gating strategies to identify the same target cell subset. Notably, proficiency panels have made it very clear that the subjectivity of gating forms a significant source of assay variability between laboratories in the absence of a harmonization program [105, 130] (see also Sect. 33.4).

With the ever-growing dimensionality of flow and mass cytometry data, it is increasingly likely that manual gating strategies will miss novel cell subsets, due to the inefficiency of exploring high-dimensional space on 2D plots. Boolean gating is an attempt to exhaustively enumerate every potential cell subset by evaluating all the Boolean combinations of individual markers partitioned into positive/negative categories. For example, the Boolean gates in an ICS assay with IFN, TNF, and IL2 would be the eight combinations IFN−/TNF−/IL2−, IFN−/TNF−/IL2+, IFN−/TNF+/IL2−, IFN−/TNF+/IL2+, IFN+/TNF−/IL2−, IFN+/TNF−/IL2+, IFN+/TNF+/IL2−, and IFN+/TNF+/IL2+. However, the number of Boolean gates grows exponentially with the number of markers, and many of these gates are empty or uninformative, making this a low-yield strategy. To increase the objectivity of flow cytometry analysis and cope with more complex data sets, automated unsupervised learning methods in which cell subsets are directly quantified by machine algorithms have been proposed [109, 131–134]. In broad terms, these algorithms have to first partition all the events in a data sample into disjoint subsets, based on properties of each individual event and its relationship to other events, then assign these subsets to biologically meaningful categories (e.g., HLA-multimer-binding CD8+ lymphocytes). In the context of cancer immunology, a specific challenge for automated approaches is the high sensitivity required, since antigen-specific responses (e.g., HLA-multimer positivity or cytokine-producing cells) may be relevant at relatively low frequencies of 0.01–0.1% [135]. Data from multiple laboratories significantly increases the challenges for automated analysis, since the algorithms have to also account for the variability across laboratories and deal with issues such as inconsistent sample annotation. For managing multicenter data, specialized cytometry data upload and management applications are helpful to ensure metadata consistency and as a platform for data sharing [136, 137].

A typical automated analysis preprocessing pipeline starts with the extraction of the essential matrix of information stored in a flow cytometer standard (FCS) file, where each row represents an event and each column represents a detector channel, either scatter or fluorescent intensity. Preprocessing algorithms may apply compensation, or specific transformations to regularize the data distribution (e.g., bi-exponential transformation). Specific channels may be explicitly excluded from analysis if they are not likely to be informative for the cell subset targets of interest. Often, a quality control filter is also applied at this stage, and data sets with inconsistent annotation, too few events, anomalous event distributions, or signatures may be flagged for manual evaluation [138].

The core of most automated analysis is the unsupervised partitioning of events into cell subsets. There are a variety of approaches that can be taken to partition or cluster events [133]. One popular approach is the use of statistical mixture models, either identifying cell subsets with individual mixture components (which are typically multivariate Gaussian, student T, or skewed

versions of these distributions) or using features of the estimated density to assign events to cell subsets [139–141]. Such probabilistic approaches provide a declarative framework to model domain knowledge, and support formal statistical inferences for structure learning, classification, and prediction. The underlying statistical model for the domain knowledge can also be naturally extended in different contexts, for example, to incorporate specific assay details for combinatorial multimer encoding [142] or to incorporate multilevel effects via hierarchical modeling [143]. The power of probabilistic models comes at a price, in that these models tend to be much more computationally demanding than non-probabilistic approaches [144–147], and the run-time for analysis of high-volume, high-dimensional data sets may be prohibitive. However, recent developments in the use of highly parallel graphical processing units [148] have accelerated run-times by orders of magnitude, making the probabilistic approaches a viable approach for many applications in cancer immunology.

The essential step in post-processing is the alignment of cell subset clusters across multiple data samples, since comparative analysis of equivalent cell subsets is a necessary requirement of flow cytometry analysis in clinical research. Perhaps the most straightforward approach is to align each data sample with respect to either a reference or a consensus clustering via an optimization routine that minimizes some distance between pairs of clusters (e.g., Euclidean distance between cluster centroids). Other possible approaches skirt the problem entirely by enforcing a common clustering across all data samples, or partition the clusters from fitting all data samples into "superclusters"—all clusters in the supercluster are then assigned to the same cell subset. The final step of assigning meaningful cell subset labels to the aligned clusters is typically done manually, although there have been recent efforts to develop heuristics that can automatically label clusters by establishing a concordance between cluster features and cell phenotype characteristics in the Cell Ontology. Innovations in the visualization of high-dimensional cytometry data have also greatly increased our ability to interpret the results of automated analysis [149–151].

The detection of antigen-specific T-cells poses a specific challenge for automated algorithms because of the extremely low frequency of these cell subsets in many patient samples—for example, as few as 0.01–0.1% of the $CD8^+$ T lymphocyte population may be specific for a particular tumor Ag and bind the relevant HLA-multimer. Two nonexclusive approaches for improving the ability of automated algorithms to improve the limit of detection are biased subsampling to enrich the sample for rare events [150, 152] or increasing of the complexity of the statistical model [143]. The development of algorithms that can accurately and robustly identify rare cell populations is a driving motivator for much current research in automated flow analysis, and we expect rapid advances in this area. Illustrative examples comparing manual and automated analysis of antigen-specific cells for HLA-multimer and ICS assays are shown in Fig. 33.1.

Visualization of data has become a critical aspect of high-dimensional flow analysis, and algorithms that can generate informative two-dimensional plots are increasingly used for both flow and mass cytometry data sets. Traditional dimension reduction techniques such as principal components analysis (PCA) or multi-dimensional scaling (MDS) for visualizing rely on linear transformations, and often fail to capture the nonlinear manifold structure of cell subset clusters in cytometry data. Recent innovations in nonlinear dimension reduction have proven to be much more capable of capturing meaningful biological properties and of generating interpretable visualizations. SPADE (SPanning-tree progression Analysis of Density-normalized Events) was an early pioneer in visualization of single-cell data, and allows direct visualization of potential lineage relationships between different cell subsets by embedding density-sampled clusters onto a minimal spanning tree (MST) [150]. Similar to SPADE, FlowSOM visualization also generates MST plots, but uses self-organizing maps, an unsupervised artificial neural network (ANN) for dimensionality reduction [153]. Perhaps the most

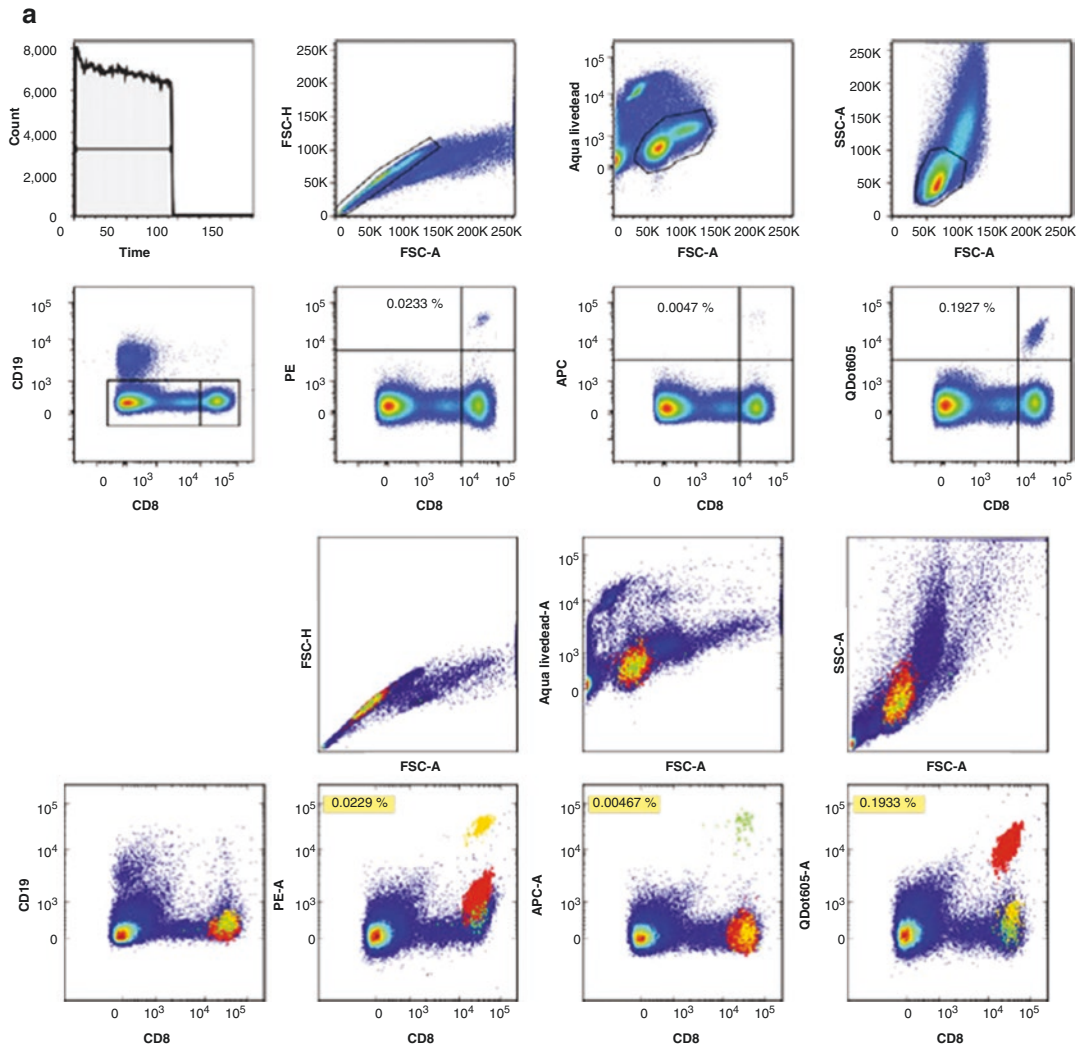

Fig. 33.1 (**a**) Manual and automated identification of antigen-specific MHC class I multimer-positive CD8+ T lymphocytes among PBMCs of a HLA-A2+ healthy donor. Top panel shows the manual gating strategy to identify CD8+ T-cells specific for three HLA-A*0201-restricted epitopes derived from the EBV, influenza, and CMV viruses using peptide-MHC tetramers. From left to right, the plots show gates to exclude artefact due to flow stream bubbles or clumps (count/time), find singlets (FSC-A/FSC-H), exclude non-viable cells (FSC-A/Aqua LiveDead), identify lymphocytes (FSC-A/SSC-A), identify CD8+ T-cells (CD8/CD19), and quantify CD8+ T-cells binding to EBV BRFL1 peptide-MHC tetramers (CD8/PE), influenza matrix peptide-MHC tetramers (CD8/APC), and CMV pp65 peptide-MHC tetramers (QDot605). Bottom panel shows the corresponding peptide-MHC-binding CD8+ T-cells identified using an automated analysis approach that fitted a Dirichlet Process Gaussian Mixture Model with 256 components to the data [143]. Essentially identical frequencies of peptide-MHC tetramer-positive cells are found with manual and automated analysis. (**b**) Manual and automated analysis of antigen-specific T-cells among PBMCs of a second HLA-A2+ healthy donor tested in an intracellular staining (ICS) assay after incubation with a synthetic peptide corresponding to an HLA-A*0201-restricted epitope of pp65 CMV. Manual analysis finds cells positive for IFN and TNF, and a few events positive for IL-2. Without further gating, it is not possible to tell if the IFN- and TNF-positive events come from two separate or a single bifunctional population. Automated analysis reveals that there is indeed a single-cell population positive for IFN and TNF, with no evidence for an IL-2-positive population. Again, the frequencies of antigen-specific events identified by expert gating and automated analysis are almost equivalent

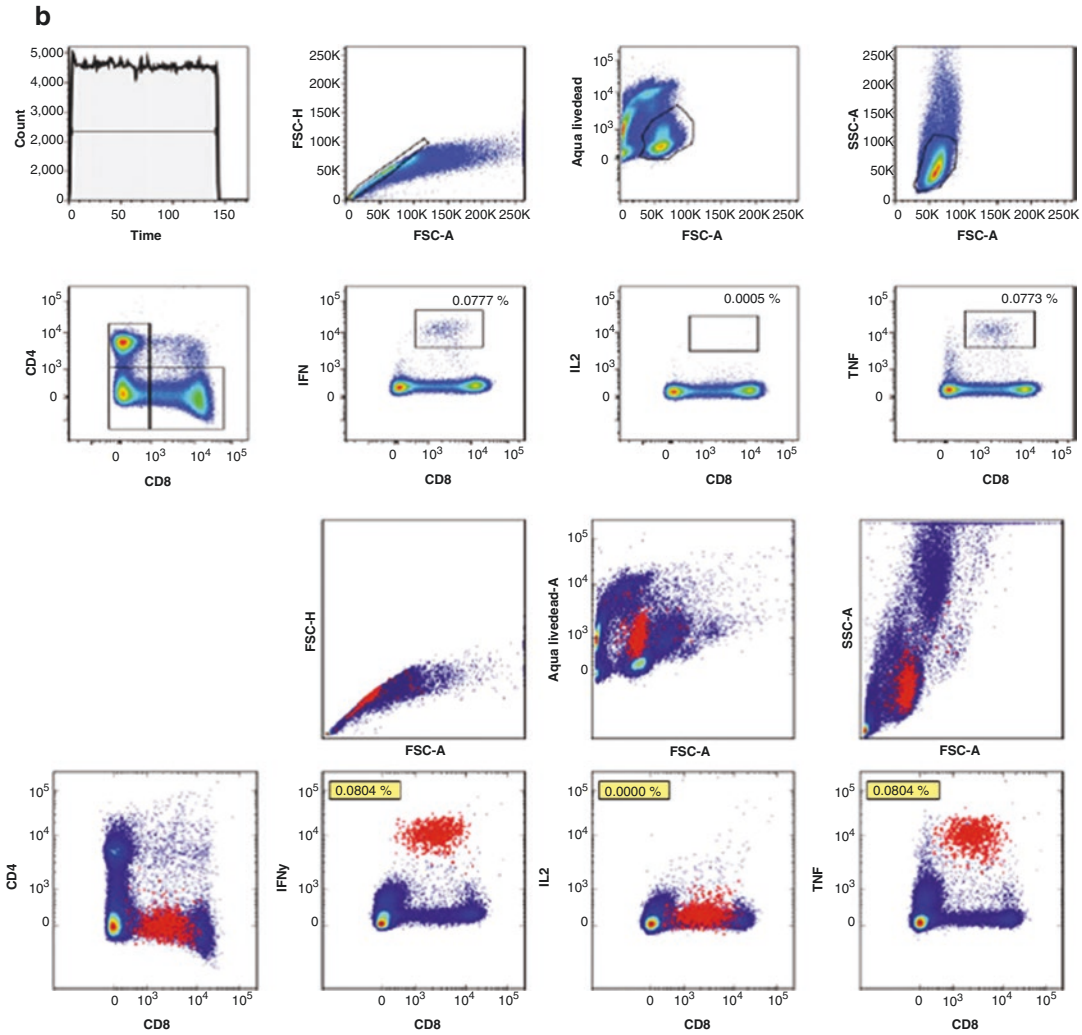

Fig. 1 (continued)

influential visualizing technique is t-Stochastic Neighbor Embedding (t-SNE) for dimensionality reduction [154], which is particularly effective at revealing local structure and minimizing crowding of individual cells, and hence at enhancing the visualization of cell subset clusters. Two t-SNE-based software packages for visualizing cytometry data on t-SNE plots pseudo-colored by marker intensities are viSNE [151] and HSNE (Hierarchical Stochastic Neighbor Embedding) [155]. Several of these visualization methods have also been implemented in cytometry analysis packages such as FlowJo [156], FCS Express [157], and cytosplore [158].

There have also been notable recent advances in the automated data-driven discovery of biomarkers and signatures from cytometry data sets. Citrus (cluster identification, characterization, and regression) is based on a pipeline that performs regularized regression on clustered data to identify cell subsets correlated with outcome. Notably, Citrus can be applied for identifying meaningful signature differences between data sets where two or more sample groups have been defined [159, 160]. A more specialized tool to identify functional cell subsets in ICS assays is COMPASS (Combinatorial Polyfunctionality Analysis of Single Cells), which uses a Bayesian

hierarchical framework to identify antigen-specific cells and directly addresses the limitations of Boolean gating [161]. Most recently there have been exciting developments in the application of deep learning algorithms to identify "interesting" features of cytometry data sets, taking advantage of the explosive growth of deep learning technology and algorithms [162]. As with other machine learning applications, these deep learning approaches are attractive as they are scalable, require minimal input data preprocessing, can be flexibly combined with outcome or confounding variable data, and typically outperform "shallow" algorithms with large data sets.

Finally, we note that most of these automated analysis tools are developed under open-source licenses, and so free to use without restriction. Some packages require a modicum of programming ability to use effectively (for example, R or Python scripting skills) and others are available online, but in general these algorithms are probably not easily used by the average flow operator in a clinical research laboratory. In the coming years, we expect that these automated analysis tools will become increasinlgy accessible to immunologists as developers of these tools continue to improve their ease of use; the most successful algorithms will be incorporated into commercial software analysis packages; and more workshops will be organized to train people in the use and potential pitfalls of these exciting new technologies.

33.8 Perspectives and New Technologies

Flow cytometry is playing an instrumental role in our comprehension of the immune system and of its interplay with human tumors. A fundamental advance in recent years is an increase in the number of parameters that can be simultaneously assessed on single cells. Access to more reagents and fluorochromes including tandem conjugates, semiconductor nanocrystals (quantum dots or eFluors), and organic polymers (brilliant violet family) [163–165], together with the wide availability of sophisticated flow cytometers, is making polychromatic analysis a routine method. Currently, the combination of 15–20 different fluorochromes represents the upper feasibility limit in expert laboratories [166], but new dyes and next-generation cytometers such as the FACSymphony (BD Biosciences), Aurora (Cytek Biosciences), ZE5 (BioRad), and Spectral Cell Analyzer (Sony) are already in use and might soon increase this limit. This, together with the development of unsupervised, automated analysis programs, is likely to broaden further the applications of flow cytometry. However, spectral overlap ultimately limits the number of fluorochromes in a single Ab panel. Mass cytometry (CyTOF, i.e., Helios and Hyperion) uses stable heavy metal ions tagged to Abs (or, e.g., HLA-multimers) in place of fluorochromes. These isotope labels are detected by time-of-flight mass spectrometry after vaporization of the cell. Although isotope labels generally produce a signal of low intensity, they have a lower background and virtually no spillover, making the simultaneous measurement of a much larger number of markers feasible.

Mass spectrometry has been reported to be qualitatively and quantitatively equivalent to flow cytometry, with the simultaneous analysis of more than 40 parameters [167, 168]. Hence, it is especially adapted for comprehensive studies of immune cell subsets in various donor/patient populations [53, 160, 169–172]. This new technology is currently available in a few highly expert laboratories and has the current following limitations as compared to traditional flow cytometry: lower label sensitivity, substantial cell loss, very low acquisition rate, and impossibility to sort living cells. Nevertheless, the method has started to reveal the complexity of immune cells and will become an indispensable technique in cancer immunology and immunotherapy, especially at the research phase or in biomarker discovery programs. As it was initiated for flow cytometry some years ago, the first standardized protocols and interlaboratory comparisons are coming [173, 174].

33.9 Conclusion

Flow cytometry is the prototypical multiparameter single-cell assay, with applications in cancer immunotherapy ranging from epitope screening to immune monitoring of clinical studies. Due to its ability to characterize complex immune phenotypes and flexibility in measuring multiple immune functions such as Ag binding, expression of activation and inhibitory markers, cytokine production, cytotoxicity, and proliferation, flow cytometry is indispensable in cancer immunology research. However, because of the complexity of the assay and the fragility of the cell sample, it is challenging to establish and maintain robustness, sensitivity, and reproducibility of the results, especially across multiple laboratories. Factors to consider when using flow cytometry in clinical research include understanding the range of flow-based assays available and introducing best practices for the reagent, sample, staining procedure, instrumentation, and data analysis, as well as regular performance controls especially participation in proficiency testing programs. The development of such expertise and measures is demanding, but more important than ever due to the increasing complexity of flow-based assays. Automated analysis with unsupervised and supervised leaning approaches has now been demonstrated to equal or improve the performance of human operators in multiple studies, and we predict that their use in clinical research will eventually be accepted as standard practice.

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