Laurence Zitvogel
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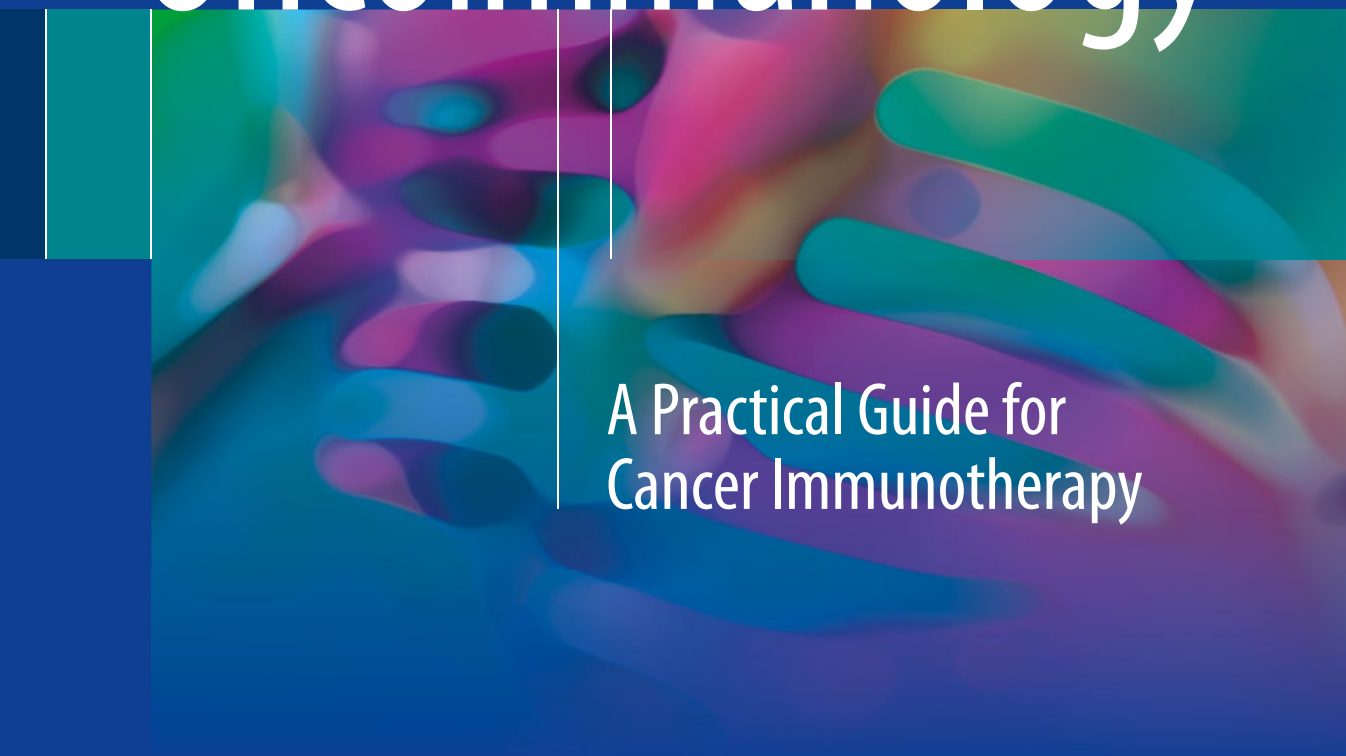
Editors

Oncoimmunology



A Practical Guide for Cancer Immunotherapy

Springer

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Our Reviewers

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And finally, we are, of course, grateful to all of the chapter contributors to this comprehensive, groundbreaking textbook in the important emerging field of immuno-oncology.

Laurence Zitvogel
Guido Kroemer

Contents

Principles of Oncoimmunology

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Laurence Zitvogel and Guido Kroemer

The history of cancer research is marked by at least three phases that each are based on different methodologies and therapeutic strategies.

During the first phase that lasts from antiquity to the eighties of the twentieth century, cancer was considered as a cellular disease resulting from the invasion of tissues by abnormal cells. Hence, the main challenge consisted in excising the tumor with its margins to make sure that all cancer cells had been removed. In addition to mutilating surgical techniques, clinical oncologists have been applying cytotoxic agents to their patients, based on the consideration that proliferating cells had to be purged from the organism. Cancer drugs were identified by their capacity to kill cultured tumor cells in vitro and then administered to patients as "chemotherapies" at the maximum tolerated doses to obtain similar effects in vivo.

The second phase of cancer research is marked by the idea that malignant disease results from genetic and epigenetic aberrations affecting the cancer cell. This phase of research has been marked by the successful identification of tumor suppressor genes and oncogenes, the development of ever-refined tools to measure gene expression and to identify mutations in the cancer genome, to classify malignancies into different molecular subcategories, and to follow the clonal evolution of cancers as they form, progress, and escape from therapy. Driven by the identification of druggable oncogene products, a myriad of 'targeted' anticancer agents

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has been developed, heralding the era of "personalized" medicine. In this yet unattained utopia, identification of driver mutations in each patient's cancer would allow a tailor-made "precision" treatment.

The third phase of cancer research is based on the discovery that cancer is not just a genetic and epigenetic disease of aberrant cells, but that it also involves a constant struggle between malignant cells (and their precursors) with the immune system. The complex relationship between cancer and the immune system has been schematically condensed to the 3E hypothesis: initial *e*limination of malignant cells by innate or acquired immune effectors, later *e*quilibrium between cancer cells and the local immune response within an often indolent neoplastic lesion, and the final and fatal *e*scape of cancer cells from immune control. This latter event, which entails the clinical manifestation of the tumor involves the selection of non-immunogenic cancer cells (a process called "immunoselection" or "immunoediting") or active inhibition of the local immune response (a process called "immunosuppression" or "immunosubversion"). In this paradigm, it appears logical that anticancer treatments should be designed in a way that they reset the relationship between cancer and the immune system from escape to equilibrium or—ideally—elimination. Several events have lent support to this idea over the last decade. Thus, it has been discovered that the density, composition, architecture, and functional state of the immune infiltrate has a major prognostic and predictive impact on cancer. Multiple studies came to the conclusion that the relative success of chemotherapy and targeted therapy was based on the reinstatement of anticancer immunosurveillance, especially if the effects of therapy lasted beyond its discontinuation. Finally, a large panel of immunotherapies have been successfully developed and applied to patients, providing proof-of-concept that reinstating immune control leads to tangible and often spectacular clinical benefits.

Of course, it is too early to proclaim that cancer research has become victorious due to its recent paradigm change. Future will tell whether the actual triumph of immunotherapies will allow us to win the war against cancer or whether we will simply obtain a pyrrhic victory. The Editors and the authors of this textbook are optimistic about the final issue of our collective adventure.

We take this opportunity to thank Professors Pierre Galanaud and François Martin for their invaluable help in editing this book. Without their patient and constant support, this textbook would not have been printed.

Part I

Fundamentals in I-O

The Human Tumor Microenvironment

2

Yann Vano, Nicolas A. Giraldo, Wolf Herman Fridman, and Catherine Sautès-Fridman

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2.1 Introduction

For a long time, cancer therapy has had as its sole objective the direct elimination of tumor cells. In case of nonmetastatic disease, this is accomplished by surgery, which removes the primary tumor. Radiotherapy and conventional chemotherapies also aimed at targeting tumor cells preferentially. The high capacity of tumor cells to divide as compared to the normal cells makes them more sensitive to agents that physically, in the case of radiotherapy, or chemically, in the case of chemotherapy, attack DNA and lead to cell death. Targeted therapies targeting mutations in tumor cells such as BRAF have been developed as well. However, these approaches also destroy the nonmalignant cells and/or have systemic consequences. To increase specificity toward the tumor cells, cytotoxic agents have been coupled to antibodies that bind to the tumor cells in order to allow their specific targeting to the tumor and not to the normal cells. However, the entry of such constructs into tumors still remains a major issue.

The progresses that have been accomplished in the field of tumor immunology in these last 20 years have led to a drastic change in the representation of primary tumors and metastases and to cancer treatments. Tumors are not anymore represented as a simple accumulation of cells that have undergone oncogenic processes but as a complex and dynamic structure made of tumor cells and inflamed tissue. Tumors are infiltrated with blood vessels that bring nutriments and all kinds of leukocytes inside the tumor and at its periphery, in the so-called tumor stroma that also contains matrix proteins such as collagen fibers. The transformation of a normal cell into a clinically detectable tumor can last for decades such as in the case of breast or colon cancers. Thus, tumors are dynamic structures that derive from this long process of carcinogenesis occurring in an inflamed and reactive tissue microenvironment.

Importantly, the last 20 years of intense research in the tumor immunology field unraveled the proof of concept of the immunosurveillance theory that was brought by McFarlane and Lewis Thomas in the 1950s (reviewed in [1]). These two scientists anticipated that immunosurveillance is a physiological mechanism that protects against nascent tumors. The description of immune cells with effector and memory functions within primary tumors and their metastases and the discovery of the correlation between their density at the site of the primary tumor and patient's survival more than 10 years ago unambiguously demonstrated that the immune system is capable of recognizing and eliminating tumor cells. The immune system uses the same basic mechanisms to fight against cancer as those used to eliminate viruses such as the influenza virus. Both the innate and adaptive arms of the immune system cooperate to mount an antitumor response leading to the development of effector $CD4^+$ T cells that produce cytokines, of effector $CD8^+$ T cells that kill the tumor cells and produce cytokines, and of B cells that differentiate into plasma cells that produce antibodies. Most importantly, so-called memory lymphocytes develop in parallel. All these cell types accumulate into tumors, and the memory lymphocytes circulate for a long time, with the possibility of transforming into effector lymphocytes very rapidly. They protect locally against tumor cells and systemically against metastatic cells that may escape from the primary tumor and circulate before nidation in distant organs, where they proliferate and become metastatic. An immune response is raised directed against tumor antigens. More than 15 years ago, it was proposed that tumors grow until an equilibrium is reached between tumor cells and the immune system. Only tumors, in which the tumor cell growth potential overcomes the pressure exerted by the adaptive immune response, can subsequently grow and metastasize into distant tissues. Indeed, tumor cells develop a series of mechanisms to evade the immune defenses including the downregulation of tumor antigens or the production of molecules that suppress immune functions. Therefore, tumor cells have long standing interactions with the immune system, especially in the microenvironment in the primary tumor and later in the metastases.

Finally, studies on the tumor microenvironment brought another major issue regarding the mounting and the regulation of the antitumor defenses. Immune cells were found to form aggregates at the tumor sites, mimicking those

found in inflamed tissues that reflect local consequences of a chronic antigenic challenge. A large body of evidences suggests that these so-called tertiary lymphoid structures play an important role to mount, maintain, and control the local and systemic immune defenses.

This deep knowledge of the antitumor defenses and of the composition of the tumor microenvironment brought a new paradigm for cancer treatment. Instead of targeting the tumor cells by using radiotherapy or chemotherapy, drugs targeting the tumor microenvironment have been developed. This major step in cancer therapy has been accomplished these last years. Drugs aiming to alleviate the immune defenses by unlocking the effector functions of the T cells, such as anti-CTLA4 or anti-PD-1 antibodies, have been developed. Other drugs targeting the tumor vasculature such as antibodies against factors favoring the growth of cells lining the blood vessels (vascular endothelial growth factor, VEGF) or molecules inhibiting the signaling pathways in the endothelial cells downstream VEGF (sunitinib) have been approved by the FDA for some cancers. Indeed the tumor microenvironment offers an array of potential new targets that can be used alone or in combination with the classical approaches preferentially targeting the tumor cells such as chemotherapy or radiotherapy which may also in some cases increase immune reactions to the tumors.

In this chapter, we will first describe the tumor natural history, how tumor cells progressively grow in a tissue that becomes inflamed, and how the tissue both facilitate the development of tumors and participate to their elimination. We will then describe the different cell types that are found in the tumor microenvironment, their function, their location, and their organization in human tumors. The prognostic impact of the different cell types of the tumor microenvironment will then be compared, and the immunotherapy approaches targeting the tumor microenvironment will be described.

Regarded for a long time as a genetic and cellular disease, cancer is now considered as a tissular and systemic disease whose outcome depends largely on interactions with the host, especially within the tumor microenvironment. The tumor microenvironment can promote or inhibit tumor invasion and metastasis. It changes during the course of the disease, and the understanding of this dynamic interaction makes it possible to identify new therapeutic prognostic factors and new therapeutic targets at all stages of the disease.

2.2 Cancer's Natural History

More than 40 year ago, Peter Nowell proposed that genetic alterations—induced by diverse mutagenic stimuli—could be responsible for the transformation of normal cells toward neoplastic states [2]. According to his theory, these random mutations confer cells with autonomous proliferative capacity and immortality. This concept has barely changed, and today we know that genetic instability is the hallmark initiating event of cancer cells. In fact, tumor cells acquire a series of mutations over time, and it is believed that the stepwise accumulation of genetic abnormalities eventually generate their malignant transformation. In average, a tumor cells exhibit 120 non-synonymous mutations [3] that not only confer them autonomous and uncontrolled proliferative capacities but also several other characteristics that allow them to survive in the hostile human body environment.

In 2011, Hanahan and Weinberg proposed the main hallmarks or essential characteristics that a cancer cells exhibit and allow them to self-support the development of a tumor mass [4]. With genetic instability and increased proliferative capacity leading the list, it is currently recognized that tumor cells also need to actively interact with surrounding endothelial, stromal, and immune cells, to guarantee their own survival. Thus, human cancers often promote angiogenesis and inflammation and commonly develop mechanisms to evade the immune system. While the stepwise acquisition of new mutations allows the development of these pro-tumoral functions, the pressure of the hostile environment leads to the selection of the more malignant and aggressive cell clones [5].

The cornerstone of tumor cell emergence and development is then genetic mutations, which can

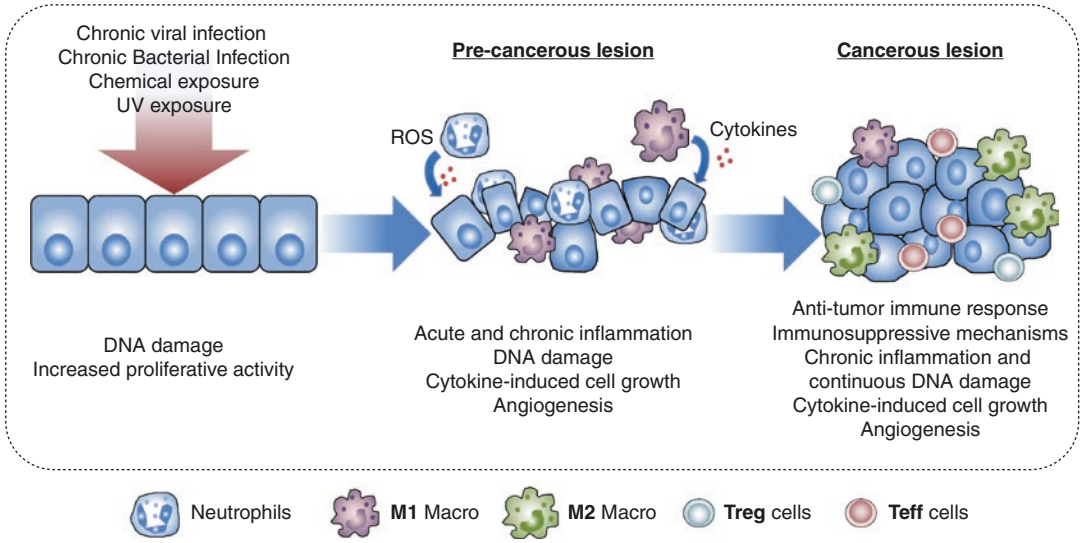

Fig. 2.1 Major immunopathological and genetic events occurring during carcinogenesis. Upon chronic inflammatory stimuli exposure, normal cells undergo transformation into precancerous cells. Local inflammation induces recruitment of myeloid-derived cells that fuel carcinogenesis via production of oxygen derivatives or cytokines. Later on, tumor growth and invasion into tissues are controlled by a balance between antitumor and immune escape mechanisms

be induced by diverse factors (Fig. 2.1). We are continuously exposed to mutagenic agent, such as UV light, pollution, or even viruses. Normal cells often possess efficient machineries that repair mutated DNA or intracellular cascades that promote cell death when the damages are irreparable [6]. Some hereditary diseases, such as xeroderma pigmentosum (associated with an extremely high risk of skin cancer at early ages due to defect in the DNA-repairing machinery), are examples of how important these proofreading systems are to prevent cancer development and how often we are exposed to mutagenic stimuli.

Inflammatory mediators are other well-known promoters of genetic alterations. In fact, many of the substances produced by the inflammatory immune cells (such as macrophages and neutrophils) can induce the direct damage of DNA in nonimmune cells. In the presence of noxious stimuli, chronic inflammation can both induce the development of driver tumorigenic mutations and promote the necessary genetic instability to allow other alterations to develop [7]. This process of cancer induced by chronic inflammation (Fig. 2.1) has been described in several pathologies, including gastric cancer in association with *Helicobacter pylori* infection, asbestos or cigarette smoke exposure and lung cancer, arsenic exposure and skin cancer, gastroesophageal reflux for cancer of the esophagus, inflammatory bowel disease for colorectal cancer, chronic pancreatitis for pancreatic cancer, and pelvic inflammatory disease for ovarian cancer [8].

Examples of inflammatory carcinogenic mediators include reactive oxygen species and matrix metalloproteinases, which can induce DNA damage and extracellular matrix disruption, respectively [9]. In addition, some cytokines can induce the growth of abnormal or preneoplastic cells, such as IL-1β for gastric carcinoma and IL-8 for melanoma. The preneoplastic potential of many other cytokines has also been described (e.g., IL-1β, IL-6, IL-23, and TNF-α).

In virus-related cancers, aside from the inflammation induced by the infection itself, the virus genetic material can integrate into the host genome and induce cell transformation by altering diverse oncogenic pathways [10]. Virus-associated cancers represent roughly 20% of all cancer types and include cervical cancer (induced by HPV), B cell

lymphoma (induced by EBV), Merkel cell carcinoma (induced by Merkel cell polyomavirus), hepatocellular carcinoma (induced by hepatitis B and C viruses), and some gastric cancer and H&N cancer (induced by EBV).

2.3 The Tumor Immune Microenvironment

As mentioned above, the tumor microenvironment is a very complex and dynamic ecosystem, where different cellular populations coexist. The major players include tumor, immune, and supporting cells (e.g., fibroblasts, stromal, and endothelial cells) [11]. Immune cells that circulate in the blood enter into tumors via transendothelial migration and are attracted by chemokines produced by tumor cells, fibroblasts, or inflammatory cells. Within the tumor mass, the immune cells locally proliferate, differentiate, exert their functions, and die, and some migrate back to the circulation. Within this population, one often can find cells related to acute inflammation (including neutrophils, basophils, and eosinophils), cells of the innate immune response (including macrophages, NK cells, and DC), and cells from the adaptive immune response (including cytotoxic $CD8^+$ T cells, Th1-/Th2-skewed T cells and B cells). We focused this subchapter in the last two populations.

2.3.1 Tumor-Associated Macrophages

Tumor-associated macrophages (TAM) represent an abundant population, and in many tumors they outnumber other immune cells [12]. Although the majority of TAM are found in the invasive margin of the tumor, we can often find also elevated densities within the tumor core [13]. TAMs exhibit an extremely plastic phenotype and function, and two main subtypes have been described: M1 TAM (induced by Toll-like receptor ligands [e.g., lipopolysaccharide and IFN-γ]) which preferentially express pro-inflammatory cytokines and inducible nitric oxide synthase and M2 TAM (induced by IL-4 or IL-13) which express arginase 1, CD206, CD163, IL-4R, TGF-β1, and PDGF [12]. Some works suggest that while M1 TAM potentiate the antitumoral Th1 response and antagonize the suppressive activities of regulatory immune cells, M2 promote angiogenesis, tumor growth, and metastasis [13].

2.3.2 NK Cells

Natural killer cells are cytotoxic effector lymphocytes of the innate immune system whose primary function is to help control infections and tumors [14]. Two major mechanisms of recognition of tumor cells by this population have been described: they can recognize cells which have downregulated major histocompatibility complex class I expression (an immunotolerance phenomenon widely described in many cancer types), or they can bind to stress-induced ligands expressed on tumor cells (e.g., MICA or MICB, which bind to NKG2D expressed on the NK cell) [14].

2.3.3 Dendritic Cells

The main function of dendritic cells (DC) is to establish a bridge between the innate and adaptive immune response. Under physiological circumstances, DC engulf and process nonself-antigens, and when they are exposed to danger or activation signals, they become activated and travel to secondary lymphoid structures in lymph nodes where they prime naïve B or T cells [15]. The DC phenotype is rather plastic, and they can produce a wide range of pro-inflammatory or immunosuppressive cytokines, as well as expressing a large series of activating or inhibition receptors, depending of the environment where they are embedded. The secondary lymphoid organs are protected environments and often provide an ideal milieu to promote a DC phenotype that effectively activates the adaptive immune response [16].

In many cancer types, tumor cells produce molecules that induce pro-inflammatory or tolerogenic DC and block their maturation at different stages.

Often, intratumor DCs exhibit an immature and inhibitory phenotype [17]. Interestingly, in recent years, several works have described the presence of tertiary lymphoid structures (TLS) in the invasive margin of many cancer types [18], where in theory the DCs are protected from tumor-produced inhibitory substances and from where they can effectively prime the antitumor immune response [19].

2.3.4 Tertiary Lymphoid Structures

TLS are highly organized lymphoid aggregates that develop in inflammatory pathologies. In cancer, TLS often develop in the invasive margin of the tumors and/or in the stroma and resemble those arising in other chronic infectious or autoimmune diseases [19]. Figure 2.2A illustrates TLS found in clear cell renal cell cancer (ccRCC). Characteristically, TLS exhibit an organization similar to secondary lymphoid organs, including a T cell zone (Fig. 2.2Aa) and a B cell follicular zone (Fig. 2.2Ab), and are often surrounded by high endothelial venules [20]. B cells in TLS form germinal centers; they undergo active proliferative machinery and somatic hypermutation [19]. T cells have a CD62L$^+$/CD45RO$^+$ central memory or a naïve phenotype, and some can be found in contact with mature DC which expresses the DC-Lamp marker (Fig. 2.2Aa) or at the periphery of B cell follicles (Fig. 2.2Ac) [20]. Follicular dendritic cells are also detected

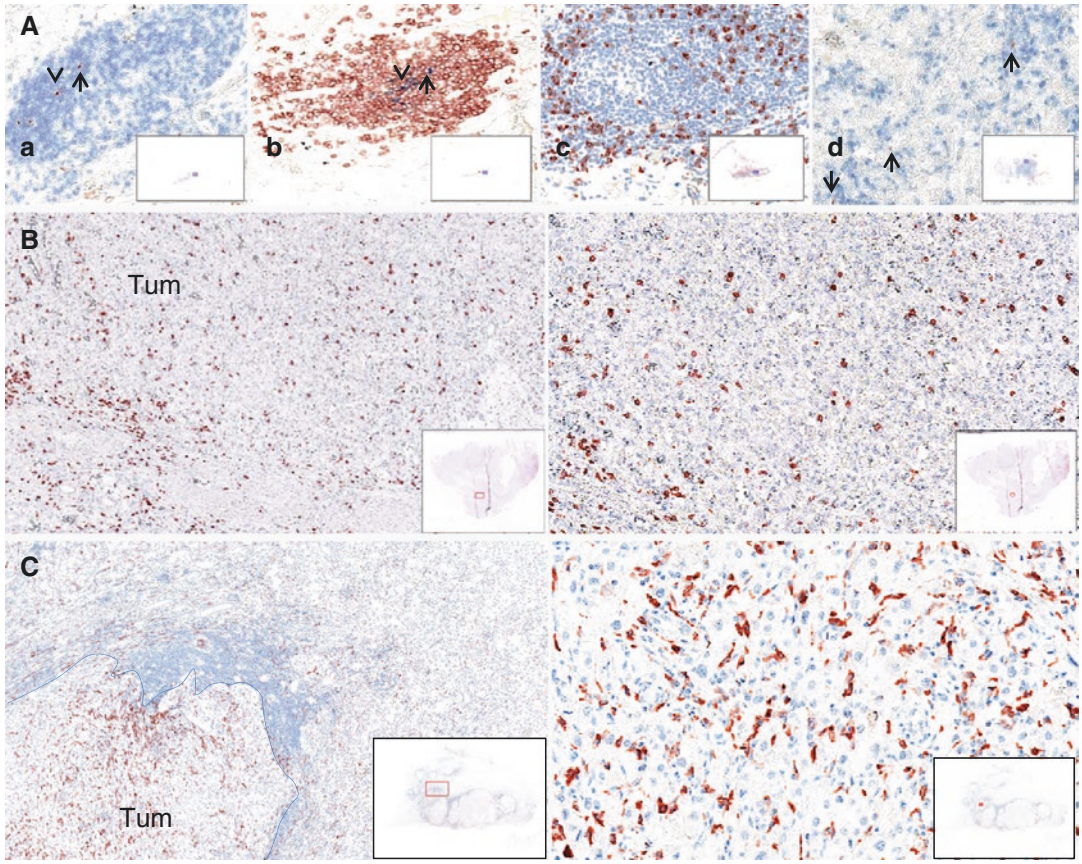

Fig. 2.2 The tumor microenvironment in human clear cell renal cell cancers as detected by IHC on paraffin sections. (**A**) Tertiary lymphoid structures: (*a*) DC-Lamp$^+$mature DC (*brown*) in the CD3$^+$T cell zone (*blue*); (*b*) CD20$^+$ B cells (*brown*) and CD21$^+$ follicular dendritic cells (*blue*) delineate the germinal center; (*c*) CD8$^+$ T cells (*brown*) are distributed around the germinal center; (*d*) non-TLS-DC-Lamp + DC (*brown*). (**B**) CD8 + T cells (*brown*) (*left* 5×, *right* 20×). (**C**) CD163+ macrophages (*red*) (*left* 5×, *right* 20×), Tum = tumor area

forming a network where immune complexes can form and be presented for selection of the high affinity B cells. Plasma cells that produce antibodies are located at the vicinity of TLS [21].

Primary tumors and metastases contain TLS at variable densities, depending on the tumor type and on the patient. As discussed below, it is assumed that TLS reflect the ongoing immune reaction within tumors. They allow the presentation of tumor antigens by mature dendritic cells to T cells leading to the differentiation of $CD4^+$ Th1 cells as reflected by the expression of the T-bet marker and the T-B cell cooperation for B cell differentiation into plasma cells. All of these events can thus occur locally, within the tumor bed. To what extent TLS bypass the need of secondary lymphoid organs to mount or control the antitumor immune reaction remains an open issue.

2.3.5 $CD4^+$ and $CD8^+$ T Cells

CD4+ T-helper cells are divided into different subtypes, including Th1, Th2, Th17, Tfh, and Treg; each subpopulation accomplishes specific roles in the antitumor immune response. Overall, a Th1-oriented response antagonizes the tumor growth and is often associated with good clinical outcome [22]. In fact, Th1-oriented cells potentiate in situ the antitumor function of cytotoxic T cells, through the production of several cytokines including IL-2 and IFN-γ. Tfh cells interact with B cells in TLS, helping antibody production.

The role of other subpopulations of tumor-infiltrating $CD4^+$ T cells (Th2, Th17, and Treg) is less well understood but is often associated with poor prognosis in different tumors [22]. Many studies suggest that Treg in cancer can dampen the antitumor immune response by two main mechanisms: (1) production of inhibitory cytokines (e.g., IL-10, TGF-β, and IL-35) and (2) suppression of DC development and maturation [23].

$CD8^+$ T cells exert a very import function in the antitumor immune response, as they are responsible of tumor cell recognition and elimination. Due to their genome instability, tumor cells often express mutant proteins at their surface. Many of these are neoantigens that can induce a tumor-specific immune response. The primed $CD8^+$ T cells are in charge of the tumor cells recognition and lysis, by mechanisms well described in the literature including the release of cytotoxic granules [24]. Interestingly, in the majority of tumors, infiltrating cytotoxic T cells express inhibitory receptors (e.g., PD-1, Tim-3, and Lag-3), whose function under physiological situations is to contract the immune response upon biding to their ligands. Many tumor cells in fact can take advantage of this inhibitory mechanism and in fact express a wide arrange of ligands (e.g., PD-L1, PD-L2) that help them escape for the T cell attack [25].

2.3.6 B Lymphocytes

In inflammatory settings other than cancer, B cells enhance T cell responses by producing antibodies and stimulatory cytokines and chemokines, serving as local antigen presenting cells and organizing the formation of TLS that sustain the immune response. In cancer, B cell can exert all of these functions and overall have an antitumor effect. In addition, recent evidence suggests they can also play an immunomodulatory role through the production of IL-10, among other cytokines [26].

2.3.7 Spatiotemporal Dynamics of the Tumor Immune Microenvironment

Chemokines ensure the local migration of these different cell types and cytokines allow their cooperation. In addition, many tumors are surrounded by a stroma containing an extracellular matrix composed of fibroblasts that form collagen fibers and produce enzymes—such as metalloproteases—that facilitate local invasion within tissues and ultimately the release of tumor cells that egress to the circulation and migrate in other tissues.

A direct consequence of these processes is that the tumor microenvironment is a tissue-dependent organized structure in which immune cells are common denominators. Figure 2.2B illustrates the presence of $CD8^+$ T cells in the

tumoral zone of clear cell renal cell cancer. A closer look into the organization of the immune microenvironment reveals that cells are not evenly distributed in the tumor area. Lymphocytes (T and B cells) are more abundant in the tissue border area called the invasive margin than in the center of the tumor [13]. They can be found dispersed or within aggregates, forming TLS in the invasive margin and/or in the stroma [18, 27]. Most of the T and B cells have a memory phenotype, $CD8^+$ T, $CD4^+$ Treg, Th1, Th2, Th17, and B cells being detected at variable densities, whereas naïve T cells and $CD4^+$ Tfh are exclusively present within TLS. NK cells are detected in the tumor stroma. Some T cells are found in close contact with tumor cells in the center of the tumor. Myeloid cells such as macrophages, myeloid-derived suppressor cells, mast cells, and neutrophils are present at high densities, both in the invasive margin and the center of the tumor. Figure 2.2C illustrates the high density of $CD163^+$ M2-oriented macrophages near the invasive margin of renal cell cancer. Immature dendritic cells are present at low densities, dispersed in the whole tumor area whereas mature dendritic cells are usually found within the TLS, in close contact with T cells (Fig. 2.2Aa). Importantly the immune composition of the tumor microenvironment evolves with the stages of tumor progression in a tumor-dependent manner. Thus, T cells are more numerous at the early stages of the disease in colorectal cancers and at their late stages in renal cell cancers [17, 28]. The density of B cells increases with tumor stage in colorectal cancers, as does that of the myeloid cells such as neutrophils, mast cells, immature dendritic cells, and macrophages. Thus, the tumor microenvironment is a complex structure, forming a tumor-dependent "immune landscape" that evolves during tumor progression.

2.4 The TME Dictates Clinical Outcome for the Patients

Quantification of immune infiltrates and its relationship with prognosis has been studied for more than 20 years. Following the observation that high T cell densities correlate with longer survival in ovarian cancer [29], the Galon, Pagès, and Fridman studies demonstrating for the first time in large cohorts of patients with colorectal cancers (CRC) the association between densities of memory T cells, early signs of metastasis, and patient's survival made a significant breakthrough in this field [28, 30]. Since then, important progresses in immunohistochemistry (IHC) with the multiplication of robust antibodies, the development of high through put technologies and of automated quantitative imaging has led to numerous studies on immune cell composition of the TME. This real enthusiasm was even more pronounced during the last 5 years with the emergence of checkpoint blockade therapy (CBT), which aims at reversing T cell exhaustion. Thus, T cell abundance in the TME and its link with outcomes and/or response to CBT is under intensive work by many teams worldwide.

2.4.1 T Cells

2.4.1.1 $CD8^+$ T Cells

T cell abundance within the TME has been extensively studied across the majority of tumor types. Our group published in 2012 a comprehensive review of the number of original articles linking immune cell populations infiltrating the tumor and prognosis [11]. We reported that high densities of $CD3^+$ T cells, $CD8^+$ cytotoxic T cells, and $CD45RO^+$ memory T cells were associated with a longer disease-free survival (DFS) and/or overall survival (OS) in most tumors (including melanoma, head and neck, breast, bladder, urothelial, ovarian, colorectal, and lung cancer) [1]. We noted at that time that clear cell renal cell carcinoma (ccRCC) was one of the rare exceptions to the rule. We updated these data last year and found similar results. In addition, we reported new tumor types such as GIST, biliary tract, thyroid, or oropharyngeal cancers where $CD8^+$ cell infiltration was associated with a good prognosis [22].

The poor prognostic value associated with $CD8^+$ T cells in ccRCC was confirmed by our group, both in kidney primary tumors [17] and in ccRCC lung metastases [31]. Besides ccRCC, studies in lung adenocarcinoma [32] and in HCC [33] also reported a poor prognostic value

associated with increased CD8+ T cell infiltration, in contradiction with other published studies. In prostatic adenocarcinoma as well, CD8+ T cell densities correlate with poor outcome [34], consistent with our own data [35].

The "Classical" Case of CRC

Colorectal cancer is the archetype of tumors where high CD8+ T cell densities are associated with good prognosis. Indeed a high infiltration of CD8+ T cells, particularly effector memory subtypes (TEM), is correlated with a low probability of metastatic spread and prolonged PFS and OS [28], suggesting T cells may control local invasion in primary tumors and confer a long-term systemic protection against metastasis. Moreover, IHC studies showed that compartmentalization of T cells in the center and the invasive margin of the tumors does matter. An immunoscore (IS) measures the density of CD3+ and CD8+ T cells in the center, and the invasive margin of the tumors has been developed by Jerome Galon's team and has been validated in a worldwide collaboration approximately 4000 CRC patients [36, 37]. Even if a high T cell density was more frequent in smaller tumors and MSI-positive tumors, the prognostic value of IS was independent from TNM stages and MSI status. Moreover IS was more accurate to predict the prognosis of patients with early stage CRC [37, 38].

The Discordant Case of ccRCC

We recently reported a clear negative association between CD8+ T cell infiltration and outcomes in ccRCC [17]. Within a cohort of 135 patients with available primary RCC tumors, we found that a high density of CD8+ cells, as assessed by IHC, was associated with a shorter disease-free survival and OS. These results were validated for OS in an independent cohort of 51 patients with (resected) lung metastases of ccRCC. The underlying mechanism for this poor prognosis value of CD8+ T cells is not fully understood. We showed that most of the intratumoral T cells have an exhausted phenotype, which may reflect impaired antigen presentation due to the presence of dysfunctional DCs with an immature phenotype (Fig. 2.2Ad). They express the DC-Lamp marker of mature DC but lack the high levels of MHC class II molecules and CD83 expressed by mature DC. They may be involved in the impairment of T cell antitumor response [17]. Consistently, in patients who have a higher density of DC within TLS, a high density of CD8+ was associated with good prognosis. Thus, antigen presentation by mature DC in the TLS seems to be a crucial event to drive antitumor response in ccRCC, in accordance with our previous observations in lung cancers [39]. Moreover, we showed by immunofluorescence (IF) that CD8+ T cells express immunoregulatory receptors such as PD-1 and/or LAG-3, suggesting a highly exhausted phenotype and both associated with poor outcomes [17].

2.4.1.2 CD4+-, Th2-, and Th17-Oriented T Cells

Consistent with CD8+ T cell infiltration, an increased in Th1-oriented CD4 T cell infiltration has been associated with favorable prognosis in almost all tumor types studied including breast cancer [40] or CRC [41].

Prognostic value of other T cell subsets (Th2, Th17) has been far less investigated first because of a low frequency in the majority of the tumors and second because of technical challenges to specifically identify these subsets.

2.4.1.3 Regulatory T Cells (Tregs)

The example of Tregs is eloquent. A high Treg density has been first associated with poor prognosis in ovarian cancer, which has been then confirmed in a variety of tumors such as in breast, lung, melanoma, or colorectal cancers (reviewed in [42]). Nevertheless, other studies reported longer survival associated with high densities of Tregs in colorectal, bladder, head and neck, or ovarian cancers. One of the reasons for these opposite results is the difficulty to identify the Treg population. Tregs are a heterogeneous population that should be ideally identified by a combination of markers (CD4+, CD25+, Foxp3+, T cells). The development of multicolor fluorescence imaging allows to increase the number of cell surface markers for their detection. Beyond the technical challenges, these results highlight that the prognostic impact of

immune cell populations depend on the tumor type and on the TME.

2.4.2 B Cells

The positive or negative role of B cells in antitumor immunity has been discussed for many years, mainly supported by mice studies. As compared to T cells, few clinical studies reported the prognostic role of intratumoral B cells. The majority of clinical studies have demonstrated that a high density of B cells within TME is associated with better prognosis including breast cancer [43], NSCLC [21], head and neck cancer [44], ovarian cancer [45], metastatic colorectal cancer [46], biliary tract cancer [47], and primary cutaneous melanoma [48]. Several nonexclusive mechanisms could explain the positive role of B cells in the antitumor immune response, some being antibody dependent by their capacity to trigger complement and antibody-dependent cell cytotoxicity (CDC and ADCC) or to form immune complexes able to activate DCs and others by acting as APC for CD4 [49] and $CD8^+$ T cell immune responses [50]. Indeed, it has been shown that B cells play a major role during initial priming and expansion of $CD4^+$ T cells [51], are able to cross-present antigens to $CD8^+$ T cells [52], and can promote cytotoxic T lymphocyte survival and proliferation [53].

On the opposite, few clinical studies reported a pro-tumoral role of B cells within the TME [54, 55]. B cells may play a pro-tumor function by the maintenance of a chronic inflammation [56], by the promotion of neoangiogenesis [57], and/or by the direct inhibition of cytotoxic T cell responses [55]. Moreover, a subpopulation of immunoregulatory B cells called "Bregs" has been described and has been shown to favor the differentiation and the recruitment of Tregs, thus amplifying the immunosuppressive environment [58].

Beyond the density of B cells, an increasing number of studies reported that the spatial localization of these cells have an impact on patient's outcome. In particular the density of B cell follicles characteristic of TLS is positively associated with outcomes. M.C. Dieu-Nojean and col. showed that an increase in B cell density within the TLS is associated with prolonged survival in NSCLC patients [21]. Similar results were reported in CRC [59] and oral squamous carcinoma [60].

2.4.3 Macrophages

Tumor-associated macrophages (TAM) are a major component of the TME, found both at the tumor core and the invasive margin. The prognostic value of TAM seems to be dependent of the tumor type. Increased density of TAMs is associated with a good prognosis in CRC [61], HCC [62], prostate [63], and cervical cancer [64]. At the opposite an increased TAM density is associated with poor prognosis in endometrial [65], gastric [66], urothelial [67], HCC [68], melanoma [69], breast [70], ovarian [71], bladder [67], NSCLC [72], and primary CRC tumors [13]. These discrepancies might be explained by the plasticity of these cells since we know that they can switch from a pro-tumoral function (M2) to an antitumoral function (M1) and vice versa [12]. M2 TAMs are associated with a shorter survival and M1 TAMs with a longer survival [22]. Unfortunately, there are no specific or consensual markers to define M1/M2 TAMs. Most of the studies used CD11c or NOS2 for M1 TAMs and CD163, CD204, or CD206 for M2 TAMs, but the use of these markers is still debated.

Tumors contain another heterogeneous subset of cells of myeloid origin, the myeloid-derived suppressor cells (MDSC). Such cells have an immature phenotype and exert profound immunosuppressive activities. Specific and robust tools are still needed for their identification in the human TME.

2.4.4 New Techniques to Estimate the Immune Cell Populations in Tumors

The most broadly used way to quantify tumor-infiltrating immune cells is to detect the protein expression of specific markers either by IHC or

IF. These techniques have been improved in the last decade, allowing to detect multiple proteins (multiplex IHC or IF) and to quantify cells automatically. Nevertheless, they remain expensive and difficult to standardize across laboratories, and available antibodies could lack sensitivity or specificity to accurately detect some of immune cell populations.

Efforts have been made to use transcriptome to estimate the composition of the TME. Nevertheless, variability in the signal has limited its applicability until recently. New methods such as CIBERSORT [13] or MCP-counter [73] aim at providing very precise quantitative information about the cell content of heterogeneous samples. Using MCP-counter, we estimated the abundance of immune cells, fibroblasts, and endothelial cell infiltrates, in transcriptomes of 25 different cancers (n = 19,000). The results showed the relative heterogeneity of the cellular composition of the tumor microenvironment in different cancers and confirmed that the inferred density of $CD8^+$ or cytotoxic T cells correlated with favorable prognosis in most cancer types [73] (Fig. 2.3).

Fig. 2.3 Estimation of the abundance of infiltrating immune and stromal cells and their prognostic significance across human solid tumors. *Left*, means of MCP-counter scores across malignant tissues (more than 19,000 tumors) in three transcriptomic platforms. *Right*, univariate prognostic values (overall survival) associated with MCP-counter scores in human solid tumors. *Green* represents significant favorable prognostic impact and *purple* significant poor prognostic impact. *Gray* represents no significant prognostic impact. Adapted from Becht E et al., Genome Biol. (2016) [73]

2.5 TME as Predictors of Response to Therapy

After decades of having targeted on tumor cells and their molecular alterations, new immuno-oncology (IO) agents such as CBT have shed a light on the crucial role of the TME. The currently approved CBT targets are CTLA-4 (ipilimumab) or the PD-1/PD-L1 axis (nivolumab, pembrolizumab, atezolizumab avelumab) [74]. These mAb block the negative signal received by T cells after their interactions with APCs or with tumor cells, thus being able to reverse T cell exhaustion.

As the main target of these agents are T cell infiltrating the tumor, efforts to predict CBT efficacy have been focusing on their characterization in terms of density, localization, phenotype and functionality, before and/or during treatment.

Other well-known and debatable candidates are still investigated as a "biomarker of efficacy" such as PD-L1 expression by IHC or the neoantigen/mutational burden, but are outside the scope of this chapter [75].

2.5.1 First Emerging Data from Checkpoint Blockade Treated Patients

2.5.1.1 Tumor-Infiltrating Lymphocytes

With the growing number of patients treated with anti-PD-1/PD-L1, translational data on the pharmacodynamics effect of these therapies on the TME are emerging. Tumeh et al. reported in patients with melanoma a higher density of CD8 TILs at baseline in responding patient to pembrolizumab (anti-PD-1) [76]. As with ipilimumab, serial biopsies on treatment showed an increased density of $CD8^+$ TILs in the responding group. In another exploratory study 53 melanoma patients who first received ipilimumab and then anti-PD-1 (pembrolizumab) at progression were serially biopsied before and on treatment. IHC analyses of the TME revealed that the increase of $CD8^+$ TIL density early on treatment was associated with response to ipilimumab, whereas baseline TIL density was not [77]. For the 46 patients who subsequently received anti-PD-1 after progression on ipilimumab, there was a statistically significant difference in the density of $CD8^+$, $CD3^+$, and $CD45RO^+$ T cells in pre-treatment samples of responders compared to nonresponders. In addition a very highly statistically significant difference in the expression of markers for T cell subsets—CD8, CD4, and CD3—and immunomodulatory molecules PD-1 and LAG3 was observed in early on-treatment tumor samples of responders versus nonresponders to therapy. Altogether these results highlight the unlocking effects of CBT on T cell response. In addition, the authors reported an increase in the ratio of $CD8^+$ TIL in the tumor center (TC) vs the IM in early on-treatment biopsies within responders compared to nonresponders suggesting an infiltration of the TILs from the IM to TC as a consequence to therapy [77]. Finally, IHC results were confirmed by gene expression analyses.

Another group performed the phenotypic analyses of TILs (flow cytometry) at baseline from 40 patients (discovery cohort and validation of 20 patients each) with metastatic melanoma treated with an anti-PD-1 [78]. CTLA4 expression by TILs was the only parameter significantly associated with a clinical response in multivariate analysis. The response rate (RR) and PFS were significantly correlated with the relative abundance of CTLA-4^{hi}PD-1^{hi} $CD8^+$ TILs.

In a multi-cohort phase I study of patients treated with atezolizumab (anti-PD-L1), both increased density of CD8 by IHC and high Teff signatures (genes regulated by interferon gamma (IFNg), including IFNg, CD8A, granzyme A, granzyme B, EOMES, and perforin) correlated with response in melanoma, but no association with clinical benefit was observed in RCC [79]. However, a higher ratio of Teff to Treg as revealed by gene expression was associated with atezolizumab response in RCC.

A translational study dedicated to investigate how VEGF blockade with bevacizumab could potentiate PD-L1 checkpoint inhibition with atezolizumab in mRCC was recently reported [80]. The authors showed that bevacizumab alone tends to increase the gene signatures associated with T-helper 1 (Th1) chemokines and CD8 T effectors, and the combination with atezolizumab further increases expression of these signatures. IHC showed similar results with an increase of $CD8^+$ density following bevacizumab, which was more pronounced with the combination. Interestingly the increased density of $CD8^+$ TILs seemed to reflect an increased trafficking into the tumor rather than

an in situ increased proliferation (unchanged ratio of Ki67+/Ki67- among CD8⁺ TIL) [80].

2.5.2 From the Molecular to the Immune Signatures

Escape to the immune surveillance has been proposed as an important mechanism of resistance to a number of systemic therapies including targeted therapies such as antiangiogenic agents [81]. Indeed, immune escape is one of the main mechanisms of resistance to VEGFR-TKI in ccRCC [82]. It was recently reported that metastatic ccRCC treated with sunitinib (VEGFR-TKI) could be classified into four distinct molecular groups (ccrcc1 to 4) using transcriptomic analysis [83]. The four groups had significantly distinct prognosis with ccrrcc1 and 4 having the poorest survival and response to sunitinib. Interestingly we found that immune cell infiltrates were different according to molecular groups [84]. For instance ccrcc4 tumors were the most highly infiltrated in T cells and had the highest expression of immunosuppressive markers such as PD-L1, PD-1, LAG-3, TIM-3, suggesting exhaustion of T cells within these tumors. Conversely, ccrcc1 tumors, which were also associated with poor prognosis, had the poorest T cell infiltration and a low expression of T CB markers. As the density of CD8⁺ infiltrating the tumor has been associated with CBT efficacy, we made the hypothesis that ccrcc4 could respond to PD-1/PD-L1 blockade alone. In contrast an anti-PD-1/PD-L1 alone might not be fully efficient in ccrcc1 due to the lack of CD8 T cells in the tumor. Another therapy able to attract T cells in tumors such as an angiogenesis inhibitor (VEGFR-TKI or anti-VEGF mAb) or CTLA4 blockade could sensitize tumors to anti-PD-1/PD-L1 therapy.

We therefore hypothesize that combination of molecular and immune signatures might be a better predictor of CBT efficacy than each signature alone. Figure 2.4 shows an example of an integrated view

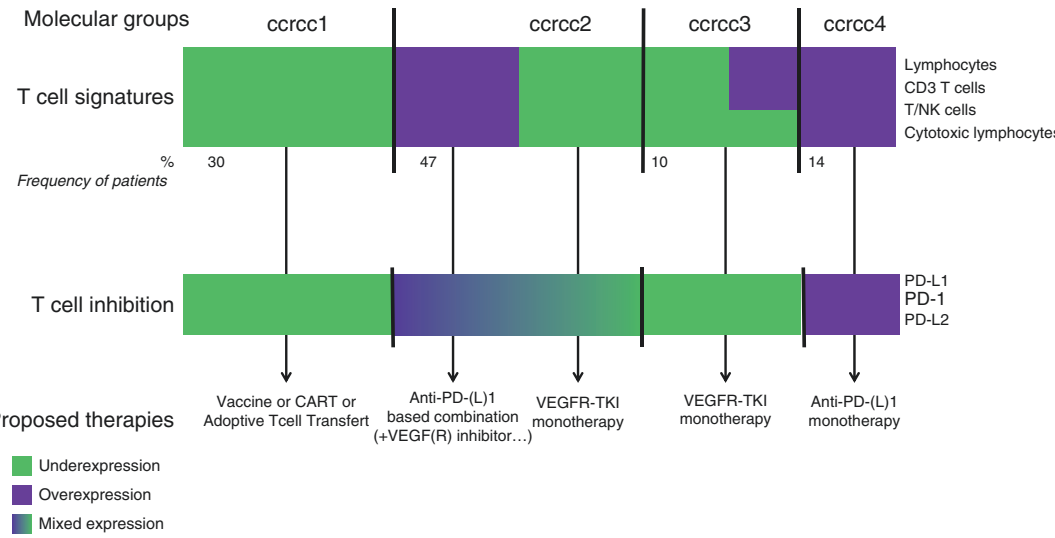

Fig. 2.4 Integrative view of biomarker-driven treatment: example of ccRCC. Using a 35-gene classifier, molecular grouping according to Beuselinck et al. [83] identified four groups of patients (ccrcc1 to 4) with distinct response to sunitinib, ccrcc3 having the best response to sunitinib. The ccrcc molecular groups have different gene expression immune profiles: immune-desert (enriched in ccrcc1), immune-competent (enriched in ccrcc3), immune-high (enriched in ccrcc4), and mixed (enriched in ccrcc2) tumors. CD8⁺ T cell infiltration evaluated by immunohistochemistry confirmed these four phenotypes [83]. T cell inhibition signatures based on the gene expression of immunoregulatory checkpoints and their ligands refine the four immunophenotypes and provide additional information to drive patient and treatment selection. ccrcc1 tumors are immune-desert and patients may benefit from a T cell attractant-based therapy such as vaccine or CAR-T cell or adoptive T cell transfer; ccrcc4 tumors are immune-high with a high density of T cells and high expression of immunoregulatory checkpoints; ccrcc4 patients may benefit from anti-PD-(L)1 alone. ccrcc3 tumors are immune-competent with a high infiltration of T cells but low expression of immunoregulatory checkpoints; VEGFR-TKI alone provides excellent results in this ccrcc3 group of patients [83]. ccrcc2 tumors are mixed in terms of T cell infiltration as well as expression of immunoregulatory checkpoints; ccrcc2 patients may be treated according to T cell infiltration and expression of immunoregulatory checkpoints

of how to combine multiple biomarkers to drive patient selection in ccRCC.

To confirm these hypotheses, we launched in March 2017 the first biomarker-driven trial to date in ccRCC called BIONIKK (BIOmarker-driven trial with Nivolumab and Ipilimumab or VEGFR tKi in naïve metastatic Kidney cancer, NCT02960906) [85]. This trial randomizes mRCC patients to receive a first line of systemic therapy with nivolumab (anti-PD-1), ipilimumab (anti-CTLA4), the combination, or a TKI according to their molecular subgroup. The primary endpoint is the objective response rate according to therapy and molecular groups. Immune infiltrates and their correlation with outcome and molecular groups will be evaluated using IHC and gene expression analyses (MCP-counter).

Conclusion

The findings of complex interactions between tumor cells and the host has led to define the concept of the immune contexture which include organization, location, density, and functional orientation of immune cells in the TME. This immune contexture helps to understand pathophysiological mechanisms that support the clinical impact of various cells of the immune response [86].

The growing approval rate of CBT targeting the PD-1/PD-L1 axis through many tumor types stimulates research teams worldwide to go deeper in the comprehension of the immune contexture to better optimize the efficacy of these agents. In addition, the high number of IO agents currently evaluated in clinical trials provides a huge competition between companies which in turn force them to understand the importance of selecting patients and to make financial efforts to support translational studies.

Many efforts are currently done to find a way to select patients who will have a durable benefit from CBT. Characterization of the tumor-infiltrating immune cells may provide one of the most promising biomarkers of efficacy. Nevertheless, some technical challenges explain why such promising biomarkers are not reproducible or difficult to assess. One of these challenges is inherent to the technique of IHC or IF. Even if major advances have been made on this field, we have to deal with high intratumor heterogeneity and lack of specific markers and to interpret a static evaluation of a dynamic process. The first two points could be partially resolved by the progress in transcriptomic analyses and particularly in the immune signatures that were recently developed such as in MCP-counter. It provides a high accuracy in defining the proportion of immune cells, is reproducible, is less dependent to tumor heterogeneity, and finally allows to compare between tumor types.

Characterization of the immune TME together with the deep characterization of malignant cells using next-generation sequencing (NGS), RNA sequencing, as well as multiplex IF will allow to treat patients with the most appropriate precision medicine and to closely monitor the dynamic changes during CBT.

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CD8+ T Cells in Immunotherapy, Radiotherapy, and Chemotherapy

3

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Contents

3.1 Introduction

Extensive studies have revealed that the infiltration of T cells, especially CD8+ T cells, into the tumor microenvironment is a favorable prognostic feature for numerous malignancies, including melanoma and head and neck, breast, ovarian, renal, bladder, urothelial, colorectal, prostatic, pancreatic, and lung cancers [1]. A high density of intratumor CD8+ T cells is associated with longer disease-free survival and overall survival. Interestingly, direct tumor contact by CD8+ T cells may not be required, as both intraepithelial and intrastromal CD8+ T cells are associated with a favorable prognosis in human breast and ovarian cancer [2–4].

CD8+ T cells are often referred to as cytotoxic T lymphocytes (CTLs) because of their

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ability to directly kill target cells. Upon antigenic stimulation, CD8+ T cells will progressively differentiate from naïve T cells into central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). The effector function increases upon CD8+ T-cell differentiation, while memory function and proliferation decrease. Effector CD8+ T cells are characterized as CCR7− CD62L−CD45RO+CD95+IL-2Rb+, in addition to expressing killer cell lectin-like receptor G1 (KLRG-1) and programmed death 1 receptor (PD-1). They secrete high concentrations of IFNγ and TNFα but secrete minimal IL2. Trafficking of CD8+ T cells is mediated through chemokine-chemokine receptor interaction, which, among others, includes the ligands CXCL9 and CXCL10 with their receptor CXCR3. Increased expression of CXCL9/10 is associated with increased number of CD8 T cells in tumor microenvironment [5, 6].

CD8+ T cells are a key component of antitumor immunity and execute tumor clearance by several mechanisms. First, CD8+ T cells recognize the specific tumor-associated antigen [7] expressed on tumor cells, release cytotoxic molecular granzyme B and perforin, which are delivered into tumor cells and induce caspase activation and ultimately apoptosis [8]. Secondly, CD8+ T cells can also induce cancer cell death through the Fas/Fas ligand pathway. It has been demonstrated that the Fas ligand is essential for tumor regression mediated by CD8+ T cells in murine models of lung cancer and B-cell lymphoma [9, 10]. Finally, IFNγ and TNFα secreted by CD8+ T cells can have antitumor activity and control tumor growth. The combination of IFNγ and TNFα can drive cancer cell into senescence [11]. IFNγ is also known to be critical for cancer immunosurveillance by enhancing antigen presentation and limiting tumor angiogenesis [12].

Although tumor-reactive CD8 T cells are often found in the tumor biopsies, cancer can still progress. It has been revealed that the immunosuppressive tumor microenvironment may drive CD8 T cells into senescence or exhaustion [13]. Senescent CD8+ T cells are characterized by short telomeres, activation of DNA damage response genes, and secretion of senescence-associated secretory phenotype (SASP) factors [14]. These cells phenotypically show downregulation of the co-stimulatory molecules CD27 and CD28 and high expression of CD57 and KLRG1. Although senescent T cells are irreversibly cell-cycle arrested, they may still retain their cytotoxic capacity [15]. Exhausted CD8+ T cells are described as cells that exhibit defects in proliferation and decreased cytokine production and cytotoxic functions, as well as display higher expression of co-inhibitory molecules, such as PD-1, CD244, CD160, CTLA-4, Lag-3, and Tim-3 [16, 17]. However, it is notable that CD8+ T cell exhaustion is reversible to some extent [18]. Blockade of CTLA-4 or PD-1 has been shown to improve CD8+ T-cell effector function, resulting in improved clinical response. In addition, adoptive transfusion of ex vivo-expanded tumor-specific T cells, especially CD8+ T cells, has achieved durable tumor remission and even cure of malignant disease. Interestingly, recent studies have revealed that antibody-based targeted therapy, radiotherapy, and chemotherapy may synergistically initiate or augment antitumor immune response. The antitumor efficacies of these therapies are at least partially dependent on CD8+ T-cell immunity.

This review focuses on the convergence of adoptive T-cell transfer, checkpoint blockade, antibody-based targeted therapy, radiotherapy, and cytotoxic chemotherapy on effector CD8+ T cells. We summarize the state of knowledge regarding how these therapies increase intratumor CD8+ T-cell infiltration, induce tumor antigen-specific CD8+ T-cell response, unleash CD8+ T-cell effector function, and sensitize tumor to CD8+ T cells. Finally, we discuss how to rationally combine immunotherapy with radiotherapy or/and chemotherapy to improve cancer patient outcomes.

3.2 CD8+ T Cells in Immunotherapy

3.2.1 Adoptive T-Cell Transfer

Adoptive T-cell therapy (ACT) for cancer is a form of transfusion therapy consisting of the infusion of various ex vivo-expanded T-cell populations. The first strategy of ACT, which has been the most extensively studied in clinical trials, is the adoptive transfer of autologous ex vivo-expanded tumor-infiltrating lymphocytes (TILs). More recently, transfer of genetically modified T cells is being developed and clinically utilized. This approach includes the utilization of peripheral blood lymphocytes (PBLs)-derived T cells expressing TAA-specific T-cell receptor (TCR) or a so-called "chimeric antigen receptor" (CAR) T cells [19, 20].

CD8+ T in Tumor-Infiltrating Lymphocytes Therapy The general protocol of ACT includes (1) collection of circulating or tumor-infiltrating lymphocytes, (2) selection and expansion of tumor-specific T-cell populations ex vivo, and (3) re-administration of T cells to patients with a conditioning regimen of lymphodepletion and IL-2 administration. Thus far, ACT of ex vivo-expanded TILs is considered to be the best available treatment for patients with chemorefractory metastatic melanoma [21, 22]. Following the harvesting of TILs from the patient, long-term ex vivo IL-2 and CD3 stimulation are used to expand CD4+ and CD8+ αβ TCR+ T cells [21]. As CD8 interacts with MHC class I expressed on tumor cells, CD8+ T cells are thought to effectuate the antitumor activity of ACT, although indirect CD4+ T-cell interaction with the tumor cannot be dismissed. A recent clinical study described three sequential trials on metastatic melanoma treated with the ACT of autologous TILs combined with lymphodepletion and IL-2. Objective response rates in the three trials using different lymphodepleting preparative regimens ranged from 47% to 72% [22]. Furthermore, the number of infused CD27+ CD8+ cells was found to correlate with objective response [22]. This corroborates other melanoma ACT trials that have also found a positive correlation between a higher number of infused CD8+ T cells and clinical response [21, 23].

Nonselective expansion of polyclonal tumor-infiltrating T cells results in a population that recognizes multiple tumor-associated antigens. These antigens include cancer testis antigens that are expressed during development and reactivated in tumors, such as NY-ESO-1 and MAGE; melanocyte lineage antigens, such as gp100, MART-1, and tyrosinase; and mutational antigens generated from the low-fidelity replication present in cancer cells. A recent study analyzed the antigens recognized by clinically effective TILs from melanoma patients that experienced durable complete regressions beyond 5 years after ACT of TILs and identified both nonmutated and mutated antigens that could be recognized by autologous TILs [24]. More recently, neoantigen-specific T cells including CD8+ T cells were successfully isolated from the blood and primary tumor in patients with melanoma [25]. A recent case report demonstrated that ACT from TILs in a patient with metastatic KRAS mutant colorectal cancer could result in durable regression of all metastatic deposits. Correlative studies revealed that four different CD8+ T-cell clones that were specifically reactive to mutant KRAS G12D mediated this response [26]. This highlights an emerging strategy where cellular immunity can be harnessed to target conserved oncogenetic mutations, which have not been conducive to pharmacologic inhibition.

In addition to TAA specificity, emerging findings indicate that the differentiation state of T-cell populations is crucial to the antitumor efficacy of ACT [20, 27]. CD8+ T cells in ex vivo-expanded TILs are a mixture of mostly T_{EM} (less-differentiated effector memory T cells), T_{EFF} (more-differentiated effector T cells), and T_{TDE} (terminally differentiated effector T cells). Relatively, very few T_{CM} (central memory) are found in the ex vivo-cultured TILs, although in preclinical and clinical models T_{CM} cells have shown increased antitumor activity compared with effector T cells in mouse melanoma models [28, 29]. Currently, little is known on which state of differentiated CD8+ T cells is optimal for ACT of TILs in human.

Genetically Engineered T Cells T cells can be genetically engineered to express a T-cell receptor (TCR) with a high affinity and specificity to target antigens. Introductions of such TCR genes are accomplished by retrovirus or lentivirus-mediated transduction. Such TCR-modified T cells have specificity for tumor-associated antigens and can be rapidly expanded ex vivo and reinfused into patients for ACT. For example, TCR transduction has been used to target MART-1 and NY-ESO-1 in clinical trials for patients with melanoma. Tumor regression and durable objective responses were observed in a subset of patients [30, 31].

Chimeric antigen receptors (CARs) are another means for providing specificity to transduced T cells. CAR molecule is an artificial receptor composed of a single-chain variable fragment (scFv) derived from antibody, fused to transmembrane and cytoplasmic domains. The scFv fragment recognizes specific surface tumor antigens in an MHC-independent fashion. The cytoplasmic domains consist of a CD3 zeta activation domain and two co-stimulatory domains, CD28 and CD137/4-1BB. Upon antigen encounter, the CAR transduces the activation signals to T cells, resulting in T-cell proliferation and expansion with cytotoxic functions [32]. Clinical trials have shown excellent outcomes for CAR-T-cell adoptive transfer therapy in patients with hematologic malignancies [33]. Almost all B-cell malignancies, as well as normal B cells, express the CD19, which is absent in other cell types. Thus, anti-CD19-redirected CAR-T cells were designed to target CD19+ B cells and have achieved impressive response rates in 60–90% of patients with relapsed or refractory lymphoblastic leukemia [34–36]. CAR-T cell therapies are also being developed to target solid tumors in a number of disease sites [37, 38]; however, these efforts have been historically hindered by off-target toxicity [39, 40].

In a manner similar to TILs, genetically modified T cells for ACT also contains both CD4+ and CD8+ T-cell populations, which both confer an antitumor response. In a recent clinical trial on patients with B-cell malignances, CD19-CAR-T cells were generated from CD8+ and CD4 T-cell subsets that were separately ex vivo expanded and infused at a 1:1 ratio. This defined composition product showed remarkable antitumor activity as 93% patient achieved bone marrow remission [41]. In contrast to polyclonal TILs, genetically modified T cells have monoclonal specificity to a single target antigen, which may facilitate tumor immunoediting and allow the development and outgrowth of antigen escape tumor subclones.

3.2.2 Checkpoint Blockade

The immune system is characterized by compensatory inhibitory mechanisms to prevent the inflammatory response from precipitating autoimmunity. Tumor-infiltrating T cells that recognize and are poised to eliminate tumor cells are held in check by negative signals that reduce their activation and effector functions. Several molecules have been identified as negative regulators or checkpoints of T-cell activation, including cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), PD-1, and PD-L1. Drugs interrupting these checkpoints can unleash the antitumor activity of T cells and mediate durable cancer regressions. Multiple therapeutic antibodies that block CTLA4, PD-1, or PD-L1 have been approved and have shown clinical benefits in a wide range of solid and liquid tumor types, including melanoma, non-small cell lung cancer, kidney cancer, and Hodgkin's lymphoma [42].

The Biology of the CTLA-4 Pathway CTLA-4 is a receptor that is expressed exclusively on T cells and primarily regulates the amplitude of the early stages of T-cell activation. The engagement of CTLA-4 downregulates the T-cell function, largely by counteracting the activity of the T-cell co-stimulatory receptor, CD28. The recognition of peptide-major histocompatibility complex (MHC) by the T-cell receptor (TCR) is insufficient for T-cell activation and must be amplified by the ligation of CD28 to its ligands, CD80 and CD86. CTLA-4 shares the same set of ligands with CD28 but with a much higher affinity; therefore, its expression on the surface of T cells damp-

ens the activation of T cells by outcompeting CD28 with regard to binding CD80 and CD86, as well as actively delivering inhibitory signals to the T cell [43]. CTLA-4 also confers T-cell inhibition via depletion of CD80 and CD86 from the antigen-presenting cell (APC) surface [44]. The essential role of CTLA-4 for maintaining normal immunologic homeostasis is demonstrated by the lethal systemic immune hyperactivation phenotype in CTLA-4-deficient mice [45, 46].

On the basis of CTLA-4 biology, ipilimumab, a therapeutic antibody against CTLA-4, has been developed and approved for the treatment of patients with advanced melanoma. Ipilimumab binds to CTLA-4 and blocks ligation with CD80 and CD86, which prevents inhibitory signal transduction and results in increased CD28-mediated co-stimulation. CTLA-4 is predominantly expressed on CD4+ T cells, and CTLA-4 blockade has been demonstrated to mediate antitumor immune response through enhancement of effector CD4+ T-cell activity, as well as inhibition of regulatory T (Treg)-cell-dependent immunosuppressive activity. In Treg cells, CTLA-4 is regulated by the forkhead transcription factor FOXP3 and therefore constitutively expressed. It has been demonstrated that anti-CTLA-4 antibody can deplete Treg population in the tumor microenvironment in a Fc-mediated manner through antibody-dependent cellular cytotoxicity (ADCC) [47].

CD8+ T Cells in CTLA-4 Blockade In addition to CD4+ T cells, CTLA-4 blockade enhances CD8+ T-cell response in the tumor microenvironment. Because CTLA-4 is also expressed on activated CD8+ effector T cells, CTLA-4 blockade is considered to directly regulate CD8+ T-cell activity. CTLA-4 regulates effector functions of CD8+ T cells through repressing the production of IFNγ and eomesodermin in individual CD8+ T cells [48, 49]. CTLA-4 blockade was shown to directly enhance the proliferation and activation of specific CD8+ T cells in vitro and in vivo, in a manner independent of CD4+ T-cell help [50]. However, studies using different mouse tumor models demonstrated that CTLA-4 blockade could also reverse CD8+ T-cell tolerance and mediated antitumor immune response by a CD4+ T cell-dependent mechanism [51, 52]. Regardless of which cell types are targeted by CTLA-4 blockade, the functional result of CTLA-4 blockade therapy is enhancement of tumor-specific CD8+ T cells and tumor regression. Ipilimumab treatment in melanoma patient results in clonal expansion of tumor-specific CD8+ T cells in the tumor microenvironment and systemic circulation, although it is related with ipilimumab-induced toxicities [53]. Ipilimumab also increases the absolute number of circulating CD8+ T cells, which correlated with improved clinical outcomes [54].

The Biology of PD-L1/PD-1 Pathway PD-1 is a cell surface receptor of the same immunoglobulin family as CD28 and CTLA-4. Similar to CTLA-4, PD-1 is absent on resting naive and memory T cells and is induced after T-cell activation. However, in contrast to CTLA-4, PD-1 expression on the surface of activated T cells is initiated at a transcriptional level and is therefore delayed [55]. Unlike CTLA-4, which primarily regulates T-cell activation at the earlier stage, PD-1 is believed to inhibit effector T-cell activity in the effector phase within peripheral tissue and tumors [56]. Ligand engagement of PD-1 results in activation of the inhibitory phosphatases SHP-2 and PP2A, which suppress the kinase signaling required for T-cell activation [55, 57].

The ligands for PD-1 are PD ligand 1 (PD-L1, B7-H1, CD274) and PD ligand 2 (PD-L2, B7-DC, CD273) [58, 59]. PD-L1 has immunomodulatory functions independent of PD-1 and can also bind CD80 on activated T cells and APCs to deliver inhibitory signals [60, 61]. The relevance of this interaction in antitumor immune resistance has yet to be determined. Additionally, PD-L1 engagement results in bidirectional signaling that "back" transmits signals into T cells and tumor cells to regulate their survival [62, 63]. Thus, PD-L1 could regulate tumor immunity by functioning as both a ligand and receptor. Similarly, PD-L2 can deliver suppressive signals through PD-1 and can also signal via repulsive guidance molecule b (RGMb) to promote respiratory tolerance [64]. The relevance of PD-L2 signaling to cancer immunity is unknown as it is not widely expressed by tumor or immune cells.

Clinically, the PD pathway blockade, including anti-PD-1 and anti-PD-L1 antibodies, has demonstrated highly durable response rates with minimal toxicity across a spectrum of different tumor types, spanning both solid tumors and hematologic malignancies [65]. In theory, targeting PD-1 may result in different biologic effects than targeting PD-L1 because of the different cellular populations that express these two molecules. In addition to activated T cells, PD-1 expression was found on B cells and natural killer (NK) cells, and, therefore, PD-1 blockade may influence the function of these cells as well [66, 67]. PD-L1 was highly expressed on tumor cells and tumor-associated APCs, including dendritic cells (DCs), macrophages, fibroblasts, and T cells [68–72]. PD-L1 on different types of cells may mediate immunoregulation through unique mechanisms. The comparative effectiveness between anti-PD-1 and anti-PD-L1 antibodies cannot yet be performed because of clinical data that has not yet matured, and biological inferences from the clinical studies may be limited by the differing degrees of chimerism and different isotype subgroups of the antibodies.

CD8+ T Cells in PD Blockade Although the cellular and molecular mechanisms are not completely defined, translational and clinical studies suggest that both PD-1 and PD-L1 blockades converge on tumor-infiltrating CD8+ T cells. In the tumor microenvironment, PD-1 is highly expressed on infiltrating lymphocytes, including tumor-specific CD8+ T cells, and engagement by PD-L1 on tumor cells or APCs results in CD8+ T-cell dysfunction. Analysis of melanoma patients treated with anti-PD-1 antibody (pembrolizumab) showed that the expansion of intratumoral CD8+ memory T cells was marked in those patients who responded to therapy [73, 74]. PD-1 blockade could enhance the proliferation of the effector memory CD8+ T cells with senescent phenotype [75]. Additionally, PD-L1 blockade was shown to reverse exhausted CD8+ T-cell function, and this could be synergized by anti-CD27 [76]. These studies suggest that both anti-PD-1 and anti-PD-L1 antibodies can enhance CD8+ T-cell proliferation and improve effector cytokine production to promote antitumor activity. Recent clinical studies on melanoma have further demonstrated that “inflamed” or “hot” tumors are highly responsive to PD pathway blockade [77]. An “inflamed” tumor is characterized by a Th1-type immune signature that includes Th1-type chemokines, CD8+ T cells, and a high level of PD-L1 expression [6, 65]. Tumor regression mediated by therapeutic PD blockade requires preexisting CD8+ T cells in the tumor microenvironment [73].

3.2.3 Antibody-Based Targeted Therapy

Antibody-based therapy for cancer has been established for more than a decade. The fundamental basis for this therapy is the differential upregulation or mutation of cell surface antigens on cancer cells, compared to normal tissues. Receptor tyrosine kinases, such as EGFR and HER2 (ERBB2), have been found to be overexpressed or mutated in various cancer types, including breast, lung, brain, head and neck, and colon tumors. Aberrant tyrosine kinase activity of EGFR and HER2 can promote cancer cell proliferation and tumorigenesis [78, 79]. Monoclonal antibodies targeting HER2 and EGFR have been approved by the FDA and are currently being utilized in a variety of disease sites [80]. These antibodies antagonize these oncogenic receptors, leading to reduced proliferation and increased apoptosis [78]. Additionally, the antitumoral effect of these antibodies is also mediated by the Fc region of antibody, which can bind to Fc receptors (FcRs) on macrophages, neutrophils, and natural killer (NK) cells and induce cell death through activation of complement-dependent cytotoxicity (CDC) and ADCC [80, 81].

Interestingly, recent studies suggested that adaptive immunity, including CD8+ T cells response, contributes to the efficacy of anti-HER2 and anti-EGFR antibodies. A murine HER2-overexpressing breast cancer model demonstrated that anti-HER2/neu antibody therapy required CD8+ T cells. Anti-HER2/neu antibody treatment increased CD8+ T-cell infiltration into

tumor and induced memory T-cell responses [82]. This result was corroborated by another immunocompetent murine HER2 breast cancer model, which demonstrated that IFNγ-producing CD8+ T cells are required for efficacy of the antibody therapy [83]. Similarly, a study using a murine EGFR+ lung cancer model showed that anti-EGFR antibody cetuximab induced a tumor-specific CD8+ T-cell response, which is required for efficacy of antibody [84]. Additionally, cetuximab was shown to promote dendritic cell maturation and CD8+ T-cell priming, leading to the activation of tumor-specific T cells in patients with head and neck cancer [84, 85].

Bevacizumab, a humanized monoclonal antibody targeting vascular endothelial growth factor A (VEGF-A), is used in the treatment of many malignancies, including colon cancer, lung cancer, glioblastoma multiforme, and renal cell carcinoma. VEGF-A is a secreted factor that is critical for tumor angiogenesis through binding to the VEGFR1 and VEGFR2 receptors. Bevacizumab binds to and neutralizes all human VEGF-A isoforms and thereby block angiogenesis [86]. Aside from its direct action on tumor vascularization, anti-VEGF-A antibody has been shown to modulate immune cells in the tumor microenvironment. Blockade of VEGF-A increased DCs maturation and inhibited infiltration of immunosuppressive cells, such as regulatory T cells and MDSCs [87]. In a mouse model of colorectal cancer, VEGF-A was reported to regulate CD8+ T-cell exhaustion by enhancing expression of PD-1 and other inhibitory checkpoints, and this phenotype could be abrogated by an anti-VEGF-A antibody treatment [88]. VEGF-A can also inhibit the infiltration of T cells by reducing adhesion molecule expression in endothelial cells. Modulation or normalization of tumor vasculature by anti-VEGF-A antibody can result in increased T-cell recruitment and infiltration into tumors [89, 90]. In patients with metastatic renal cell carcinoma, bevacizumab therapy increased intratumoral CD4+ and CD8+ T-cell infiltration [91]. Increased intratumoral T cells were also observed in a combination therapy of bevacizumab with anti-PD-L1 antibody in renal cell carcinoma [92].

3.3 CD8+ T Cells in Radiotherapy

3.3.1 Radiation Therapy Induces Immune Responses

Radiotherapy is a highly effective treatment modality used for the curative and palliative management of almost all cancer histologies. It is frequently combined with other treatment modalities, including surgery, chemotherapy, and more recently immunotherapy, to maximize the chance of disease control [93]. Radiotherapy is a noninvasive localized therapy that applies ionizing radiation (IR) to a tumor. This induces single- and double-stranded DNA breaks in the irradiated tissue. As cancer cells are more sensitive to DNA damage-induced cell death than normal cells because of deficiencies in DNA repair pathways, ionizing radiotherapy can selectively damage cancerous cells [94].

Consistent with this, classical radiobiologic models have shown that radiotherapy induces tumor cell intrinsic mitotic catastrophe and cell death [95]. However, recent studies have highlighted the cell extrinsic mechanisms through which radiation modulates local or systemic immune responses and highlight the challenges and promise of combining radiotherapy with immunotherapy. Low-dose total body radiotherapy was used prior to hematopoietic stem cell transplant to create an immunosuppressed host with stem cell niche availability [96]. The hematopoietic compartment, which is comprised of hematopoietic stem cells, progenitor cells, and the vast majority of innate and adaptive immune cells, is vulnerable to radiation due to a rapid cycling time. Thus, even low doses of radiation are sufficient to induce cell death and damage in mature NK cells, T and B cells, as well as bone marrow stem cell precursors of monocytes and granulocytes. Low-dose radiotherapy was also historically used for the management of benign inflammatory conditions with moderate efficacy [97]. Finally, fractionated courses of radiotherapy had often been delivered in which small doses of radiotherapy are delivered on consecutive days for a duration up to 7 weeks to allow normal tissue healing and minimize treatment-

associated toxicity. When fractionated radiotherapy is given to a large area with concurrent chemotherapy, incidental lymphopenia can result for several months, which can compromise ongoing efforts to promote tumor immunity [98].

In contrast to low-dose total body irradiation, emerging evidence demonstrates that high-dose localized radiation often initiates or enhances antitumor immune response, and the efficacy of radiotherapy even relies, in part, on the host innate and adaptive immunity [99, 100]. Over the last decade, advances in diagnostic imaging and radiotherapy delivery allow for more conformal treatments to a smaller volume without compromising local control. Radiotherapy techniques, including intensity-modulated radiotherapy, have been shown to decrease the toxicity of treatment at many disease sites, including the risk of lymphopenia [95]. Further, hypofractionated approaches, including stereotactic body radiotherapy and stereotactic radiosurgery, which provide equivalent or superior outcomes in one to five total treatments, are increasingly utilized in a variety of disease sites. Biologically, radiation induces immunogenic cell death by causing the release of tumor antigens and danger-associated molecular patterns (DAMPs), such as calreticulin, ATP, and high-mobility group protein B1 (HMGB1). DAMPs are endogenous molecules that induce immunostimulatory effects upon release or exposure during cell death and act by binding to pattern recognition receptors (PRR) expressed on innate immune cells. Simultaneously, radiation can create an inflammatory microenvironment by the induction of cytokine and chemokine production, which leads to infiltration of DCs, macrophages, cytotoxic T cells, and some immunosuppressive cells. Released DAMPs work on APCs through TLR4 signaling to promote efficient processing and cross-presentation of tumor antigens [101]. Mature APCs can migrate to the draining lymph node, where T-cell priming is augmented to initiate a systemic antitumor immune response.

3.3.2 CD8+ T Cells in Radiotherapy

Emerging evidences have demonstrated that radiotherapy can induce tumor-specific CD8+ T cell responses that are critical for radiation-mediated tumor reduction. Using a mouse B16 melanoma model, Lee et al. showed that ablative hypofractionated radiation induces significant tumor regression dependent on CD8+ T-cell activation and recruitment [102]. Radiation has also been shown to induce activation of tumor-associated DCs that support tumor-specific effector CD8+ T cells. The efficacy of radiotherapy depends on DCs and CD8+ T cells, whereas CD4+ T cells or macrophages are dispensable [103, 104]. More recent study suggested that CD8+ T cells and IFNγ contributed to radiation-induced tumor equilibrium in two animal models. Depletion of CD8+ T cells or neutralization of IFNγ leads to tumor regrowth, and blockade of PD-L1 augments CD8+ T-cell response and leads to tumor rejection [105].

Concomitant with increased T-cell activation, radiotherapy can diversify the TCR repertoire of tumor-infiltrating CD8+ T cells. Radiation increases the expression of MHC class I and the production of novel proteins to favor neoantigen presentation [106]. Another study has shown that radiation increases the expression of cancer testis antigens, which promotes the immunological recognition of cancer cells by T cells [107]. In a more recent study involving melanoma patients and a mouse melanoma model, TCR sequencing revealed that high-dose radiation increased diversity of TCR clonotypes of CD8+ TILs. The optimal antitumor response was achieved by the combination of the three treatment modalities: high-dose radiation, CTLA-4 blockade, and PD-L1 blockade [108].

Radiation could also promote tumor infiltration of CD8+ T cells through alterations in tumor vascularity and improved T-cell homing. Radiation induces a pro-inflammatory milieu including inductions of IFNγ as well as many other cytokines and chemokines. This leads to

the infiltration of different immune cell subsets, including CD8+ T cells. Radiation-induced chemokines include CXC-motif chemokine 9 (CXCL9), CXCL10, CXCL11, and CXCL16, which binds to corresponding receptors on CD8+ effector T cells, resulting in migration of T cells into the tumor microenvironment. Moreover, type I IFNs were demonstrated to be required for the CXCL10 production within tumor after radiation treatment. Radiation-induced CXCL10 expression correlated with intratumor CD8+ T-cell numbers [109].

3.4 CD8+ T in Cytotoxic Chemotherapy

3.4.1 Chemotherapeutic Agents Activate Immune Responses

Cytotoxic chemotherapy is another efficacious treatment modality used for the management of many advanced cancers. Cytotoxic chemotherapy functions by inducing tumor cell death or inhibiting tumor cell reproduction. Based on their principal mechanism of action, conventional chemotherapeutic agents can be organized as several categories:

1. Alkylating agents or DNA-damage agents, which cause DNA strand cross-link by adding alkyl groups to the electronegative groups and result in DNA-damage-induced cell death (e.g., cyclophosphamide and cisplatin)
2. Antimetabolites, which function as the building blocks by imitating purine or pyrimidine to inhibit the synthesis of DNA and RNA (e.g., 5-fluorouracil)
3. Spindle poisons, which interfere microtubule function and mitotic spindle assembly, resulting in cell-cycle arrest (e.g., paclitaxel and taxanes)
4. Topoisomerase inhibitors, which prevent the correct unwinding of DNA during replication, transcription, and repair (e.g., irinotecan and etoposide)
5. Antitumor antibiotics, which are made from natural products of soil fungus *Streptomyces* and exert antineoplastic effects by various mechanisms, including DNA intercalation, altering membrane fluidity, and generation of oxygen radicals (e.g., doxorubicin and bleomycin) [95]

The integration of cytotoxic chemotherapy with immunotherapy is the subject of several ongoing clinical trials.

Similar to radiotherapy, cytotoxic chemotherapy has historically been considered immunosuppressive because most chemotherapeutic agents indiscriminately impair cellular division and thus impact tumor cells, effector lymphocytes, and homeostasis of innate leukocytes [110, 111]. Cytotoxic chemotherapy is now the main backbone for conditioning regiments to generate lymphodepletion prior to HSCT and ACT [20]. However, recent studies demonstrated that select chemotherapy agents might also augment tumor immunity [112, 113]. Chemotherapy can initiate or promote antitumor immune response through two major mechanisms. First, chemotherapy induces immunogenic cell death on tumor cells [114]. Similar to radiotherapy, chemotherapy-induced ICD involves the release of tumor antigens and the emission of DAMPs in the tumor microenvironment. Immunogenic chemotherapy-associated DAMPs include calreticulin (CRT), heat shock protein HSP70 and HSP90, ATP, annexin A1, and HMGB1, although different drugs may correlate with different DAMPs [115–117]. For example, Obeid et al. reported that during anthracycline-induced cell death, CRT was exposed to the cellular surface and facilitates their engulfment by DCs, which leads to tumor antigen presentation and tumor-specific CTL response [115, 118]. The antitumor efficacy of many chemotherapy drugs has been demonstrated to partially rely on the induction of ICD. Secondly, chemotherapy agents could activate systemic immunity. Some chemotherapy drugs could directly stimulate the effector activity

of myeloid or lymphoid cells. Paclitaxel was shown to promote DC maturation and cross-priming in mouse breast cancer model [119] and enhance the infiltration of NK cells in a cohort of breast cancer patients [120]. Cyclophosphamide has been shown to favor Th17 and Th1 memory response through altering the composition of microbiota in the small intestine [121]. Gemcitabine resorted defective cross-presentation of tumor antigen in DCs [122]. Finally, cytotoxic chemotherapy may also preferentially target immunosuppressive cells to indirectly enhance antitumor immune response. Gemcitabine [123], 5-FU [124], docetaxel [125], oxaliplatin [126], and paclitaxel [127] have all been shown to deplete blood-borne or tumor-infiltrating Treg cells or MDSCs.

3.4.2 Chemotherapy Enhances the Antitumor Function of CD8+ T Cells

Among innate or adoptive immune cells associated with chemotherapy, cytotoxic CD8+ T lymphocytes are considered a crucial mediator for tumor regression. The antitumor efficacies of these agents even rely in part on CD8+ T cells. In a mouse sarcoma model, the depletion of CD8+ T cells using anti-CD8+ antibody abolished anthracycline-mediated tumor regression, suggesting CD8+ T cells are indispensable for the anticancer efficacy of anthracyclines [128]. Using lung adenocarcinoma mouse models, the chemotherapy of oxaliplatin combined with cyclophosphamide was shown to induce antitumor response relied on innate immune sensing through TLR4 signaling and ultimately depended on CD8+ T-cell immunity [129]. Tumor regression induced by paclitaxel combined with blockade of IL-10 receptor was dependent on CD8+ T cells in a mouse model of orthotopic PyMT-derived tumors. Correlative studies in human breast cancer have found expression of CD8A to be predictive of pathological complete response [130] to neoadjuvant paclitaxel, and patients who achieve a pathological complete response (pCR) have improved clinical outcomes [131].

Additionally, chemotherapy induces tumor antigen-specific CTL response. When chemotherapy induces tumor cell death, tumor-associated antigens are released by dying cells and are taken up by APCs and presented to T cells, resulting in increased T-cell responsiveness and expansion of tumor-specific CD8+ T cells. In an ovalbumin-expressed murine mesothelioma model, cisplatin and gemcitabine have been shown to enhance the presentation of specific epitopes and amplify the CTL response [132]. In a breast cancer patient treated with gemcitabine and radiotherapy, ex vivo analysis of the TCR-Vβ repertoire of TAA-specific T cells in blood and TILs revealed the expansion of TAA-specific CD8+ T [133]. Dacarbazine combined with peptide vaccination in melanoma patients increased the antigenic repertoire of T cells and induced greater tumor reactivity compared to the vaccine alone [134].

Chemotherapy can also increase the infiltration of CD8+ T cells. Increased tumor-infiltrating lymphocytes have been also observed after certain chemotherapy regimens, and this is explained by the induction of chemokine expression in cancer cells. Dacarbazine, temozolomide, and cisplatin were all able to induce expression of T-cell-attracting chemokines, including CCL5, CXCL9, and CXCL10 in human melanoma cell lines in vitro. Using a genetically modified mouse model of melanoma, the authors demonstrated that chemotherapy-induced intratumoral expression of these chemokines increased T-cell infiltration into cutaneous tumors. In patients with melanoma, these chemokines were also increased in chemotherapy-sensitive lesions and correlated with T-cell infiltration and patient survival [135]. The antitumor effects of anthracyclines were known to partially rely on T-cell immune response. Anthracyclines rapidly stimulate the production of type I IFNs by malignant cells. Type I IFNs then trigger autocrine and paracrine signaling on cancer cells resulting in the release of CXCL10, a potent chemotactic factor for CD8+ T cells [136].

Finally, chemotherapy sensitizes tumor cells to the killing effect of CD8+ T cells. In an earlier study that combines vaccinia viral vaccine with conventional chemotherapy, the treatment with

cisplatin or cyclophosphamide after vaccination led to complete regression of the established tumors. These chemotherapy drugs augment the antitumor effect of the tumor-specific CD8+ T cells that were induced by vaccinia virus [137]. Chemotherapy with cyclophosphamide was also shown to sensitize tumor cells to TRAIL-dependent CD8+ T-cell-mediated apoptosis in a mouse model of malignant mesothelioma [138]. Moreover, doxorubicin, cisplatin, and paclitaxel sensitized tumor cells to the cytotoxic effect of CD8+ T cells through increasing the permeability of tumor cells to granzyme B. This effect was mediated by chemotherapy-induced upregulation of mannose-6-phosphate receptors on the surface of tumor cells [139].

3.4.3 CD8+ T Cells Sensitize Tumor Cells to Chemotherapy

While most studies have focused on the effects of chemotherapy on TILs, the reciprocal relationship may also be important. A recent study demonstrated that effector CD8+ T cells could abrogate fibroblast-mediated chemoresistance in ovarian cancer. Fibroblasts in tumor microenvironment inhibit the therapeutic efficacy of cisplatin by release of cysteine and glutathione, which are both utilized by tumor cells to protect them from cisplatin-induced apoptosis. CD8+ T cells restore the cisplatin sensitivity by IFNγ-mediated alterations of glutathione and cystine metabolism in fibroblasts. The presence of CD8+ T cells is positively associated with chemotherapy response and patient survival with ovarian cancer [4]. Also in ovarian cancer model, miR-424 was shown to directly regulate PD-L1 and CD80 expression in tumor cells and enhance the efficacy of chemotherapy by activating CD8+ T cells and reducing regulatory cytokine secretions [140].

3.5 Future Directions

Immunotherapies including adoptive T-cell transfer and checkpoint blockade are efficacious in a broad spectrum of cancers and can induce durable clinical responses. Unfortunately, this result is achieved in a minority of patients. No benefit has been seen in certain cancer histologies, including ovarian, mismatch intact colorectal cancer, and pancreatic cancer. Emerging evidence suggests that immunotherapy most benefits patients with preexisting tumor-infiltrating CD8+ T cells. A current challenge is to turn "non-inflamed" tumor to "inflamed" tumor to increase CD8 T-cell infiltration in hopes that this will augment antitumor efficacy. Scientists and clinicians are now looking for the optimal strategy to achieve this goal.

One promising strategy is to enhance effector T-cell trafficking through epigenetic reprogramming. Effector T-cell tumor infiltration correlates with the level of intratumoral Th1-type chemokines, CXCL9 and CXCL10, which are frequently epigenetically repressed by histone modification and DNA methylation in tumor cells. Treatment with epigenetic modulators can enhance tumor Th1-chemokine production, increasing CD8+ T-cell tumor infiltration and augmenting antitumor efficacy of PD-L1 blockade and adoptive T-cell transfer in preclinical models [7]. Moreover, DNA methyltransferase inhibitor 5-azacitidine was shown to increase immunostimulatory genes including interferon signaling, antigen presentation, and cytokines/chemokines in several human epithelial cancers [141]. Another DNA methyltransferase inhibitor decitabine could also increase chemokine production and CD8+ T-cell infiltration in a murine ovarian cancer model [142]. Thus, epigenetic therapy may be able to increase Th-1-type chemokines, IFN signature genes, and CD8+ T-cell immunity and ultimately sensitize to checkpoint blockade therapy.

Another promising strategy is the merging immunotherapy with radiotherapy or chemotherapy. The basic scientific rationales of combining traditional cancer treatment modalities with immunotherapy have been demonstrated in many preclinical studies. Unfortunately, much of this work relies on immunocompetent murine models, and there are important distinctions between rodent and human immunology and cancer biology [143]. The successful combination of

immunotherapy with traditional cancer modalities will require both empiric discernment and rational mechanistic administration. To thoroughly assess the combination with chemotherapy, optimization of the sequencing, timing, and chemotherapy agent selection will be required. From a radiotherapy standpoint, the dosage, target, and timing will need to be assessed and optimized. Additionally, rigorous preclinical models and clinical trials will be required to realize the hope of combining the efficacious therapies already in the clinic with ground-breaking immunotherapy to unleash CD8+ T cells and achieve the best disease control and cure for cancer patients.

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Mutant Epitopes in Cancer

4

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4.1 Introduction

Targeted immunotherapy in cancer is a rapidly expanding and evolving field with a developmental history spanning at least three decades. Beginning with the identification and characterisation of tumor-specific antigens (TSA)—protein molecules which are exclusively associated with transformed cells—and very recently the dawn of neoantigen-specific immune-cell reactivity—championed by immune checkpoint blockade therapy—demonstrates that immune-based interventions will substantially shape the future of cancer therapy. Neoantigens arise from naturally processed mutated host protein molecules—eventually presented as immunogenic peptides to the immune system. However, a deeper understanding concerning the generation and recognition of neoantigens is indispensable in order to better understand the immunological and biological underpinnings in diagnostics and therapeutic applications to enhance healthcare for patients with cancer. We briefly introduce the reader to the antigen processing and presenting machinery in cancer, and provide a condensed history of cancer antigen discovery, touching upon seminal findings. Last but not least, we discuss the latest

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development in cancer immunotherapy—with a strong focus on neoantigen-directed strategies, which may be improved for the time to come in the context of clinical translation and therapy. We limit the focus in this chapter to active cellular therapy (ACT) for patients with cancer and the potential of using mutant epitopes in combination witih cellular therapy.

Harnessing the potential of neoepitope-specific T-cell subsets is highly attractive, due to their ability to recognize and respond to tumor cells with limited off-target toxicity, superior efficiency, and with the capacity to provide durable and clinically meaningful outcome in patients with cancer [1]. This anti-cancer reactivity directed against transformed cells is in essence a targeted but productive autoimmune response and dependent on the presence of a T-cell receptor T-cell receptor (TCR) repertoire capable of recognising mutant targets. Some cancer antigens have been identified as 'cancer antigens' due to their selective tissue expression or overexpression in malignant/transformed cells, i.e. mesothelin, or cancer testis antigens (discussed later in this chapter). In other cases, mutations that occur in otherwise normally expressed and functional proteins may cause them to become cancer-inducing agents. These mutated host molecules may be involved in cancer initiation (oncogenesis), disease maintenance, or in metastasis. Since some mutations are crucial for malignant transformation and for tumor cell survival, they may also be instrumental in immune escape mechanisms, either by selecting tumor-promoting T-cell responses, or - not mutually exclusive, 'blinding' anti-cancer immune responses by inducing loss of immune - 'fitness'.

Recent findings in cancer research show that the success of immune - based therapies requires a T-cell receptor repertoire capable of recognizing mutant targets along with anti-cancer directed cellular immune responses (e.g. cytotoxicity, Th1 - type immune responses, see Fig. 4.1). In line with this, T-cell-based cancer immunotherapy is gaining momentum since the most successful novel interventions against solid tumors rely on cancer-specific T-cell activity and their mobilisation to sites of disease, i.e. immune checkpoint inhibitors, chimeric antigen receptors (CARs) and T-cell receptor (TCR)-modified T-cell products [2, 3]. Local activation of antigen-specific tumour-infiltrating T lymphocytes, known as TILs, allows for recirculation of cells, robust killing of tumour cells, reduction in tumour mass and orchestration of anti-tumour responses in tissue. Monoclonal antibodies targeting PD-1 and CTLA-4 have thus revolutionised cancer therapy, with signs of potential use in treating chronic infectious diseases such as viral hepatitis, human immunodeficiency virus (HIV) infection, malaria and tuberculosis [4–7]. In particular, anti-PD-1 therapy has been shown to activate CD8 T cells specific for mutated antigens (neoantigens) associated with cancer progression in metastatic melanoma [3]. Patients showing durable responses following immunotherapy had increased numbers of neoantigen-specific T-cells in their blood, signifying the underlying mechanism of anti-PD-1 therapy.

4.2 Antigen Processing and Presentation in Cancer

In order to gain an understanding of the dynamics driving the generation and 'visibility' of antigens to the immune system, it is advantageous to provide an overview about antigen processing and presentation to immune effector cells. Antigens can be generally viewed as being either intrinsic or extrinsic in nature; they are biochemically processed within cells and presented to various T-cell subsets, B cells as well as natural killer (NK) cells [8]. The essential molecule associated with presenting antigens to the immune system is termed as the major histocompatibility complex (MHC), or specifically in humans, the human leukocyte antigen (HLA) [8]. The function of MHC/HLA system was discovered and first described by Rolf Zinkernagel and Peter Doherty in the early 1970s, the seminal work for which they were awarded the Nobel prize in Medicine and Physiology in 1996 [9–12].

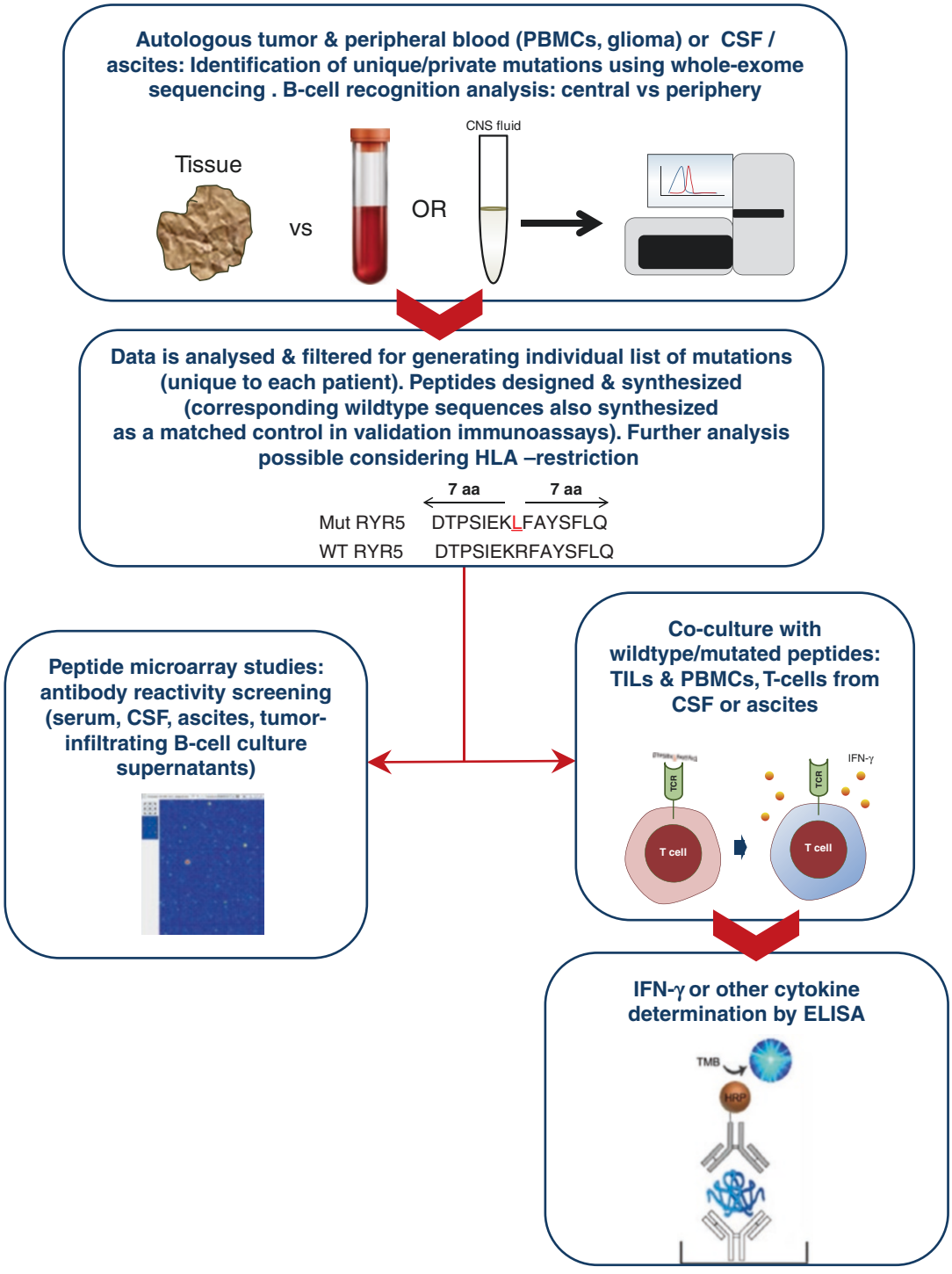


Fig. 4.1 Strategies to identify mutant epitopes from transformed cells, CSF: cerebrospinal fluid

Antigens can derive from whole pathogens, i.e. bacteria, viruses and parasitic organisms, or by non-mutant, or mutant proteins associated by transformed cells. Intrinsic antigens, also called 'endogenous' antigens, are processed and presented to the immune system in the form of specific peptides called epitopes. This pathway is termed the MHC/HLA class I pathway (hereafter referred to as the 'HLA class I pathway'), and plays a crucial role in eliciting immune responses to viruses (viral components synthesised within the host cell), intracellular bacteria as well as to cancer - associated antigens - which relies on the immune system's capacity to recognized 'self' or 'mutant self' antigens [8]. All cells of the body (with the exception of erythrocytes) are capable of processing and presenting antigens via the HLA class I pathway. The processing of antigens in this pathway involves a crucial step, where the immunoproteasome (occurring in the cytosol) cuts up denatured (unfolded) protein structures into small peptide sequences between 8 and 10 amino acids long. The amino acid junctions at which the proteasome enzymatically cuts a protein decides on which peptide or epitopes are naturally presented to immune cells. Epitopes presented by HLA class I molecules are recognised by CD8+ T-cells, which can respond by i) proliferation, ii) cytokine production and / or iii) production of cytotoxic molecules, capable of killing transformed cells [8]. CD8+ T-cells may produce perforin, granzymes, and granulolysin (that can be easily measured using an CD107a induction assay), or - not mutually exclusive - IFN-gamma in response to transformed cells [13]. If (cancer) target epitopes are identified using the 'reverse immunology strategy', i.e. that epitopes are selected based on their predicted capacity to bind to MHC class I or class II molecules, it cannot be assumed with a very high degree of certainty that T-cells will also recognise the naturally processed and presented epitopes on tumour cells—a scenario which was described more than two decades ago [14]: T-cells that were shown to be peptide specific were not able to react against naturally processed and presented peptides on tumor cells. One of the reasons driving this phenomenon is that the specialised, or 'skewed' antigen processing and presentation machinery in transformed cells may be different compared to professional and non-professional antigen presenting cells [15] that are responsible for activating and expanding antigen-reactive T-cells. Alternatively, epitopes may have been created via post-translational modifications (such as phosphorylation) that could not be predicted from the primary structure of the wildtype and/or the mutant protein [16].

Antigens that are taken up from the external environment by professional antigen presenting cells or APCs (i.e. dendritic cells, macrophages), including B-cells, that have also professional APC functions, are usually processed and presented to the immune system via the HLA class II pathway. Whole pathogens, as well as proteins, e.g. generated via destruction of cancer cells by antibody-mediated mechanism, NK or CD8 T-cells, are actively taken up by APCs in endocytic vesicles called phagosomes, after which proteolytic enzymes contained within lysosomal compartments fuse with the phagosome to digest the antigen to yield smaller peptide sequences, usually 13–17 amino acids in length. These epitopes are then presented to CD4+ T-cells, which are also termed as helper T-cells (Th), and have an indispensable role in orchestrating immune responses mainly by producing effector cytokines, i.e. IFN-γ, tumor necrosis factor alpha (TNF-α), interleukin (IL)-2 (Th1 cells), IL-4, IL-10 (Th2 cells) and in some cases, IL-17 (Th17 cells). Cytotoxic activity is not exclusively attributed to CD8+ CTLs; cytotoxic CD4+ T-cells have also been reported to mediate biologically relevant immune responses in cancer as well as in viral infections [17–19].

The T-cell receptor (TCR) on the surface of T-cells binds to the HLA-epitope complex, along with co-receptors CD8 or CD4, to initiate an immune synapse. Interactions between T-cells and tumor cells are governed by HLA-restriction—the alleles encoding a person's HLA repertoire and matching TCRs available in the tissue microenvironment and/or in blood, which dictates the nature and strength of the immune response. HLA allele-restriction of epitopes and immune cross-reactivity thereof plays an indispensable role in dictating

the nature of immune responses. For example, HLA-DQ variants have been associated with increased susceptibility to certain infectious diseases; mutations in the β57 subunit of HLA-DQ may perpetrate progression to pulmonary disease [20]. Interestingly, mutations in HLA-DQ alleles have been attributed to susceptibility to contract type 1 diabetes mellitus (T1DM). While HLA-DQ is highly prevalent among Caucasians in the Americas as well as Europe, East Asians and Africans are much less likely to express these alleles [21]. Indeed, individual HLA alleles may also favour certain immune-recognition profiles, independent of the peptide repertoire displayed by the nominal restricting MHC element, i.e. HLA-DQ0602 favours IL-17 production independent of binding peptides, as shown in the transgenic murine model of multiple sclerosis [22]. This IL-17-centric reactivity represents a double-edged sword; it may more effectively contain certain bacterial infections [23, 24] and IL-17 may be beneficial to attract immune cells to the tumor site [25] while the chronic exposure to IL-17 may rather promote malignant transformation [26–28]. Therefore, the nature, quality and quantity of immune responses following vaccination appear to greatly depend on an individual's HLA profile, which shapes the quality and quantity of ensuing cellular immune responses, including increased or decreased risk for infections, autoimmune responses or the ability to present (neo) epitopes to T-cells dependent on the restrictions imposed by the MHC–peptide complex and the responding TCR repertoire. For instance, even if neoepitopes are generated during malignant transformation, they may not be visible to the cellular immune system, if they are not processed and ultimately complexed to the respective HLA molecule and presented to responding T-cells.

Th1–Th2 Responses and MHC Restriction Most studies use IFN-gamma as the readout of T-cells responding to wildtype and mutant epitopes provided from cancer cells, yet Th2 responses, with the signature cytokines IL-4, IL-5 and IL-13 may also be present, either as an 'original' Th2 response or as a result of partial agonist peptides, imposed by the mutational event (see below) that may turn Th1 T-cells into Th2 cytokine-producing T-cells [29]. Th2-type T-cell responses may not *per se* signify an unproductive and potentially 'tolerizing' immune response; more recent reports indicate that Th2-type immune responses may also be able to mediate clinically relevant anti-cancer immune reactivities [30]. In a preclinical model, antigen-specific Th2 cells eradicated myelomas without the help of CD8 T-cells, leading to massive inflammation at the tumor site [30]. Th2-mediated tumour destruction has been shown to be associated with IL-1, TNF-alpha (Th1) and Th2 cytokine (IL-4, IL-5, IL-13) production in situ, while passively transferred Th2 cells were able to confer long-lasting cellular anti-cancer directed immune responses. CD8-independent and antigen-specific T-cells in Th2-mediated immune responses were shown to be eotaxin- and STAT6-dependent [31–36]. In general, Th2 infiltrates in human cancers have not been studied extensively and some studies even suggested a better outcome with Th2-type cytokines [36]. The nature of Th2 responses in recognising mutant epitopes is not well explored at this time. The more detailed association of CD4 Th2 responses may also benefit from closer association of T-cells with the restricting MHC class II elements. For instance, previous studies reported Th1/Th2 CD4+ T-cell responses against NY-ESO-1 in DPB1*0401/0402-positive patients with ovarian cancer [37]. Much more information is available concerning the nature of the cellular immune response directed against peptides presented by the rather less variant (as compared to HLA-DR) HLA-DP molecules from infectious pathogens, e.g. Hepatitis B or MHC class II molecules that pre-dispose humans to certain autoimmune diseases (e.g. gluten-associated colitis) [37–45]. The impact of variant epitopes in association with certain MHC alleles that are associated with certain cytokine production patterns (IL-17, Th1, Th2) is unexplored up to now. Table 4.1 provides an overview of wildtype and mutant target epitopes recognised in TIL from patients with glioma, demonstrating that Th2 responses exist in the TCR repertoire from individual patients directed against mutant epitopes.

Table 4.1 TIL reacting against wildtype and mutant epitopes—Th1 and Th2 patterns

ID	Patient code	Wildtype sequence	IFN-γ wildtype	IFN-γ mutant	TNF-α wildtype	TNF-α mutant	IL-17A wildtype	IL-17A mutant	IL-4 wildtype	IL-4 mutant	IL-5 wildtype	IL-5 mutant	IL-13 wildtype	IL-13 mutant	Mutated sequence	Gene ID
1	GBM-1	ALYDICSKTLKLPTP	193.09	570.95	64.68			22.69			11.75	36.71	45.35	33.71	ALYDICSRTLKLPTP	TUBB8
2	GBM-1	SSGGCCGSSSGGCCS		63.38							4.92	9.07		3.96	SSGGCCGSSSGGCCS	LCE1F
3	GBM-1	AKQTSNCVLEICAEQ									17.37	4.74		2.31	AKQTSNCILEICAEQ	ESPNP
4	GBM-1	REQEEKMWRQEEKIR					126.84	85.39			3.94	30.39	4.95	11.85	REQEEKMRRQEEKIR	NCKAP1L
5	GBM-1	REDAGAGGEDVGAGG		88.74								37.28		1.98	REDAGAGEEDVGAGG	GOLGA6L1
6	GBM-1	IREQEEMLREQEAQR	105.48	754.23				35.73			18.34	53.61			IREQEEMiREQEAQR	GOLGA6L2
7	GBM-1	PPTWSGRRAPGDRDN		270.84						69.22				19.06	PPTWSGRHAPGDRDN	LOC645752
8	GBM-1	QFLIPTSLSVSSNSV		30.48											QFLIPTSFSVSSNSV	DSPP
9	GBM-2	WPSFSEAHGTSGSDE	428.21	747.66							76.27	86.03		28.36	WPSFSEARGTSGSDE	MSRB2
10	GBM-2	TATASSTQATAGTPH				196.06									TATASSTRATAGTPH	MUC5B
11	GBM-2	TATATTTGATGSVAT				31.21									TATATTTRATGSVAT	MUC5B
12	GBM-2	NLKEKCFLTQLAGFL					9.24								NLKEKCFVTQLAGFL	NBPF8
13	GBM-2	LLTPDEPDKSQGQDL					18.28	3.1	116.92						LLTPDEPEKSQGQDL	NBPF12
14	GBM-2	PDAVGKCRSAGIKVI	353.94	865.82							15.74	60.5		36.45	PDAVGKCGSAGIKVI	ATP1A2
15	GBM-2	ARCSSEDDDSKESTC		412.16			47.28				7.53	147.1		26.11	ARCSSEDSKESTCSP	PHOX2A
16	GBM-2	RWEEWNKRLEEVKRE					30.69								RWEEWNK	AK7
17	GBM-2	PGEGHGGEHLDSEGE					7.41								PGEGHGGDHLDSEGE	GOLGA8DP
18	GBM-2	PSDLRRHVRTHTGEK					81.47	24.61			42.07	30.96			PSDLRRHMRTHTGEK	ZNF764
19	GBM-2	EGGPAAPRLGSRTAP		288.05											EGGPAAPHLGSRTAP	LINC00273
20	GBM-2	NRPTSGPWQRHTRRS		495.14						19.38					NRPTSGPRQRHTRRS	LINC00273
21	GBM-2	ADPIPSGLSPGPCGA				31.21									ADPIPSGRSPGPCGA	LINC00273
22	GBM-2	MKDCQLRKQQNENVS					41.12								MKDCQLRRQQNENVS	SLFN12L
23	GBM-2	VKRNPPKTAKVSEPG	246.07	1275.82			9.24		60.58		84.52	169.86		49.05	VKRNPPKKAKVSEPG	HOXB1
24	GBM-2	SAFEPEGVLANVLGL								65.93				25.85	SAFEPEGGLANVLGL	CYB561
25	GBM-2	GSGPSCRRWEKKLAS	376.6								25.49				GSGPSCRQWEKKLAS	PIK3R5
26	GBM-2	DMYGTGQESLYS		472.45								69.15		31.08	DMYGTGQQSLYS	CDH7
27	GBM-2	QSYKNDFNAEYSEYR		385.13							37.04				QSYKNDFSAEYSEYR	ELL
28	GBM-2	ARKAKYNVHATVRYQ		376.14							3.01				ARKAKYNLHATVRYQ	NCAN
29	GBM-2	MRVMKFSVSPVVRVA			258.99										MRVMKFSISPVVRVA	EEF2
30	GBM-2	YAPCGDLSGMLQERG						23.08							YAPCGDLNGMLQERG	SBK3
31	GBM-2	GQLAVSKRLALEVTV			304.12		39.39								GQLAVSKCLALEVTV	SIRPG
32	GBM-2	QRAAAIARQKAEIAA		889.28			112.28								QRAAAIACQKAEIAA	JPH2

33	GBM-2	ISPSRAARQLMERTQ	606.87								120.34				ISPSRAACQLMERTQ	ELMO2
34	GBM-2	FARKLKDVHETLGFP		410.66											FARKLKDIHETLGFP	TTN
35	GBM-2	EPDNIKYVISEEKGS		361.16											EPDNIKYMISEEKGS	TTN
36	GBM-2	DNHCEQLRVKIRKLK	>1864.49	>1864.49							184.5				DNHCEQLGVKIRKLK	ANKRD36C
37	GBM-2	LTELKDNHCEQLRVK	495.5	561.95	1759.72							80.89		34.27	LTELKDNLCEQLRVK	ANKRD36C
38	GBM-2	DFSVIIMAYVSENIK	669.42												DFSVIIMVYVSENIK	SCN5A
39	GBM-2	GKGVMLAVSQGRVQT	328.34	676.71		702.12						69.23		53.48	GKGVMLAISQGRVQT	TENM3
40	GBM-2	ESRGLLQRRAQAAQE									214.08				ESRGLLQHRAQAAQE	CFAP99
41	GBM-2	SRELCPGRWRAGPWS	467.9		1023.03						52.66				SRELCPGHWRAGPWS	ADAMTS2
42	GBM-2	WDLTDALRLAALSIE			423.55										WDLTDALWLAALSIE	LAMA4
43	GBM-2	SHLIAASNCHSLELQ	993.08								146.98	33.34			SHLIAASSCHSLELQ	CAPN11
44	GBM-2	EDVKWPPTLQPPTLR			682.55										EDVKWPPPTLQPPTL	IRF5
45	GBM-2	NGMEWNGMEWNRIES	343.39												NGMEWNGIEWNRIES	PCSK5
46	GBM-2	AATSHPKPTTGHIKP	307.31	1000.95					14.03		134.72	109.23		33.29	AATSHPKHIKPATSH	GPR50

TIL were harvested from 2 patients with glioma (GBM1 and GBM2) using IL-2, IL-15 and IL-21 without restimulation with autologous tumor cells. TIL were tested against individual mutant epitopes and the corresponding wildtype sequences, followed by detection of Th1 and Th2 cytokines by ELISA. Note a focused T-cell recognition pattern against a low number of mutations in TIL from GBM1 versus a diverse spectrum of recognition in TIL from GBM2 exhibiting as well Th2-cytokine production against mutation epitopes. This test was performed with synthetic epitopes and does not necessarily imply that the wildtype or the mutant target is naturally processed and presented by tumor cells. Nevertheless, TIL reactivity is detectable that falls into the categories that either the mutant epitope is exclusively recognized, or both the wildtype and the mutant epitope sequence are recognised. Numbers are picogram (pg) cytokine production 1×10^5 TIL.

Processing and presentation of neoantigens may yield mutant epitopes (neoepitopes) that are shared as well as patient-specific ('private'). This of course depends on the location of the mutation, i.e. point mutation which might disrupt the naturally occurring cleavage site and the nature of the mutation itself i.e. point mutation vs. chromosomal deletion vs. premature stop codons. A comprehensive analysis of somatic mutations in the HLA class I pathway, using DNA isolated from tumour and non-tumour tissue from patients representing 20 different cancer types, revealed a high likelihood for loss-of-function mutations occurring in the N-terminus of the HLA class I molecule, which abrogates transport of the peptide-HLA complex to the cell surface [46]. Furthermore, in all cancers tested, the most frequent mutations were found to occur in the α3 region of the HLA class I molecule, which is required for binding of the CD8 co-receptor on T-cells during an immune synapse for subsequent activation of the CD8- TCR complex [8].

4.3 Cancer Antigens and Epitopes: From Discovery to Therapeutic Application

Preclinical studies in the mouse model of human cancer, in particular melanoma, provided the first insights into cancer antigen discovery and functional characterisation, in the context of tumour rejection. Thierry Boon and colleagues had shown in the late 1980s that the tumor antigen P19A, heterologously expressed in mouse P815 tumour cells (isolated from DBA/2 mice bearing methylcholanthrene-induced sarcoma), contains an HLA class I epitope (within a 13-mer sequence harbouring a point mutation) capable of eliciting potent CTL responses and lysis of target cells [47].

Epitope mining in the human cancer setting was first performed using tumour tissue derived from human melanoma lesions, spearheaded by groups in Europe and the United States. Thierry Boon, Pierre Coulie and colleagues at the Ludwig Institute in Brussels, Belgium discovered the first tumour-associated antigen (TAA) in 1991, after in vitro characterisation of CTL responses using melanoma cell lines derived from an anonymous patient MZ2 who had metastatic disease [48]. This TAA, first annotated as MZ2-E and later renamed as melanoma-associated antigen 1 (MAGE-1, cancer testis antigen), was recognised by an autologous CTL line and induced lysis of the tumour cell line expressing the MAGE-1 DNA and restricted by HLA-A1 [48]. Further work with a cell line from the same patient led to the discovery of MZ2-F, or as it is known today, G antigen 1 (GAGE-1) [49]. Much of the ongoing work at the time focussed on discovering novel immunogenic HLA class I-restricted antigens that mediated CTL reactivity and lysis of melanoma cells from patients, with a strong interest to first understand and then to develop immune-based interventions; Melan-A (HLA-A2+ epitope) [50]; MAGE-3 (HLA-A1+ epitope)-specific CTL response in a patient vaccinated with MAGE-3.A1 peptide [51].

Simultaneous efforts by researchers in Europe and the United States revealed another important cancer antigen, the cancer testis antigen NY-ESO-1, which was discovered by serological analysis of expression cDNA libraries (SEREX) (indicating the presence of antibody responses), using cDNA prepared from human oesophageal squamous carcinoma cells [52]. NY-ESO-1 was later shown by Elke Jäger and co-workers (Frankfurt) to contain biologically functional CD8+ (HLA-A2/B51) and CD4+ (HLA-DRB*1) T-cell epitopes, based on seminal studies performed on human melanoma cells as well as transfected T2 cells as a model [53–56]. The afore-mentioned T2 cells harbour a defect in the transporter associated with antigen processing (TAP), which in turn inhibits them to present endogenous cytosolic cytosolic peptides (except for some leader peptide sequences loaded onto HLA-A2 molecules), but accommodates the introduction of exogenously added HLA class I epitopes for CTL recognition assays [57].

Steven Rosenberg and colleagues at the Surgery Branch, National Cancer Institute (NCI, National Institutes of Health (NIH), Bethesda, MD) made pivotal contributions to antigen discovery in human melanoma, in particular those that induce reactivity among TILs: the tyrosine related protein 1 (TRP-1) or gp75 restricted by the HLA-A31 molecule in 1995 [58]; HLA-A31-restricted

TRP-2 peptide LLPGGRPYR, which was a major target of TILs infused into a patient with metastatic melanoma who thereafter showed disease regression [58]; epitopes from TRP-1 and TRP-2 ($TRP_{197–205}$) restricted by HLA-A31 as well as HLA-A33 [59]; a mutated epitope derived from triosephosphate isomerase restricted by HLA-DR1 and recognised by CD4+ TIL and cell division cycle protein 27 homolog (CDC27) epitope restricted by HLA-DR4 [60, 61]. Collectively, these early efforts (over a span of 15 years, from the late 1980s to early 2000s) provided an excellent foundation which lead to the expansion of the field of targeted cancer immunotherapy.

A whole series of other molecules were identified to be associated with transformed cells. For instance, mesothelin was discovered as a marker of several important solid cancers, i.e. mesothelioma, ovarian cancer, pancreatic ductal adenocarcinoma based on serological (a murine 'Ki antibody' recognising human mesothelin) and genetic analyses [62–64]. Further exploration of the clinical significance of this molecule in ovarian cancer, mesothelioma and squamous cell carcinomas, and in conjunction with measurable mesothelin as well as antibody responses in sera of patients, indicated the immunogenic potential of mesothelin and its designation as a legitimate cancer antigen [65, 66]. An experimental immunotoxin developed based on the mesothelin-binding region of the K1 antibody was among the earliest attempted targeted immune-based interventions, with preclinical studies performed in a murine model of human carcinoma xenografts [67].

Work implemented in the later part of the 1990s placed a greater focus on studying mutated proteins in human cancer cells, and the possibility of discovering mutated antigenic determinants (neoepitopes) presented by HLA restricting elements, with biological and clinical relevance in therapy. An early example is a neoepitope derived from melanoma ubiquitous mutated 1 protein (MUM-1, initially named LB33-B, after the patient from whom the melanoma tumour was obtained, LB33 [68]), which is restricted by the HLA-B*44*02 allele. This 9-mer neoepitope was identified following in vitro cytotoxicity studies directed against the autologous melanoma cell line LB33-MEL.A-1; the same cytolytic activity was not seen with the wildtype peptide sequence [69]. A 10-mer neoepitope (amino acids 23–32) from mutated cyclin-dependent kinase 4 ($CDK4_{R24C}$) protein, restricted by HLA-A*0201, was also shown to mediate cytolytic activity by autologous CTLs in a dose-dependent manner, when exposed to T2 cells transfected with the $CDK4_{R24C}$ cDNA [70]. A caspase 8-derived mutated peptide restricted by HLA-B*3503, which showed potent cytolytic activity against the autologous head and neck cancer cells as well as tumour cDNA-transfected B-cell lines [71] further strengthened the field of neoepitope mining from human cancer cells.

A high-throughput analysis of whole genomic as well as exomic DNA from clinical tumor samples representing thirty different human cancers revealed the unique mutational burden in each cancer type, in addition to specific mutational signatures characterising these cancers [72]. Although this provides an elegant view of the general landscape of mutational burden in human cancers, the mutational signature in each patient varies—thus giving rise to a 'compendium' of private mutational signatures involved not only in driving and maintaining malignant transformation, but also in the activation and expansion of immune effector cells.

The mutated form of the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, or known as KRAS in short, is a well-established neoantigen implicated in the pathogenesis of pancreatic, colorectal and lung cancers [73–76]. Native KRAS was discovered in 1982 following gene sequencing of human lung adenocarcinomas, and is a guanine triphosphatase involved in cellular signal transduction [77]; however, mutations at positions 12, 13 and 16 are associated with oncogenesis, thus making it a proto-oncogene in humans.

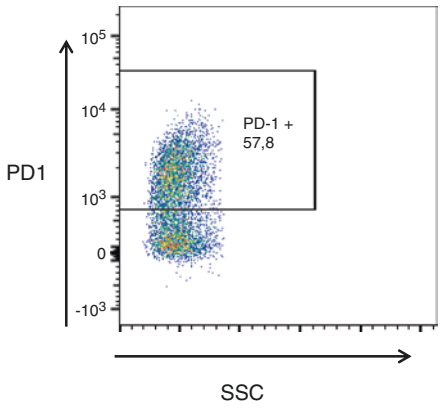
Steven Rosenberg and colleagues at the Surgery Branch, National Institutes of Health (Bethesda, MD) recently developed a cutting-edge approach to screen for neoepitope-specific T-cell responses for individual patients. This method has been termed the 'tandem minigene (TMG)' approach, which first requires whole-exome sequencing data of genomic DNA isolated from patients' tumor tissue samples. The sequencing data then yield all non-somatic mutations contained within gene-coding DNA of the patient. This allows for

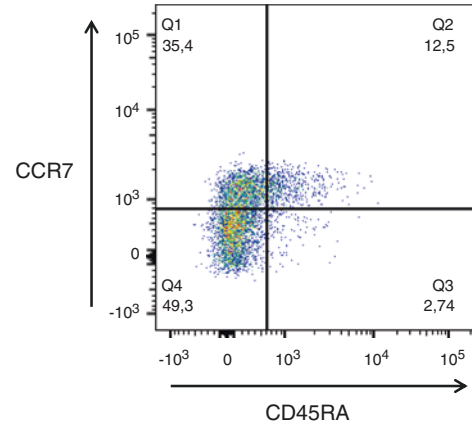
constructing a personalised library of the patient's 'private' mutations that potentially code for neoepitopes. These short gene sequences are then put together, an artificial construct, and inserted into an expression plasmid, which is subsequently transfected into a lentiviral vector for infection of APCs, i.e. dendritic cells (DCs) from a patient. Autologous TILs (from the same patient) are then co-cultured with the TMG-bearing DCs to allow induction of immune-reactivity. A positive response, represented by IFN-γ production by the TILs, would signal that the co-cultured DCs harbour a TMG that includes a neoepitope-encoding sequence(s) that is/are naturally processed and presented to the immune system [78].

Mutations may lead to different, not mutually exclusive effects on the responding T-cell population based on the prerequisite that a mutant epitope is indeed processed and presented to T-cells: (1) a T-cell may be newly recruited that would exclusively recognize the mutant sequence, (2) potential T-cell receptor (TCR) cross-reactivity between wildtype and mutant epitope sequences (if both, the wildtype and the mutant epitopes are being processed and presented to T-cells). It is biologically relevant whether there is already a T-cell population expanded that recognizes the wildtype epitope and then, following malignant transformation, recognizes the mtuant target, since this situation may lead to differential TCR triggering and subsequently to differential T-cell effector functions.

Mutant Epitopes as the 'Biological Scalpel' Against Cancer Cells Increasing immune effector functions by recruiting T-cells that recognise mutant epitope sequences is a clinically attractive attempt to improve and broaden the TCR repertoire directed against mutations that exclusively exist in malignancies and would therefore represent the 'ideal' cancer-associated antigen - a 'biological scalpel' that would only target cancer cells and not harm non-transformed cells. One method is to modify peptides at residues that do not interact with the nominal MHC restricting molecule, yet with the TCR contact residues: these variants are called 'heteroclitic' analogues and are able to trigger the nominal TCR with differential T-cell effector functions, e.g. cytotoxicity, quality and quantity of cytokine production, as well as proliferation [79]. This approach has been used to induce T-cells that react to wildtype peptides, e.g. to p53, yet are elicited with a variant peptide. If single mutations occur in epitopes, it could yield peptides that are naturally processed and presented by tumour cells to TCRs. What could potentially happen if a T-cell response, directed against a wildtype target, will also be able to able to react to the corresponding mutant target epitope, presented by the identical, nominal HLA-restricting element?. The following scenarios may occur, which have been described already in the early 1990s from several groups: In general, a single ligand specificity for each individual TCR appears to be rare. In contrast, the TCR recognition has been shown to be flexible, induced by altered peptide ligands, grouped into antagonists, partial agonists and superagonists. Mutations within peptide targets can induce differential phosphorylation of the TCR/CD3 complex with a differential downstream signalling pathway configuration [80]. Mutations may therefore—in case the wildtype peptide is also recognized—lead to abrogation of T-cell recognition simply because the ligand is not processed and presented. Alternatively, the T-cell ligand may well be processed and presented, but the T-cell signal may be abrogated potentially due to cellular anergy [29]. Partial agonists, i.e. by inducing a single amino acid residue, will still be able to stimulate the T-cells directed against the wildtype peptide, even across a similar dose range of the nominal epitopes: as the T-cells with the wildtype TCR ligand react with proliferation, cytokine production and cytotoxicity, peptide variants may either induce cytotoxicity and/or cytokine production, in the absence of T-cell proliferation [81, 82]. Of note, a similar observation may be true for TCRs directed against the mutant epitope that would react with a qualitatively and quantitatively differential T-cell reactivity pattern. Single amino acid exchanges may also turn T-cell clones from a Th1 into a Th2 cytokine production pattern, or lead to T-cell clones with abrogated cytokine production, yet strong cytotoxic T-cell responses as shown for viral pathogens [81–83]. Mutations in nominal targets, associated with differential signalling events, may also be crucial for the differentiation status of T-cells reacting to wildtype as well as to mutant targets, first described in preclinical models of thymocyte

differentiation and maturation [84, 85]. Differential triggering of the nominal TCR is associated with T-cell maturation and differentiation—a quality that is important for long-term immune memory, access to (tumor) tissue as well as for clinical efficacy of T-cell therapy. The passive transfer of immune cells directed to TAAs has been shown to be clinically relevant as the transfer of the T-cell product leads to the generation of central memory T-cells [86]. The biochemical signals that govern T-cell memory rely not only on the cytokine environment, yet also on the signal strength delivered by the TCRζ chain complex; the quality and quantity of T-cell responses including T-cell memory is strikingly shaped by the strength of the MHC/peptide–TCR interaction [87], which may in part be relevant for CD8+ T-cells. A decreasing potential model has been proposed, gauging the signal strength delivered by the target epitope to the corresponding TCR that is dictating whether the T-cell most likely enters the T-cell memory pool [88, 89]. This 'signal strength' model will need to take into account the locally produced cytokines and pro-inflammatory signals associated with moving T-cells into the diversity of the memory T-cell pool. In general, weaker TCR signals are sufficient in order to move T-cells into a memory T-cell program [90, 91]. Not mutually exclusive, the length of the TCR signalling (i.e. shortening the TCR stimulation) will also decide whether T-cells enter the memory T-cell pool [92, 93]. The observation that point mutations within peptides affect the contact with the nominal TCR also impairs CD8+ T-cell memory development, mediated in part by TCR-dependent NFκB signalling [94]. This may partly explain why T-cell clones targeting the identical (mutant) tumor epitope exist in heterogeneous differentiation states (see Fig. 4.2).





Clone ID	PD1+ TIL	CD45RA+ CCR7+	CD45RA - CCR7+	CD45RA - CCR7 -	CD45RA+ CCR7 -
A	57,8	12,5	35,4	49,3	2,74
B	4,94	47,5	2,1	38,6	11,8
C	0,9	1,91	5,1	79	14
D	30,6	2,45	0,22	89,5	7,8

Fig. 4.2 Different T-cell clones (A: VB5.1, B-D: VB9) recognize a naturally processed and presented (mutant) target on autologous pancreatic cancer cells. Note that the cancer - directed T-cell clones expresses different T-cell homing and differentiation markers, defined by CD45RA and CCR7 expression, i.e. CD45RA+CCR7+ T-cells are precursor T-cells, CD45RA-CCR7+ central memory T-cells, CD45RA-CCR7- T-cells memory effector T-cells and CD45RA+CCR7- T-cells represent terminally differentiated effector T-cells. Note that the majority of cells derived from clone B reside in the central memory T-cell subset that has been shown to be associated with increased responsiveness in the cellular therapy of cancer

The role of gamma-delta (γδ) T-cells in cancer is now also being revisited, due to their non-classical recognition of antigens. γδ T-cells recognise non-peptide structures, i.e. phosphoantigens such as derivatives of the eukaryotic isoprenoid (mevalonate) pathway presented by the CD1d molecule [95]. The Vγ9Vδ2 (Vδ2+) subset of γδ T-cells, which are found in peripheral blood. They have also been described to express the CXCR3 surface marker which is crucial for tissue penetration, an important feature in accessing transformed cells or tissue-residing pathogens. An interesting feature of Vδ2+ γδ T-cells is that they express the CD16 co-receptor, which can bind to FcγRIII present on tumour cells in addition to the killer receptor NKG2A [96]. Thus, like NK cells, γδ T-cells can also orchestrate antibody-dependent cellular cytotoxicity (ADCC), which is implicated in the therapeutic activity of several monoclonal antibody-based cancer drugs, i.e. rituximab, trastuzumab, ofatumumab and alemtuzumab [97–99]. An intermediate of the isoprenoid pathway, isopentenyl pyrophosphate (IPP), is strongly recognised by Vγ9Vδ2 T-cells, as shown in the context of zoledronic acid-treated human cancer cells [100]. Zoledronic acid induces accumulation of IPP in cancer cells, thus stimulating the activity of Vγ9Vδ2 T-cells, subsequently promoting the production of IFN-γ as well as cytotoxic molecules. This effect can be further enhanced in the presence of IL-2 and/or IL-15 conditioning. Although altered/mutated forms of IPP are yet to be reported, the significance of γδ T-cells in targeted cellular therapy should be explored further. There have also been reports of the recognition and killing of overexpressed human heat shock protein 60/70 on cancer cells by γδ T-cells, indicating that the overall T-cell repertoire in human which recognizes tumor antigens is rather generous [101, 102].

4.4 Clinical Significance of Neoepitope-specific Immune Responses

The clinical value of neoantigen-specific responses is most evident in immune checkpoint blockade therapy. Case reports of patients with melanoma or non-small cell lung cancer treated with anti-PD-1 and anti-CTLA-4 monoclonal antibodies showed that the repertoire of neoantigen-directed CD8+ T-cell responses (based on the diversity of TCRs recognizing mutated peptides) is is associated with clinical responses [103–108]. The most relevant examples are the T-cell responses from patients with metastatic melanoma or non-small cell lung cancer NSCLC, whereby the number of PD-1+ circulating T-cells directed against neoepitopes (visualised by flow cytometry) associates with clinical outcome in patients [105, 109, 110]. Furthermore, more recent clinical observations indicate that neoepitope-specific immune responses in peripheral blood can be used as a prognostic marker for several solid cancers [107, 110, 111].

Tissue scarring, arising from inflammatory processes processes, associated with infection(s), may lead to to genetic aberrations, which in time may perpetrate oncogenesis. Observations in patients with lung adenocarcinomas who had previously contracted *M. tuberculosis* infection in the lung showed that immune responses to mycobacterial antigens ('old' tuberculosis (TB) lesions) caused mutational changes to the gene encoding epidermal growth factor receptor (EGFR), in association with cancer development [112]. More strikingly, these patients had a worse 1-year survival prognosis compared to those who did not have 'old' TB lesions in the lung at cancer diagnosis. Patients presenting with 'old TB lesions' and adenocarcinomas in the same lung did not harbour the $EGFR_{L858R}$ mutation (occurring in exon 21 of the EGFR gene, which encodes the intracellular tyrosine kinase domain of the receptor), which is implicated in positive clinical outcomes in patients with lung cancer who are treated with the tyrosine kinase inhibitor gefitinib [113]. The $EGFR_{L858R}$ mutation has been shown to give rise to neoepitopes that induce antibody responses in patients with NSCLC who received gefitinib therapy [114]. Another EGFR-associated mutation, $EGFR_{T790M}$, which is found in approximately 60% of patients with NSCLC, yields HLA-A2-restricted neoepitopes that are linked to favourable anti-tumor immune responses that could be implemented for designing better immunotherapies [115, 116].

The agonistic activity of peptides, namely their ability to stimulate T-cell activation can in fact shape the cellular immune response milieu due to mutational changes in their molecular structure. Paul Allen and co-workers had elegantly shown in the mid-1990s that peptide analogues of staphylococcal enterotoxin A, derived from haemoglobin, can abrogate the effector functions while inhibiting the proliferation of T helper cells (CD4+ T-cells with a Th1 or Th2 phenotype) [117]. While some mutations in the haemoglobin peptides inhibited T-cell proliferation, other mutations did not have a deleterious effect on the T-cell. Further research showed that partial phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM), which forms an indispensable component of the intracellular TCR zeta (ζ) chain, can either totally abrogate or even lead to T-cell death during an immune synapse [118]. Importantly, this phenomenon can be due to the binding of TCR with HLA molecules presenting mutated peptides, and more importantly, the nature of the mutation itself and the very position of the mutation within the epitope sequence. It is undeniable that the local inflammatory milieu in cancer lesions (such as those described in chronic infections [119]) may also contribute to chromosomal aberrations resulting in strong downregulation or loss of the TCRζ chain. These seminal findings were first reported in a preclinical murine model of colon carcinoma and later in TILs from patients with renal cell carcinoma and peripheral blood T-cells from patients with non-Hodgkin's lymphoma [120–122].

Preclinical studies of infectious disease models may provide an insight into TCR repertoire shaping in relation to neoepitope-specific immune responses. Analyses of splenic and bronchoalveolar lavage fluid-derived T-cells from mice primed with a wildtype strain of influenza A virus (HK/PR8) by intraperitoneal infection showed that animals' CD8 TCRVβ repertoire was shaped by primary viral challenge to efficiently recognize and respond to a secondary challenge with another wildtype strain but not a *mutated* version of either virus (HK/PR8-NPN3A) [123]. Also, while challenge with a wildtype virus strain provided a broader TCRVβ repertoire, the mutant strain of the virus induces a more focussed and narrow antigen-specific T-cell compartment, with subtle TCR re-arrangement patterns. Furthermore, an immunogenic epitope from the wildtype virus (NP_{366}, ASNENMETM) induced a measurable CD8+ T-cell response among mice primed and re-challenged with a mutated viral strain. Conversely, the mutated version of the NP_{366} epitope, harbouring only a single amino acid change ($NPN3A_{366}$, ASAENMETM), did not promote strong binding between MHC and TCR among T-cells from mice challenged with a wildtype virus, exhibiting a high 'off-rate' (large percentage of mutated epitope-bearing tetramers dissociating from the TCR within minimal time), requiring greater dependence on the CD8 co-receptor binding to MHC to elicit an immune response. This is of relevance to immune responses in cancer; T-cell reactivity to neoepitopes may be subdued owing to poor binding kinetics between the HLA-restricting element and the TCR. However, vaccination with a broader array of personalised neoepitopes may help prime the immune system to either re-awaken the smaller populations of central memory T-cell that are tumor-reactive, or not mutually exclusive, generate a fresh pool of (as yet not activated) antigen-specific T-cells. [124].

4.5 Harnessing Basic Immunology to Improve Clinical Immunotherapeutic Approaches

The effect of gut commensal bacteria on shaping (and re-shaping) immune responses in health and disease has been at the heart of current immunological research. It was recently shown that induction of T-cell responses to select, 'immunogenic' intestinal bacteria (*Bacteroides thetaiotaomicron* and *B. fragilis*) driven by anti-CTLA-4 therapy correlates with clinically beneficial outcomes in patients with metastatic melanoma [125]. In a murine model highly susceptible to tumors, the introduction of *Bifidobacterium sp.* notably improved cytotoxic lymphocyte-dependent control of tumor burden [126]. Combination of *Bifidobacterium* inoculation and anti-PD-L1 monoclonal antibody administration further

enhanced tumor control in these animals, thereby underlining the critical role of gut microbiota in dictating anti-cancer immune responses. Thus, supplementing biologically active material from intestinal bacteria with immune blockade therapy or T-cell immunotherapy may potentially improve neoantigen-specific immune responses in patients with advanced cancer.

Small molecules and cytokines that target the activation of fatty acid oxidation (FAO) in CD8+ T-cells and promote maintenance of cellular memory can be used as an adjunct to mainstream therapeutic regimens in cancer and infectious diseases. For example, the antidiabetic drug metformin activates 5′ adenosine monophosphate-activated protein kinase (AMPK) and improves FAO in memory CD8+ TILs, as shown in a proof-of-concept study in a murine model of chemically induced skin cancer [127]. IL-15 also promotes lipid metabolism by upregulating mitochondrial biogenesis and inducing the expression of carnitine palmitoyl transferase, an enzyme that is critical for mitochondrial beta-oxidation [128]. This process has been shown to be upregulated in memory CD8+ T-cells in mice, and enhances their survival. In cancer therapy, IL-15 has already been evaluated as an instrumental adjuvant with pronounced effects on proliferation of TIL and enhanced cytotoxic activity of tumour-antigen specific T-cells [129–134].

Although immunological tolerance of T-cells is necessary to prevent overt pathology, increased numbers of regulatory T-cells (Tregs) have significant implications for the success of cell-based immunotherapies. While infusion of mesenchymal stromal cells for downregulation of severe inflammation requires subsequent TGF-β production and Treg activation [135], T-cell products reinfused into patients with cancer are allowed to contain only minimal Treg populations in order to optimise anti-tumor activity mediated by cancer-specific T-cells [136]. In addition, IL-17 production in response to chronic inflammation in the tumor microenvironment can induce TGF-β production and suppression of CD8+ T-cell responses [27]. Tregs could also be stimulated by TAAs, i.e. NY-ESO-$1_{157–170}$-specific Treg responses in patients with melanoma given the NY-ESO-1/ISCOMATRIX™ therapeutic vaccine [137]; NY-ESO-$1_{119–143}$ and TRAG-$3_{34–48}$ (derived from another cancer testis antigen, Cancer/Testis Antigen Family 24) can induce the expansion of both Th1 cells and FoxP3+ Tregs in patients with melanoma [138], and therefore contribute to immune evasion. More research is needed to better understand whether certain mutations would represent the nominal epitopes for Tregs directed specifically against cancer mutations.

New information arising from basic research needs to be considered for inclusion into preclinical (pre-GMP) evaluation of T-cell products. For example, analysis of BTB Domain and CNC Homolog 2 (BACH2), a transcription factor that promotes the generation and maintenance of regulatory as well as central memory T-cells in the host while repressing immune effector mechanisms could be a useful tool in characterising the T-cell populations which may persist in the patient to fight transformed cells [139]. Mice lacking BACH2 were able to mount a strong T-cell response in the tumor microenvironment (marked by CD4+ and CD8+ T-cell proliferation and IFN-γ production), concomitant with reduced numbers of FoxP3+ regulatory T-cells, which subsequently allowed for allowed for improved tumor control. BACH2 deficiency also increased gene transcription of cytotoxic molecules, i.e. granzymes and perforin. From a translational viewpoint, this finding has direct implications for enhancing the T-cell-mediated anti-tumor effect in targeted cellular immunotherapy. Regulating the expression of BACH2 in neoantigen-reactive T-cells in diseased tissue, i.e. TILs as well as peripheral blood T-cells may improve the quality and efficacy of immune cells for exploitation in clinical therapy [140].

Exploiting novel technology platforms to screen for TCR specificities in diseased tissue, i.e. deep (TCR) sequencing, peptide microarrays, cellular microarrays and TCR-epitope docking studies are contributing substantially to our current knowledge of disease mechanisms and immune dynamics. This may allow to characterise in greater detail the immune repertoires crucial for orchestrating long-term immunological protection

against cancer. Combining mathematical knowledge and biological understanding of cancer dynamics and tumor development algorithms will benefit this field greatly. Furthermore, implementing comparative studies using these techniques with clinical samples from various anatomical sites of healthy individuals and patients will deliver new information for the development of next-generation biotherapeutics. The differential TCR repertoires in TIL versus PBMCs, in addition to the mutational load in a patient with cancer relate to the success of checkpoint inhibitors. This is evident in patients with metastatic melanoma and NSCLC, who have among the highest mutational burden and respond well to anti-PD-1 and/or anti-CTLA-4 therapy [3, 103–105, 107]. 'Mining' biologically and clinically relevant TCRs targeting cancer mutations may lead to the generation of T-cell products for therapy by cloning and transferring specific TCRs to PBMCs; at present a viable and pursuable platform, as already shown in a patient with metastatic colorectal cancer [86, 141, 142]. These examples underline the importance of a topic that has been discussed for decades, e.g. in the field of cellulary immune responses directed against HIV: How much focus and how much diversity should a tailored immune response directed against mutant epitopes afford? How much diversity, with regard to focus on single epitopes, is biologically and clinically relevant taking into account the (1) similarity of mutations in primary tumors versus relapse, (2) the mutational diversity displayed by the primary tumour and distant metastasis as well as (3) the 'local' imprint of gene expression (bearing in mind that not all gene-encoding DNA may at all, or at some points be translated into RNA and then subsequently into protein), associated with the tissue environment (e.g. lung versus liver-metastases). These questions will represent a matter of clinically relevant research with impact on the design of biologically and clinically relevant studies. For instance, an educated decision will take into account the similarity and dissimilarity of the primary tumor versus the corresponding relapsed malignancy, if TIL would be immediately available from the primary tumor upon clinical detection of a relapse.

The humoral immune response to cancer antigens, and thus its significance in mediating clinically relevant and beneficial anti-tumour responses in patients calls for greater emphasis [114, 143–145]. Along these lines, peptide microarray studies possess the sensitivity and specificity to discover naturally presented epitopes recognised by circulating antibodies in serum as well as those derived from patients' B cells in culture. In serum derived from patients with cancer, disease-associated epitopes may include those belonging to neoantigens, and can be screened for using the high-content peptide microarray (HCPM) platform. The HCPM is a novel technology used for profiling antibodies in many research areas, which has been more recently been developed, including our research group, in order to visualize an unbiased view of serum reactivity to a wide range of epitopes. This sophisticated technology allows to display on each individual microarray slide 2.9 million peptide sequences (spots), corresponding to unique epitopes. Using only a small sample volume (i.e. 4 μL of biological fluid per slide), it is possible to identify immune-recognition patterns associated with relevant endpoints on a HCPM microarray chip containing the whole human proteome, at the highly detailed level of 16-mer peptides. Well-documented experience in the use of the HCPM platform with regard to chip design, pre-processing and methods of analysis as well as techniques [146–151], and different applications of HCPM in various clinical settings, i.e. bacterial infections [149, 152, 153], viral infections [154–156], sarcoidosis [157] and pertussis [151] (further references for readers: 146–157) strongly suggests that this platform is also able to pick up very specific serum reactivities directed against mutant versus wildtype target molecules. This technique can also be used to detect humoral immune responses to 'private' neoepitopes in the peripheral blood of patients with cancer [158]. Results from HCPM studies can contribute to developing novel antibody-based therapies including the identification of novel, clinically relevant cancer - associated targets for CARs, or augment cellular immune responses directed against (intracellular) mutant

antigens via ADCC. On the other hand, B cell-dependent immune responses in disease may aid to modulate a T-cell driven 'immunopathological' milieu, such as that observed in patients with post-transplantation lymphoproliferative disease (PTLD) [159]. The early studies and identification of cancer-associated antigens supported the hypothesis that strong B-cell responses point to the existence of strong anti-cancer T-cell responses in patients with cancer. For instance, the cancer testis antigens MAGE or NY-ESO-1, which are clinically relevant T-cell targets, were identified via B-cell responses and are currently used in clinical protocols to induce disease-modifying T-cell responses targeting NY-ESO-1+ cancer lesions [132, 160–164].

Where Could Anti-Mutation-Reactive T-cells be Harvested? Immune cells from peripheral blood express tissue-specific homing markers of their surface (e.g. VLA-4 for the central nervous system, CXCR3 for the lung, CCR6 for the gut) that can be instrumental in gauging circulating T-cells among PBMCs that are travelling either *from* or *to* the respective target organ [165]. Enrichment, e.g. for VLA-4+ T-cells, will result in selecting T-cells trafficking to and from the tumor lesions in the patient, which can then be tested for their recognition of neoepitopes and potential immunoreactivity targeting transformed cells. Furthermore, some of the epitopes recognised in target organs overlap with the recognition patterns observed in PBMCs, while others do not and are either exclusively recognised in PBMCs or TILs [109]. In line with this, 15 out of 20 HLA-A2+ patients with breast cancer whose tumor and blood samples were analyzed were shown to harbor 18 TCR specificities shared between TILs and PBMC-derived T-cells [166]. Sim et al. reported in 2016 that the complementarity determining region (CDR) 3 of the TCR, the portion of the complex which binds to the HLA-peptide complex on targets cells, is greatly diverse between PBMCs and TILs among patients with glioma [167]. Importantly, this research consortium also found a unique TCR signature present in peripheral blood of the patients exhibiting a minimally divergent TIL TCR repertoire concomitant with low-grade glioma, while patients with glioblastoma showed a wider selection of TCRs. We have also noticed this among patients with glioblastoma, where some of the somatic mutations are recognized by PBMCs but not TILs, and vice versa (Liu et al., unpublished data). Thus, information arising from such studies is already translated into clinical products for patients with advanced cancer, i.e. genetically transferring the TCR repertoire associated with better prognosis into PBMCs, for re-infusion as adjunctive therapy, given the feasibility of using advanced gene transfer technologies [141, 142, 168]. Dr. Rosenberg's group at the NIH has in fact treated patients with advanced cancer harbouring particular mutations using autologous T-cell products expressing specific TCRs directed against neoepitopes. Pivotal examples include the treatment of a patient with metastatic cholangiocarcinoma with CD4+ IFN-γ+ TILs reognizing an HLA-DQ*06-restricted neoepitope derived from the receptor tyrosine-protein kinase erbB-2 (ERBB2), or HER-2 interacting protein (ERBB2IP) [169], and a patient with metastatic colorectal cancer, who received a TCR-transferred T-cell product specific for the $KRAS_{G12D}$ mutation (HLA-C*08*02-restricted) driver mutation, with subsequent regression of metastases expressing the $KRAS_{G12D}$ mutation [86]. The latter strategy was initiated by a TMG screen performed on TILs isolated from tumour tissue samples obtained from 10 patients with metastatic gastrointestinal cancers, in the quest to detect neoepitope-specific reactivity [170]. Patients with the HLA-C*0802 allele had TIL responses to their tumor cells (directed to KRAS) underlining that the restricting HLA element in the patient may limit the therapeutic targeting of KRAS, as other (KRAS) mutations may not be visible to the cellular immune system, since these mutations may not be naturally processed and ultimately be presented to the patients cellular immune repertoire.

Identification and verification of clinically and biologically relevant neoepitopes remains a challenge, i.e. whether the epitopes are expressed in a representative fashion, whether they are processed and presented on tumour cells, and whether a 'fit' TCR repertoire is available and capable of reacting to it—leading to immune effector functions that

will most likely facilitate a strong and long-lasting anti-tumor immune response. A possible way is to design and generate potential neoepitopes based on whole genome sequences from patients with cancer. Once these neoepitopes are chemically synthesised, they could be submitted for large-scale in vitro screening of T-cell cultures to carefully select for high quality, mutation-specific T-cells that could be expanded and reinfused into patients. In a non-mutually exclusive fashion, the TCRs exclusively targeting mutant TAAs, and not the wildtype peptide sequence, may be cloned and subsequently transferred into recipient target cells (T-cells) for the active cellular therapy of patients with cancer [86, 103]. PBMCs may serve as a very good starting point to screen for populations of neoepitope-specific T-cells circulating (and re-circulating) in the patient. The fact that peripheral T-cells are able to recognise mutant epitopes/neoepitopes has been heralded by the Rosenberg group, pointing to PD-1+ T-cell populations that are enriched for tumor neoantigen-specific T-cells that rather reflect the repertoire of antigen-experienced and not only 'exhausted' T-cells [109, 171]. This echoes earlier findings in TIL from patients with metastatic melanoma: PD1+ TIL recognize 'private' mutations presented by tumor cells. However, the heterogeneity of cancer lesions needs to be further investigated concerning the anatomy of the 'diversity of mutanomes' and the diversity of the corresponding immune effector cells that could be harvested from individual cancer lesions [140]. An essential point for TCR transfer targeting commonly shared or private mutations is that the HLA-restriction element presenting cancer epitopes differs from individual to individual. Thus, not all patients would benefit from a single TCR-HLA-matched T-cell product/TCR transfer but rather a more personalized approach, taking into account the neoepitope structure, the corresponding TCR sequence(s) as well as the HLA-restricting element. An illustrative example in this regard is the occurrence of PTLD associated with Epstein–Barr virus (EBV)-induced inflammatory T-cell responses [159]. Individuals with an HLA- A2, A11, B5, B18, B21, Bw22 and B35 background suffer a greater risk of PTLD onset following solid organ transplantation compared to those an HLA-A03 or HLA-DR7 (CD4+ T helper cell response) genetic background, while individuals with an HLA-A1, B8 or DR8 appeared to be protected against PTLD [172–174]. Careful selection of specific TCRs and HLA restriction is also being pursued in targeted immunotherapy of patients with hepatitis B virus (HBV)-induced hepatocellular carcinoma, with a focus of HBV-specific epitopes and potentially, neoepitopes [175]. Ton Schumacher's research group at the Netherlands Cancer Institute in Amsterdam recently showed that since naturally occurring neoepitope-specific T-cell responses may be silenced in patients with cancer and that T-cells isolated from peripheral blood from healthy individuals may contain neoepitope-recognising TCRs [176], the latter option may represent the starting point of generating anti-mutant epitope reactive T-cells. Five different HLA-A2-restricted neoepitopes were recognized by CTL lines, established from PBMCs obtained from different healthy donors, and produced IFN-γ and/or CD107a in response to patient-derived melanoma cell lines that expresses the cognate (mutant) T-cell epitope. Also, T-cells which recognized the neoepitopes as well as the tumor cells expressed epitopes which exhibited strong peptide-HLA binding kinetics (half-life of MHC-class I–β_2 microglobulin–epitope interaction) Specific TCRs could then be cloned and heterologously expressed in T-cells from an individual who is in need of an 'improved' TCR repertoire tailored to target mutation-specific T-cells. This has been shown in PBMCs from patients with cancer using T-cells from MHC-matched donors [176] and has been shown to be clinically feasible in the context of infectious diseases, i.e. by transferring CMV-specific T-cells from an allogeneic-donor matched for the HLA class I-restricting allele, to patients with CMV infection (and a non-functional immune system) after allogeneic hematopoietic stem cell transplantation [177, 178]. Similar observations have been generated in our laboratory using HLA class I-matched TIL (see Table 4.2), that have been shown to react—i.e. without restimulation and expansion—to mutant target epitopes that are reognized on autologous tumor cells from an HLA-B*2705 matched patient, suggesting that precursor T-cells exist, even in TIL, following exposure to epitope arising from 'driver' mutations, i.e. KRAS, SMAD4, p53 or even commonly shared mutations among individuals [1, 86, 179].

Table 4.2 Shared recognition of mutant epitopes between MHC class I matched TIL from different patients

Gene ID	Wildtype sequence from GBM-alpha	IFN-γ wildtype (GBM-alpha TILs)	IFN-γ mutant (GBM-alpha TILs)	IFN-γ wildtype (GBM-beta TILs)	IFN-γ mutant (GBM-beta TILs)	Mutated sequence from GBM-alpha
TUBB8	ALYDICSKTLKLPTP	193.09	570.95	4494.32	2791.76	ALYDICSRTLKLPTP
LCE1F	SSGGCCGSSSGGCCS		63.38			SSGGCCGSSSGGCCS
GOLGA6L1	REDAGAGGEDVGAGG		88.74			REDAGAGEEDVGAGG
GOLGA6L2	IREQEEMLREQEAQR	105.48	754.23	2568.97	1826.17	IREQEEMIREQEAQR
LOC645752	PPTWSGRRAPGDRDN		270.84		390.47	PPTWSGRHAPGDRDN
DSPP	QFLIPTSLSVSSNSV		30.48			QFLIPTSFSVSSNSV

TIL were harvested after IL-2, IL-15 and IL-21 driven expansion from patients GBM alpha and GBM beta and epitopes were identified from the tumor from patient GBM alpha. TIL from patient GBM alpha were tested for recognition, defined by IFN gamma production, directed against the patient's own mutations (and corresponding wildtype sequences). The epitope mutant PPTWWSGRHAPGDRDN is exclusively recognized by TIL from patients GBM alpha—and not the wildtype sequence: a similar situation for TIL from patient GBMbeta that recognize the identical mutation which is restricted by HLA-B*2705. Numbers are picrogram (pg) cytokine production in 1×10^5 TIL.

Preparing the Ground: Enhancing the TCR Repertoire to Mutant Targets The activation of the innate immune compartment using standard anti-cancer drugs, i.e. gemcitabine, decitabine, cisplatin and doxorubicin can prompt the activation of APCs such as macrophages and dendritic cells, and facilitate antigen-processing and orchestrating pro-inflammatory immune response, i.e. IL-12 production [180]. In addition, chemoimmunotherapy with decitabine has also been shown to induce potent anti-cancer cytotoxic responses mediated by CD8+ T-cells [181], while its use in patients with pancreatic cancer along with cytokine-activated killer cells leads to improved progression-free survival compared to chemotherapy alone [182]. Increase in neoantigen-specific cellular immune responses following adjuvant cancer therapy has not been explored in depth, yet could be the focus of future evaluations - directed towards enhancing the 'visibility' of the patients' mutanome to their immune repertoire. Several approaches could be explored, which is beyond the scope of this overview. A strong argument that anti-cancer immune responses can be increased with 'standard therapeutic manipulations' is the observation that in patients with brain metastases who underwent whole-brain radiation therapy (WBRT), immune responses were activated that resulted in tumor regression at distant lesions [183]. Patients with advanced melanoma-related brain metastases appear to have a greater survival advantage when they are treated with ipilimumab (anti-CTLA-4) in addition to WBRT [184]. Along these lines, preclinical evaluation of radiotherapy-induced CTL responses have shown that the mutational landscape shifts alongside modifications in HLA class I antigen processing and presentation, subsequently concomitant with enhanced control of the tumor burden [185]. In general terms, ionising radiation promotes immune stimulatory events such as (1) increased HLA molecule expression, MHC-I expression, (2) generation of specific peptides involved in cytotoxic T-cell recognition and (3) promotion of cytotoxic T-cell activity by the release of tumor associated antigens [186, 187]. The latter processes lead to the activation and trafficking of effector cells promoting in situ cellular immune responses, although distant (abscopal) responses may also occur [186–190]. In this particular context, dose and fractionation seem to play a determining role for eliciting anti-tumor immunological effects. The preclinical studies of the Demaria group on breast- and colon cancer models showed a clear indications of anti-tumor T-cell responses when combining local fractionated radiation schedules (8gy × 3, 6gy × 5) in addition to CTLA-4 blockade [186, 187, 191, 192]. Other preclinical/clinical studies lend support to the relevance of such combined approaches [186, 187,

193]. Considering the inhibitory effects of monoclonal antibodies on CTLA-4 and PD-1-modulated immune suppressive actions taking place in metastatic non-small cell lung cancer, melanoma and renal cancer, further studies aiming to identify the potential adjunctive effects of radiation on local and distant sites are certainly necessary [186]. Postow et al. reported a case of a patient diagnosed with metastatic melanoma treated with ipilimumab; at a stage, the patient underwent hypofractionated radiotherapy to treat a paraspinal metastasis; the patient was treated with 28.5 Gy delivered in three fractions over a period of 7 days. Four to five months later, the paraspinal mass and a group of previously identified distant hilar lymphadenopathies and splenic lesions (not targeted by radiation) had considerably diminished. CT-imaging 10 months after treatment still showed a stable condition [194]. Reproducing these latter described effects in a larger group of patients requires consideratino of many biological and clinical variables, among others mathematical models able to provide qualitative and quantitative predictive data on radiation-induced immune responses. Image analysis that may even reflect the 'mutational load' and T-cell infiltration may also provide a prognostic tool, yet also an instrument to gauge how 'focused' and narrow or broad a T-cell response directed against mutated antigens should be (see Fig. 4.3) in order to provide increased survival.

Although (limited) data suggests that a very focused immune recognition is clinically more favourable (as defined by survival), more basic and clinical research needs to be undertaken to visualize a link between mutational load, the number of targets recognized, the possibility to tailor the T-cell graft targeting mutations and, subsequently, clinical responsiveness. Thus, it is plausible that activation of neoepitope-specific T-cells in patients after a combination of radiotherapy and immune checkpoint blockade therapy is key to improved clinical outcomes, and thus warrants further exploration in well-controlled clinical settings. Full-scale analyses of surface tissue-homing markers as well as memory markers on neoantigen-reactive T-cells, the relationship between these readouts and the anatomical locations of metastasis in the patient as well as the functionality of the T-cells are of paramount importance to enrich our understanding of targeted cellular immunotherapies in cancer [195]. These immunological analyses can be performed on clinical samples obtained from patients undergoing therapy, i.e. peripheral blood drawn at various time points during 'standard' or immunological treatment strategies. Another major conundrum in further optimising targeted T-cell-based therapies lies in the lack of our understanding of antigen processing and presentation, i.e. which epitopes are naturally presented, what is their respective tissue expression pattern, their corresponding HLA restriction and their capacity to drive antigen-specific T-cell responses, based on the TCR repertoire capable of reacting to individual mutant target epitopes. Further research into the 'immunological fitness' of antigen-specific T-cell populations may impact on the quality of cell-based therapies and further aid to tailor T-cell products. For instance, recent advances in T-cell therapy for cancer, viral infections and autoimmune diseases highlight the broad therapeutic potential of T-cell engineering. Even as site-specific genetic manipulation in primary human T-cells remains challenging, they hold great clinical promise to tailor T-cell products, e.g. genome editing in T-cells using the CRISPR and TALEN approaches [196] along with the detailed analysis of asymmetric T-cell division in order to better understand and define the quality of mutational epitopes that would give rise to immediate immune effector cells, as well as to long-term memory T-cells [197]. New and clinically relevant insights as to how exposures to pathogens and 'environmental factors' may impact on TCR repertoires and ultimately disease susceptibility [198] will aid to decipher the molecular 'decision-making process' in adaptive cellular immune responses targeting mutant epitopes in patients with cancer [199, 200] and provide the necessary tools to enhance treatment decisions to offer more effective, multi-layered and long-term cellular immune responses for patients with malignancies.

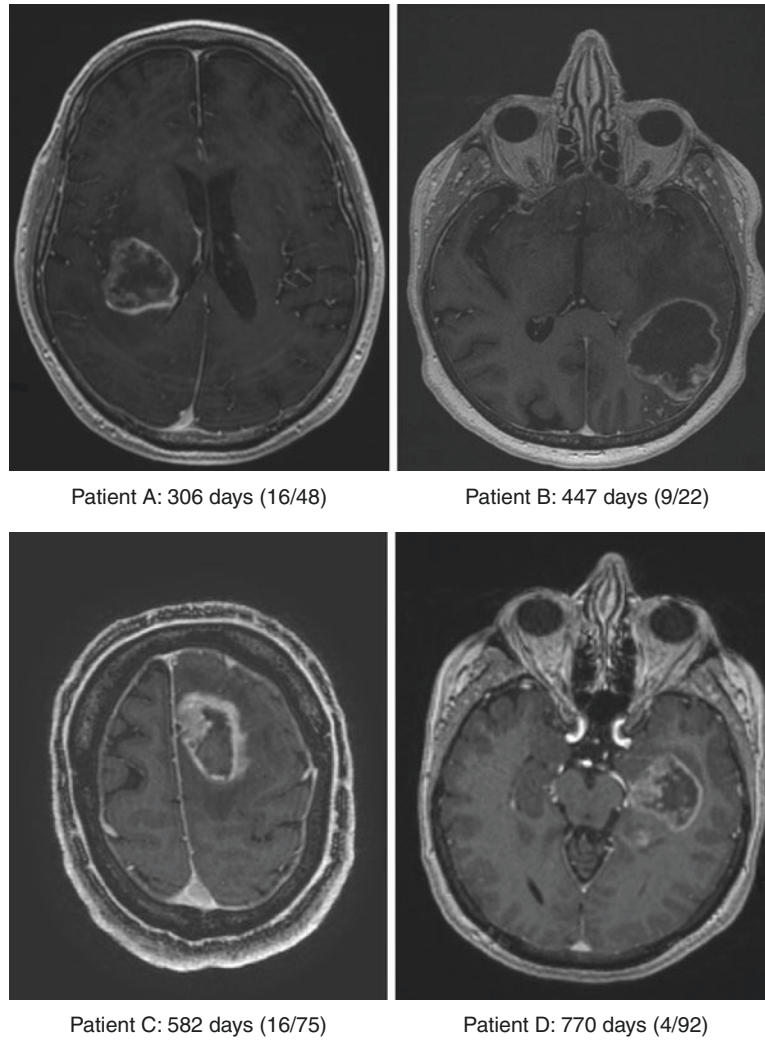


Fig. 4.3 MRI scans of 4 patients with glioma (grade 4, WHO classification) with (i) survival time in days after surgery and (ii) the number of mutant epitopes recognized by the patients' TIL defined by IFN-gamma production in relation to the total number of mutations recognized. A focused TIL response appears to be associated with increased survival, an observation that needs to be followed up in larger studies

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The Secrets of T Cell Polarization

5

Thaiz Rivera Vargas and Lionel Apetoh

Contents

5.1 Introduction

Genetic mutations can trigger cell transformation, possibly leading to the development of a tumor. However, the immune system, as initially postulated by Burnet in 1957, usually prevents the formation of clinically detectable tumors. Actors involved in the so-called immunosurveillance process, which is discussed elsewhere in this guide, include innate effectors and innate molecules such as gamma delta T-cells [1], natural killer cells (NK) [2], dendritic cells (DCs) [3], natural killer T-cells (NKT) [4], TRAIL [5], perforin/granzyme [6] as well as members of adaptive immune responses (B- and T-cells) [7]. Accordingly, mice lacking the recombination-activating gene 2 (RAG2), which is essential for B and T cell generation, are more prone to develop spontaneous and chemically induced tumors compared to wild-type mice. T cells, which are essential for clearing viral, protozoan, and intracellular bacterial infections [8], are

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indeed key actors in the immunosurveillance of cancer. In preclinical cancer models, antibodies against CD8 were shown to effectively block the spontaneous rejection of transplantable tumors [9]. Subsequently, adaptive immune responses were demonstrated to be crucial to limit growth of mutagen-induced spontaneous tumors [7, 10]. Interestingly, depletion of CD4 and CD8 T cells during the equilibrium phase of cancer growth, where cancer cells persist but are kept in check by the immune system [11], reinstates cancer progression, underscoring the importance of T cells in controlling cancer growth over long time periods [12].

T cells have also been shown to be essential effector cells in the context of anticancer therapies. We and others have shown that the anticancer efficacy of various anticancer agents relies on their ability to not only kill cancer cells but also induce T cell-dependent anticancer responses [13–17]. Thus, an interesting feature of anticancer treatments is their potential ability to induce anticancer immune responses targeted to tumor antigens. Indeed, while radiotherapy and chemotherapy regimens induce potent direct tumor cell death, the release of cellular components from dying cells such as "damage-associated molecular patterns" [18] has previously been shown to elicit T cell dependent immune responses and contribute to tumor elimination [19–21]. The induction of T cell responses relies on a close cooperation between innate and adaptive immune cells. First, the capture of tumor antigens by dendritic cells (DC) is indispensable for the subsequent antigen processing in late endosomes by DC. Second, upon activation DCs migrate from tumor beds to draining lymph nodes and become fully mature [22]. This then results in the presentation of antigenic peptides on DC major histocompatibility (MHC) class I and II molecules to antigen specific CD4 and CD8 T-cells and triggers their activation (signal 1). The recognition of co-stimulatory molecules (signal 2) and the production of cytokines by mature DC (signal 3) lead to the polarization and full activation of T cells [23, 24]. Finally, DC drive the expression of chemokine receptors on T cells, thereby enabling their migration to the tumor bed, leading to tumor eradication [25, 26].

One of the ways to categorize T cell subsets is to study their expression of CD62L and CCR7 [27, 28]. T cells strongly expressing these two markers home to secondary lymphoid organs and possibly become activated after encountering DC. Naive T cells (T_N), which have not yet been activated by antigen, belong to this category as well as two other T cell subsets that have already met their antigen, central memory cells (T_{CM}), and T memory stem cells (T_{SCM}). These cells preferentially locate in lymphoid organs and feature strong proliferative abilities. By contrast, other T cell subsets, including effector memory (T_{EM}) and effector T cells (T_{EFF}) locate in peripheral tissues where they execute their effector functions. These cells, which have strongly downregulated CD62L and CCR7 expression, harbor potent and rapid secretion of proinflammatory cytokines following activation and can exhibit cytotoxic activity against antigen-expressing targets. The role of these different cell subsets in a cancer setting has been reviewed [16, 29].

Naive CD4 T cells differentiate into several subsets, which express distinct transcription factors, secrete different cytokine panels, and exert various immune functions. This has led to the concept of functional heterogeneity among different CD4 T cells. Th1 cells secrete IFN-γ and limit infections caused by intracellular bacteria while Th2 cells secrete IL-4, IL-5, IL-10, and IL-13, and skew the immune response toward humoral immunity [30]. Since the initial description of the Th1/Th2 dichotomy, novel subsets of effector and regulatory CD4 T cells have been characterized such as Th17 cells, which secrete IL-17 and promote inflammation, Foxp3 regulatory T cells (Tregs), which express the Foxp3 transcription factor and suppress immune responses, and IL-10-secreting Tr1 cells that also regulate immune responses and rely on the transcription factors c-Maf and AhR for their development [31]. Th9 cells, which can be differentiated from naive CD4 T cells in the presence of TGF-β and IL-4, were next characterized as CD4 T cells expressing the PU.1 transcription factor and secreting the cytokine IL-9. While Th9 cells were originally shown to promote inflammation in colitis, asthma, and experimental autoimmune encephalomyelitis (EAE),

in the mouse model for multiple sclerosis, we and others have recently shown that these cells exhibit potent anticancer properties upon adoptive transfer in vivo [32–34]. Overall, the ability of T cells to differentiate into different types of effector cells will shape the quality and magnitude of adaptive immune responses with a clear relevance to a vast number of diseases. Here, we will review the regulation of T cell differentiation with a focus on the molecular mechanisms that dictate T cell fate. The direct exploitation of T cell properties for the treatment of cancer will not be discussed here as this segment is dedicated to presenting the molecular bases of T cell differentiation. The relevance of T cell manipulation to treat cancer will be abundantly discussed in segments 2, 3, 4, and 5. We will however conclude this chapter by presenting the pathological consequences of defective T cell differentiation for human diseases.

5.2 Transcriptional and Epigenetic Regulation of T Cell Differentiation

5.2.1 A Network of Pioneer and Master Regulators, Transcription Factors, Governs CD4⁺ T Cell Differentiation

Differentiation of T helper (T_H) and regulatory T (Treg) cells from CD4⁺ T cells represents a relatively late-stage of differentiation. Antigens and cytokines are key extracellular signals sensed by T cells through T cell receptors (TCRs) and cytokine receptors. Ligation of antigens to TCRs activates transcription factors (TFs) such as nuclear factor-κB (NF-κB), nuclear factor of activated T cells (NFAT), and activator protein 1 (AP-1), whereas cytokines binding to their cognate cytokine receptors leads to activation of factors such as signal transducer and activator of transcription (STAT) [35–40]. The affinity of a TCR for its cognate antigen on MHC molecules, combined with co-stimulatory receptor–ligand interactions, generates variable intensities of cytosolic signals that govern the activities of downstream pioneer TFs. Variations in TCR signaling intensities influence the differentiation of CD4⁺ T cell subsets by tuning their response to different cytokines, inducing the expression of specific cytokine receptors or by impinging on the activation of specific STATs [41, 42]. In general, a strong TCR signal is known to favor T_H1, T_H17, and T_{FH} cell differentiation, while weak TCR signal promotes development of T_H2 cells and induced Treg cells (*i*Treg) [43–46] (Fig. 5.1a). The cytokines signal through type I/II cytokine receptor superfamily that uses Janus kinase (JAK)–STAT signaling pathway to convert environmental signals into intrinsic signals that initiate specific gene expression programs [122]. STATs are DNA-binding regulatory proteins able to drive selective gene expression program that determines specification of a relevant T_H-cell subset [123, 124]. Sensing of the extracellular environment through TCR and cytokine receptor signaling activates a coordinated network of pioneer TFs that regulate global chromatin state and the expression of T_H specific master regulators. Notably, AP-1, interferon regulatory factors (IRFs), STATs and NFAT, among other factors, recruit co-activators such as chromatin remodelers, histone acetyltransferases, like p300, and enzymes responsible for histones methylation, like H3K4me1. These chromatin-modifying factors act to increase accessibility of regulatory elements [125, 126]. Finally, this activated network of pioneer factors builds a specific epigenetic landscape essential for CD4⁺ T cells master regulators subsequent binding to mediate lineage-specific gene expression programs (Fig. 5.1a).

Initially, CD4⁺ T cell master regulators were described as TFs associated with different functional lineages essential and sufficient for driving specific cell fates. T-bet is the master regulator for T_H1 cells, GATA3 for T_H2 cells, PU.1 for T_H9 cells, RORγt for T_H17 cells, BCL-6 for T_{FH} cells, and FOXP3 for T_{reg} cells [127] (Fig. 5.1a). Interestingly, several groups showed that the epigenetic signature of T_H1 cells or T_H2 cells is perturbed by deletion of T-bet or GATA3, respectively, although transcription of mRNA encoding the lineage defining cytokines is reduced in cells deficient for T-bet and GATA3 [128, 129]. These data indicate that while TFs

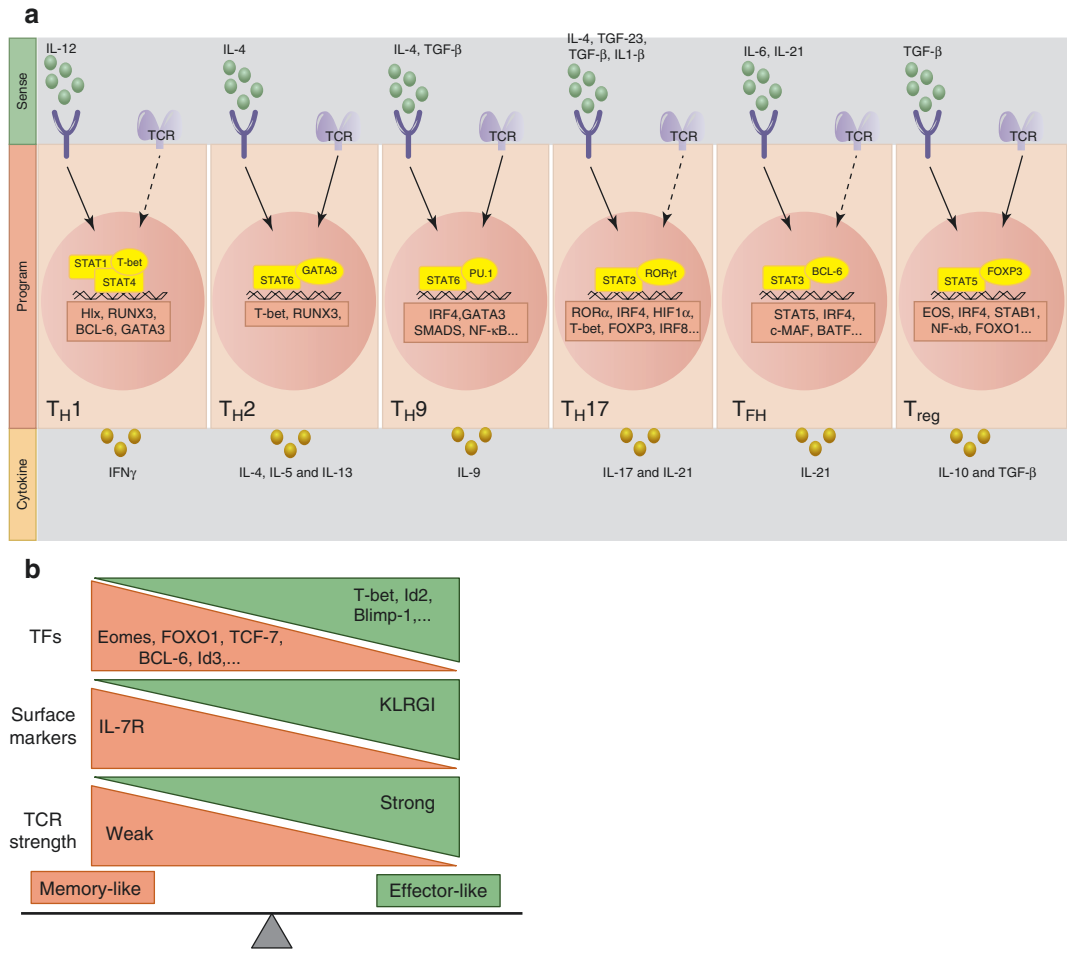


Fig. 5.1 T cell differentiation. (**a**) CD4$^+$T cell differentiation: *T_H1*: Coupled with a strong TCR activation IL-12 initiates T_H1 cell differentiation through phosphorylation and nuclear translocation of STAT4, which then binds the regulatory elements of target genes such as IRF1 and IFNγ. IFNγ induces phosphorylation of STAT1 and activates the transcription of the T_H1 master regulator T-bet (*Tbx21*) that in turn acts in a positive feedback loop to amplify the T_H1 differentiation. STAT4 further promotes specification in T_H1 cell lineage by negatively regulating the genes favoring T_H2 cell differentiation [47–49] while T-bet stimulates transcription of *Ifng* by establishing a positive feedback loop and further potentiates the IL-12 signaling [50, 51]. However, STAT4 is required for T-bet to achieve IL-12-dependent specification of T_H1 cell lineage [52]. Moreover, T-bet interacts with other transcriptional regulators of T_H-cell differentiation, for instance with the members of Ets, and Hlx families, RUNX3, BCL-6 [53, 54], GATA3 [55, 56], RUNX1 [57, 58], and IRF4 [59] to oppose the alternative cell lineages by negatively regulating the expression of their lineage defining genes. At the later stage of T_H1 cell differentiation, T-bet–BCL-6 complex represses *Ifng* transcription to keep the production of IFNγ in control as excessive production of IFNγ could cause autoimmunity [60]. *T_H2*: Coupled with a weak TCR activation IL-4 initiates T_H2 cell differentiation by phosphorylating STAT6, which then translocates to the nucleus and activates transcription of *Il4* and *Gata3* the key cytokine and TF, respectively, needed for T_H2 cell lineage specification. GATA3 is the master regulator of T_H2 cell differentiation that auto-regulates its own expression by binding to its regulatory elements to further amplify T_H2 differentiation [61]. GATA3 promotes T_H2 differentiation and maintains the cellular identity through distinct mechanisms. GATA3 induces transcription of T_H2-specific cytokine genes (*Il4, Il5*, and *Il13* genes)

through interacting with co-factors, and by inducing epigenetic modifications [62, 63]. Recent reports on genome-wide mapping of GATA3-binding sites suggested that GATA3 directly controls the expression of a large number of genes involved in T_H2 differentiation [64, 65]. For instance, GATA3 cooperates with STAT6 for its binding to regulatory sites of its target genes in T_H2 cells [65]. GATA3 also acts as repressor of transcription of genes important for lineage specification and commitment of the alternative Th-cell lineages [66]. For example, GATA3 interacts with T-bet and RUNX3 to repress T_H1 differentiation [55, 66, 67]. *T_H9:* A combination of TGF-β and IL-4 cytokines coupled with TCR activation initiates T_H9 cell differentiation in naive $CD4^+$ T cells by inducing the expression of PU.1 (purine-rich box 1) and IRF4 directly regulating the transcription of the *Il9* gene through direct binding to its regulatory elements [68]. In T_H9 cells, IL-4 activates *Stat6* and *Irf4* expression, while TGF-β stimulates the expression of PU.1, which is the master regulator of T_H9 cells. PU.1 inhibits the transcription of *Tbx21* and *Gata3* while induces IL-9 expression [69]. Enforced expression of PU.1 in $CD4^+$ T cells greatly enhanced T_H9 cell development by TGF-β and IL-4, while deficiency of PU.1 aborted T_H9 cell differentiation [70]. Similar experimental approach revealed that IRF4 had a similar effect on T_H9 development as PU.1 [71]. Both PU.1 and IRF4 bind to the *Il9* promoter to induce transcription of *Il9* gene [70, 71]. Computational analysis of regulatory sites at *Il9* locus has identified binding sites for several other TFs, such as AP-1, NF-κB, NFAT, GATA3, GATA1, STATs, SMADs, and NOTCH [69]. Moreover, studies have indicated the role of NF-κB, Notch receptors, BATF, and Smad2/Smad3 in regulating T_H9 responses [72, 73]. However, the mechanisms by which these TFs regulate T_H9 development remain to be further studied. *T_H17:* Strong TCR activation and cytokines (IL-6, IL-23, TGF-β and IL-1β) stimulation induce pioneer and lineage-specific and other TFs that control the specification and commitment of developing T_H17 cells. STAT3 initiates lineage specification by directly regulating the transcription of several target genes required for T_H17 development including lineage-specific TFs, RORα, and RORγt [74, 75]. RORγt is the master regulator of T_H17 cells [76–78]. However, several other TFs expressed in T_H17 cells were shown to positively or negatively regulate T_H17 cell differentiation. For example, TFs including RORα, RUNX1, BATF, IRF4, and HIF1α promote T_H17 differentiation through various signaling pathways [68, 77–87]. TFs that suppress T_H17 development include T-bet, FOXP3, and IRF8 [40, 88–98]. *T_{FH}:* Differentiation of T_{FH} cells is induced by a strong TCR activation, IL-6 and IL-21. T_{FH} cells express BCL-6 as their master regulator [99–103]. Depletion of BCL-6 in $CD4^+$ T cells results in a failure to produce T_{FH} cells, whereas BCL-6 overexpression promotes T_{FH} cell development indicating that BCL-6 is necessary and sufficient for T_{FH} cell differentiation [39, 104, 105]. Furthermore, BCL-6 is a transcriptional repressor acting on the transcription of lineage-specific TFs of alternative T_H cell lineages, such as *Tbx21*, *Rorγt*, and *Gata3* [106]. However, expression of BCL-6 is not restricted to T_{FH} cells, but expressed in other T_H lineages as well [107]. Other regulators of T_{FH} cell differentiation include STAT3/5, IRF4, c-MAF, and BATF. STAT3 depletion significantly reduced the $CXCR5^+$ T_{FH} cells as well as caused defective germinal center responses and B cell helps both in human and mouse [108, 109] *T_{reg}*: FOXP3 is a master regulator important for the development and homeostasis of T_{reg} cell. FOXP3 expression is required for T_{reg}-mediated tolerance both in mice and human because FOXP3-deficient T_{reg} cells have been linked with severe autoimmunity [110–112]. Global mapping of FOXP3-binding sites in T_{reg} cells revealed that FOXP3 is actually only partly accountable for T_{reg} signatures [113–117] suggesting the role of other TFs in the regulation of Treg cell development [113, 118]. In fact, FOXP3 interacts with other nuclear factors to cooperate in determining the T_{reg} signature and functions [119]. TFs, such as EOS, IRF4, SATB1, LEF1, and GATA1, can work together with FOXP3 to form a transcriptional network governing T_{reg} cell differentiation [120]. Moreover, TCR signaling induced TFs such as NF-κB, NFAT, AP-1, and FOXO1 were shown to regulate development and function of T_{reg} [119, 121]. *A dashed arrow* represents a strong TCR signal and a *thin arrow* a weak TCR signal. (**b**) TFs in the terminal-effector versus memory $CD8^+$ T lymphocyte fate decision: Prominent examples of factors that can promote effector-like or memory-like differentiation. For instance, the amounts of TFs pairs T-bet-Eomes, Id2-Id3, and Blimp-1-BCL-6 are key, and a gradient effect in which increasing amounts of T-bet, Id2, and Blimp-1 favors increasing differentiation into terminal effector cells

Table 5.1 Overview of effector CD8⁺T-cell subpopulations, their polarizing cytokines and the transcription factors important for their differentiation

Type	Polarizing cytokine in vitro	Transcription factor	References
Tc1	IL-2, IL-12	T-bet, Blim-1, Id2, IRF4	[133–135]
Tc2	IL-4	GATA3	[136–138]
Tc9	TGF-β, IL-4	IRF4	[139–141]
Tc17	TGF-β, IL-6, IL-21	RORγt, RORα, IRF4	[142–144]
CD8⁺Treg	TGF-β	FOXP3	[145–147]

such as T-bet and GATA3 control expression of some of the key genes associated with T_H1 and T_H2 cells, the fundamental identity of these lineages is established by other transcriptional events, directed by upstream pioneering factors that regulate accessibility of the entire transcriptional network to key sites of gene regulation. However, despite the relatively small regulatory footprint of master regulators factors, in vivo loss-of-function studies support the importance of master regulators in heritable maintenance of cellular phenotype, environmental responsiveness, and plasticity. For instance, in vivo, FOXP3 is critical for T_{reg} cell identity and loss of FOXP3 in mature T_{reg} cells results in their dedifferentiation, acquisition of alternative T-cell subset phenotype and extensive immunopathologies [130, 131]. In addition, master regulators engage in positive feedback loops for core lineage TFs expression and augment expression of cooperatively regulated genes, thereby adapting and stabilizing the transcriptional program. Master regulators simultaneously inhibit the expression of genes instructing alternative lineages such as other master regulators factors or cytokines instructing opposing lineages.

5.2.2 An Expression Gradient of Multiple Factors to Control the Balance Between Effector-Like and Memory-Like T Cell Differentiation

An activated CD8⁺ T cell is exposed to a myriad array of signals, such as cytokines, growth factors, and environmental cues. How these signals are transmitted into cells and translated into gene expression patterns that promote effector differentiation yet also preserve a long-lived and multipotent pool of cells that can self-renew is an important question. Despite the fact that CD8⁺ T lymphocytes exhibit less functional diversity than CD4⁺ T, TFs specifying CD8⁺ T cell decision into Tc1, Tc2, Tc9, Tc17 or CD8⁺ T regulatory fate were described. Indeed a growing list of TFs has been linked to various aspects of effector or memory T cell biology. For Tc1 differentiation, the expression of BCL-6, Blimp-1, Eomes, Id2, Id3, TCF-7, Foxo1 and T-bet is decisive for the effector versus memory fate. Additionally, IRF4 has been shown to be crucial for effector and memory Tc1 differentiation. Similar to its function in CD4⁺ T cells, IRF4 is essential for the differentiation of CD8⁺ T cells to Tc9 and Tc17 cells while only a minority of CD8⁺ Treg cells express the lineage-specific factor Foxp3 [132] (Table 5.1).

An important conceptual model in the transcriptional regulation of CD8⁺ T cell differentiation is that pairs of transcription factors operate in opposing ways to facilitate the terminal-effector versus memory CD8⁺ T lymphocyte fates (Fig. 5.1b). For instance, T-bet expression is induced initially by TCR signaling and augmented by IL-12 signals in activated CD8⁺ T cells [148, 149]. In virus-specific CD8⁺ T cells, T-bet expression was found to be elevated in the KLRG1^{hi}IL-7R^{lo}shorter lived effector CD8⁺T cells relative to the KLRG1^{lo}IL-7R^{hi} memory precursor effector CD8⁺ T cells [148, 150]. This finding suggests that an expression gradient of T-bet acted like a rheostat to control the balance between terminal effector CD8⁺ T-cell differentiation and memory cell potential in effector CD8⁺ T cells. Higher amounts of T-bet instructed KLRG1^{hi}IL-7R^{lo} terminal effector cell formation, but lower amounts appeared to permit normal memory cell formation.

Eomesodermin (Eomes), another T-box factor expressed in activated CD8+ T cells, is also important for CD122 and perforin expression in CD8+ T cells [148, 150, 151]. On one hand, T-bet and Eomes appear to cooperate in cytotoxic T lymphocyte (CTL) function and memory T-cell homeostasis. T-bet and Eomes coordinate the expression of CD122 in memory CD8+ T cells [148, 150] and CD8+ T cells that are doubly deficient in both genes are incapable of generating CTLs during lymphocytic choriomeningitis virus (LCMV) infection. Instead, *Tbx21*$^{-/-}$*Eomes*$^{-/-}$CD8+ T cells abnormally differentiated into IL-17-producing CD8+ T cells that caused excessive neutrophil infiltration and a lethal inflammatory syndrome [152, 153]. On the other hand, in contrast to T-bet, IL-12 paradoxically suppresses Eomes expression and Eomes expression preferentially increases relative to T-bet as memory CD8+ T cells form and mature [149]. This counterbalance also applies to the transcription factor pair Id2 and Id3 [154, 155] and the pair Blimp-1 and BCL-6 [156–159] and perhaps other pairs (Fig. 5.1b). While the paradigm described above has been mainly characterized in CD8+ T cells, this same system also seems to control CD4+ T cell memory. Indeed, the pair Blimp-1 and BCL-6 has been reported to influence effector versus memory subsets of CD4+ T cells [157–159].

The classic notion that a single TF or pair of TFs can control a whole differentiation program is clearly an oversimplification. Key TFs such as those described above actually operate with numerous locally bound cofactors [160, 161]. The mapping of combinatorial interactions among known TFs and the correlation with tissue-specific expression led to the estimation that tissue-type specification would be accomplished by networks of approximately 15 TFs [162]. Thus a particular TF may be expressed in multiple tissues, but only the coexpression and colocalization of a specific set of TFs in a given tissue enables their interaction and the specification of a unique fate. Indeed, like in CD4+ T cells [35, 79, 163], BATF and IRF4 act as pioneer factors in CD8+ T cells [164]. Loss of either BATF [164–166] or IRF4 [133, 165, 167–169] greatly perturbs the early phases of the CD8+ T cell immune response, which results in collapse of the effector phase after initial proliferation. Interestingly, this is accompanied by disrupted regulation of a large percentage of genes associated with CD8+ T cell activation, including genes encoding key TFs and molecules that control metabolism, as well as molecules associated with effector functions [164]. However, additional work in deciphering the roles of BATF and IRF4 in regulating chromatin accessibility during the CD8+T cell response is crucial to elucidate how the activation of a naive T cell establishes a chromatin landscape suitable for lineage-specific gene expression. There is robust evidence that cells with strong memory potential are established very early in the immune response, how this may be influenced by early modifications to gene accessibility is a key question. Another important component of understanding how heterogeneity arises in the effector population and contributes to memory formation will probably include a description of the enhancers and regulatory regions of different CD8+ T cell subsets, as well as their accessibility at different stages of the immune response, and identification of the many factors that bind those regions [64, 170–172]. A major challenge for the field will be to integrate this information with data on TFs such as those described above, microRNAs and long noncoding RNAs, to generate comprehensive understanding of the events that determine heterogeneous lymphocyte fates.

5.2.3 ncRNAs in the Epigenetic Control of T Cell Differentiation

Non-coding RNAs (ncRNAs) are classified into two major categories [173]. For instance, miRNAs belong to the small ncRNAs category and regulate gene expression by binding to the coding or untranslated regions (UTRs) of target mRNA transcripts and resulting in either mRNA degradation or inhibition of translation [174]. Long ncRNAs show high degree of tissue and species-specific expression and there are reports on their role in gene regulation [175].

Depletion of microRNA-processing endonucleases *Drosha* and *Dicer* genes caused disturbances in the stability and function of T_H cells, indicating a role of regulatory ncRNAs in T-cell differentiation [176–178]. Different studies build a "microRNome" or "lncRNome" to categorize a set of microRNAs and lncRNAs regulating lineage commitment during T_H-cell differentiation both in mouse and human lymphocytes [179, 180]. Several other studies have focused on identifying unique miRNAs that regulate the development and function of the T_H cells (Table 5.2).

lncRNAs use a range of different molecular mechanisms to modulate gene expression. Several lncRNAs have been associated with T cell differentiation and function. For instance, NRON lncRNA regulates NFAT function [208], lncRNA, GAS5 halts T cell growth [209], and NeST lncRNA is selectively expressed in T_H1 cells and drives *Ifnγ* expression [210, 211]. Global analysis of lncRNAs in mouse $CD8^+$ T cells identified several lncRNAs that potentially regulate their activation and differentiation [211].

Table 5.2 Overview of microRNAs relevant to T-lymphocyte differentiation

MicroRNAs	Target genes	Function	References
miR-17-92	*Bim, Pten, Pd1, Btla*	Modulates DN to DP transition. Skews differentiation toward short-lived terminal effector cells by increasing mTOR signaling, promotes effector $CD8^+$ T-cell expansion. Controls T_H1 differentiation	[174, 181–183]
miR-10a	*Bcl-6*	Regulates the flexibility of T_H cells	[184]
miR-21	*Ifng*	Regulates $T_H17$differentiation Highly expressed in effector and memory $CD8^+$ T cells	[185–187]
miR-125	*Ifng, Il2R β, Il10Rα, Blimp1*	Maintains the naive state of T-cell. Involved in T_{reg} differentiation	[188, 189]
miR-125b	*Ifng, Il2R β, Il10Rα, Prdm1*	$CD4^+$ naive T-cell–specific microRNA with a crucial role in the process of naive T-cell differentiation	[177]
miR-126	?	Modulates T_H2 responses	[190]
miR-142-3p	*Rac1, Rock2*	Repressed by FOXP3 leading to increased suppressor function of T_{reg} cells. Highly expressed in $CD8^+$ T-cells	[187, 191–193]
miR-146a	*Irak1, Traf6* *Stat1*	Highly expressed in T_{reg} and T_H1 cells Plays a role in Treg cell-mediated T_H1 responses	[174, 194, 195]
miR-146b	?	Regulates $T_H17$differentiation	[185]
miR-147	?	Modulates T_H1 differentiation	[196]
miR-150	*Notch3*	Modulates maturation from DP to SP stage of thymocyte	[174, 197–199]
miR-155	*c-Maf, IfnγRα* *Socs1*	Knockout mice have increased T_H2 cell generation and reduced T_H17 and T_H1 cells Regulates the development of T_{reg}	[197, 200, 201]
miR-181a	*SHP2, PTPN22* *DUSP5, DUSP6* *Bcl-2, CD69* *TCR alpha*	Increases TCR signaling in thymocytes and peripheral T-cells, modulates T cell sensitivity in response to antigens, and regulates T cell and B cell development positive selection in thymocyte	[183, 202, 203]
miR-181c	*Il2*	Negatively regulates $CD4^+$ T-cell activation	[204]
miR-182	*Foxo1*	Increases clonal expansion of activated T_H	[205]
miR-210	*Hif1-α*	Regulates T_H17 cell differentiation	[206]
miR-301a	*Pias3*	Contributes to the development of the T_H17 subset via targeting the IL-6/23–STAT3 pathway	[186]
miR-326	*Ets-1*	Promotes T_H17 differentiation	[207]

5.3 Metabolic State and T Lymphocyte Differentiation

T cell lineage differentiation is closely linked to changes in their cellular metabolic programs. Key metabolic processes such as glycolysis, fatty acid, and mitochondrial metabolism are crucial players in T cell activation and differentiation, and their modulation can differentially affect their fate and function.

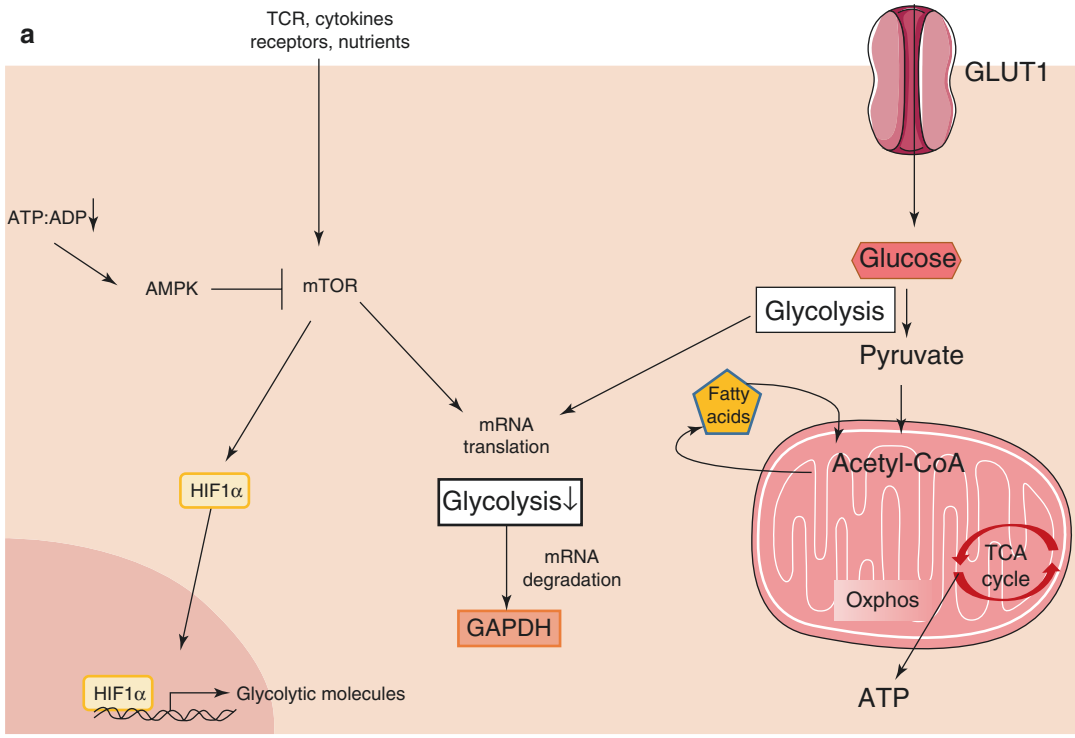
5.3.1 Metabolic Reprogramming During T Cell Activation

In terms of metabolic activity, naive T cells are in a quiescent state [212, 213]. Upon antigen recognition, $CD4^+$ and $CD8^+$ naive T cells become highly proliferative and differentiate. Upon TCR stimulation the expression of glycolysis-associated genes is upregulated, including the expression of transporters for extracellular nutrients, leading to a metabolic reprogramming during which the cell strongly increases the uptake of nutrients, especially glucose [214–216]. This metabolic reprogramming is under the control of the mechanistic target of rapamycin (mTOR) complex [216–220]. mTOR is a protein kinase that acts as a central integrator of various environmental cues and is able to regulate multiple cellular processes accordingly [221–225] (Fig. 5.2a). Among them are autophagy, glucose uptake and consumption (glycolysis), and the control of protein and lipid synthesis, all key processes during T cell activation [226, 227]. Activated T cells engage aerobic glycolysis. Intriguingly, in T cells, glycolysis is important for the regulation of GAPDH that functions as a metabolic checkpoint by linking T cell effector function to glucose availability. Indeed, in the absence of its substrate, GAPDH interferes with the translation of IFNγ in T_H1 cells. Importantly, most enzymes involved in glycolysis can enter the nucleus, like GAPDH, and appear to exert alternative functions that can directly affect the transcription and stability of factors associated with cell proliferation [228]. Fatty acid synthesis is also essential for T cell proliferation and differentiation by providing lipid supply [216, 229–231]. Fatty acid synthesis also participates in post-translational modification of proteins implicated in T cell activation [232–234]. For instance, myristoylation, a process that affects protein–lipid interactions and influences the cellular localization of proteins, is necessary for the events downstream of TCR signaling [232]. Thus it is possible that lipid-dependent modifications of, for example, TFs also influence their cellular localization, such as nuclear translocation, where they can initiate specific transcriptional programs. Despite the fact that T effector cells are highly glycolytic, Sena et al. demonstrated that their activation and proliferation can be sustained entirely by a functional mitochondrial metabolism [235]. Mitochondria also supports T cell activation by regulating its cytosolic calcium (Ca2+) uptake upon TCR engagement [236–240]. It was also shown that mitochondrial biogenesis is upregulated upon T cell activation [241]. Thus, mitochondrial remodeling and the increase of mitochondrial metabolism may actively support cell growth and proliferation following T cell activation.

5.3.2 Metabolic Checkpoints and Pathways Controlling T Cell Differentiation

T cells must meet certain metabolic criteria in order to properly differentiate. Accordingly, key metabolic regulators such as mTOR and AMPK help the cell to sense its metabolic state, controlling if and how activation and differentiation takes place. Additionally, the modulation of lipid metabolism also plays an important role in T cell differentiation (Fig. 5.2a).

The mTOR protein integrates signals that indicate the presence of nutrients and essential factors for cell growth and division, and links them to the appropriate metabolic processes [221–225, 242]. Importantly, genetic or pharmacological mTOR inhibition impairs effector T cell differentiation [243–245]. Indeed, mTOR activity seems to support T_H17 development by the expression of S6 K2, which binds RORγt and


a
TCR, cytokines
receptors, nutrients
GLUT1
ATP:ADP
AMPK
mTOR
Glucose
Glycolysis
Pyruvate
Fatty acids
mRNA translation
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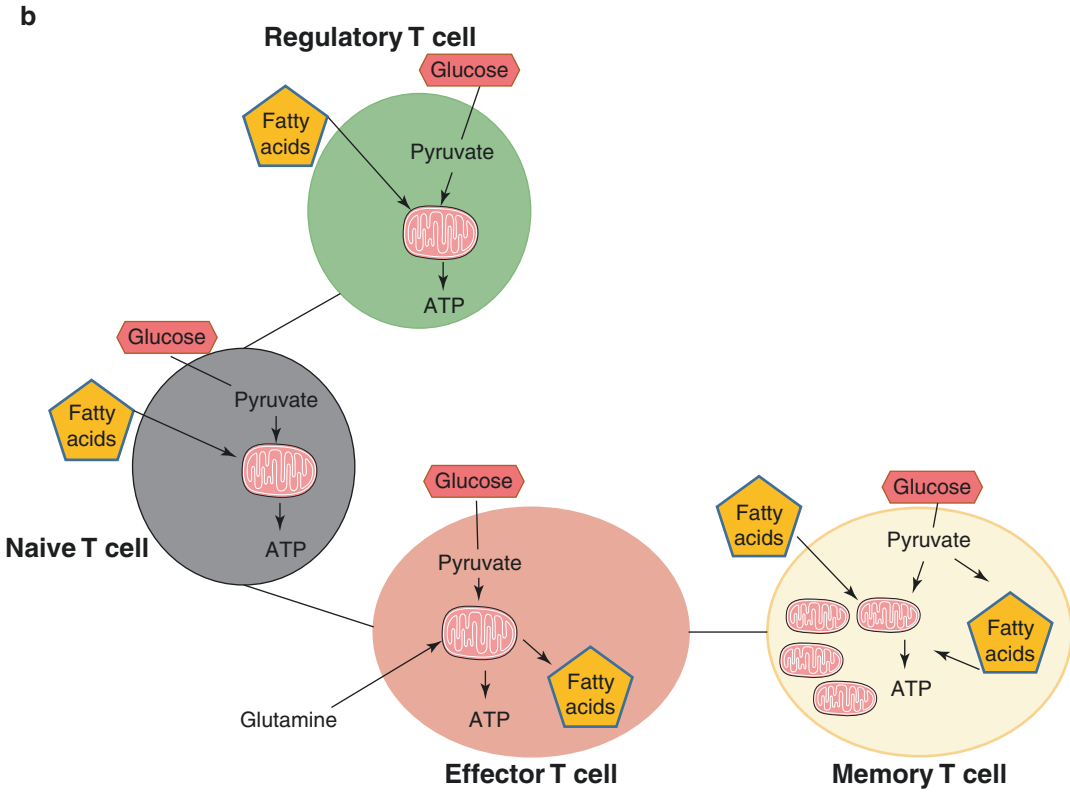
Glycolytic molecules
ATP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Regulatory T cell
Glucose
Fatty acids
Pyruvate
ATP
Glucose
Fatty acids
Pyruvate
ATP
Naive T cell
Glucose
Pyruvate
Fatty acids
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Glutamine
Effector T cell
Glucose
Fatty acids
Pyruvate
Fatty acids
ATP
Memory T cell

facilitates its transport inside the nucleus [246, 247]. Most studies so far confirm mTOR as a prerequisite for T_H17 differentiation, however, its impact on other T lineages is less clear, and may strongly depend on the experimental model [248, 249]. While in vitro, naive T cells lacking mTOR expression spontaneously develop into T_{regs} even in the absence of exogenous TGF-β [250], in vivo, mice lacking mTOR develop general autoimmune responses [251]. This paradox was clarified by observations that T_{regs} proliferation needs transient mTOR inhibition [252]. In vitro, T_{regs} are exposed to a medium rich in nutrients and cytokines, which results in constant mTOR activation, leading to defective T_{regs} proliferation. In vivo, exposure to constantly changing conditions in the microenvironment might correlate with changing mTOR activity thus enabling T_{regs} proliferation [252, 253]. This result suggests that environmental signals that shape mTOR activity modulate the metabolic profile of T_{regs} in vivo [254]. Additionally, autophagy, a self-degradative intracellular process initiated under nutrient stress conditions, was shown to support the stability and survival fitness of T_{regs} by influencing the mTOR signaling [255]. T_{regs} that were unable to perform autophagy due to their Atg7-deficiency exhibit decreased lineage stability as compared to autophagy-competent Tregs. Upon adoptive transfer, Atg7-deficient T_{regs} lost FOXP3 expression and upregulated IFNγ or IL-17-production. These defects were associated with mTOR hyperactivation after TCR signaling, c-Myc activation and upregulation of the glycolytic metabolism, together resulting in a shift towards effector T cell differentiation. Importantly, inhibition of mTOR by exposing the cells to rapamycin restored their lack of stability upon adoptive transfer [256]. Interestingly, data from our group show that, in T_H9 cells, genetic and pharmacological inhibition of autophagy enhance T_H9 cell differentiation in an mTOR independent fashion. Indeed, the absence of autophagy leads to an increased stabilization of T_H9 master regulator PU.1. mTOR also influences cell fate decisions during short-lived effector and long-lived memory $CD4^+$T and $CD8^+$T cell differentiation. Asymmetric mTOR activity in T_H cells that replicate resulted in lower mTORC1, a member of the mTOR complex, activity in one of the daughter cells, which was associated with a memory-like phenotype such as higher longevity. Hence, these findings indicate that the levels of mTOR activity can determine whether a cell will proceed to develop into an effector or memory phenotype already at an early time point after TCR stimulation and cell division [257].

AMP-activated protein kinase (AMPK) is activated in response to nutrient shortage or other physiological stress, making AMPK an important sensor of the cellular energy levels [258, 259] (Fig. 5.2a). Studies using metformin, an activator

Fig. 5.2 Metabolic state and T cell differentiation. (**a**) Integration of metabolic state and gene expression: mTOR is activated by environmental cues and signaling via receptors (TCRs, costimulatory receptors, and cytokine receptors) in T cells via kinase-dependent pathways. mTOR regulates cell growth, survival, and metabolism via multiple mechanisms, such as the induction of glycolysis through the stabilization of HIF1α, as well as lipid and protein biosynthesis. mTOR promotes translation initiation and protein synthesis. The AMPK complex is activated when cellular energy levels decrease (ATP:AMP) and suppresses cell growth by blocking biosynthetic pathways and inhibiting mTOR. AMPK can induce fatty acid oxidation and suppress glycolysis. mTOR can induce activity of HIF1α transcription factor that coordinates the cellular response to low oxygen tension, including induction of the expression of many molecules required for glycolysis. As an example of a metabolic enzyme that can also function as an RNA-binding protein and regulate mRNA translation, GAPDH has been shown to regulate the translation of mRNA encoding effector molecules such as IFNγ. GAPDH is engaged as a metabolic enzyme during glycolysis; however, when not engaged in glycolysis and when the cell generates ATP via oxidative phosphorylation (OXPHOS), GAPDH can bind the 3′ untranslated region of cytokine-encoding mRNA and diminish translation. (**b**) Different T cell subsets and their main metabolic characteristics: T_{regs} and naive T cells have similar metabolic profiles, with most glucose and fatty acids being fully oxidized in the mitochondria for the generation of ATP. During the transition from naive to effector, there is a major upregulation of glucose uptake. Glutamine uptake also increases and fatty acid synthesis is engaged to nourish activation. In memory T cells, the mitochondrial mass is increased and a futile cycle of fatty acid synthesis and oxidation has been reported

of AMPK, showed that T_H1/ T_H17 differentiation was impaired after exposure to this compound [260, 261]. Despite these findings, AMPK appears to be dispensable for T cell differentiation under normal metabolic conditions, since T cells which lack a functional AMPK are still able to acquire effector functions [262, 263]. Considering that T cells are exposed to highly variable microenvironments, these findings suggest that AMPK works as a shield against suboptimal environmental conditions [264–266]. Additionally, generation of memory T cells is dependent on AMPK since AMPK-deficient $CD8^+$ T cells, after a second exposure to antigen, are defective in their ability to undergo population expansion [267].

Lipid biosynthesis pathways also influence T-cell differentiation. During glycolysis the generated acetyl-coA gives rise to de novo FAS. While FAS is an anabolic process that supports cell proliferation, fatty acids can also be used by a catabolic process designated as fatty acid β-oxidation (FAO). Following activation, T cells switch from FAO to FAS [216, 229–231]. Accordingly, $CD4^+$ T and $CD8^+$ T cell proliferation was impaired by inhibition of FAS [230, 268, 269]. While inhibition of de novo FAS in T cells impaired T_H17 and T_H1 differentiation, T_{regs} development was enhanced. Moreover, blocking FAS even induced a shift from T_H17 towards T_{reg} induction under T_H17 culture conditions [230, 268, 270]. It was further shown that inhibition of FAS in T_H17 cells resulted in changed nuclear localization of RORγt and a decreased binding of this TF to enhancer regions in the *Il17a* locus [270]. Interestingly, the fatty acid and cholesterol composition of the cell was shown to modulate RORγt binding to different gene promoters such as *Il17* and *Il10* [271, 272].

5.3.3 Metabolic State and T Cell Differentiation

T_{regs} and memory T cells have metabolic phenotypes close to naive T cells. In vitro differentiated T_{regs} have been reported to be less glycolytic and more reliant on FAO and oxidative phosphorylation (Oxphos) when compared to effector T cells [230, 273, 274] (Fig. 5.2a, b). Unlike effector T cells, memory T cells do not proliferate and produce little or no cytokines and display in general a catabolic metabolism, relying essentially on oxidation of glucose and fatty acids [231, 275]. However, memory T cells have several characteristics, which distinguish them metabolically from naive T cells. For example, $CD8^+$ and $CD4^+$ memory T cells possess an increased mitochondrial mass [276, 277] with the $CD4^+$ T subset also displaying an enhanced spare glycolytic capacity such as higher cytosolic concentration of GAPDH [277]. Interestingly, memory $CD8^+$ T cells simultaneously engaged FAS and FAO [278]. In general, the engagement of such a futile cycle is prevented to prevent waste of energy. Memory T cells may activate both processes likely to ensure immediate metabolic fitness in the event of restimulation [279]. Complementary studies have shown that manipulation of the effector $CD8^+$ T cell metabolic profile also affects the differentiation of effector and memory subsets during the $CD8^+$ T cell response. Inhibition of mTOR activity in effector $CD8^+$ T can impair the production of effector molecules such as IFNγ, granzyme B, and perforin [218, 280–282]. BCL-6, a memory T cell–associated transcription factor, has been shown to repress glycolytic molecule-encoding genes in $CD4^+$ or $CD8^+$ effector T cells [283], which provides a transcriptional link to the metabolic changes observed during memory formation. Thus, in multiple contexts, promoting mitochondrial fatty acid oxidation or suppressing glycolysis favors the emergence of antigen-experienced $CD8^+$T cells with prolonged survival and antigen recall; conversely, driving glycolysis supports a more effector-like fate (Fig. 5.2b).

5.4 T Cell Transcriptional Plasticity

Lineage-tracing systems in mice have shown that endogenously polarized $CD4^+$ T cells from many subsets change their phenotype during their lifespan [284–287] while $CD8^+$ T cells studies have demonstrated that a single naive $CD8^+$ T lymphocyte is able to give rise to both effector and memory lymphocytes [288, 289]. Subsequent studies showed that individual $CD8^+$ T lymphocytes

exhibit highly disparate tendencies to yield effector progeny heterogeneous in their proliferative capacity, cytokine production, and expression of phenotypic markers, including KLRG1 and IL-7R [290–293]. Curiously, the degree of heterogeneity appears to be somewhat tissue specific [290]. In humans, the combination of phenotypic analyses and sequencing of TCRs of clonal descendants of single cells reveals a high degree of heterogeneity in the type of T cell response generated from single T cells [294, 295]. All these observations revealed that during the course of its life cycle the phenotype of a single T cell is not stable. Indeed, a single T cell has the ability to take on characteristics of many T cell subsets simultaneously or at different times giving rise to the concept of T cell plasticity [132, 296–298]. Importantly, the ability to generate many various functional T cells associated with different T cell subsets from individual T cells seems advantageous for host immunity.

The prevalence of phenotypic plasticity in T cell immunity is well exemplified by T_{reg} cells, which, in response to different contexts, can polarize similar to other inflammatory T cell subsets. The phenotypic plasticity of T_{regs} that mirrors each T helper cell subset supports the hypothesis of an inherent flexibility of T cells, both inflammatory and regulatory, to adapt their function to changing environments. Similar to $CD4^+$ T cells, $CD8^+$ T cells also show some lineage plasticity. Indeed, Tc1 and $CD8^+T_{reg}$ cells seem to possess relatively stable fates while Tc2, Tc9, and Tc17 cells tend to acquire qualities of other subpopulations. Tc2 and Tc17 cells maintain their cytokine profile, but acquire additional characteristics of Tc1 cells. Tc9 cells appear to be relatively unstable in vivo with plasticity toward the Tc1 or Tc1/Tc2 fates [132].

5.4.1 Cytokines, the Master Regulators of Plasticity

Cytokines have a dominant role in driving the plasticity between $CD4^+$ T cell subsets [299]. T_H1 cells cultured with IL-4 or in the context of helminth infection in vivo repolarize the cells to produce IL-4 and to extinguish the expression of IFNγ [300, 301]. T_H2 cells express T_H1-type cytokines when incubated with IL-12, IFNγ, and type I IFNs [302, 303]. The discovery of T_H9 cells further illustrates the plasticity among $CD4^+$ T cells. Veldohen et al. showed that TGF-β was able to reprogram the differentiation of T_H2 cells into IL-9 producing T cells while Dardalhon et al. demonstrated that IL-4 is able to block FOXP3 induction in T_{regs}, thereby inducing a population of T helper cells that predominantly produce IL-9 [68, 304]. Importantly, members of the IRF family are able to regulate T_H9 cell plasticity. We actually show that IL-1β, besides increasing IL-9 secretion from T_H9 cells, also enhances IL-21 secretion levels from differentiating T_H9 cells [34]. The underlying mechanism involves STAT1-dependent activation of IRF1 and further illustrates the plasticity of the T_H9 program. Intriguingly, adoptive transfer experiments indicated that the IL-1β-induced T_H9 cells maintained their transcriptional program in vivo, suggesting that the IL-1β-induced expression of IRF1 contributes to stabilize the T_H9 cell transcriptional program. The capacity of the T_H17 cell subset to gain IFNγ expression or convert fully to T_H1 cells by losing the expression of IL-17 and RORγt requires the cytokines IL-12 or IL-23, both of which can activate STAT4 [286, 305, 306]. Polarized T_{FH} cells from mice can be induced to make T_H1-, T_H2-, or T_H17-type cytokines, in addition to IL-21, by culturing them in the presence of IL-12, IL-4 or IL-6 and TGF-β, respectively, whereas T_H1, T_H2, and T_H17 cells can express IL-21, CXCR5, and programmed cell death 1 (PD-1) by culturing in T_{FH} cell conditions with IL-21 and IL-6 [307]. Finally, these reprogramming effects can be mimicked by specific deficiencies in suppressor of cytokine signaling (SOCS) genes that oppose the activity of specific STATs through various mechanisms, highlighting the importance of cytokine signals in driving plasticity [308]. Importantly, the cytokine environment can even influence plasticity between inflammatory and regulatory programs. For instance, TGF-β is crucial for the conversion of T_H17 cells towards a regulatory phenotype through the promotion of FOXP3 or IL-10 expression [309–311] (Fig. 5.3).

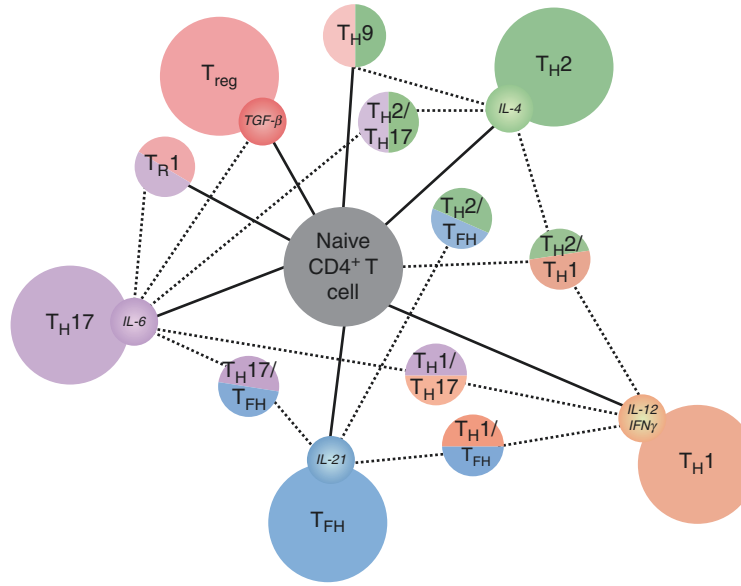

Fig. 5.3 Cytokine-driven T cell plasticity. The key cytokines IFNγ, IL-12, IL-4, TGF-β, IL-6, and IL-21 alone or in concert drive naive $CD4^+$ T cells towards differentiation. This cytokines are able to promote polarization or plasticity between subsets. *Black lines* linking the subsets depict known transitions. Reprogramming between subsets seems to occur by transitioning through intermediate stages in which cells exhibit phenotypes of many subsets

5.4.2 Transcriptional and Epigenetic Modulation of T Cell Plasticity

Following the importance of cytokines in tuning T cell plasticity, STATs drive T_H cell plasticity in direct response to the binding of cytokines to receptors. Moreover, the observation that T cell master regulators have a substantial, but often incomplete, role in setting the transcriptional programs is also crucial to enable a plastic system [129, 312–314]. Nevertheless, the expression of specific STATs and master transcription factors is not sufficient for polarization or plasticity of T helper cell subsets [120, 315]. Additional transcription factors, such as the nuclear receptor 4A (NR4A) family, transcription regulator protein BACH2, RUNX proteins, retinoic acid receptors, and aryl hydrocarbon receptor (AHR), clearly have essential roles in the maintenance of polarized states, as the disruption of these transcription factors leads to enhanced plasticity between subsets [31, 316–319]. Epigenetic modulation of chromatin can also influence T cell plasticity. For instance, in the absence of HATs, T_{regs} cells lose FOXP3 expression and gain IL-17 expression [320]. Importantly, once the polarized functions of effector T cells are established relaxed methylation allows for substantial plasticity following restimulation in the quiescent memory phase [321, 322]. The generation of heterochromatin by complexes, such as HDACs and the H3K27me3-associated polycomb repressive complex 1 (PRC1), also has a central role in preventing phenotypic plasticity between the polarized subsets [323, 324]. For example, PRC1 is crucial for T_H2 cell function and for limiting their plasticity towards T_H1 cell phenotypes [323]. Global analyses of histone modifications associated with transcriptionally accessible (H3K4me3) or repressed (H3K27me3) loci in different polarized T cell subsets provide further information into how T cells can both acquire specific functions and retain plasticity. Whereas the cytokine loci of different T cell subsets exhibit either H3K4me3 or H3K27me3 marks, the chromatin structure at most polarizing transcription factor loci contains both marks, indicative of a permissive chromatin state, allowing for the induction of transcription factors from opposing subsets to initiate cellular reprogramming [172]. Thus chromatin- and DNA-modifying enzymes have important roles in T cell plasticity, often through cooperation with the polarizing transcription factors or lncRNAs in each subset. Finally, T cell plasticity is also regulated by microRNA-mediated post-transcriptional regulation of a wide

variety of genes such as those involved in cytokine signaling, TCR and co-stimulatory signaling, and transcriptional regulatory pathways (reviewed in [325]).

5.4.3 Metabolic Regulation of Plasticity

T cell plasticity can be controlled by the metabolic programs of the cell that respond dynamically to fluctuations in the nutrients, oxygen levels, and energy sources present in the environment. Such factors are likely to be important when T cells migrate between distinct microenvironments during immune responses, such as between lymphoid organs and tissue sites or tumor microenvironments [326]. The phosphatidylinositol 3-kinase (PI3K) AKT-mTOR pathway has a central role in the regulation of plasticity by environmental cues. For example, AKT function is blunted in T_{reg} cells by the activity of phosphatase and tensin homologue (PTEN) [327–329]. PTEN deficiency results in severely compromised T_{reg} cell stability and in their conversion into T_H1 and T_H17 cells [330, 331]. In addition, although a requirement for some mTORC1 activity in T_{reg} cells has been demonstrated [251], rapamycin treatment actually promotes T_{reg} cell stability [332, 333], and hyperactivation of mTORC1 in T_{reg} cells drives IL-17 production and loss of FOXP3 expression [334]. Interestingly, glutamine is an important biosynthetic precursor that tips the balance between T_H1 and T_{reg} cell polarization in an mTORC1-dependent manner [335]. Finally, HIF1α activity in T_{reg} cells leads to ectopic IFNγ production and reduced FOXP3 expression [220, 336, 337].

5.5 T Cell Differentiation Defects and Human Diseases

As discussed above, differentiated T cell subsets are essential to maintain host integrity and fight against infections. This concept is further exemplified in humans harboring genetic defects resulting in defective T cell differentiation. Activated T cells express CD40L, which engages CD40 present on macrophages and dendritic cells, resulting in their IL-12 secretion and Th1-skewing [338, 339]. Mutations in the gene encoding CD40 ligand not only result in the development of hyper IgM syndrome [340], where B cells are severely compromised in their ability to secrete all antibody isotypes expect IgM, but also impair Th1 immunity to infections by pathogens [341]. In line with these observations, patients with mutations in the gene encoding IKKγ, which regulates NF-κB activation following CD40 engagement, presented with monocytes with impaired IL-12 secretion and thus reduced IFN-gamma secretion by T cells [342]. Importantly, these patients featured susceptibility to mycobacterial diseases, thereby establishing a direct molecular link between defective induction of Th1 cell differentiation and susceptibility to disease [342].

Another example of disease that is linked to defective T cell differentiation is the hyper-IgE syndrome (HIES). This disease manifests with recurrent skin and pulmonary infections triggered by bacteria, candidiasis caused by fungi as well as high concentration of IgE in the serum. The pathogenesis of HIES was shown to rely on dominant-negative mutations in the STAT3 gene [343]. As discussed above, the TF STAT3, whose expression can be induced by IL-6, IL-22, and IL-23, is central for Th17 cell differentiation. Defective Th17 cell polarization results in reduced secretion of IL-17A and IL-22, which respectively favors neutrophil recruitment and the secretion of microbial peptides from epithelial cells. The enhanced levels of IgE might be explained by augmented Th2 responses that occur in the absence of functional Th17 cells. Altogether, the HIES illustrates how defective Th17 cell polarization results in disease development. These observations are in line with early findings pointing to the crucial role of IL-17A and F signaling in chronic mucocutaneous candidiasis disease (CMCD), which is triggered by *Candida albicans* and presents with recurrent or persistent infections of the skin, nails, and oral and genital mucosae [344]. Interestingly, gain-of-function mutations of STAT1 leading to reduced Th17 cell polarization in response to STAT3-activating

cytokines were also associated with CMCD [345]. Overall, these two diseases illustrate that the dysregulation of CD4 T cell polarization can be highly relevant in a clinical situation.

The relevance of Th2 cell polarization in asthma development has been documented in several preclinical studies. Th2-driven IL-4 secretion indeed promotes B cell class-switching to IgE, an event that supports the development of asthma [346]. These findings are relevant in clinical situations. In this regard, candidate susceptibility genes for asthma that are directly related to Th2 cell biology have been identified. These include genes encoding the β subunit of the high affinity IgE receptor FcεR1 and IL-3, IL-4, IL-5, IL-9, IL-13, and GM-CSF. Interestingly, all the later cytokines enhance IgE class switching, eosinophil survival, and mast-cell proliferation, thereby supporting the development and the maintenance of an IgE-driven allergic response. Accordingly, genetic variation in the promoter region of the gene encoding IL-4 favors enhanced IgE levels in atopic individuals. In addition, functional variations of the IL-4 receptor have been associated with asthma [347]. These findings altogether demonstrate that enhanced Th2 cell polarization can contribute to asthma development and/or exacerbate this disease.

Regulatory T cells are central to the maintenance of tolerance. Thus, defects in Treg cell differentiation have profound consequences in living organisms. As discussed before, the TF Foxp3 is central for Treg cell biology. Accordingly, mice carrying the scurfy mutation, which results in the loss of the DNA-binding domain of Foxp3 [348], die within a few weeks after birth because of massive lymphoproliferation and autoimmune response. The human relevance of these mouse results has been underscored in individuals suffering from the IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome. This autoimmune disease, which can be fatal, is caused by mutations in the FOXP3 gene [348, 349]. Interestingly, individuals with IPEX do not present with reduced circulating Treg numbers compared to healthy individuals. However, their suppressive ability is compromised, explaining the development of autoimmunity.

Defects in the execution of effector CD8 T cell functions can also result in disease development. For instance, the hemophagocytic lymphohistiocytic (HLH) syndrome is a life-threatening disease that is caused by defective release of cytotoxic granules by CD8 T cells and NK cells. However, cytokine secretion from these two cell types is preserved in patients with HLH and actually contributes to enhance disease severity because of an increased release of proinflammatory cytokines [350]. Variants of HLH have also been characterized and are named familial hemophagocytic lymphohistiocytosis (FHL). Interestingly, these variants all affect specific proteins in the CD8 T cell cytolytic pathway. In this regard, inherited deficiencies of the cytolytic granule perforin, which forms a pore in the target cell thereby enabling its killing, are associated with FHL2 [351]. Likewise, defects in the proteins syntaxin 11 and Munc18-2, which are required for the fusion of the vesicles containing the cytotoxic granules and the plasma membrane, are respectively associated with FHL4 and FHL5. Defects in CD8 T cell cytotoxic activity also manifest in the Chediak–Higashi syndrome (CHS), which is caused by mutations in the gene encoding LYST protein. CTLs from patients with CHS have cytotoxic granules unable to degranulate [352]. Abnormal granules accumulate in T lymphocytes, which have a defective cytolytic activity. Affected individuals suffer from severe bacteria and fungi infections. Overall, defects in CD8 T cell cytotoxicity are directly linked to several diseases in humans.

5.6 Concluding Thoughts

T cells differentiate into various effector and regulatory subsets that harbor specialized functions. The high degree of plasticity of some T cell subsets is also important not only to protect the host integrity against a diverse range of infections but also during the course of a given disease. An illustrative example is the infection course of Salmonella, which initially colonizes

the intestinal epithelium, thereby eliciting Th17-dependent responses specific for bacterial flagellin that results in IL-22-dependent release of antimicrobial proteins that restrain bacterial growth. Flagellin-specific Th1 cells are also detected during this phase of the infection and may arise from ex-Th17 cells. Bacteria subsequently downregulate flagellin expression to evade killing by IFNγ-activated macrophages and spread systemically. However, during the systemic phase of the infection, there is an adaptation of the T cell response towards the bacterial antigens that enable survival within macrophages, resulting in Th1 cell polarization and bacterial clearance [353]. This time and context-dependent adaptation of the immune response underscores the importance of understanding the key processes responsible for the differentiation of immune effector and regulatory T cell subsets. In this regard, kinetic functional genomic analyses and single cell analyses of differentiating T cell subsets will surely permit the identification of novel druggable targets for therapeutic purposes [35, 271, 354–357].

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Regulatory T Cells: Their Role, Mechanism of Action, and Impact on Cancer

6

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Contents

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6.1 Introduction

Generating antitumor immunity by using therapeutic monoclonal antibodies to block immune checkpoint receptors expressed on the surface of T cells has led to a revolution in the treatment of several solid tumors and hematologic malignancies [1]. T cells upregulate expression of immune checkpoint receptors following prolonged antigen stimulation, and expression of these receptors is associated with T cell dysfunction. Monoclonal antibodies targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), or programmed death-ligand 1 (PD-L1) have been successful in the clinic. These advances in immuno-oncology have led to prolonged survival in some patients with aggressive cancers, such as metastatic melanoma and non-small cell lung carcinoma [2]. Despite the success of immuno-oncology, there are still many patients that do not derive benefits from

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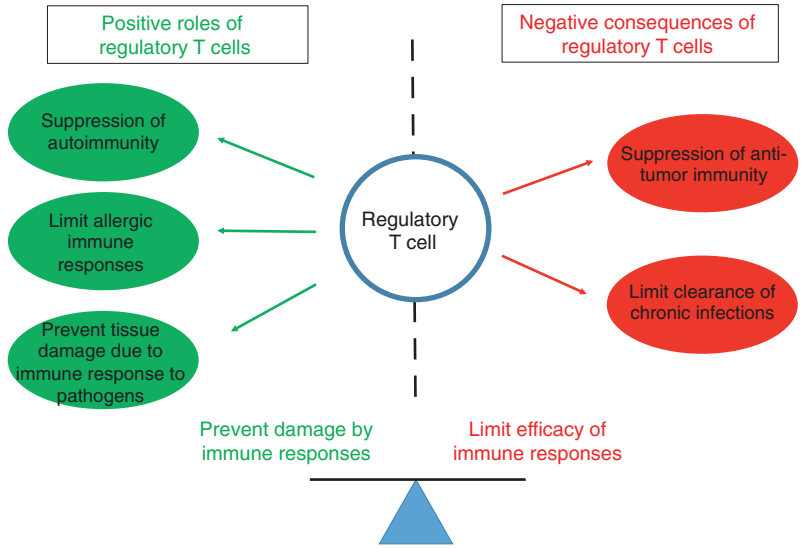

Fig. 6.1 Regulatory T cells (T_{regs}) limit self-directed immune responses but also suppress antitumor immunity and clearance of chronic viral infections. T_{regs} are essential for the maintenance of peripheral tolerance and control immune homeostasis through suppression of autoimmunity, limiting allergic immune responses and preventing damaging immune responses to pathogens. However, because of these normal immunosuppressive functions, T_{regs} also have negative consequences in that they can prevent effective antitumor immunity and limit clearance of chronic viral infections

blockade of inhibitory receptors, suggesting that additional immune mechanisms may need to be targeted to elicit an effective antitumor response. Regulatory T cells (T_{regs}) maintain peripheral tolerance by limiting inflammation and autoimmunity. However, T_{regs} also inadvertently limit the clearance of chronic viral infections and lead to tumor tolerance because of their homeostatic role in limiting tissue damage (Fig. 6.1). In this chapter, we discuss T_{regs} in the context of immuno-oncology, beginning with the discovery of T_{regs} and T_{reg}-specific cell markers, describing the immunosuppressive mechanisms of T_{regs}, and presenting evidence for the roles T_{regs} play in limiting antitumor immunity.

6.2 Discovery of a T Cell Population that Regulates Autoimmunity

T_{regs} were first discovered as a subpopulation of $CD4^+$ T cells that were responsible for preventing autoimmunity. Subsequent work over several decades has elucidated molecular pathways and surface receptors associated with T_{regs} and has led to an appreciation for the central role they play in maintaining peripheral tolerance *in vivo*. As such, research into the role of T_{regs} in cancer continues to expand, especially in the context of immunotherapeutic targeting. An initial discussion of the discovery and elucidation of surface markers for the identification of this T cell population is warranted to establish a basic understanding of the role of T_{regs} in immune homeostasis.

6.2.1 Suppression of Autoimmunity by $CD4^+$ T Cells

The importance of T_{regs} was first described in studies of autoimmunity in animal model systems dating back to the mid-1960s. These early studies showed that removal of the thymus from neonatal mice led to severe autoimmunity in many organs, including hematological disorders, endocrinopathies, gastritis, and oophoritis/orchitis [3]. These studies demonstrated an important role for T cells derived from the thymus in suppressing immune responses in a variety of tissues.

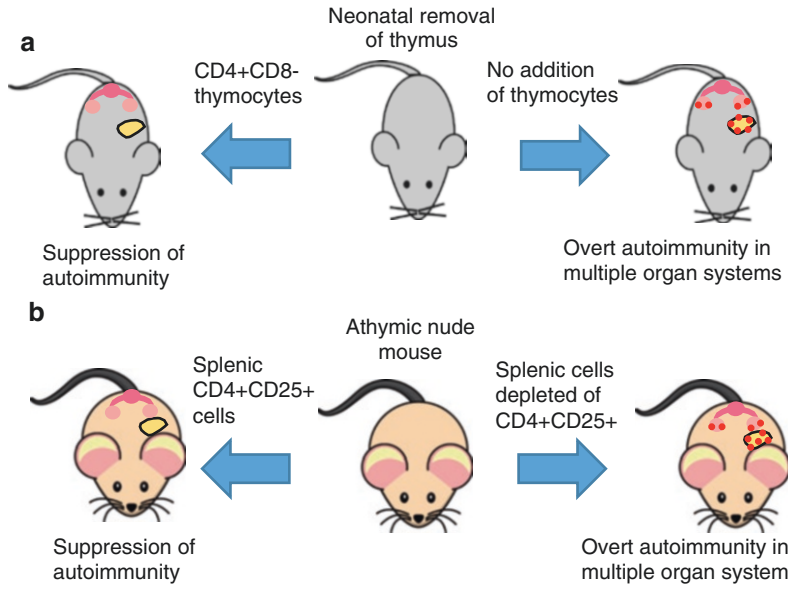

Fig. 6.2 Discovery of a T cell population that suppresses autoimmunity. (**a**) When the thymus is removed from neonatal mice within 3 days of birth, overt autoimmunity occurs in diverse organ systems such as the ovaries and pancreas. However, this autoimmunity can be prevented when $CD4^+CD8^-$ thymocytes are used to reconstitute these athymic mice. (**b**) Subsequent work showed that autoimmunity occurred in multiple organ systems when naturally athymic nude mice were reconstituted with splenic cells depleted of $CD4^+CD25^+$ T cells. Conversely, reconstitution of nude mice with splenic $CD4^+CD25^+$ T cells led to the suppression of autoimmunity. Collectively, these experiments demonstrated that thymus-derived $CD4^+CD25^+$ T cells that develop soon after birth are responsible for the suppression of autoimmunity

Importantly, these early studies also demonstrated that a T cell subpopulation was required to prevent autoimmunity as the transfer of $CD4^+CD8^-$, but not $CD4^-CD8^+$, thymocytes was sufficient to abrogate autoimmunity (Fig. 6.2a).

6.2.2 Markers of Regulatory T Cells

Subsequent studies sought to define cell-intrinsic markers of this suppressive cell population. Studies to identify markers of T_{regs} first focused on the observation that T_{regs} appeared to be an activated T cell population. A cell surface marker that is associated with T cell activation and function is the high-affinity IL-2 receptor α-chain (IL2Rα or CD25). The importance of CD25 in T_{reg} biology was discovered by comparing reconstitution of athymic nude mice with $CD4^+CD25^+$ versus $CD4^+CD25^-$ splenocytes. Reconstitution of nude mice with the $CD4^+CD25^+$ subpopulation led to suppression of autoimmunity, while the $CD4^+CD25^-$ T cell-reconstituted mice succumbed to autoimmunity (Fig. 6.2b) [4]. This study was the first to highlight the importance of CD25 as a marker of T_{regs}.

Advancing these observations demonstrated that both CD25 and IL-2 are essential for T_{reg} development and survival [5]. Although IL-2 contributes to survival and function of all T cells via CD25, it is critically important for T_{regs}, so much so that $CD4^+CD25^+$ T cells are highly enriched for T_{regs}. Importantly, T_{regs} do not secrete IL-2 themselves and therefore require paracrine production of IL-2 by other cell types to exert their effector function. Following the identification of $CD4^+CD25^+$ T cells as a suppressive cell population, additional studies sought to further define markers of T_{regs}.

The discovery of a genetic mutation that led to a severe autoimmune disease in mice (known as scurfy), and a similar disease in humans (known as immunodysregulation polyendocrinopathy enteropathy X-linked syndrome or IPEX), led

investigators to consider the role of this gene in T_{regs}. This single X-linked gene (now known as *Foxp3*) was found to encode a key transcription factor (FOXP3) that directs the formation and function of T_{regs} [6]. FOXP3 has been shown to be critical in driving the development and suppressive function of T_{regs} by controlling the transcription of key genes required for the maintenance and function of T_{regs}. FOXP3 drives or enhances the transcription of genes associated with suppression, such as CD25 and CTLA-4, while simultaneously suppressing the transcription of inflammatory genes, such as IFN-γ and IL-2. Expression of FOXP3 itself is driven by the epigenetic hypomethylation of the *Foxp3* promoter, which is considered a hallmark of T_{regs} [7]. While FOXP3 is a specific marker for T_{regs}, it should be noted that FOXP3 can also be transiently expressed in activated human $CD4^+$ T cells [8]. This transient expression of FOXP3 following activation can cause some effector T cells to appear to be $FOXP3^+$ T_{regs}, and therefore phenotypic identification of human T_{regs} should rely on a combination of markers (i.e., coexpression of CD25 and FOXP3 or the absence of CD127, as described below). Additionally, FOXP3 cannot be used to sort T_{regs} from unmanipulated samples from mouse or human donors, as it is a transcription factor that is expressed in the nucleus. Consequently, murine studies in which $FOXP3^+$ cells are purified routinely rely on the use of genetic reporters.

More recently, the absence of the interleukin-7 (IL-7) receptor (CD127) on $CD4^+CD25^+$ T cells has been described as a population that is enriched for $FOXP3^+$ T_{regs}. Conventional memory T cells require signals from IL-7 for their maintenance and as such express high levels of CD127 [9]. Conversely, T_{regs} do not express CD127 because FOXP3 suppresses transcription of the *Il7r* gene, leading to the absence of CD127 on cells that express FOXP3 [10]. In addition to being enriched for expression of FOXP3, cells that are $CD4^+CD25^+CD127^-$ are highly suppressive *in vitro*, demonstrating that this population is functionally T_{regs}. Collectively, these markers have facilitated the purification and analysis of T_{regs}. However, identification of additional markers would be beneficial.

6.2.3 Regulatory T Cell Origins: Thymus Versus Periphery

The role of T_{regs} in preventing autoimmunity was described using thymus-derived T_{regs} (tT_{regs}). However, populations of suppressive $CD4^+FOXP3^+$ T cells can also be generated outside of the thymus [11]. T_{regs} that develop *in vivo* outside of the thymus are known as peripheral T_{regs} (pT_{regs}). pT_{regs} differentiate from naïve $CD4^+$ T cells in the periphery following activation of naïve CD4+ T cells with suboptimal doses of antigen in the presence of transforming growth factor β (TGF-β). Activation of naïve CD4+ T cells under these conditions leads to the induction of FOXP3, the inability to secrete the effector cytokines IFN-γ and IL-2, and the ability to suppress proliferation of effector T cells *in vitro*. While tT_{regs} clearly limit autoimmunity *in vivo*, the role that pT_{regs} play is less clear [12]. One proposed function of pT_{regs} is to suppress immune responses to potentially damaging antigens, such as the gut microbiota, that are not recognized by the self-directed T cell receptor repertoire of tT_{regs}. Alternatively, pT_{regs} may be important for controlling immune responses in specific situations, such as in response to mucosal inflammation [13] or in controlling fetal-maternal tolerance [14]. It is clear that pT_{regs} can be induced in specific situations or to specific antigens *in vivo*, but their contribution relative to tT_{regs} in controlling autoimmunity and ultimately their role in cancer immunology requires further investigation. Taken together, tT_{regs} are indispensable for limiting autoimmunity *in vivo*, while pT_{regs} most likely play a role in controlling immune activation in specific scenarios where exogenous antigen-specific T_{regs} are required.

The identification of a $CD4^+$ T cell population that is essential to the prevention of autoimmunity has led to an entire branch of immunology dedicated to their study. Progress over several decades has led to substantial insight into the role of T_{regs} in suppressing autoimmunity, the origin and development of T_{regs}, and the appreciation for the essential role that FOXP3 plays in driving T_{reg} development and function. Identification of cell surface markers for T_{regs} has also accelerated their analysis *in vitro* and *in vivo*.

6.3 Regulatory T Cell Suppressive Mechanisms

Considerable attention has been devoted to understanding the mechanisms by which T_{regs} suppress immune responses. Broadly speaking, this can be broken down into two classes: mechanisms that are contact dependent and mechanisms that are mediated by soluble factors (Fig. 6.3) [15]. Contact-dependent mechanisms rely on direct interaction of T_{regs} with the cell types that are being actively suppressed. Soluble suppression mechanisms depend on either T_{reg} secretion of cytokines or metabolic inhibition of effector cells by T_{regs}. Both types of suppressive mechanisms can also be modulated and potentiated by the local microenvironment and cell-extrinsic pathways, as discussed below.

6.3.1 Contact-Dependent Suppression of Immune Responses

Early studies suggested that T_{regs} required direct contact with effector T cells or antigen-presenting cells to mediate suppression [16, 17]. Through physical interaction with either conventional CD4+ CD25- T cells or antigen-presenting cells, T_{regs} limited the production of IL-2 from effector T cells and prevented co-stimulation of effector T cells by antigen-presenting cells. T_{reg}-mediated suppression was lost following the addition of IL-2 or anti-CD28, underscoring that suppression by T_{regs} relied on deprivation of IL-2 and co-stimulation. These initial studies laid the framework for more in-depth analysis of the contact-dependent mechanisms used by T_{regs} to suppress immune responses.

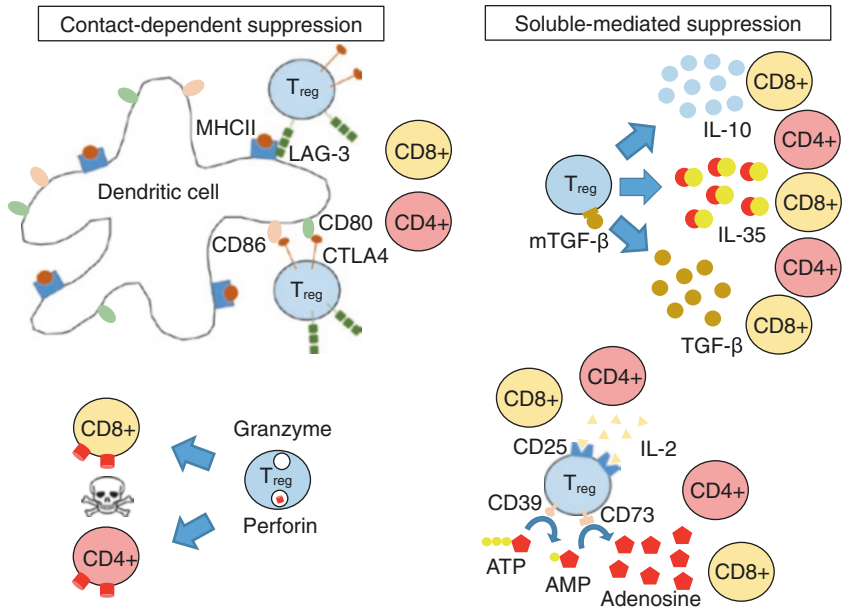

Fig. 6.3 Immunosuppressive mechanisms used by T_{regs}. T_{regs} suppress immune responses through either contact-dependent mechanisms or soluble mediators. Contact-dependent inhibition is achieved through interaction of CTLA-4 on T_{regs} with CD80/CD86 on dendritic cells or through interaction of LAG-3 on T_{regs} with major histocompatibility complex II on dendritic cells. The interaction of CTLA-4 with CD80/CD86 on dendritic cells prevents co-stimulation of CD28 on effector T cells with CD80/CD86, while LAG-3 prevents TCR/CD3-mediated activation of effector T cells. Expression of granzyme B and perforin in T_{regs} can lead to the suppression of immune responses through contact-dependent direct cytolysis of effector T cells. T_{regs} also can suppress effector T cells through soluble cytokines, such as IL-10, IL-35, and TGF-β. Alternatively, T_{reg} suppression with soluble mediators also occurs through metabolic disruption of effector T cells. This occurs through preferential uptake of IL-2 by T_{regs} due to high expression levels of CD25 (IL2Rα). The presence of the ectoenzymes CD39 and CD73 on the surface of T_{regs} can catalyze the breakdown of ATP into adenosine, which then can suppress effector T cells or dendritic cells

One surface molecule expressed by T_{regs} that mediates contact-dependent suppression is cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). CTLA-4 is required to prevent systemic autoimmunity, and knockout of this gene in mice leads to fatal autoimmune-mediated destruction of multiple tissues [18]. CTLA-4 competes with CD28 for binding to the dendritic cell-expressed co-stimulatory molecules CD80 and CD86. Compared with CD28, CTLA-4 binds to CD80/CD86 with a higher affinity, effectively depriving conventional T cells of co-stimulation. Importantly, CTLA-4 is constituteively expressed by T_{regs} and mediates one form of contact-dependent suppression. T_{regs} can also further deprive effector T cells of co-stimulation through trans-endocytosis and subsequent degradation of CD80/CD86 from antigen-presenting cells [19]. In head and neck cancer patients, treatment with targeted chemotherapy led to an increase in intratumoral CTLA-4$^+$ T_{regs}, which was associated with poor clinical outcome [20]. Contact-dependent immunosuppression by CTLA-4$^+$ T_{regs} is necessary to maintain immune homeostasis, and CTLA-4$^+$ T_{regs} are likely to play a role in suppressing antitumor immunity.

Another molecule expressed on T_{regs} that contributes to contact-dependent suppression is lymphocyte-activation gene 3 (LAG-3) [21]. In addition to expression on T_{regs}, LAG-3 is upregulated on the surface of conventional T cells following activation. LAG-3 is associated with the T cell receptor (TCR) on the surface of T cells and binds major histocompatibility complex class II (MHC-II) molecules. This interaction between LAG-3 and MHC-II leads to inhibition of TCR/CD3-mediated T cell activation. LAG-3 can also directly modulate dendritic cell function by interacting with MHC-II and preventing dendritic cell maturation by depriving them of activating signals from conventional CD4$^+$ T cells. Consistent with a role for LAG-3 in contact-dependent suppression by T_{regs}, *in vitro* or *in vivo* blockade of LAG-3 reduces suppression by T_{regs} [22]. Further, genetic deletion of *Lag3* in mice also led to reduced suppressive activity [23]. In melanoma and colorectal cancer patients, LAG-3$^+$ T_{regs} are expanded in peripheral blood compared with healthy donors and are present at a higher frequency within lymph nodes containing tumor metastases compared with normal lymph nodes [24]. Furthermore, FOXP3$^+$LAG-3$^+$ cells were found to secrete IL-10 and TGF-β and to potently suppress proliferation in a contact-dependent manner *in vitro*.

A separate contact-dependent mechanism by which T_{regs} can exert effector function is through the release of cytolytic granules containing granzyme and perforin. Although this is a feature that is normally restricted to CD8$^+$ T cells, T_{regs} can express granzyme and perforin and can eliminate autologous cells through a perforin-dependent pathway [25]. T_{regs} expressing granzyme and perforin are therefore able to suppress immune responses through the direct elimination of effector T cells. Finally, one study demonstrated the importance of granzyme and perforin in the suppression of antitumor immunity in a murine cancer model, underscoring the importance of this contact-dependent mechanism in promoting tumor growth [26].

6.3.2 Suppression of Immune Responses via Soluble Factors

The second general mechanism by which T_{regs} can exert their suppressive function is either by secretion, uptake, or generation of soluble molecules. As discussed earlier, T_{regs} are characterized by constitutive expression of the IL-2 receptor CD25. This high expression of CD25 causes T_{regs} to preferentially bind IL-2, depriving conventional T cells of this important stimulatory cytokine. Effector T cell deprivation of IL-2 at inflammatory sites subsequently leads to loss of their effector function and apoptosis [27].

A second soluble mechanism used by T_{regs} to suppress immune responses is the secretion of cytokines, such as interleukin-10 (IL-10) [28]. In particular, IL-10 production by T_{regs} plays a role in controlling inflammation at mucosal sites, and mice that lack the *Il10* gene in T_{regs} develop spontaneous colitis and inflammation of the skin and lungs [29]. IL-10 can directly inhibit effector T cells through interaction with the

hetero-tetrameric IL-10 receptor complex, leading to activation of STAT3 and transcription of anti-inflammatory genes [30]. In addition to direct suppression of effector T cells, IL-10 can also suppress immune responses by limiting the ability of macrophages to produce inflammatory cytokines [31]. Similarly, IL-10 also prevents the maturation of dendritic cells and inhibits their expression of co-stimulatory molecules [32]. While T_{regs} are noted for their ability to secrete IL-10, other cell types, such as macrophages under certain conditions, also secrete IL-10 [28]. Although IL-10 is a highly pleiotropic cytokine, it has a clear role in suppression of immune responses by T_{regs}.

Another important cytokine produced by T_{regs} that has been shown to play a broad and important role in the immune system is TGF-β [33]. Unlike other cytokines, TGF-β is initially translated as an inactive protein that requires proteolysis for activation. Inactive TGF-β is non-covalently bound to latency-associated peptide (LAP) through an association with GARP on the surface of T_{regs} [34]. This membrane-bound form of TGF-β is then activated through several possible proteolytic pathways, allowing the activated form of TGF-β to perform its immunosuppressive function [35]. One of the first descriptions of a connection between TGF-β and T_{regs} was in a model of experimental autoimmune encephalitis (EAE) in mice, where oral tolerance was induced by feeding mice myelin basic protein [36]. Analysis of the $CD4^+$ T cells that infiltrated the nervous system to facilitate tolerance revealed that these cells produced TGF-β and prevented EAE. As with other T_{reg} molecules, knockout of TGF-β from murine T_{regs} leads to induction of autoimmune disease, underscoring the importance of TGF-β in immune homeostasis [37]. TGF-β suppresses effector T cell responses in several ways, including inhibiting IL-2 production and IFN-γ and perforin production in CD8+ T cells [38]. In head and neck cancer patients, an important role of TGF-β secreting T_{regs} has been described [39]. Taken together, secretion of TGF-β by T_{regs} plays an important role in maintaining immune homeostasis and can inhibit antitumor immunity.

Another important cytokine produced by T_{regs} to facilitate immunosuppression in murine models is interleukin-35 (IL-35) [40]. IL-35 is a member of the IL-12 family of heterodimeric cytokines and consists of one IL-12α subunit and one IL-27β/Ebi3 (Epstein-Barr virus-induced gene 3) subunit [41]. These cytokine genes are constitutively expressed in a subpopulation of murine T_{regs}, but not conventional T cells, and are upregulated following T_{reg} activation. IL-35 confers suppressive activity on naïve CD4+ T cells and directly suppresses division of conventional cells. Like other inhibitory cytokines, IL-35 can also drive the development of an induced T_{reg} population, called iTr35, that can suppress effector T cells via IL-35 [42]. IL-35 mediates signaling via a unique IL12rβ2:gp130 receptor heterodimer and a STAT1:STAT4 heterodimer [43]. In murine cancer models, IL-35 has recently been shown to play an important role in promoting tumor growth by contributing to T cell exhaustion in the tumor microenvironment [44]. Consistent with increased IL-35 production from highly activated T_{regs}, an IL-35 reporter mouse revealed enrichment of $IL\text{-}35^+$ T_{regs} in the tumor microenvironment, and neutralization of IL-35 or T_{reg}-specific genetic deletion of *Ebi3* led to enhanced antitumor immunity, which was mediated via enhanced cell proliferation and effector function and improved memory cell generation of effector T cells. T_{reg}-restricted deletion of *Ebi3* also led to reduced expression of the inhibitory receptors PD-1, LAG-3, and TIM-3, suggesting that IL-35 may promote exhaustion through upregulation of multiple inhibitory receptors [44].

Finally, T_{regs} can also mediate immunosuppression via the generation of adenosine, a labile, highly suppressive molecule [45]. Extracellular adenosine accumulates at sites of ischemia and inflammation *in vivo*. In the extracellular space, adenosine is generated by T_{regs} via breakdown of ATP. The extracellular ectoenzymes CD39 and CD73 on T_{regs}, or cells in close proximity, in tandem catalyze the breakdown of ATP to adenosine. While CD73 is broadly expressed on activated T cells and other cell types, CD39 expression is largely restricted to T_{regs}. Increased levels of adenosine at sites of inflammation

inhibit immune responses through interaction with either the adenosine A_{2A} receptor on effector T cells or the adenosine A_{2B} receptor on antigen-presenting cells. The interaction of extracellular adenosine with either receptor leads to increased intracellular levels of cAMP and limits the release of inflammatory cytokines from both effector T cells and antigen-presenting cells. T_{regs} can therefore limit the production of inflammatory cytokines locally by breaking down ATP into adenosine through extracellular ectoenzymes.

As described in this section, T_{regs} use multiple contact-dependent and contact-independent/contact-soluble mechanisms to suppress effector T cell responses and antigen-presenting cell development and function. Given the detrimental effects of autoimmunity and excessive immune responses and the diversity of cell populations and effector mechanisms they need to control, T_{regs} likely have evolved multiple immunosuppressive mechanisms to adequately control autoimmunity and inflammation in a variety of settings. An important question is whether certain mechanisms are more dominantly or preferentially utilized by T_{regs} in tumors and thus may be targeted therapeutically without substantially impacting the ability of T_{regs} to maintain immune homeostasis and peripheral tolerance.

6.3.3 Potentiation of Suppression and Survival

T_{regs} function in diverse environments and suppress a variety of cell types. Consequently, their function and survival are likely modulated or potentiated by a variety of environmental cues, many of which are likely poorly understood or have yet to be defined. Early studies suggested that T_{reg} suppression was contact dependent [16, 17]. However, this notion was inconsistent with the growing appreciation of the importance of cytokines in mediating T_{reg}-dependent suppression. This conundrum was resolved when a more recent study showed that it was not suppression by T_{regs} per se that was exclusively contact dependent but rather the boosting/potentiation of their suppressive activity that was contact dependent [46]. This study found that co-culture of T_{regs} with fixed or live conventional $CD4^+$ T cells or antigen-presenting cells was sufficient to boost the capacity of T_{regs} to suppress effector T cells across a permeable transwell membrane via IL-10 and IL-35.

The potentiation of T_{reg} function and survival was found to be mediated by neuropilin-1 (Nrp1) on the surface of T_{regs} via interaction with Sema4a [47]. Nrp1 is involved in normal neural and vascular development and also plays a role in tumor angiogenesis [48]. Signaling through the Nrp1/Sema4a interaction is necessary for T_{reg} suppression by soluble cytokines *in vitro* [47]. Nrp1 on T_{regs} limits Akt (protein kinase B [PKB]) activity via phosphatase and tensin homolog (PTEN), which in turn stabilizes the T_{reg} phenotype and enhances their survival and function. Consistent with a requirement for Nrp1 to mediate T_{reg} potentiation, genetic ablation of *Nrp1* in murine T_{regs} led to a significant enhancement of antitumor immunity *in vivo* but did not lead to overt autoimmunity or peripheral inflammation [47, 49]. These observations highlighted the unique role of Nrp1 in stabilizing and potentiating the survival and suppressive function of T_{regs} in the tumor microenvironment. However, it remains to be determined if the Nrp1 pathway is only utilized in the tumor microenvironment and if so why and if there are other mechanisms that regulate T_{reg} fate and function.

6.4 Relationship Between Regulatory T Cells and Cancer

T_{regs} are indispensable *in vivo* for their control of immune homeostasis through suppression of autoreactive T cells. Tumor tissue originates from healthy tissue, and as such T_{reg} suppression of autoreactive immune responses likely limits antitumor immune responses because of their normal role in protecting tissue from damage caused by overt inflammation. Experimental evidence from murine models has highlighted the importance of T_{regs} in suppressing antitumor immunity, while the presence of T_{regs} in human tumors correlates with poor prognosis.

6.4.1 Role of Regulatory T Cells in Suppression of Antitumor Immunity

The role that T_{regs} play in the suppression of antitumor immunity has been demonstrated in several mouse models. Mutation or elimination of the *Foxp3* gene in mice and humans leads to fatal autoimmunity, so germline deletion of *Foxp3* in mice cannot be used to study antitumor immunity. While T_{regs} can be limited or depleted in adult mice with antibodies targeting CD4 or CD25 or drugs such as cyclophosphamide, these treatments also impact activated effector T cells, confounding experimental interpretation [50]. Instead, inducible T_{reg}-targeting genetic systems have been used to transiently deplete T_{regs} in adult mice to study their role in suppressing antitumor immunity. One model that has been utilized in mice is insertion of the human diphtheria toxin receptor (DTR) under control of the *Foxp3* locus (*Foxp3*^{DTR}) [51, 52]. Following the administration of diphtheria toxin, all cells expressing FOXP3 are depleted, allowing for a direct assessment of the role of T_{regs} in suppressing antitumor immunity.

Using the *Foxp3*^{DTR} mice, depletion of FOXP3$^+$ T_{regs} has been performed in mice with a variety of implanted tumors. Following depletion of T_{regs}, mice had reduced tumor growth and prolonged survival compared with littermates that did not have their T_{regs} depleted [47, 53]. These mechanistic studies in mice have demonstrated that T_{regs} play an important role in suppressing antitumor immunity and that specific depletion of T_{regs} is sufficient to prevent tumor growth and prolong survival in mice. However, depletion of T_{regs} following administration of diphtheria toxin in the *Foxp3*^{DTR} mice is not specific for T_{regs} in the tumor microenvironment, and these mice quickly succumb to autoimmune disease despite their antitumor immune responses. This once again highlights the importance of T_{regs} in maintaining peripheral tolerance throughout life and suggests that systemic depletion of T_{regs} may not be a viable treatment option for cancer patients.

6.4.2 Local Expansion of Regulatory T Cells in Tumors

T_{regs} are present in many healthy tissues, and as tumors grow, T_{regs} can possibly expand locally through increased antigenic stimulation and subsequent proliferation (Fig. 6.4a). In the context of a tumor, tissue-resident T_{regs} specific for self-peptides presented on MHC receive additional anti-

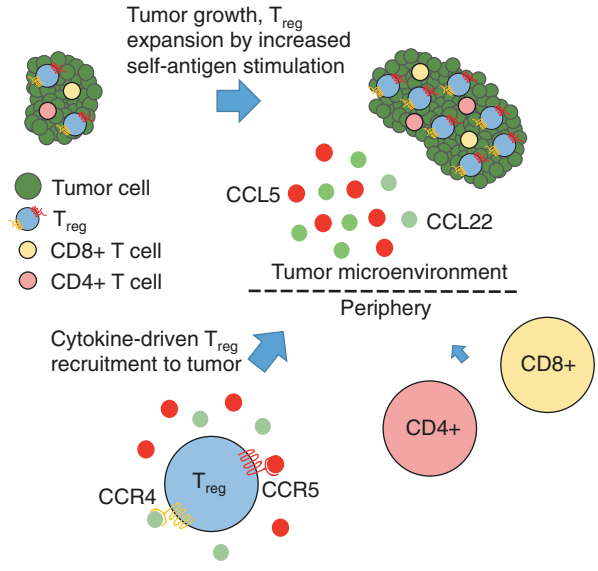

Fig. 6.4 Origins of T_{regs} in the tumor microenvironment. T_{regs} can increase in frequency within the tumor microenvironment as the tumor grows by increased self-antigen presentation and subsequent proliferation of T_{regs}. Enhanced recruitment of T_{regs} to the tumor microenvironment from the periphery can also occur through interaction of chemokine receptors on T_{regs} with chemokines produced by tumor cells, tumor-associated macrophages, or CD8$^+$ effector T cells within the tumor microenvironment. This chemokine signaling leads to the preferential accumulation of T_{regs} within the tumor

genic stimulation as the tumor grows, leading to expansion of local T_{regs}. In support of this concept, studies have found that T_{regs} within tumors have a distinct T cell receptor repertoire from conventional $CD4^+$ T cells [54] and that the T cell receptor repertoire of T_{regs} is largely skewed toward a few clonally expanded populations [55]. Also, T_{regs} are often the most proliferative immune cell type in tumors [47]. Expansion of T_{regs} specific for antigens present in normal tissue may partially explain the increased presence of T_{regs} within tumors, although this mechanism could exist in conjunction with enhanced trafficking of T_{regs} to tumors.

6.4.3 Regulatory T Cell Trafficking to Tumor Tissues

To perform their effector function, activated T_{regs} need to traffic to sites of inflammation within tissues. Trafficking of leukocytes is generally controlled via chemotactic cytokines known as chemokines. These chemokines interact with a specific array of cell surface transmembrane G protein-coupled receptors. For example, under normal physiologic conditions in the lymph node, expression of the chemokine receptor CCR7 on T_{regs} leads to their recruitment to T cell zones, where they have access to abundant IL-2 [56]. The recruitment of T_{regs} to T cell zones within lymph nodes highlights the function of chemokine receptors and ligands to target trafficking to specific anatomical locations. Similarly, secretion of specific chemokines by tumors or other immune cells within the tumor microenvironment can actively recruit T_{regs} through interaction with specific homing receptors on T_{regs}.

In cancer, specific chemokine/receptor interactions that recruit T_{regs} to the tumor are dependent on the tissue origin of the tumor and the cytokine milieu produced in the tumor microenvironment. The most commonly reported mechanism by which T_{regs} are recruited to the tumor microenvironment is through interaction of the chemokine CCL22 with CCR4 expressed on T_{regs}. First described in breast cancer, this pathway has been found to play an important role in recruiting activated T_{regs} to other tumor types including ovarian cancer, colorectal cancer, and head and neck cancer [57]. While tumor cells may actively secrete chemokines, tumor-associated macrophages (TAMs) are another major source of chemokines. TAMs were shown to be the major source of chemokines responsible for the recruitment of T_{regs} in ovarian cancer [58]. CCL22 secretion by tumor-infiltrating $CD8^+$ T cells can also drive T_{reg} recruitment into the tumor [59].

In the normal setting of inflammation, T_{regs} are recruited to limit tissue damage from the ensuing immune response. This is analogous to recruitment of T_{regs} to tumors via local expansion or chemokine-mediated recruitment. This highlights the co-opting of a normal biological process to promote tumor-induced tolerance. Studies in a wide variety of murine tumor models have demonstrated that T_{regs} play a central role in preventing antitumor immunity.

6.4.4 Regulatory T Cells and Prognosis

Many studies have evaluated associations between the frequency of T_{regs} in the tumor microenvironment and clinical outcome, including in head and neck [60], ovarian [58, 62], breast [63], pancreatic [64, 65], gastric [66, 67], lung [68], renal [69, 70], and liver cancers [71] and melanoma [61]. Studies assessing the frequency of T_{regs} within tumors have generally used tissue sections and have looked for FOXP3+ cells in the presence or absence of additional markers. Other studies have looked at the frequency of T_{regs} within tumors as a ratio of $CD8^+$ T cells to T_{regs} [72–75]. A recent meta-analysis of 17 different types of cancer across more than 15,500 cancer cases found that a higher frequency of T_{regs} in tumors was associated with poorer overall survival when considering all cancer types [76] (Fig. 6.5). Many of these studies used histology of tissue sections and have relied on identifying T_{regs} using staining for FOXP3 alone or with one or two other markers.

However, studies looking at the frequency of T_{regs} and their association with outcome have occasionally yielded conflicting results, with

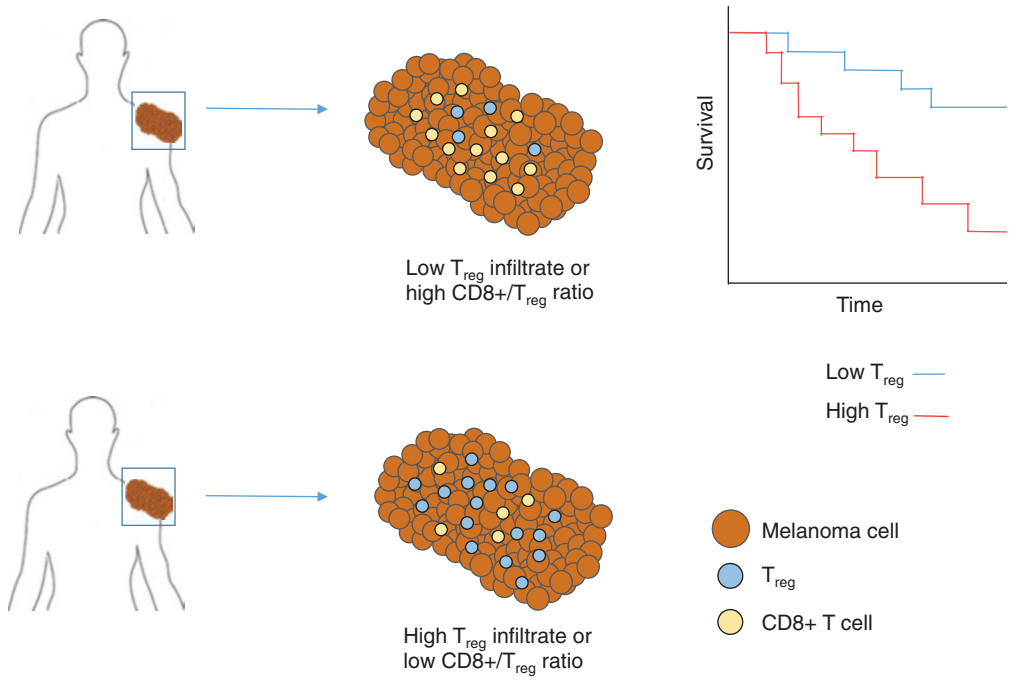

Fig. 6.5 Higher frequency of T_{regs} in tumors is associated with poorer prognosis. High absolute counts of FOXP3$^+$ T_{regs} within tumors or a low ratio of CD8$^+$ T cells to FOXP3$^+$ T_{regs} in tumors is associated with poorer outcomes (i.e., shorter overall survival) compared with low counts of FOXP3$^+$ T_{regs} or a high ratio of CD8$^+$ T cells to T_{regs}

some studies concluding that a higher frequency of T_{regs} is associated with poor clinical outcome and others showing that a higher frequency of T_{regs} is associated with better clinical outcome. These conflicting results have been found in studies looking at colorectal [77, 78], breast [79, 80], ovarian [81], and gastric [82] cancers.

Given the lack of clarity in the relationship between the frequency of T_{regs} in tumors and prognosis, recent work has attempted to further elucidate the role of FOXP3$^+$ T_{regs} in tumors. As discussed above, FOXP3 is predominantly expressed by T_{regs} but is also transiently expressed at lower levels in activated T cells. Given that human CD4$^+$FOXP3$^+$ T cells can contain both T_{regs} and activated effector T cells, studies that rely on FOXP3 histological analysis using no additional markers can have difficulty in accurately identifying T_{regs}. Erroneous identification of effector T cells as T_{regs} could underlie the variable conclusions reported. A recent study evaluating the role of CD4$^+$FOXP3$^+$ T_{regs} in colorectal cancer has sought to evaluate the role of FOXP3$^+$ T_{regs} versus FOXP3$^+$ effector T cells in controlling the prognosis in colorectal cancer [83]. These authors found that there were two distinct classes of colorectal cancer immune infiltrates: inflammatory and suppressive. Intriguingly, the authors found the role of FOXP3 expression was critically dependent on the class of immune infiltrate. In patients with suppressive tumors, a high frequency of FOXP3$^+$ T_{regs} was observed, and in this group, higher expression of FOXP3 was associated with poorer clinical outcome. In the second group of patients, with inflammatory tumors, a higher frequency of FOXP3$^+$ effector T cells was found. In this inflammatory group, higher expression levels of FOXP3 was associated with better overall survival. This important study highlights that not all human CD4$^+$ T cells that express FOXP3 are T_{regs} and that studies aimed at assessing the role of T_{regs} in tumors need to carefully classify FOXP3$^+$ cells as T_{regs} versus activated effector T cells. Additional studies in other cancer types are needed to fully understand the prognostic significance of T_{regs} in human cancer.

6.5 Immunotherapeutics and Regulatory T Cells

6.5.1 Altering the Balance Between Regulation and Inflammation

A hallmark of immunotherapy is the reinvigoration of the immune response against tumors. Exhausted $CD8^+$ T cells in the tumor microenvironment bear transcriptional hallmarks of dysfunction. $CD8^+$ T cells expressing inhibitory receptors exist on a spectrum of dysfunction, with higher levels of PD-1 expression, in conjunction with expression of additional inhibitory receptors such as LAG-3, TIM-3, and TIGIT, associated with the most dysfunctional cells [84]. Blockade of inhibitory receptors results in either partial or full functional reinvigoration of previously exhausted cells or a prevention of further exhaustion. However, not all patients respond to checkpoint inhibition. One potential explanation for the failure of patients who express PD-L1 within tumors to respond to PD-1/PD-L1 blockade is that their tumors may be enriched for T_{regs}. Increased frequencies of T_{regs} in $PD\text{-}L1^+$ tumors could lead to the failure of exhausted cells to be converted to effector cells due to the presence of suppressive cytokines, lack of co-stimulation, or inability to access presented antigens. Also, secretion of IL-35 by T_{regs} leads to expression of multiple inhibitory receptors on $CD8^+$ T cells, potentially rendering $CD8^+$ T cells unresponsive even in the presence of PD-1/PD-L1 blockade. Expression of multiple inhibitory receptors on $CD8^+$ T cells could significantly contribute to the lack of response following immunotherapy in patients, and many clinical trials are now currently investigating simultaneous blockade of multiple inhibitory receptors.

6.5.2 Potential Direct Effects of Therapeutics on Regulatory T Cells

Antitumor immunity is enhanced in some patients following blockade of CTLA-4 or PD-1/PD-L1. Much focus has been devoted to understanding the molecular dysfunction of effector $CD8^+$ T cells that express the inhibitory receptors CTLA-4 and PD-1 and the ways in which blockade of these inhibitory receptors improves the function of these effector cells. However, $CD8^+$ effector T cells are not the only cells that express these inhibitory receptors. Both effector $CD4^+$ T cells and T_{regs} can express inhibitory receptors, and their blockade, particularly on T_{regs}, may affect their frequency and function. While the most well-understood mechanisms governing immunotherapy are those that are controlled by effector $CD8^+$ T cells, potential effects of blockade of inhibitory receptors on T_{regs} are also an area of highly active research. PD-1 can be expressed on T_{regs}, but the effect of PD-1 signaling in T_{regs} is still unclear. CTLA-4 is also expressed on T_{regs}, and the role played by CTLA-4 in suppressing immune responses by T_{regs} is well described. However, the relative role that blockade of CTLA-4 on T_{regs} versus effector T cells has on antitumor immunity is an area of active research.

CTLA-4 is constitutively expressed by T_{regs} and is one of their key contact-dependent immunosuppressive mechanisms. Despite the appreciation of the role that $CTLA\text{-}4^+$ T_{regs} play in mediating immunosuppression, the enhancement of antitumor immunity has largely been attributed to the effects of blockade of CTLA-4 on conventional $CD8^+$ T cells [85]. The notion that the efficacy of CTLA-4 blockade is largely achieved through $CD8^+$ T cells is consistent with findings demonstrating that ligation of CTLA-4 limits activation of T cells in a cell-intrinsic manner. However, it is also possible that administration of anti-CTLA-4 may either deplete or alter the function $CD4^+FOXP3^+CTLA\text{-}4^+$ T_{regs}, leading to enhanced antitumor immunity.

Experimental evidence in support of the depletion of T_{regs} by CTLA-4 blockade comes from a mouse model in which the Fc receptor (FcR) portion of the antibody (the portion of the antibody that mediates antibody-dependent cellular cytotoxicity) of the CTLA-4 blocking antibody was mutated. In the absence of FcR binding, the effect of CTLA-4 blockade on tumor growth was largely lost, suggesting that antibody-dependent cellular cytotoxic elimination of T_{regs} may contribute to

the efficacy of CTLA-4 therapy [86, 87]. Secondly, emerging studies in humans have suggested that the efficacy of CTLA-4 therapy is dependent on the elimination of T_{regs} within the tumor microenvironment [88]. These studies in mice and humans demonstrate that depletion of CTLA-4$^+$ T_{regs} may play a role in the response following blockade of CTLA-4, but it is also possible that blocking this ligand on T_{regs} may affect their function. For example, CTLA-4 on T_{regs} interacts with CD80/CD86 on dendritic cells, leading to expression of indoleamine 2,3-dioxygenase (IDO). IDO-expressing DCs potently suppress T cell activation [89]. However, if CTLA-4 is blocked or CTLA-4+ T_{regs} are depleted, this may prevent upregulation of IDO on DCs, leading to enhanced antitumor immunity. Overall, the mechanisms of action by which CTLA-4 and PD-1/PD-L1 blockade enhanced antitumor immunity are areas of active investigation. It will be important to fully assess the impact of checkpoint inhibition of T_{reg} frequency and function.

6.6 Perspectives on the Importance of Regulatory T Cells in Immuno-Oncology

T_{regs} are an essential CD4$^+$ T cell subpopulation that control peripheral tolerance and immune homeostasis. However, T_{regs} can also limit antitumor immunity as demonstrated in a wide variety of mouse cancer models. There is also growing support for the importance of T_{regs} in limiting antitumor immunity in a broad range of human cancers. Consequently, effective therapeutic targeting of T_{regs} in cancer will likely be restricted to mechanisms that are selectively or preferentially utilized by intratumoral T_{regs}, without inducing detrimental autoimmune or inflammatory consequences. While new potential therapeutic targets for the selective modulation of intratumoral T_{regs} have recently been described, further T_{reg}-focused discovery efforts are clearly warranted. Whether T_{regs} limit the efficacy of checkpoint blockade (i.e., PD-1/PD-L1 or CTLA-4 blockade) is another important question that remains unresolved. The central role that T_{regs} play in normal physiology and cancer immunology suggests that future immunotherapeutics must carefully consider their impact on T_{reg} function in tumors and in the periphery.

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Purinergic Receptors: Novel Targets for Cancer Immunotherapy

7

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Contents

Key Points

- Solid tumors display regions of hypoxia, and this triggers cells in the tumor microenvironment (TME) to release ATP and adenosine. ATP through its action on P2 purinergic receptors (P2R) predominantly exerts pro-inflammatory signals to inhibit tumor cell growth. By contrast, activation of the P1 purinergic receptors (P1R) by adenosine favors pro-tumor and anti-inflammatory immune functions.
- Of the known P2 receptors, the pre-clinical and clinical role of P2X7R is most extensively studied. P2X7R exhibits bi-functional phenotype; depending on the concentration of extracellular ATP and cell type it can either promote or inhibit tumor growth.
- Activation of adenosine receptors A2AR and A2BR is known to promote tumor growth and metastases via distinct immune and non-immune cell mechanisms. In the TME, the pro-tumor effects of A2AR are largely mediated

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through its activation on immune cells, while expression of A2BR on tumor cell is demonstrated to be critical for tumor progression and metastases.
- Ecto-nucleotidases (CD39 and CD73) are the major source of extracellular adenosine in the TME. Increased expression of CD39 and CD73 is associated with poor prognosis in patients with cancers. They are ubiquitously expressed on tumors and immune cells, and have been identified to mediate immunosuppression through pathways acting on both these cell types.

7.1 Introduction

Tumor hypoxia has been a topic of immense interest to oncologists ever since its discovery by Thomlinson and Gray in 1955. In their study, the authors observed that tumors from human lung cancer patients were radio-resistant and upon histological analysis, exhibited a non-uniform distribution of oxygen [1]. In 1977, Reinhold et al. extended on these initial findings and concluded that the architecture of blood vasculature encapsulating tumors was disordered, which subsequently led to fluctuations in oxygen levels [2]. Currently, we know that a hypoxic tumor microenvironment (TME) is genetically unstable [3] and undergoes constant metabolic re-wiring [4, 5]. This hypoxic environment triggers cells in the TME to release the pro-inflammatory adenosine triphosphate (ATP), which is subsequently dephosphorylated by Ecto-nucleotidases (CD39 and CD73) to immunosuppressive adenosine [4]. Work by Geoffrey Burnstock was instrumental in demonstrating that extracellular ATP and adenosine were able to associate with plasma membrane-bound purinergic receptors in an autocrine/paracrine manner. In 1978, his team classified purinergic receptors into two sub-groups, one selective for ATP/adenosine diphosphate (ADP) (called the P2 receptors) and the other selective for adenosine (called the P1 receptors) [6]. In the last decade, the P2 and P1 purinergic receptors (P2R, P1R) have been demonstrated to influence tumor development via multiple non-redundant, immune and non-immune mediated mechanisms. Seminal work has now conclusively demonstrated that the activation of several of these purinergic receptors on immune cells as well as on tumors can promote tumor growth and metastasis [7–9]. Particularly, hypoxia and adenosine can downregulate the expression of major histocompatibility complex (MHC) class II molecules and other co-stimulatory molecules on antigen presenting cells (APC). This impairs the activation and effector functions of T cells, which are central for the immune control of many cancers [10]. Our group and others have further demonstrated that expression of some of these purinergic receptors may directly or indirectly suppress T cell and natural killer (NK) cell effector functions, thereby hampering immune responses against both primary tumors and their metastases [11, 12]. Adenosine can also promote the proliferation and immunosuppressive function of regulatory T cells (T_{regs}) and myeloid cell populations [13, 14]. Besides its role in immune cells, several of these ATP and adenosine receptors are overexpressed in human cancers, and their activation promotes tumor cell proliferation, invasion, migration, and angiogenesis [9, 10]. Currently, two of these purinergic receptors (CD73 and A2AR) are in clinical trials as targets for blockade in the treatment of solid cancers (www.clinicaltrials.gov), thus highlighting the importance of this pathway in tumor progression. This chapter therefore, describes the mechanisms by which ATP and adenosine change the dynamics of a TME to influence tumor growth and development. Importantly, we outline therapies that target various receptors in the purinergic pathway and have also demonstrated synergistic effects in combination with current approved immune checkpoint inhibitors, such as anti-PD-1/PD-L1 and anti-CTLA-4. Overall, evidence from mouse and human studies suggests the merit in developing agents that can specifically target various aspects of the purinergic pathway as a new avenue for cancer treatment.

7.2 Extracellular ATP and Adenosine in Cancer

Oxygen is a microenvironmental factor that is essential in regulating cellular metabolism, as well as in the maintenance of normal tissue homeostasis. The diffusion of oxygen from blood vessels into any given tissue and the proximity to these vessels determine the level of oxygenation in that tissue. Typically, the diffusion limit for oxygen is approximately 100–200 μm and tissues residing beyond this radius are classified as hypoxic [15]. In solid tumors, the poor architecture of the tumor vasculature coupled with uncontrolled proliferation of transformed cells often results in uneven oxygen availability. As a result, areas of hypoxia exist in most solid tumors, occurring either acutely or chronically. For example, in patients with squamous cell carcinoma, up to 30% of the tumor mass was identified to be hypoxic [16]. Tumors adapt to hypoxia by initiating a series of response mechanisms that favor tumor cell angiogenesis, invasion, and metabolic reprogramming [4]. Hypoxic tumors are genetically unstable and show increased frequencies of DNA breaks and DNA replication errors [3]. In addition, hypoxia modulates the expression of several genes including the multidrug resistance gene 1 (MDR1) and multidrug resistance associated proteins 1 (MRP1). This makes these tumors refractory to radiotherapy and chemotherapy [17], and therefore in the clinic, hypoxia is a negative prognostic factor [18].

Hypoxia activates hypoxia-inducible factor-1 α (HIF-1α) that in turn induces the upregulation of several biosynthetic intermediates necessary for energy (ATP) generation [4]. In tumors, extracellular ATP can be released by necrotic or inflammatory cells as well as from cancer cells themselves through mechanisms involving either the ATP-binding cassette transporters, pannexins, or connexins (outlined in Fig. 7.1) [19]. ATP being highly unstable is rapidly catabolized to ADP or adenosine monophosphate (AMP) and finally to adenosine (Fig. 7.1). Increased levels of hypoxia can therefore induce elevated levels of adenosine in the TME. This observation was first reported by Busse and Vaupel in 1996 where levels of adenosine in the TME correlated with tumor mass [20]. Since then, several studies have confirmed elevated adenosine levels in many mouse and human tumors. As an example, Zhang et al. showed that hypoxia promoted the development of tumor promoting M2-type macrophages through activation of the signaling molecule, extracellular signal-regulated kinase (ERK). This resulted in increased vascularization and enhanced progression of 3LL Lewis Lung Carcinoma growth in mice [21]. Likewise, extracellular levels of adenosine were found to be increased manyfold in various human solid adenocarcinomas such as human colon (T-84 and HT-29) and lung (A549) carcinoma as well as in mouse MC38 colon cancer compared to surrounding normal tissue [22].

During neoplasia, extracellular adenosine is generated predominantly via the sequential activation of two Ecto-nucleotidases. These include CD39, which hydrolyzes ATP and ADP to AMP, and CD73 that dephosphorylates AMP further to adenosine (Fig. 7.1). Transcription factors Sp-1 and HIF-1α mediate the upregulation of CD39 and CD73 expression, [23]. Besides CD39 and CD73, alkaline phosphatases (ALP) and several members of the nucleotide pyrophosphatase and phosphodiester family including NPP1/CD203a can also contribute to the production of extracellular adenosine. However, the involvement of this pathway in cancers is poorly understood, although it has been reported to be activated in gliomas, melanoma, prostate cancer [24], and multiple myeloma [25]. Importantly, adenosine can be further degraded to inosine in the presence of adenosine deaminase (ADA) through its association with CD26 (Fig. 7.1). A study by Tan et al., however, showed that elevated levels of adenosine itself could potently inhibit the activities of ADA and CD26 on HT-29 colorectal cancer cells [26]. It remains to be determined whether ADA is critical during cancer development.

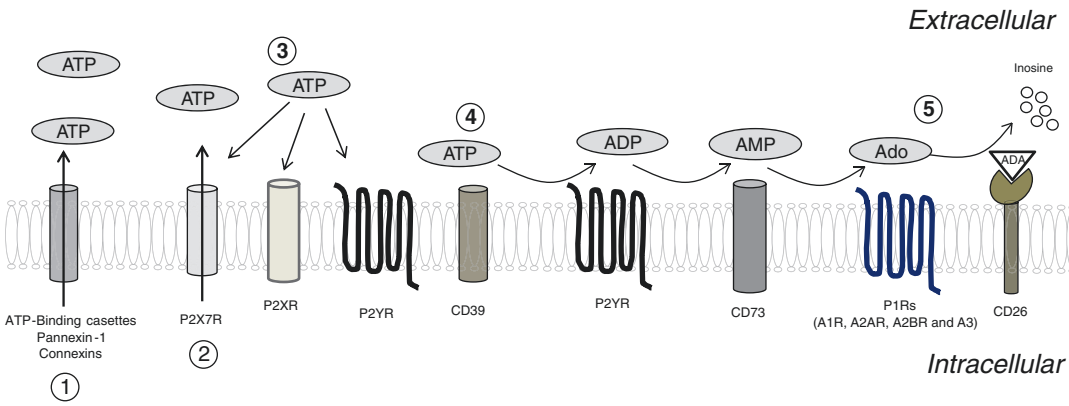

Fig. 7.1 Generation of extracellular ATP and adenosine in the tumor microenvironment (TME). (*1*) In response to hypoxia, cells in the TME release ATP through either ATP-binding casettes, Pannexin-1 or connexins. (*2*) In addition, the purinergic receptor, P2X7R can also contribute towards the secretion of ATP in the TME. (*3*) Accumulation of ATP in the milieu stimulates the P2 purinergic receptors; P2XR and P2YR. (*4*) The sequential action of CD39 and CD73 degrade ATP to ADP/AMP and adenosine (Ado) respectively, thus contributing majorly to the generation of an immunosuppressive TME. (*3*) Some P2YR including, P2Y1R, P2Y12R and P2Y13R are able to bind ADP and trigger downstream signaling. Ado modulate tumor growth by acting through P1 receptors, A1R, A2AR, A2BR and A3R. (*5*) The accumulated adenosine can be subsequently catabolized to inosine in the presence of adenosine deaminase (ADA) and its receptor CD26. *ATP* adenosine triphosphate, *ADP* adenosine diphosphate, *AMP* adenosine monophosphate

7.3 Purinergic Receptors in Immunity and Cancer

The biological actions of ATP and adenosine rely on the activation of purinergic receptors that are expressed abundantly on both tumors and the infiltrating immune and non-immune cells. ATP binds to P2Rs while adenosine binds P1Rs. The P2Rs can be further classified into seven P2X (P2X1-P2X7) and eight P2Y (P2Y1–2, P2Y4, P2Y6, P2Y11–14) receptor subtypes. P1Rs bind adenosine and comprise of four G-protein coupled receptors (GPCRs), namely A1R, A2AR, A2BR, and A3R [10]. Importantly, the concerted action of the two Ecto-nucleotidases, CD39 and CD73 constitute the major source of extracellular adenosine in the TME. In this section, therefore, we discuss how activation of the purinergic receptors as well as Ecto-nucleotidases on tumor cells and immune cells influences cancer growth and metastasis.

7.3.1 Effect on Tumor Cells

7.3.1.1 ATP Sensing P2 Receptors: P2XR and P2YRs

The high ATP concentration in the TME can directly affect the expression and function of P2Rs during tumor development. This is evident by the differential expression of P2Rs on several human and mouse cancer cell lines (summarized in Table 7.1). Typically, the expression of P2XRs on tumor cells is associated with anti-tumor functions (see Table 7.1). However, P2X7R has seemingly opposite effects on the growth and survival of tumor cells. For example, activation of P2X7R facilitated the migration of PC9 lung carcinoma and T47D breast cancer cells and siRNA knockdown of P2X7R in these cells reversed their migration ability [38]. Conversely, exogenous addition of ATP to P2X7R overexpressing acute myeloid leukemia (AML) cells repressed its expression and resulted in the inhibition of cell-cycle genes, migration, and adhesion molecules [71]. Likewise, activation of P2X7R on B16F10 melanoma cells resulted in reduced proliferation and survival of these cells [72]. A possible explanation for the disparities in these findings could be attributed to the levels of ATP used for in vitro stimulation in these studies. This contention was supported by Giannuzzo et al., who found the concentration of ATP could have opposite effects on P2X7R activity. Specifically, high levels of ATP activated P2X7R on human pancreatic ductal adenocarcinoma cells (PDAC) resulting in their reduced proliferation and survival. By

Table 7.1 Expression of purinergic receptors on cancer cells

Type of purinergic receptor	Receptor	Human cancer cells	Mouse cancer cells	Functions	References
P2XR	P2X3	Colon carcinoma (SW480)		- Inhibition of apoptosis	[27]
	P2X4	Mammary carcinoma (MDA-MB-231), non-small cell lung cancer (NSCLC) patient tumors	Mammary carcinoma (4T1.2)	- Activation of autophagy and ICD	[28, 29]
	P2X5	Lymphoid malignancies, melanoma (A431)		- P2X5R^{+} lymphoid tumors are easily lysed by Lrh-1^{+} CTLs - Inhibition of cell proliferation	[30]
	P2X7	Mammary tumors (MDA-MB-231, MCF-7, MCF-10A, T47D) NSCLC patient tumors Pancreatic ductal adenocarcinoma (PDAC) Mesothelioma Prostate cancer Lung cancer cells (pc-9, H292) Pancreatic cancer (Panc-1, MIA PaCa-1) Ovarian cancer (SK-OV3, Caov-3, MM14.Ov)	Mammary carcinoma (4T1.2) Melanoma (B16F10) Colon carcinoma (MCA38)	- Activation of autophagy and ICD - Increased cell migration and invasion - Promotes proliferation - Promotes epithelial-mesenchymal transition (EMT) - Promotes cell death - Increases Ca^{+2} levels	[28, 29, 31–39]
P2YR	P2Y1	Prostate cancer (PC-3)		- Increase in cell number - Increase migration through ERK pathway	[30, 40]
	P2Y2	Melanoma (A431), Mammary tumors (MDA-Mb-231, MCF-7) Prostate cancer (pc-3 M) Ovarian cancer (SK-OV3) Lung epithelial carcinoma (A549)		- Increase in cell number - Increase migration through ERK pathway - Increased IL-8 and invasion - Activation of EGFR and induction of EMT - Increase expression of adhesion molecules	[30, 40–43]
	P2Y6	Mammary carcinoma (MDA-MB-231)		- Invasion and cancer migration	[39]

Table 7.1 (continued)

Type of purinergic receptor	Receptor	Human cancer cells	Mouse cancer cells	Functions	References
Ecto-nucleotidase	CD39	B cell lymphoma (BL-41, B104, Ramos) Melanoma (SK-Mel-5, SK-Mel-28) B CLL (EHEB, MEC2) Ovarian cancer (OAW-42, Sk-OV3)		- Increased ATPase activity - Inhibition of $CD4^+$T cell and $CD8^+$T cell proliferation - Inhibition of NK cell cytotoxicity	[42, 44]
	CD73	Endometrial cancer (G1 EEC, HEC-1A, HEC-1B) Breast cancers (MDA-Mb-231, LM3)	Melanoma (SM1WT1, B16F10[a]) Mammary tumors (4T1.2)	- Increased expression on tumor core in A2AR-deficient mice - In endometrial cancers - CD73 is essential to prevent tumor cell migration and invasion - In B16F10 cells, CD73 inhibited cell migration and adhesion - Promotes extravasation and colonization in breast cancer cells	[11, 45–48]
Adenosine receptor	A1R	Endometrial cancer (G1 EEC, HEC-1A, HEC-1B) Glioblastoma patients Colon cancer (CW2) Breast cancer (MCF-7)	Glioblastoma (Gl261)	- In endometrial cancers, CD73 is essential to prevent tumor cell migration and invasion via A1R - Reduced tumor cell proliferation and induction of glioma cell death - Regulates expression of estrogen receptor-α on MCF-7 cells and induces tumor proliferation	[48–51]
	A2AR	Ovarian cancer (OVCAR-3, Caov-4, SCOV-3) NSCLC cell lines Melanoma (A375)		- cAMP induction - Induction of tumor cell proliferation	[52–54]
	A2BR	Mammary cancer (MDA-MB-231) Gastric cancer (HGC-27) Ovarian cancer (OVCAR-3, Caov-4) Prostate cancer (PC-3, DU145, LnCaP)	Melanoma (B16F10) Mammary tumor (4T1.2, E0771)	- Induces FGF-2 and B16F10 cell proliferation - A2BR is a target of Fos-related antigen-1 (Fra-1) and mediates metastasis - Promotes proliferation, migration, and invasion	[55–60]
	A3R	Ovarian cancer (OVCAR-3, Caov-4) Melanoma (A375) Colon cancer (HCT 116, HT-29) Renal cell carcinoma (RCC4-VHL, ACHN) Glioma cells (A172) Bladder cancer cells (253 J and 5637, T24) Breast cancer (MCF-7, MDA-Mb-231) Prostate cancer (DU-145, PC3, LNCap-FGC-10)	Colon cancer (CT-26) Melanoma (B16-BL6) Lewis lung carcinoma (LLC) Leukemia (HL60)	- Inhibition of tumor growth - Promote tumor cell apoptosis via caspase-3 and caspase-9 - Majorly inhibits but is shown to promote growth of HT29 cells - Inhibition of renal cell carcinoma growth - Increased tumor death via increased ROS production and intracellular Ca^{+2} - Reduced tumor cell migration	[61–70]

[a]High levels of intracellular but not surface CD73 was detected in B16F10 cells
ICD immunogenic cell death

contrast, low or basal levels of ATP promoted their cell migratory and invasive properties [37]. Such opposing P2X7R effects were also noted in cancer patients, whereby expression of P2X7R correlated favorably to overall survival in patients with gliomas, non-small cell lung cancer (NSCLC), and hepatocellular carcinoma (HCC). The reverse was, however, seen in patients with renal cell carcinoma (RCC) (Table 7.2).

Expression of P2YRs on tumors largely favors tumor growth, migration, and survival (Table 7.1). P2Y2Rs were detected on the human breast cancer cell line MCF-7 and its activation potentiated migration and proliferative capability [40]. Similarly, the activation of P2Y2R on MDA-MB-231 cells increased the expression of adhesion molecules intercellular adhesion molecule-1(ICAM-1) and vascular cell adhesion molecule (VCAM). This subsequently resulted in increased adhesion of tumor cells to endothelial cells, thereby facilitating tumor cell migration and invasion [92]. By contrast, the migration of AML cells in response to the chemokine CXCL12 was blunted following incubation with P2Y2R and P2Y6R agonists [71].

7.3.1.2 Adenosine Generating Ectonucleotidases: CD39 and CD73

Co- or differential expression of CD39 and CD73 has been reported on many human cancers of solid and hematological origins, as summarized in Table 7.1. Accumulating evidence now suggests that the expression of CD39 and CD73 on tumor cells can potently trigger tumor cell growth and survival. It is hence not surprising that cancer patients with high expression of CD39 and/or CD73 show poor survival and recurrence-free survival outcomes across several cancer types (Table 7.2). In pre-clinical experiments, co-culture of human $CD39^+CD73^+$ SK-MEL5 melanoma cells with human peripheral blood mononuclear cells (PBMCs) impaired the proliferation of $CD4^+$ and $CD8^+$ T cells, as well as hampered the cytolytic activity of $CD8^+$ T cells. Pre-treatment of cancer cells with a CD39 inhibitor rescued this suppressive phenotype and increased the effector functions of $CD8^+$ T cells and NK cells [42]. Similarly, CD39 expression on human ovarian cancer cells was reported to suppress T and NK cell effector functions [93]. CD73 expressing ID8 mouse ovarian cancer cells also inhibited the proliferation of $CD8^+$ and $CD4^+$ T cells and the production of the effector cytokine IFN-γ [94]. Exosomes isolated from mesothelioma patients were also found to express CD39 and CD73, which exhibited potent enzymatic activity leading to production of local adenosine. Functionally, these exosomes suppressed human $CD4^+$ and $CD8^+$ T cell proliferation and cytokine

Table 7.2 Role of purinergic receptors in human cancers

Type of purinergic receptor	Receptor	Cancer type	Major finding	References
ATP receptors	P2X3	Hepatocellular carcinoma (HCC)	- Low expression of P2X3R was associated with increased OS and RFS in these patients (n = 188)	[73]
	P2X7	Non small cell lung cancer (NSCLC)	- No association between P2X7R expression and survival in these patients (n = 93)	[74]
	P2X7	NSCLC	- High expression was associated with better RFS and OS (n = 96)	[75]
	P2X7	Glioma	- High expression of P2X7 was associated with longer survival and increased sensitivity to radiotherapy (n = 343)	[76]
	P2X7	HCC	- Increased peritumor P2X7 was associated with favorable prognosis in patients after surgical resection (n = 273)	[77]
	P2X7	Renal cell carcinoma	- Low intratumoral P2X7R was associated with better RFS in patients (n = 273)	[78]
	P2Y13	HCC	- High expression was associated with increased OS and RFS (n = 188)	[73]

Table 7.2 (continued)

Type of purinergic receptor	Receptor	Cancer type	Major finding	References
Ecto-nucleotidase	CD39	High-grade serous (HGS) ovarian cancer	- High CD39 expression showed a trend ($p = 0.0507$) towards poor OS (meta-analysis)	[79]
	CD39	HCC	- High CD39 expression in HCC tumors from 326 patients correlated with poor OS and RFS	[80]
	CD39	Gastric cancer	- Overexpression of CD39 in tumor but not peritumor was associated with poor prognosis in these patients - Similarly high CD39 expression on $CD8^+$T cells was associated with poor survival outcomes ($n = 84$)	[81]
	CD39	Chronic lymphocytic leukemia (CLLL)	- Patients with high CD39 expression on T cells but not B cells showed poor survival ($n = 68$)	[82]
	CD73	Breast cancer	- High CD73 expression was correlated with poor OS and DFS ($n = 136$)	[83]
	CD73	Gastric cancer	- High CD73 expression was associated with poor prognosis in these patients ($n = 68$)	[84]
	CD73	Triple negative breast cancer (TNBC)	- High CD73 expression was associated with poor prognosis in TNBC patients but no association was identified in patients with HER2+ tumors or luminal breast cancers	[85]
	CD73	NSCLC	- High CD73 expression was associated with poor prognosis ($n = 653$)	[86]
	CD73	Human rectal adenocarcinoma	- High tumoral CD73 was associated with worse outcomes ($n = 90$)	[87]
	CD73	Ovarian cancer	- High CD73 expression was a poor prognostic marker in these patients - This correlated with increased infiltration of $CD73^+CD8^+$T cells in the tumor (meta-analysis)	[79]
	CD73	Prostate cancer	- High expression was associated with shorter RFS and shorter bone-metastasis free survival in these patients ($n = 285$) - These observations correlated with increased CD73 expression on tumor infiltrating $CD8^+$T cells	[88]
	CD73	Oral squamous cell carcinoma (OSCC)	- High CD73 expression was associated with poor RFS and overall survival in patients with OSCC ($n = 113$)	[89]
	CD73	Head and neck squamous carcinoma (HNSC)	- High CD73 impacted OS in these patients ($n = 162$) and was associated with high lymph node metastasis	[90]
	CD73	Urothelial bladder cancer	- High CD73 expression reduced the rate of progression of cancer in these patients ($n = 174$)	[91]
Adenosine receptor	A2AR	NSCLC	- Better OS and RFS was observed in patients with high A2AR expression ($n = 653$)	[86]
	A2BR	TNBC	- High A2BR expression was associated with poor survival in TNBC patients but not in HER2+ or luminal breast cancer patients (meta-analysis)	[59]

OS Overall survival, *RFS* recurrence-free survival

release [95]. While CD73 is well characterized for its pro-tumor role, interestingly, a group recently identified an anti-tumor role for CD73 on human endometrial carcinoma. In this study, loss of CD73 on these tumors impaired actin polymerization, thus hampering cell-cell adhesion and loss of endometrial epithelial cell integrity. As a result, these tumors became highly migratory and invasive [48].

7.3.1.3 Adenosine Binding P1 Receptors: A1, A2a, A2b, and A3

P1Rs, similar to the P2YRs are widely expressed by tumor cells in mouse and humans (Table 7.1). Under physiological conditions, adenosine activates the high affinity A1, A2A, and A3 receptors. In contrast, activation of A2BR, a low affinity receptor occurs in conditions when adenosine concentration is high, which is frequently seen under pathological conditions such as the TME. Signaling via A1R and A3R inhibits intracellular cyclic AMP (cAMP), while A2AR and A2BR activate adenylyl cyclase and protein kinase A, leading to increased levels of (cAMP). High levels of cAMP is generally associated with profound immunosuppression and therefore A2AR and A2BR are viewed as tumor promoting adenosinergic receptors [96].

The suppressive function of A2AR has largely been investigated in the context of immune cells within the TME, and there exists limited understanding on how A2AR expression on tumors might influence tumor cell functions. A recent study reported that high expression of A2AR tumor biopsies of in adenocarcinoma patients correlated favorably with survival [86]. Paradoxical to this observation, inhibition of A2AR in human A375 melanoma cells reduced cell survival and proliferation in these tumors [10, 86]. Hence, the precise reason underlying these opposing results is presently unknown. A2BR, on the other hand, largely exerts its suppressive functions on tumor rather than immune cells. This receptor is detected in human and mouse triple negative breast cancer (TNBC) cells and its expression increased survival of these cells. In line with these findings, overexpression of A2BR was associated with poor survival in patients with TNBC, multiple myeloma, AML, and liposarcoma [59]. Wang et al. recently showed that microRNA-128b repressed the expression of A2BR. In patients with gastric cancer, microRNA-128b is downregulated leading to increased expression of A2BR, and this consequently increased tumor cell proliferation, migration, and survival [58].

Activation of A1R increases estrogen receptor-α (ER-α) expression on breast cancer cells and subsequently supports cancer growth [50]. The expression of A3R on tumor cells has contrasting effects. A3R is observed in primary human glioblastoma and prostate cancer, and in vitro stimulation inhibits proliferation of PC3 prostate carcinoma, HCT-116 colon carcinoma, and MIA-PaCa pancreatic carcinoma cells [97]. Paradoxically, activation of A3R on HT29 and CaCo2 colon cancer cells induces tumor cell proliferation [98]. Similarly, treatment with an A3R agonist CI-IB-MECA on human A375 melanoma cells induced the secretion of an angiogenic factor, Angiopoeitin-2 (Ang-2) [99].

7.3.2 Effects on Immune Cells

7.3.2.1 ATP/ADP Binding P2 Receptors

P2XRs mediate the transport of Na^+, K^+, and Ca^{2+} ions across the plasma membrane [10]. As a result, it was assumed that these receptors were not involved in the regulation of anti-tumor immunity. Therefore, although P2XRs and P2YRs were identified around the same time, the role of P2XRs in immune regulation is only now becoming apparent [10]. The expression of individual P2XRs on various immune cell types is outlined in Table 7.3. Of the known P2XRs, the expression and function of P2X7R has been most comprehensively investigated, in both immune and tumor cells. Cockcroft and Gomperts were the first to identify P2X7R expression in mast cells from rats [142]. They initially termed P2X7R as "ATP^{4-} receptor," because of its

Table 7.3 Expression of purinergic receptors on human and mouse immune cells

Type of purinergic receptor	Receptor	Human immune cell subsets	Mouse immune cell subsets	Function(s)	References
P2XR	P2X1R	B cells neutrophils, DC	ND	- Chemotaxis - DC maturation	[100–104]
	P2X4R	B cells, T cells, Dc, Monocytes Macrophages	Macrophages	- T cell activation - DC maturation - Calcium mobilization - Induction of autophagy	[100–106]
	P2X5R	T cells B cells	ND	- T cell activation	[107]
	P2X7R	B cells $CD4^+$ T cells $CD8^+$T cells Macrophages DC T_{regs}	Macrophages $CD4^+$ T cells $CD8^+$ T cells T_{regs} B cells	- CD62L shedding on T cells - Inflammasome activation - T_{regs} proliferation - T cell homeostasis - Shedding of CD23 on B cells	[102, 108–113]
P2YR	P2Y2R	Neutrophils Macrophages T cells	Macrophages Neutrophils DC Inflammatory monocytes	- Release of granules, activation of neutrophils - Neutrophil migration, release of inflammatory mediators - T cell activation - ROS production	[114–119]
	P2Y6R	Monocytes, Macrophages, Neutrophils	Eosinophils	- Neutrophil migration - Release of pro-inflammatory cytokines - Increases responsiveness of leucocytes to CCL2 - Induce chronic allergic inflammation	[118, 120–122]
	P2Y11R	Neutrophils NK cells DC	Only present in humans	- Activation marker - NK cell cytotoxicity and chemotaxis - Inhibition of IL-12	[123–125]
	P2Y14R	Neutrophils	T cells Neutrophils	- Chemotaxis - Inhibition of T cell proliferation	[126, 127]
Ecto-nucleotidase	CD39	Neutrophils T_{regs} B cells Tr-1 cells $CD8^+$ T cells	B cells T_{regs} MDSC NKT cells T cells	- Inhibition of IL-8 - Induction of local immunosuppression - T cell exhaustion - Survival and cytokine release by NKT cells	[128–133]
	CD73	T_{regs} B cells Tr-1 cells Monocytes NK cells	B cells T_{regs} $CD8^+$ T cells MDSC T cells NK cells	- Induction of local immunosuppression - Polarization to M2 macrophages - Inhibition of T cell cytokines - Inhibition of NK cell cytotoxicity	[131, 132, 134, 135]

(continued)

Table 7.3 (continued)

Type of purinergic receptor	Receptor	Human immune cell subsets	Mouse immune cell subsets	Function(s)	References
Adenosine receptor	A1R	Monocytes neutrophils		- Secretion of VEGF by monocytes - Chemotaxis	[136, 137]
	A2AR	Monocytes neutrophils	Macrophages $CD4^+$ T cells $CD8^+$ T cells NK cells	- M2-macrophage polarization - Inhibition of T effector functions - Chemotaxis - Inhibition of NK cell cytotoxicity	[11, 137–139]
	A2BR	Monocytes DC $CD4^+$ T cells $CD8^+$ T cells	MDSC DC	- M2-macrophage polarization - T_H2 polarization - Deactivation of T cells	[63, 139, 140]
	A3R	B cells neutrophils		- Inhibit B cell proliferation - Reduce neutrophil chemotaxis	[108, 141]

association to fully dissociated ATP (ATP^{4-}) [142]. In 1996, this "ATP^{4-} receptor" was cloned by Surprenant et al. and subsequently re-named as P2X7R, owing to its homology to other P2XRs [143]. P2YRs, by contrast, are G-protein coupled receptors that respond to ATP/ADP or even uridine 5′-phosphate (UTP) and influence the activation of (cAMP), which can either have pro- or anti-inflammatory functions [10]. Similar to P2XRs, P2YRs are also expressed in mouse and human immune cells, with the P2Y6R subtype being investigated more thoroughly than the others [10] (Table 7.3).

Similar to the effects on tumors, the activities of P2 receptors are largely modulated by the levels of extracellular ATP (Table 7.3) (Fig. 7.2). For example, low to moderate levels of ATP triggered mouse dendritic cell (DC) maturation and induction of T helper 2 (T_H2) cells via the activation of P2Y11R [144]. High levels of ATP, by contrast, activated P2X7R on human alveolar macrophages and this interaction resulted in the release of the inflammatory cytokine IL-1β [115]. Vishva Dixit's group later showed that activation of the inflammasome was responsible for the P2X7R-mediated IL-1β secretion [145]. Signaling via P2X7R can also activate several key macrophage function including production of reactive oxygen species (ROS), nitric oxide (NO), induction of phagocytosis, and formation of multinucleated giant cell formation [146]. Similar induction of migratory and chemotactic functions has been reported for P2Y2R, P2Y11R, and P2Y14R signaling on human and mouse neutrophils [147], and P2Y2R and P2Y6R on human monocytes, mouse macrophages, and DC [19]. On human NK cells, engagement of P2Y11R suppresses NK cell responsiveness to the chemokine CX3CL1, by abolishing their migratory and cytotoxic functions [124]. T cell receptor (TCR) stimulation can also trigger ATP release from T cells, which then mediates autocrine activation of P2X1R, P2X7R, and P2X4R to augment T cell proliferation and signaling [148]. Importantly, signaling via the P2YRs on human endothelial cells promotes angiogenesis through transactivation of vascular endothelial growth factor receptor-2 (VEGFR-2) [149, 150].

7.3.2.2 Adenosine Generating Ecto-nucleotidases: CD39 and CD73

Gregory et al. were the first to describe CD39 expression on B cells. In their study, CD39 was identified as an activation marker on tonsillar B cells from patients with Burkitt's Lymphoma [151]. Since then, expression of CD39 has been

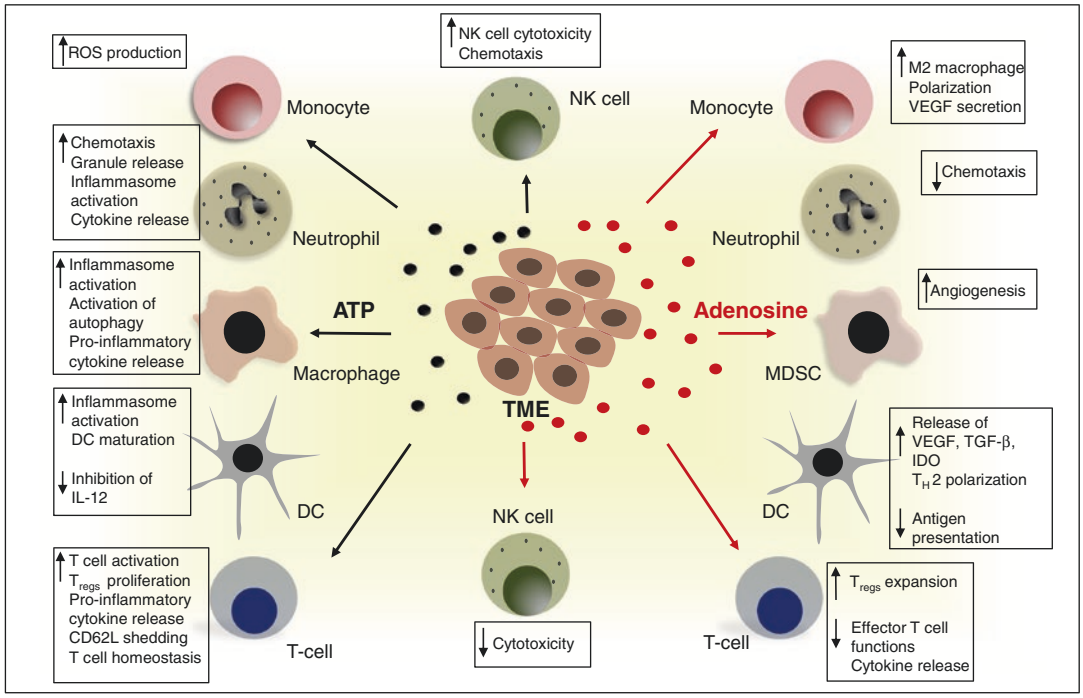

Fig. 7.2 The ecto-nucleotidase activities of CD39 and CD73 shifts the tumor dynamics from an ATP-driven inflammatory niche to an adenosine-rich immunosuppressive TME. ATP released from tumors and tumor infiltrating cells activates the P2 purinergic receptors. This primarily induces anti-tumor effects through the activation of the inflammasome, autophagy, increased cytokine release and activation of the immunogenic cell death (as shown in *left*). In contrast, the generation of adenosine through the CD39-CD73 axis negatively influences the TME and promotes tumor growth and metastasis via mechanisms such as increased angiogenesis, expansion of immunosuppressive immune populations (MDSC and Treg) and impaired cytotoxic functions of NK cells and CD8+ T cells (shown in *right*). *ATP* adenosine triphosphate, *DC* dendritic cell, *NK* natural killer, *ROS* reactive oxgen species, *MDSC* myeloid-derived suppressor cells, *VEGF* vascular endothelial growth factor, *TGF-b* transforming growth factor, *IDO* indoleamine 2,3 dioxygenase, *Treg* regulatory T cell

found to be constitutively expressed in spleen, thymus, lung, as well as on primary tumors (Table 7.3). Cytokines, oxidative stress, and hypoxia-induced transcription factors such as Sp-1, STAT3, and zinc finger protein growth factor independent-1 transcription factors (Gfi1) can all upregulate CD39 expression [24]. The expression pattern of the other ecto-nucleotidase, CD73 on immune cells and tissues also closely resembles that of CD39 [9, 24] (Table 7.3).

The immunosuppressive effects of CD39 and CD73 can be attributed to the relatively high expression of these receptors on anti-inflammatory cells including tumor-associated macrophages (TAM), T_{regs}, type-1 regulatory (Tr-1) cells, and suppressive myeloid populations (Table 7.3). In the TME, the major functions of CD39 and CD73 lie in triggering the rapid catabolism of ATP to immunosuppressive adenosine to prevent the desensitization of P2 receptors and promote tumor development. Indeed, when co-incubated with $CD4^+$ T cells, $CD73^+CD39^+$ TAM significantly suppressed the proliferation of T cells through production of adenosine. Blocking the activity of either CD39 or CD73 on TAM was sufficient to rescue this T cell effect [152]. Similarly, co-culture of $CD39^+$ Tr-1 cells with $CD73^+CD4^+$ T cells further contributes to the suppressive activity of these cells [131]. The CD39/CD73 axis is also essential for efficient activation, adhesion, and chemotaxis of neutrophils, which have been reported to have tumor promoting functions [24]. The majority of human T_{regs} are $CD39^+$ but $CD73^-$, however; CD73 in

these cells is abundantly present in the intracytoplasmic granules [131]. The expression and activity of CD73 on T_{regs} is enhanced following TCR engagement [131].

7.3.2.3 Adenosine Binding P1 Receptors: A1, A2a, A2b, and A3

P1Rs are widely expressed by immune cells of both the myeloid and lymphoid lineage in mouse and humans (Table 7.3). Amongst the P1 receptors, A1R and A2BR expression is more restricted to myeloid cells, while A2AR and A3R are more ubiquitously expressed on both myeloid and lymphocyte populations [19, 96].

Expression of A1R and A3R on immune cells primarily activates anti-tumor immune responses. For example, pre-treatment of mice with CPA, an A1R agonist resulted in enhanced proliferation of splenic $CD4^+$ T cells and increased production of IL-2 [153]. Similarly, activation of A3R using the agonist Cl-IB-MECA, enhanced NK cell functions, and improved anti-tumor immunity in mice [154].

The anti-inflammatory role of A2AR was first demonstrated by Sitkovky's group in 2001. In their study, A2AR-deficient mice demonstrated exacerbated hepatitis and more severe tissue damage compared to their wild type (WT) counterparts [155]. Currently, we understand that A2AR signaling in both myeloid and lymphoid cells can participate in mediating local immunosuppression in the TME (Fig. 7.2). A2AR signaling suppresses macrophage differentiation, maturation, and activation [156]. Furthermore, Cekic et al. showed that the expression of A2AR on myeloid cells can increase anti-inflammatory IL-10 cytokine production by TAM and tumor-associated DC (TADC) in the TME. Additionally, conditional deletion of A2AR on myeloid cells significantly enhanced infiltration, activation, and effector functions of $CD8^+$ T cells and NK cells in the tumor [12]. Likewise, the activation of A2AR on NK cells inhibits their ability to produce IFN-γ [157], while activation with A2AR agonist CGS21680 increased the proportions of T_{regs} in mixed lymphocyte cultures [158].

A2BR stimulation also promotes immunosuppression, but primarily on myeloid cells. Specifically, A2BR inhibits the differentiation of hematopoietic progenitor cells into mature myeloid cells and results in the increased accumulation of immature cells. These cells exhibit immunosuppressive functions through the production of vascular endothelial growth factor (VEGF), IL-8, TGF-β, and indolamine 2, 3-dioxygenase (IDO) [13]. Similarly, treatment of mice with an A2BR agonist, Bay60-6583 increased the frequencies of myeloid-derived suppressor cells (MDSC) infiltrating the B16F10 melanoma primary tumor, while depletion of these suppressor cells promoted anti-tumor effects in these mice, thus implying that A2BR signaling on MDSC promotes tumor progression [159].

7.4 Pre-clinical Evidence for Purinergic Signaling in Cancer

7.4.1 Extracellular ATP in Cancer

Given the extremely high concentration of extracellular ATP within the TME coupled with increased expression of P2 receptors on tumors and immune cells, it is not surprising that the purinergic pathway plays important roles in directly modulating tumor growth and anti-tumor immune responses. Interestingly, the P2X7R can have direct and opposite effects on cancer growth. Several lines of studies indicate that the engagement of P2X7R directly on tumor cells potentiates their growth whereas the same engagement on host cells such as DC, macrophages, and T cells activate potent anti-tumor responses. The pro-tumor effects of P2X7R include stimulating tumor cell growth, release of immunosuppressive factors and cytokines by MDSC, as well as stimulation of factors including VEGF to mediate tumor neovascularization [146]. To this end, P2X7R silencing in prostate cancer cells downmodulates genes that are involved in epithelial/mesenchymal transition including Snail, E-cadherin, Claudin-1, IL-8, and matrix-metalloproteases (MMP-3) [33]. Similarly, mice inoculated with mouse melanoma or human neuroblastoma cells and knocked down for P2X7R

had slower tumor growth and significant reduction in metastases [33]. Inhibition of P2X7R using the selective inhibitor, AZ10606120 also caused a strong inhibition of tumor growth and reduction in VEGF production in B16F10 inoculated mice, compared to untreated controls [160].

By contrast to the above-mentioned studies, activation of P2X7R on DCs can promote antitumor activity. P2X7R has long been known for its role in the activation of the NLRP3 inflammasome and IL-1β release. Virgilio's group demonstrated that P2X7R knockout mice exhibited accelerated B16F10 melanoma growth and lung metastases over their WT littermates. These effects were attributed, in part, to the low intratumor IL-1β that led to inefficient recruitment and infiltration of anti-tumor immune cells into the tumor [161]. Thereafter, studies confirmed that in the TME, the activation of NLRP3 inflammasome in DC through P2X7R positively influenced its antigen presenting capacity, and enhanced anti-tumor immunity in mice [162]. Additionally, IL-1β production through the inflammasome axis can, in conjunction with IL-23, promote IL-17 release by γδ T cells. This further promotes the release of IFN-γ by $CD8^+$ T cells [162, 163]. Importantly, binding of ATP to P2X7R or P2Y2R triggers an immunogenic signal such that dying cancer cells are able to exert a potent anticancer vaccine effect [164]. This was elegantly demonstrated by Zitvogel and Kroemer, where they showed that chemotherapeutic drugs such as mitoxantrone or oxaliplatin induce release of ATP from dying tumor cells. This resulted in an enhanced infiltration of DC and T cells, which promoted potent anti-tumor immunity. Importantly, this immunogenic cell death (ICD) was abrogated in mice deficient in P2X7R and these mice were unable to mount tumor-specific $CD8^+$ T cell responses [162]. Similarly, clinical observations corroborated this finding whereby loss-of-function polymorphism in P2X7R negatively affected disease outcome in breast cancer patients undergoing treatment with anthracycline-based chemotherapy [162].

Similar to P2X7R, P2Y2R can have direct pro-tumor effects. P2Y2R activation on MDA-MB-231 breast cancer cells increased their proliferation, ability to adhere to endothelial cells, and their production of MMP-9 and VEGF [92]. This study further showed that knockdown of P2Y2R suppressed invasion and migration of prostate cancer cells [165]. Interestingly, patients with NSCLC harboring mutations in the anaplastic lymphoma kinase (ALK) are often treated with ALK inhibitors, such as crizotinib. The overall response rate in these patients was 74% and progression free survival was a median of 10.9 months. Despite initial responses, resistance to crizotinib occurs in the majority of these patients [166]. Crizotinib resistant-NSCLC cell line, H3122 demonstrated an increased expression of the P2YR subtypes (P2Y1R, P2Y2R, and P2Y6R). These P2YRs upon activation led to increased protein kinase C (PKC) activity and production of endothelial growth factor receptor (EGFR) [167]. Overall, these findings suggest that P2YR inhibitors could be putative anticancer drug in the treatment of ALK-dependent NSCLC patients who develop resistance to ALK inhibitors.

7.4.1.1 Adenosine Generating Ectonucleotidases in Cancer Progression

Feng et al. demonstrated a role for CD39 in promoting tumor growth using mice lacking CD39. In this study, B16F10 melanoma growth and metastasis were markedly reduced in CD39 knockout mice compared with their corresponding WT littermates. In addition, endothelial cells in the CD39 knockout mice were impaired and were unable to influence tumor cell proliferation [72]. A similar observation was also reported by Sun et al. where they showed loss of host CD39 abrogated experimental liver metastases of B16F10 melanomas and MC38 colon tumors. Mechanistically, they showed using elegant adoptive T cell transfer setup experiments that CD39 expression on T_{regs} was a critical determinant in tumor rejection. Particularly, transfer of T_{regs} from WT donors into host CD39 KO mice but not vice versa resulted in the suppression of NK cell cytotoxic functions. This study also provided proof-of-principle that targeting CD39 activity could ameliorate tumor growth and

metastases. Indeed, POM-1, a pharmacological inhibitor for CD39, prevented B16 melanoma and MC38 colon tumor growth. Importantly, the anti-tumor effect of POM-1 was similar to those observed with the CD39 knockout mice [168]. Given the short half-life of POM-1 in the circulation, several groups have now developed CD39-blocking monoclonal antibodies (mAbs). Human anti-CD39 mAb (clone 9-8B) has shown promising activity in inhibiting the enzymatic function of CD39 and improving survival in a lethal metastatic patient-derived sarcoma xenograft model [169]. Similarly, Bastid and co-workers recently described another anti-human CD39 mAb (clone BY40) that showed inhibition of CD39 enzymatic activity in SK-MEL5 human melanoma cells. Addition of this mAb to SK-MEL5 and T cell co-cultures resulted in efficient blocking of CD39 activity and increased the proliferation of $CD4^+$ and $CD8^+$ T cells. The therapeutic efficacy of this anti-CD39 mAb, however, remains to be tested in vivo [42]. In mice overexpressing CD39 and challenged with MC-26 colon cancer, significantly larger metastatic tumors compared with WT or heterozygous controls were observed [170]. Thus, CD39-blocking antibodies are potentially useful in alleviating immunosuppression and restoring immunity to cancers. By contrast to these encouraging observations, aged CD39 deficient mice were reported to spontaneously develop hepatocellular carcinoma owing to high accumulation of ATP and mechanistic target of rapamycin (mTOR) signal activation [171].

Similar to CD39, the importance of CD73 in promoting tumor growth through several host and tumor mediated pathways is also well understood. Using CD73 knockout mice, Stagg et al. demonstrated that the loss of host CD73 conferred resistance to B16F10 primary subcutaneous growth and experimental lung metastases. Such protection in the absence of CD73 was also seen with other tumor models including MC38 colon cancer, EG7 lymphoma, AT-3 mammary, EL4 lymphoma, and ID8 ovarian tumors. The effect of host CD73 loss was found to be dependent on the infiltration and activation of $CD8^+$ T cells [14]. Additionally, two independent studies in 2011 observed that T_{regs} from CD73 knockout mice compared to WT mice were functionally inefficient in suppressing IFN-γ production from effector T cells [14, 172]. In the transgenic adenocarcinoma mouse prostate (TRAMP) model, absence of host CD73 protected these mice from de novo prostate cancer development; IFN-γ, NK cells, and $CD8^+$ T cells were required for this protective effect [173]. Interestingly, increased protection to primary tumor was seen with the EG7 lymphoma model (over the parental EL4 lymphoma) and B16F10-SIY (over the parental B16F10) when inoculated into CD73 deficient mice, indicating a role for tumor immunogenicity in mediating optimal anti-tumor effects [172]. By contrast, the anti-metastatic effect seen in CD73-deficient mice is mediated independently of hematopoietic cells and possibly through endothelial cells. In vivo pharmacological blockade of CD73 with a selective inhibitor, APCP, or anti-CD73 mAb (clone TY/23) significantly reduced lung metastases [14]. Terp et al. demonstrated that anti-human CD73 mAb (cloneAD2) reduced the number of human breast cancer MDA-MB-231 and LM3 metastases by inducing the internalization of surface CD73. Importantly, the anti-metastatic effect was independent of CD73 catalytic function [46]. Furthermore, we now understand that the optimal protection to metastasis by the anti-CD73 mAb is largely dependent on the activation of Fc receptors by the host and the expression of CD73 on tumors, as recently demonstrated by Young and colleagues [45].

7.4.2 Adenosine Receptors in Cancer Progression and Metastasis

The effect of A1R on tumor cells has been mostly investigated in vitro with limited data in vivo investigating the role of this receptor in cancer progression and metastasis. One study by Synowitz et al. showed that adenosine acting via A1R on microglial cells impaired GL261 glioblastoma growth [49]. Treating mice with the A1R agonist, N6-cyclopentyladenosine (CPA) significantly decreased tumor size, and reduced MMP-2 levels. Given that MMP-2 is associated

with promoting glioblastoma invasiveness, this study concluded that A1R signaling on microglia was essential in optimum inhibition of glioblastoma tumor development [49].

Antagonists to A2AR as putative anti-tumor therapy has attracted attention since 2006, whereby Ohta et al. observed complete rejection of the CL8-1 melanoma and RMA T cell lymphoma cell lines, in a large proportion of A2AR gene-targeted mice [174]. Using synthetic (ZM241385) and natural ((1,3,7 trimethylxanthine (caffeine)) A2AR antagonist, this study revealed that the anti-tumor efficacy in the A2AR-deficient mice was attributed to the increased IFN-γ production by T cells, and the inhibition of angiogenesis in these mice, altogether leading to tumor cell apoptosis [174]. Similar observations were seen with the rejection of EL4 lymphoma grown in the A2AR-deficient mice [175]. A recent study has also demonstrated that A2AR-deficient mice showed increased suppression of primary subcutaneous tumors—SM1WT1 melanoma and AT-3 mammary tumor growth, in a $CD8^+$ T cell-dependent fashion. Absence of A2AR in host was crucial for mediating protection to LWT1 melanoma lung metastases compared to WT controls [45]. Importantly, loss of host A2AR improved memory T cell responses, as shown by Waickman et al. In this study, subcutaneous inoculation with a low-dose of EL4 lymphoma cells led to tumor rejection in both A2AR-deficient mice and WT mice, however, a subsequent re-challenge with a lethal dose of the same tumor resulted in tumor rejection only in the A2AR-deficient mice, but not WT mice [175]. Furthermore, incubation of tumor-specific $CD8^+$ T cells with the A2AR inhibitor ZM-241385 prior to adoptive transfer into tumor bearing mice enhanced their ability to mediate tumor rejection. Similarly, siRNA gene-targeted knockdown of A2AR on T cells resulted in better suppression of lung metastases and prolonged survival in RMA-inoculated mice [174]. In a number of studies, A2AR inhibitors were shown to be effective in reducing metastasis in CD73-overexpressing B16F10 melanoma with NK cells shown to play a dominant role in limiting metastatic growth in these mice [11, 45, 176].

Similar to A2AR, Morello's group showed that activation of A2BR using the A2BR agonist BAY60-6583 promoted growth of B16F10 melanoma [159]. Extending on these initial findings, this group further demonstrated that A2BR stimulation with agonistic BAY60-6583 induced expression of fibroblast-associated protein (FAP) and fibroblast growth factor-2 (FGF-2) by host melanoma-associated fibroblasts. These FAP^+ fibroblasts triggered angiogenesis by releasing the chemokine CXCL12 which subsequently increased the number of $CD31^+$ endothelial cells within the tumor [60]. Similarly, administration of the A2BR inhibitor, PSB1115 arrested tumor growth and these effects were primarily mediated by the reduced accumulation of MDSC in the tumor [177]. Linden's group showed that blockade of A2BR with a selective antagonist ATL801 slowed in vivo growth of MB49 bladder and 4T1 mammary carcinomas, and this was due to increased adaptive immune response through the IFN-γ-CXCR3 axis [178]. Likewise, A2BR-deficient mice showed delayed tumor growth and prolonged survival in a mouse model of Lewis lung carcinoma, compared to WT control mice. This suppression was mediated due to the inability of A2BR-deficient mice to secrete VEGF, thus preventing angiogenesis [179]. In a similar in vitro experiment, knockdown of A2BR by siRNA in human A375 melanoma cells inhibited the release of the pro-angiogenic growth cytokine IL-8 [180]. Recent work by Mittal et al. demonstrated that the expression of A2BR on tumor cells, specifically the mouse triple negative mammary carcinomas, E0771 and 4T1.2, and human triple negative breast carcinoma, MDA-MB-231, was critical for promoting lung metastases and siRNA knockdown of A2BR in these tumors significantly reduced metastases. By contrast, in these studies host A2BR was without significant effect [59].

A3R, as distinct from A2AR and A2BR has been reported to have tumor suppressing function. This was demonstrated in xenograft models, where mice treated with the A3R agonist LJ-529 showed enhanced anti-tumor effect by inducing apoptosis and downregulation of the Wnt signaling molecules Akt, Cyclin D1, and

GSK-1β. Interestingly, an A3R agonist was also able to downregulate the expression of ER-α on ER^+ breast cancer tumors including MCF7 and T47D [181]. In another study, when mice were treated with the A3R agonist CF101, prior to chemotherapy, increased numbers of neutrophils and other leukocytes were observed and this enhanced the therapeutic efficacy of chemotherapy in the human colon carcinoma model [182]. Lastly, A3R activation using agonistic CI-IB-MECA inhibited B16F10 melanoma growth and increased serum IL-12, a pro-inflammatory cytokine, essential for the activation of NK cells. Additionally, when splenocytes derived from CI-IB-MECA-treated mice were adoptively transferred; a significant reduction in metastases was observed, implying an anti-tumor role for A3R in this model [183].

7.5 Combination Therapies

Clinical data together with results from pre-clinical mouse tumor models have demonstrated that multiple immunosuppressive pathways exist in tumors and that their co-targeting increases the efficacy of host anti-tumor immunity. The emerging role for the purinergic pathway in driving cancer growth and metastasis makes it an attractive new target for cancer immunotherapy. We now discuss some pre-clinical studies demonstrating how targeting of this pathway synergizes with either chemotherapy or other immune checkpoint molecules.

Allard et al. found that anti-CD73 mAb (clone TY/23) significantly enhanced the activity of both anti-CTLA-4 and anti-PD-1 mAbs against mice bearing subcutaneous MC38-OVA (colon) or RM-1 (prostate) tumors, as well as in mice bearing the spontaneously metastatic 4T1.2 mammary carcinoma cells. This effect was mediated by activation of $CD8^+$ T cells and IFN-γ [184]. Similar findings were reported by two other groups. Lannone et al. reported that co-treatment with the CD73-specific inhibitor, APCP, and anti-CTLA-4 mAb significantly retarded B16F10 melanoma growth compared with monotherapy alone [185]. Hay et al. also showed that treatment with a mouse/human cross-reactive anti-CD73 mAb, MEDI9447, and anti-PD-1 retarded CT26 colon adenocarcinoma growth and prolonged survival of these mice [186].

Studies by Stagg have shown the effectiveness of combining CD73 or A2AR blockade with cytotoxic chemotherapy. Mice receiving a combination of anti-CD73 mAb and doxorubicin demonstrated enhanced anti-tumor immune response in 4T1.2 mammary cancers over monotherapy. A similar effect was observed when a specific A2AR blocking inhibitor, SCH58261, was used in combination with doxorubicin [85]. Similarly, co-treating mice with the A2BR inhibitor, PSB1115, and chemotherapeutic agents dacarbazine or gemcitabine significantly suppressed B16F10 melanoma growth, through reducing the accumulation of MDSC within the TME [177]. A prolonged tumor-free survival and overall survival was seen in A2AR-deficient mice compared to WT mice when treated with a soluble B7-DC/Fc fusion protein, which specifically targeted the PD-1 receptor, expressed on dendritic cells resulting in T cell activation. In mice bearing experimental lung metastases, Mittal et al. reported that co-treatment with SCH58261 and anti-PD-1 resulted in significantly lower levels of metastases compared to monotherapy alone [176]. Interestingly, a recent study by Beavis et al. showed that a combination of anti-PD-1 with either A2A-deficient chimeric antigen receptor (CAR)-T cells, or treatment of WT CAR T cells with the antagonist SCH58261, significantly improved the anti-tumor efficacy of these T cells against HER2-expressing mouse tumors [187]. Similarly, Iannone et al. showed that pharmacological blockade with the A2AR antagonist ZM241365 improved the efficacy of anti-CTLA-4 therapy in B16F10 melanoma models. In this study, the authors also reported that the A3 agonist, CI-IB-MECA synergized with anti-CTLA-4 to suppress tumor growth in these melanoma-bearing mice, compared to monotherapy alone [185]. Lastly, Young et al. recently showed that co-inhibiting A2AR with anti-CD73 mAb significantly reduced tumor growth and

lung metastases in mice compared to monotherapy alone [45]. This improved anti-metastatic efficacy upon co-targeting molecules within the purinergic pathway suggests the non-redundant nature of these molecules in mediating anti-tumor immunity.

7.6 Ongoing Clinical Trials

One of the simplest approaches to reduce microenvironmental hypoxia within tumors is to increase oxygen levels. Early experimental studies demonstrated that tumor-bearing mice that were allowed to breathe in air containing high levels of oxygen (95% oxygen +5% carbon dioxide), before and during irradiation, displayed significantly enhanced response to radiotherapy compared to mice that received 100% oxygen or air alone [188]. However, recent studies have shown that high concentration of supplemental oxygen can cause oxygen toxicity as well as non-specific inflammatory responses [189]. Sitkovsky's group showed that supplementing mice with 60% oxygen, to induce respiratory hyperoxia, significantly reduced the number of pulmonary MCA205, B16F10, and 4T1 tumor metastases in these mice. As respiratory hyperoxia is widely applied in the clinical settings, the authors in this study concluded that supplementing 60% oxygen with other available immunotherapies could represent a new strategy for treatment of cancers [190].

In addition to hypoxia, there is immense interest in the clinic to target the purinergic receptors that influence the generation of extracellular ATP and adenosine. Currently, several studies have assessed the expression levels of purinergic receptors in human cancers and this expression has been correlated with recurrence-free survival (RFS) and overall survival (OS) in these patients (Table 7.2). These association studies coupled with strong pre-clinical studies have resulted in the initiation of many clinical trials to investigate the anti-tumor efficacy of targeting the purinergic pathway in human cancers (www.clinicaltrials.gov.au). Specifically, a phase I clinical trial using polyclonal sheep anti-P2X7 Ab (BIL010t) was completed in 2014 (NCT02587819). Topical administration with BIL010t in 21 patients with basal cell carcinoma for 28 days resulted in reduction in lesion area in 65% of these patients. Histopathology of the excised lesion revealed complete response in 2 patients, partial responses in 9, and no response in 8 patients [191]. Additionally, MedImmune Inc. recently reported the development of MEDI9447, a mouse/human cross-reactive anti-CD73 mAb to enhance anti-tumor immunity in pre-clinical tumor models [186]. A phase I trial using MEDI9447 in combination with MEDI4736 (anti-PD-1) is currently underway (NCT02503774). Lastly, Corvus Pharmaceuticals, Inc. and Palobiofarma have developed A2AR inhibitors, namely CPI-444 (NCT02655822) and PBF-509 (NCT02403193) for the treatment of cancers. Importantly, CPI-444 will be co-administered with Atezolizumab (anti-PD-L1) in patients with advanced tumors, while Palobiofarma aims to test the combinatorial blockade of PBF-509 with anti-PD-1 treatment in patients with NSCLC.

Conclusions

It is now becoming apparent that ATP and adenosine are fundamental components of the TME that can affect tumor growth and immune cell activation. Current strategies to target the purinergic receptors involve mechanisms that not only can inhibit tumor cell growth, but importantly can also boost host anti-tumor response. In addition to the purinergic receptors, the enzymatic activities of ecto-nucleotidases (CD39, CD73) contribute equally, or perhaps, even more towards the generation of an immunosuppressive TME, by switching tumor dynamics away from inflammatory ATP and towards an immunosuppressive adenosine-rich microenvironment. Importantly, the non-redundancy of the purinergic receptor signaling as seen with co-targeting A2AR and CD73 indicates that targeting multiple pathways involved in adenosine receptor signaling could be more efficacious than targeting an individual molecule or pathway within the same pathway. Furthermore, because P1Rs are G-protein coupled receptors (GPCR), and thus

a difficult target for immunization, no therapeutic antibodies have yet been reported that can effectively block the functions of these receptors. To circumvent this, Jaakola et al. showed that binding of A2AR antagonist, ZM241385 to the A2AR hinders the activation of A2AR through competitive inhibition [192]. Similar studies have been conducted to define the binding pocket of the A1R inhibitor, DU172 to A1R [193]. Such understanding is expected to provide valuable insights to allow the design of more selective agonists and antagonists that could be used in cancer immunotherapy. To date, A2AR inhibitors demonstrated good safety profiles and were well-tolerated in the clinical trials for neurodegenerative diseases [194], thus showing promise for its use in cancer immunotherapies. There have also been no reports of immune related adverse events being enhanced by A2AR inhibitors in combination with immune checkpoint blockade in mice. Overall, targeting of the purinergic pathway represents a very promising approach to use in combination cancer immunotherapies.

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Competing Interest MJS has scientific research agreements with Bristol Myers Squibb, Corvus Pharmaceuticals, and Aduro Biotech. MJS is also on the scientific advisory boards of F-star, Kymab, and Arcus Biosciences. All other authors declare that they have no competing interest.

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8 Plasmacytoid DC/Regulatory T Cell Interactions at the Center of an Immunosuppressive Network in Breast and Ovarian Tumors

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Contents

8.1 pDC Are Deficient for IFN-α Production in Primary Tumor Environment

8.1.1 pDC Are Specialized in Type-I IFN Production

Plasmacytoid dendritic cells (pDCs) are a unique population of bone marrow-derived immune cells that bridge the innate and adaptive immune systems ([1]). They serve two professional roles, one as type-I interferon (IFN-I)-producing cells and the other as antigen-presenting cells (APCs). Although accounting for only 0.3–0.5% of peripheral blood mononuclear cells, pDCs are responsible for over 95% of IFN-I (IFN-α/β/ω) produced by circulating lymphocytes. Activation of pDCs and the subsequent production of IFN-I occur as the result of a signaling cascade that ini-

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tiates through pattern recognition receptors, such as toll-like receptors (TLRs). pDCs are primarily activated through the engagement of endosomal TLR7 and TLR9, by ssRNA or nonmethylated CpG DNA, respectively, which are common to microbial genomes, such as viruses or their replicative intermediates. TLR7 and TLR9 utilize the universal adapter protein MyD88, which acts via the transcription factor IRF7 and the inflammatory transcription factor NF-κB, thereby initiating transcription of type-I and -III IFN, or inflammatory cytokines and chemokines, respectively [2, 3]. Upon activation, pDCs also undergo phenotypic changes resulting in the upregulation of co-stimulatory molecules (CD40, CD86) [4]. They ultimately develop into more "conventional" dendritic cells with classical DC (cDC) morphology and the ability to present antigens to activate naïve and memory T cells. When dysregulated, pDCs can drive autoimmunity, since complexes of self-nucleic acids with autoantibodies also trigger sustained production of IFN-I, exacerbating autoimmune flairs and B cell proliferation [5]. In this pathophysiological context, HMBG1, a nuclear DNA-binding protein and LL37, a cationic anti-microbial peptide, both released by dying cells, work in concert with circulating anti-DNA autoantibodies to deliver self-nucleic acids inside pDC, leading to IFN-I production in a TLR7/9-dependent manner. However, in the right context, such as airway or oral antigen delivery, pDC can also potentially contribute to peripheral tolerance [6] by favoring regulatory T cell (Treg) differentiation and expansion (for review [7]) (Fig. 8.1). The duality of pDC in immune responses likely depends on their IFN-I production and their activation by interferogenic stimuli.

pDCs are essential for recognition of altered self and thus may contribute to immune responses directed towards transformed cells. It would, therefore, be expected that an increased presence of pDCs in tumor tissues should promote activation, immune recognition of tumor antigens and, in turn, lead to tumor rejection.

8.1.2 Evidence for a Role of Type-I IFN in Cancer Immunosurveillance

Although type-II IFN has emerged early as a key mediator of tumor immunosurveillance, it is only very recently that the role of IFN-I has been discovered [8–10]. Indeed, gene-targeted mice lacking the type-I IFN receptor (IFNAR1)

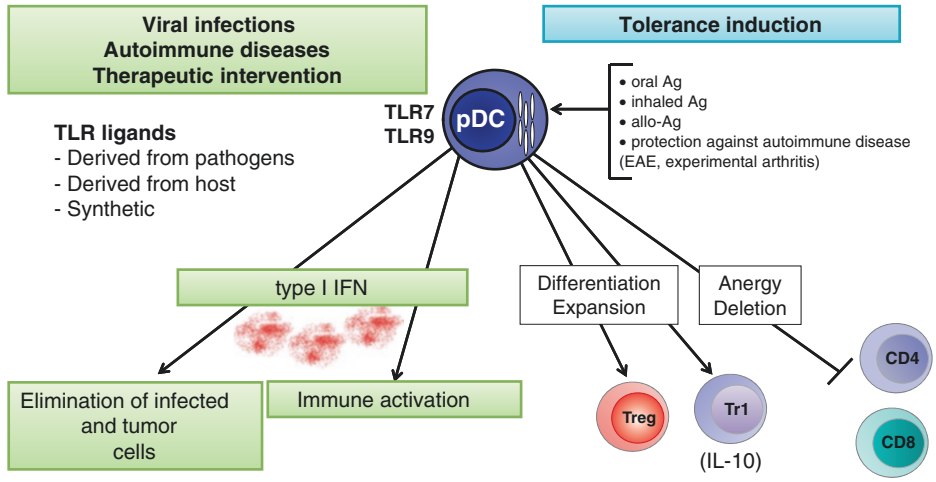

Fig. 8.1 pDC plasticity. During viral infection and certain autoimmune diseases (Lupus, Psoriasis) pDCs contribute to immune response through TLR-7/9 engagement by viral or endogenous nucleic acid leading to their production of IFN-I and phenotypic maturation. However, in other contexts, such as airway or oral antigen delivery, in absence of TLR-7/9 engagement, pDC can also contribute to peripheral tolerance by favoring Treg differentiation and expansion

develop more carcinogen-induced primary tumors than WT control mice [9, 11] and antibody-mediated blockade of the IFNα/β receptor in WT hosts abrogated rejection of immunogenic transplanted tumors [9]. Several recent reports have shown that host-derived IFN-I responses are critical in early steps of tumor immunosurveillance [8–10, 12] as well as in the efficacy of radiotherapy [13] or of certain chemotherapeutic treatments [14].

Moreover, in humans, exogenous administration of recombinant IFN-α has shown efficacy in the treatment of cancer patients [15] and high intratumor expression of IFN-I responsive genes has a positive prognostic value in cancers [16–18]. In line with this observation, it has been shown recently that downregulation of IFNAR1 in tumor stroma stimulates tumor development and growth, playing a key role in the formation of an immune-privileged niche, and predicting poor prognosis in human colorectal cancer patients [19].

In a mechanistic view, recent studies in mouse models have shown that IFNs contribute to antitumor immunity via stimulating specific CD8α$^+$ DCs to cross-present tumor-derived antigens to cytotoxic T lymphocytes (CTLs) [8, 9]. In addition, IFN-I provides a signal to stimulate the clonal expansion of CTLs [20] and increase their viability [21]. Furthermore, recent studies have identified the cGAS/STING pathway of DNA sensing as a critical route for type-I IFN induction during cell transformation and chemo or radiotherapy treatment (for review [22]). STING agonists are currently in clinical trial aiming at increasing IFN-I production in the tumor to limit neoplastic growth and promote specific antitumoral immune responses. However, the nature of the IFN-I-secreting cells in most cancer models remains undetermined and other tumor-derived ligands and innate sensors are likely to contribute to induction of endogenous IFN-I. In this perspective, pDCs represent prime suspects with regard to their presence in the diseased tissue, their ability to sense host-derived factors through TLR7/9, and their IFN-I producing specialization.

8.1.3 pDCs in Breast Tumors

Breast cancers are the most common malignant tumors and the first leading cause of cancer death in women. Among several types of breast cancers, the so-called triple negative (non-amplified Her2neu, ER^{neg} and PR^{neg}) (TN) is the most aggressive one. Breast cancers are considered as immunogenic tumors as (1) CD8$^+$ T cell responses as well as humoral responses against TAA (Her2neu, p53, Muc1) have been demonstrated [23–27] and (2) CD8$^+$ T cell infiltration has been recently reported to correlate with better prognosis [28–30]. Moreover we recently demonstrated that primary breast tumors (BT) are largely infiltrated by immune cells involved in innate sensing, i.e., NK cells, dendritic cells (DC), and macrophages (MΦ) that display an activated phenotype [31, 32] suggesting their stimulation within the BT environment. We and others also reported the presence of strong CD4$^+$ [33] and CD8$^+$ [24–27] T lymphocytes infiltrates in BT suggesting that all the players required to set up an efficient anti-tumor response are present within the BT environment. However, when tumors are clinically detected, this immune response is, in most cases, unable to counteract cancer development because tumors have developed immunosubversion processes.

Several studies have pinpointed that the tumor microenvironment subverts the function of immune cells and favors immunosuppression, avoiding the establishment of anti-tumor immunity. Recent works including from our group [32, 34] have also shown that pDC accumulate in several types of solid cancers, but very limited studies analyzed their function within the tumor microenvironment.

In this context, we have observed that BT infiltration by pDC is associated with an adverse clinical outcome [32], suggesting that they might contribute to the tumor immune evasion and ultimately to its outgrowth.

In a prospective study analyzing 79 newly diagnosed breast cancer patients, we observed a preferential accumulation of pDC in aggressive BT with a high mitotic index and a TN phenotype

[35]. These results strengthen our previous observation on the deleterious impact of TApDC on breast cancer patients' outcome [32]. Such tumor-associated (TA)-pDC exhibited a partially activated phenotype (CD40, CD83, CD86, and HLA-DR intermediate levels) when compared to patients' associated blood pDC. This partially activated phenotype may be related to the local action of GM-CSF on TApDC [36] or to endogenous nucleic acid as TLR7/9 ligands. Of note, the concomitant increase of GM-CSF and pDC was significantly associated with relatively more aggressive breast cancer subtypes. In addition, similarly to tonsil pDC, TApDC activated in vitro by TLR7 and 9 agonists retain their capacity to mature and induce the proliferation of naïve $CD4^+$ T cells and their secretion of IL-10 and IFN-γ. In contrast, TApDC were strongly impaired in their ability to secrete IFN-α upon TLR7/9 stimulation in vitro. This functional defect was specific to IFN-α and occurred selectively at the tumor site. Indeed, the production of [1] inflammatory molecules such as CXCL10 in TApDC [35] as well as [2] IFN-α in patients' blood pDC was not affected [35, 37].

8.1.4 pDCs in Ovarian Tumors

Ovarian cancer (OC) is the most frequent and aggressive gynecologic cancer. This is due, at least in part, to its diagnosis at advanced stages (III/IV) in the majority of patients with peritoneal carcinosis and malignant ascites [38, 39]. We investigated the clinical significance of the presence of pDC in tumor mass and malignant ascites by conducting a systematic comparison of pDC number, phenotype, and function in blood, tumor, and ascites. We observed an accumulation of pDC in most of malignant ascites and their presence at high frequency in 36% of primary tumors. Importantly, as in breast tumors, accumulation of pDC in tumors was an independent prognostic factor associated with early relapse.

These results obtained on 33 OC patients in whom pDC were identified by flow cytometry as $CD4^+BDCA2^+CD123^+$ cells [38] were confirmed on a larger series of OC patients (n = 97) by immunohistochemistry (IHC) [39]. In this study, TApDC were identified as $BDCA2^+$ cells on formalin-fixed, paraffin-embedded (FFPE) tissues using tissue microarray (TMA) allowing the analysis of the impact of the presence of pDC in both cancer epithelium and lymphoid aggregates present in cancer stroma. $BDCA2^+$ TApDC were present in only 18/97 tumors (18%). In univariate analysis, the presence of TApDC within cancer epithelium was associated with early relapse and shorter median overall survival. In multivariate analysis, in addition to clinical prognostic factors (advanced stage, debulking surgery, and residual tumor), the presence of TApDC remains an independent prognostic factor associated with shorter PFS [39].

Thus, using two different and complementary methods (flow cytometry and IHC) on two independent cohorts, we observed a deleterious impact of the presence of TApDC within OC tumors on patient's outcome. These data corroborate our findings in breast cancer [32] and others in melanoma [34] showing that TApDC accumulation correlates with poor prognosis. Collectively, these results suggest that TApDC may contribute to immune tolerance and tumor progression.

Like in breast tumors, we showed that, unlike ascites pDC, TApDC from ovarian cancer (1) expressed a semi-mature phenotype as evidenced by high levels of CD40 and CD86 and (2) were strongly impaired for their IFN-α production in response to CpG-A (TLR9 ligand) known to induce huge amounts of type-I IFNs [38]. These results suggest that TApDC respond to endogenous signals delivered by the tumor microenvironment by maturing rather than by producing IFN-α, suggesting a preferential activation of NFκB rather than IRF7 pathway [40, 41].

8.1.5 Mechanisms Leading to TApDC Functional Defect

The similarity in the biology of TApDC between breast [32, 35, 42] [5] and ovarian [38, 39] tumors strengthens the importance of our work and indicates that common inhibitory mechanisms could

occur between epithelial cancers with different localizations.

In both breast and ovarian cancers, we demonstrate that besides IFN-α, the production of IFN-β and TNF-α but not CXCL10 nor MIP1-α/CCL3 by TLR-activated healthy pDC is impaired by the breast and ovarian tumor environments [38, 42]. Importantly, we identified TGF-β and TNF-α as major soluble factors involved in TApDC functional alteration (Fig. 8.2). Indeed, recombinant TGF-β1 and TNF-α synergistically blocked IFN-α production of TLR-activated pDC, and neutralization of TGF-β and TNF-α in tumor-derived supernatants restored pDCs' IFN-α production [38, 42]. The involvement of tumor-derived TGF-β was further confirmed in situ by the detection of phosphorylated Smad2 in the nuclei of TApDC in breast tumor tissues [42]. Mechanisms of type-I IFN inhibition did not involve TLR downregulation but the inhibition of IRF-7 expression and nuclear translocation in pDC after their exposure to tumor-derived supernatants or recombinant TGF-β1 and TNF-α.

Recently, E. Gatti and collaborators (CIML) showed that the molecule BAD-LAMP (LAMP5) is uniquely expressed by non-activated human pDCs among hematopoietic cells [43] and that BAD-LAMP controls TLR9 trafficking to LAMP1+ late endosomes, reducing IFN-I production in pDCs activated by CpG DNA and favoring TNF-α. Conversely, BAD-LAMP silencing allows spontaneous TLR9 activation in

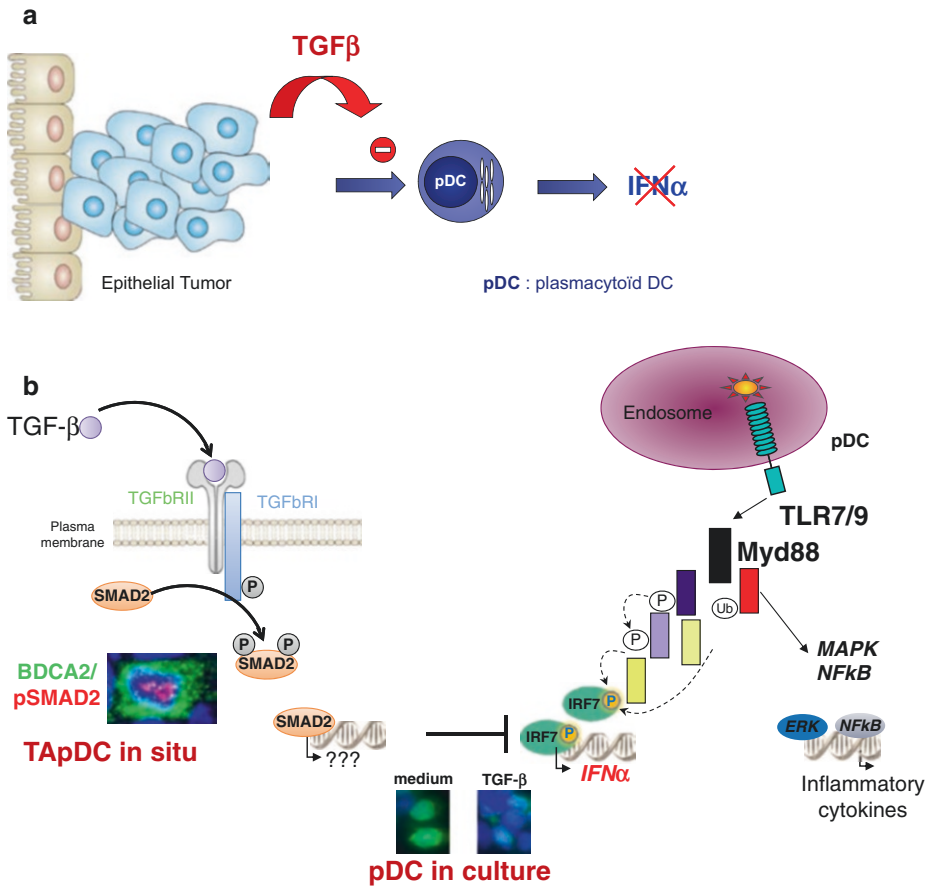

Fig. 8.2 TApDC inhibition of type-I IFN production through TGF-β. (**a**) TGF-β produced in breast and ovarian tumor environment potently suppressed type-I IFN production by pDC. (**b**) In situ TApDC expressed nuclear phosphorylated-Smad2 demonstrating their exposition to TGF-β, and leading to blockade of IRF7 nuclear translocation

absence of exogenous TLR agonists. Together, we further demonstrated that breast TApDCs display increased BAD-LAMP expression that contributes to their inability to produce IFN-I. BAD-LAMP expression therefore limits IFN-I expression in pDCs by promoting TLR9 sorting to late endosomes at steady state and in response to TME (Combes, under revision). "BAD-LAMP controls TLR9 trafficking and signaling in human plasmacytoïd dendritic cells" by Alexis Combes, Voahirana Camosseto, Prudence N'Guessan, Rafael Argüello, Julie Mussard, Christophe Caux, Nathalie Bendriss-Vermare, Philippe Pierre, and Evelina Gatti

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As IFN-I plays important anti-tumor functions, either directly or through immunosurveillance activation [44], our work suggests that the inhibition of TApDCs' IFN-I production by BT might confer a selective advantage to tumor cells and represent a novel escape mechanism.

The alteration of IFN-α production by pDC in response to TLR ligands has been previously described in chronic viral infections such as HIV and HCV contributing to the failure of an efficient immune response [45]. In the context of cancer, previous works have also reported this alteration in breast [35], lung [46], and head and neck [47] cancers, and chronic myeloid leukemia [48].

Our findings indicate that targeting TApDC to restore their IFN-α production, by combining TLR7/9-based immunotherapy with TGF-β antagonist, might be an achievable strategy to induce anti-tumor immunity in breast cancer.

8.2 Treg in Breast Tumors

The presence of regulatory T cells (Treg) has been described in a large panel of solid tumors. However, their impact on tumor progression differs according to the tumor type analyzed [49]. We recently obtained evidence in breast carcinoma that Treg localized within lymphoid aggregates, but not in the tumor bed, have a negative impact on patients' survival [33]. The poor impact of Tregs has been confirmed by others in invasive breast carcinoma [50] and the reduction of Treg during neoadjuvant chemotherapy correlates with improved prognosis [51]. Moreover, we showed selective Treg recruitment through CCR4/CCL22 in the lymphoid aggregates upon contact with mature DC, where they became strongly and selectively activated (HLA-DR^{high}, inducible co-stimulatory molecule (ICOS)^{high}) and block conventional T-cell response.

8.2.1 Treg Recruitment

Several studies including from our group demonstrated the infiltration of BT by immune subsets involved in immune tolerance, i.e., pDC [32] and CD4$^+$CD25^{high}CD127^{neg}FoxP3$^+$ Treg [30, 33, 52] and type-2 MΦ [for review, see [53], Ramos RN et al. submitted] that are all of poor prognosis for overall survival (OS) in primary BT.

An in-depth ex vivo analysis demonstrated that tumor-associated Treg (TATreg) (1) are activated as they express ICOS, HLA-DR, GITR, and CTLA-4, (2) are functional as they suppress CD4$^+$ T cells proliferation and IFNγ secretion, (3) proliferate in situ in contrast to the resting non regulatory CD4$^+$ memory T cells and CD8$^+$ T cells detected within BT [33].

In contrast to associated patients' blood Treg, TATreg present a selective loss of membrane CCR4, consecutive to an active recruitment through CCL22 secreted within the BT environment [33]. In line with this, (1) CCL22, but not CCL17 induced the CCR4 downregulation and (2) BT lacking CCL22 expression are not infiltrated by TATreg independently of their production of CCL17, the other CCR4 ligand.

In primary BT context, independently of the molecular subtype of the tumor, CCL22 expression is strongly increased compared to peritumoral breast tissue as assessed not only by IHC but also by ELISA within the BT dilacerations supernatants [31].

Interestingly, at the systemic level, we observed a gradual increase in CCL22 plasmatic levels from healthy subjects, patients with primary BT, first metastatic relapse or with more advanced BT [54] that could reflect the tumor burden.

Using BT epithelial cell lines but also primary BT specimens, we demonstrated the major role of immune infiltrate in the selective induction of CCL22 but not CCL17 by tumor epithelial cells [31]. In vitro experiments using (1) inhibitory antibodies against cytokine receptors and/or cytokines or (2) exogenous recombinant cytokines demonstrate the preponderant role of a dialogue between epithelial tumor cells and tumor-infiltrating NK cells and MΦ for this CCL22 production [31]. Through these studies we propose the following sequence of events (Fig. 8.3): (1) NK cells detecting tumor cells secrete IFNγ, (2) IFNγ activates MΦ favoring their secretion of IL-1β and TNFα, (3) these 3 cytokines act together to increase CCL22 production by epithelial tumor cells. This was further confirmed in ex vivo experiments using primary BT specimens demonstrating the cooperation of MΦ and NK cells to favor CCL22 production by freshly purified tumor cells [31].

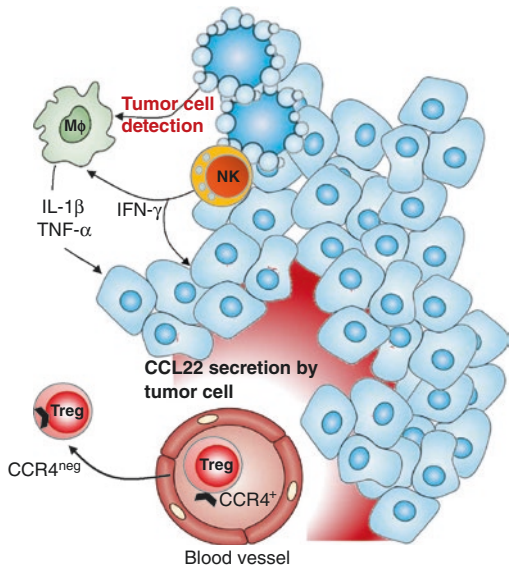

Fig. 8.3 IFNγ, TNFα, and IL-1β secretion by NK and MΦ leads to CCL22 secretion by transformed cells attracting CCR4+ Treg in breast tumor. Healthy breast epithelial cells secrete low levels of CCL22 in a polarized manner within the luminal acini, their transformation favor their recognition by infiltrating NK cells leading to IFNγ secretion. IFNγ promoted MΦ activation that will secrete TNFα and IL-1β after interaction with breast epithelial tumor cells. Combined action of IFNγ, IL-1β, and TNFα will induce strong CCL22 non polarized secretion by tumor cells that will induce the recruitment of CCR4+ Treg from periphery, leading to CCR4 internalization

This illustrates a mechanism allowing the transformed breast epithelial cells to counteract the local inflammation involving NK and MΦ to favor Treg recruitment through CCL22 secretion as previously described in chronically inflamed colon [55]. In turn, TATreg may also favor tumor progression via (1) the inhibition of NK cytolytic functions (for review [56]) (2) the conversion, as recently demonstrated in HIV context [57] of type-1 MΦ into type-2 MΦ that have pro-tumor functions through production of factors promoting angiogenesis, tumor cell proliferation, and favoring immunosuppression (for review [53, 58]).

All together, these data strongly indicate that CCL22 participates to the immunosubversion in BT and will favor disease progression. In this context, CCR4 antagonists (small molecule (AF-399)) have been validated to block in vitro and in vivo CCL22-mediated recruitment of human Treg and Th2 cells [59].

8.2.2 pDC-Mediated Treg Expansion

High numbers of TATreg is an independent factor of poor prognosis for BT patient's survival. Furthermore, BT infiltrating $CD4^+FoxP3^{neg}$ T cells (TATconv) are of memory phenotype ($CD45RO^+$) and very few are activated contrasting with highly activated TATreg ($ICOS^{high}$, $CTLA\text{-}4^+$, $GITR^+$, $HLA\text{-}DR^+$), that proliferate in situ ($Ki67^+$) and display suppressive activity in vitro [33]. Furthermore, ICOS, belonging to the CD28 family is highly expressed by TATreg as reported in melanoma [60] and ovarian tumors [61]. Despite their Ki67 expression in situ, TATreg did not proliferate in vitro under classical stimulation with anti-CD3/anti-CD28 agonist Ab coated beads, in the presence of exogenous IL-2, in contrast to blood Treg or Tconv either from BT or blood that proliferated strongly. Of importance, infiltration of BT by pDC, also of poor prognosis for patients' survival [32], correlates with Treg infiltration and both cell subsets co-localized within tumor [62] and especially within TNBT [35]. We thus wondered whether TApDC might

contribute to TATreg expansion within the BT microenvironment. We observed that TApDC as well as healthy donors pDC preconditioned with BT-derived supernatants were very potent in inducing [1] the selective expansion of Foxp3$^+$ Treg and [2] the differentiation of IL-10-secreting CD4$^+$ T cells [62]. Interestingly, exogenous IFN-α reverted immunosuppressive CD4$^+$ T cell responses induced by TApDC and BT environment [35], indicating that such TApDC tumor-promoting capacity is strongly amplified in tumors as a result of their impaired IFN-α production.

In order to understand the negative impact of TApDC, we developed an orthotopic murine mammary tumor model that closely mimics the human pathology, including pDC and Treg infiltration [63]. We showed that TApDC are mostly immature and maintain their ability to internalize antigens in vivo and to activate CD4$^+$ T cells in vitro. Most importantly, TApDC are specifically altered for cytokine production in response to TLR9 ligands in vitro while preserving unaltered response to TLR7 ligands. In vivo pDC depletion delayed tumor growth and reduced intratumoral Treg frequency, showing that TApDC provide an immunosubversive environment most likely through Treg activation favoring tumor progression.

pDC were previously shown to regulate growth of multiple myeloma (MM) cells [64] and more recently to favor bone metastasis of breast cancer cells [65].

Whereas healthy donors pDC overexpressed ICOS-L, the unique ligand of ICOS, after in vitro activation by TLR7 or TLR9 agonists, TApDC despite their activated phenotype (CD40$^+$HLA-DR$^+$CD86$^+$), lacked ICOS-L expression in breast and ovarian tumor dilacerates [38, 62]. Interestingly, in vitro ICOS-L engagement by activated ICOS$^+$ CD4$^+$ T cells led to its downregulation at pDC membrane and 24 h culture of tumor cell dilacerate suspensions in the presence of a blocking anti-ICOS mAb restored ICOS-L expression on TApDC [62]. These data demonstrate that ICOS/ICOS-L interaction occurs in BT during Treg/pDC contacts.

Interestingly, allogeneic reactions of [pDC + TA-CD4$^+$ T cells] co-cultures led to a strong enrichment and proliferation of FoxP3$^+$ Treg and enhanced IL-10 secretion. This immunosuppressive T cell response to pDC stimulation was highly dependent on ICOS as the addition of a neutralizing anti-ICOS mAb selectively inhibited both Treg proliferation and IL-10 secretion. Furthermore, myeloid DC (mDC, Lin^{neg}HLA-DR$^+$CD11c$^+$BDCA2^{neg}) that did not overexpress ICOS-L after activation were not associated with Treg enrichment nor strong IL-10 secretion [62]. In agreement with our observations on TATreg, proliferation in response to pDC of ICOS$^+$ Treg issued from ovarian tumor ascites [61] or ICOS expressing natural Treg (nTreg) from thymus [66] was also dependent on ICOS/ICOS-L interaction. In addition, pDC were also reported to increase IL-10 production by CD4 T cell through ICOS/ICOSL interaction [67, 68]. Furthermore, we recently confirmed, in collaboration with D Olive team, the in vivo downregulation of ICOS-L on Follicular Lymphoma B cells and the ICOS/ICOS-L-dependent expansion of Treg in this tumor environment [69].

Importantly, neither IL-2, IL-17 nor IFN-γ was detectable in BT dilacerates in contrast to IL-10 [62]. ICOS neutralization only slightly reduced IFN-γ secretion and proliferation of Tconv and did not impact T cell response to mDC. This suggests that ICOS favors TATreg expansion and IL-10 production but does not participate in the induction of immune effectors in primary BT. Of most importance, on a retrospective cohort of BT patients, ICOS expression was mainly detected on Treg by IHC and ICOS$^+$ cell infiltration correlated with reduced PFS and OS in univariate analysis [62], in agreement with results in ovarian tumors [61].

ICOS constitutes a critical regulator of humoral immune responses, mainly as it stimulates follicular helper T cell (Tfh) activation, as illustrated in ICOS-deficient mice and patients [70]. This said, a few reports also suggest that ICOS may contribute to anti-tumor cellular immunity. Indeed, in melanoma patients, an increased proportion of IFN-γ-producing

$CD4^+ICOS^+$ T cells has been observed in patients responding to anti-CTLA-4 (ipilimumab) treatment [71], and ICOS-deficient mice bearing B16 tumors do not respond properly to anti-CTLA-4 therapy.

$ICOS^+$ Treg have been described in several human and mouse tumors and our in vitro experiments demonstrated that $ICOS^+$ TATreg are strongly dependent on ICOS for their amplification, contrary to Treg from peripheral blood [62]. ICOS dependency could reflect a particular subpopulation of Treg, either linked to a particular origin similarly to thymic $ICOS^+$ nTreg [66] or to their microenvironment and/or activation status. In this context, it is not clear whether TATreg are nTreg or are induced from naïve T cells in the periphery. Deciphering whether ICOS blockade displays a differential impact between nTreg, iTreg, and TATreg could be of importance in the perspective to revert $TACD4^+$ T cell immunosuppressive response in breast cancer patients.

Taken together, our data suggest that ICOS blockade might be a promising strategy to eradicate TATreg and IL-10-producing $CD4^+$ T cells in primary BT. The ICOS neutralization might need to be transient in order to abrogate Treg amplification while leaving unperturbed the restoration of effector cells potentially expressing ICOS [71].

8.2.2.1 Conclusion

Collectively our results show that BT microenvironment inhibits type-I IFN production by TApDC through TGF-β and TNF-α that confers them enhanced capacity to promote $FoxP3^{high}$ TATreg expansion and IL-10-secreting T cells via ICOS-ICOSL interaction in vivo. This favors the accumulation of immunosuppressive $CD4^+$ T cells at the tumor site preventing anti-tumor immune responses (Fig. 8.4). Our observations pave the way for the development of new therapeutic strategies for breast cancer patients by (1) restoring TApDCs' IFN-α production using a combination of TLR-7/9 ligands with TGF-β and TNF-α antagonists, for the induction of a potent anti-viral-like anti-tumor immunity, or (2) neutralizing ICOS/ICOS-L interaction between pDC and Treg.

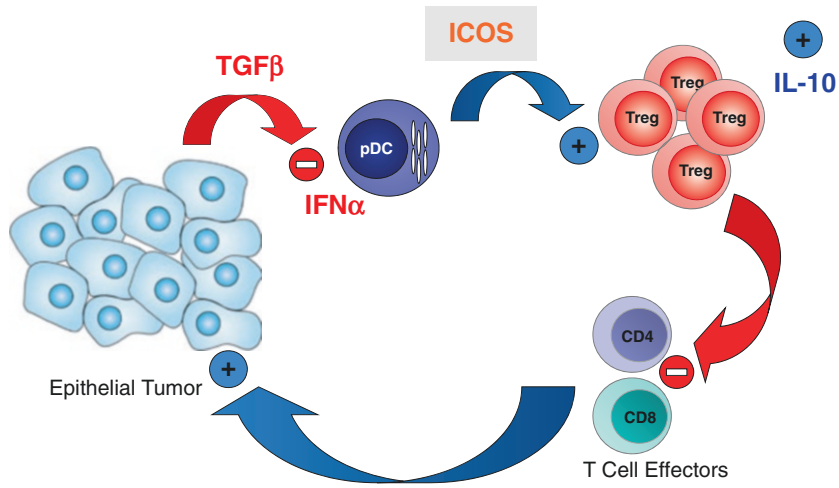

Fig. 8.4 pDC/Treg at the center of immunosuppressive networks in breast tumor environment, role of ICOS/ICOSL. Through ICOS-L expressed on TApDC, ICOS engagement participates in Treg expansion and IL-10 secretion in BT and $ICOS^+$ cells are associated with poor prognosis for patients' survival. This demonstrates that ICOS participates in T cell mediated immunosubversion and suggests ICOS neutralization on Treg as a new cancer immunotherapy strategy

8.2.3 Treg-Mediated Suppression

Treg can suppress most immune cells including $CD4^+$ and $CD8^+$ T cells, DC, B cells, MΦ and NK cells. In vivo and in vitro studies suggest Treg-mediated suppression could be operated through multiple mechanisms and that various molecules could be secreted or expressed at cell surface and actively participate simultaneously and synergistically to their suppressive functions on these different cell subsets (Fig. 8.5).

- *IL2:* IL-2 may increase Treg suppressive function by upregulating FOXP3 expression via STAT5. Moreover, Treg could deprive local IL-2 by consumption through their high-affinity IL-2Rα receptor (CD25).
- *Cytokine secretion:* IL-10 and TGF-β contribute to nTreg and iTreg-mediated suppression.
- *Granzyme-dependent cell cytolysis:* Human activated Treg express perforin and granzyme A after activation and can kill activated $CD4^+$

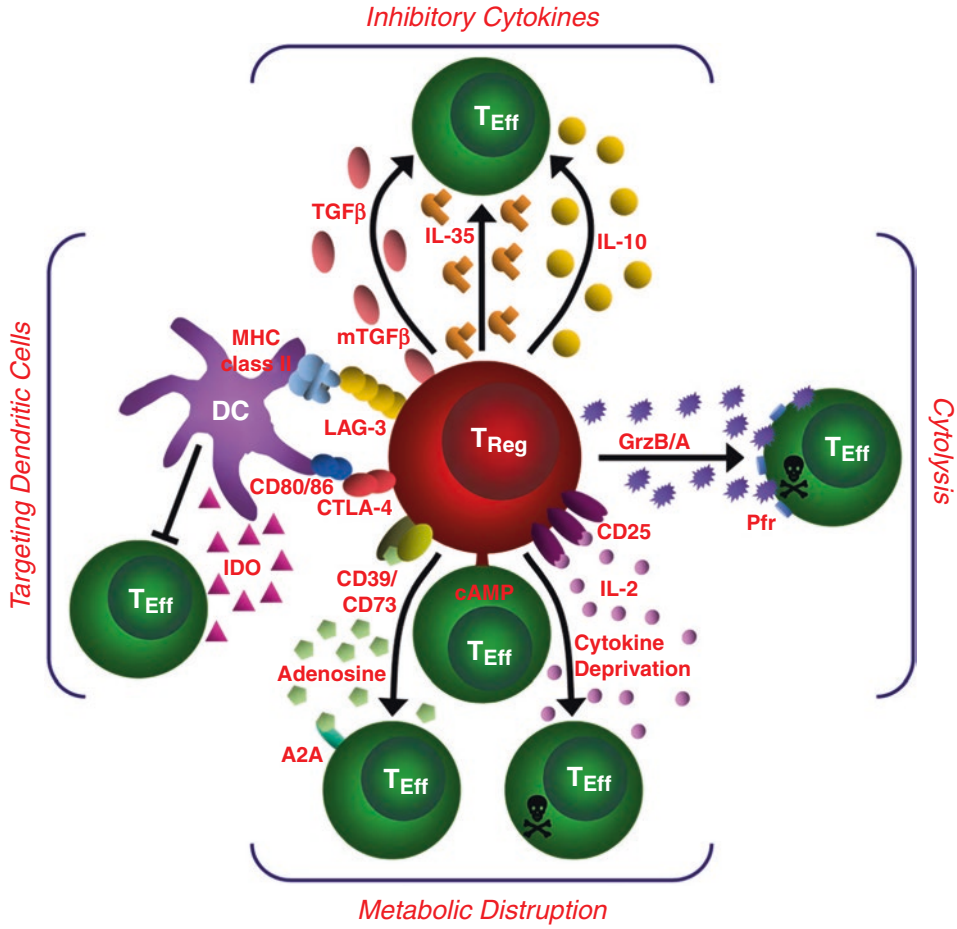

Fig. 8.5 Mechanisms of Treg suppression. This schematic depicts the various T_{reg} mechanisms arranged into four basic modes of action. "Inhibitory cytokines" include IL-10, IL-35, and TGF-β. "Cytolysis" includes granzyme-A- and granzyme-B-dependent and perforin-dependent killing mechanisms. "Metabolic disruption" includes high-affinity IL-2 receptor α (CD25)-dependent cytokine-deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition, and CD39- and/or CD73-generated, adenosine–purinergic adenosine receptor (A2A)-mediated immunosuppression. "Targeting dendritic cells" include mechanisms that modulate DC maturation and/or function such as LAG3 (also known as CD223)–MHC-class-II-mediated suppression of DC maturation, and CTLA4–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive enzyme, by DCs

and CD8+ T cells and other cell types independently from Fas/FasL [72–74].

- *Extracellular nucleotide metabolism* via *CD39 and CD73:* CD73 cooperates with CD39 to generate adenosine (Ado) from ATP [75]. CD39 predominantly catalyzes conversion of ATP into AMP, which in turn is degraded by CD73 to produce Ado. To mediate suppression, Ado binds one of the four distinct AdoR (A1, A2A, A2B, A3). A2A and A2B receptors are coupled to Gs subunit that activates adenylate cyclase and protein kinase A (PKA) thereby increasing cAMP levels. Binding to A2B and A2A suppresses the function of both innate and adaptive immune cells (for review [76]). Indeed, through A2B receptor, Ado alters the maturation of APCs (monocytes and DC) and their secretion of pro-inflammatory cytokines and favors an immunosuppressive environment through induction of IL-10 secretion by monocytes [77]. Furthermore, through A2A receptor, Ado reduces cytotoxic capacity of NK cells [78], decreases IFN-γ secretion, and favors secretion of immunosuppressive cytokines (IL-4 and TGF-β) by NKT cells [79], inhibits BCR-induced NFκB activation [80] and reduces major functionalities of effector CD4+ and CD8+ T cells such as IL-2 and IFNγ secretion, proliferation, and cytolytic function and alters their survival [81]. Together Ado will alter the development of an effective anti-tumor immune response.

  The degradation of ATP into Ado by CD39 in tandem with CD73 represents a mechanism that is used by Treg to induce the production of peri-cellular Ado. However, whereas most studies reported CD39 and CD73 co-expression on murine Treg [82–85], our work showed that *in human* peripheral blood, lymphoid tissues or breast tumor tissues, Treg never expressed CD73 either on the membrane or in the cytoplasm (Gourdin et al. submitted). Similarly to mouse [86], in human breast tumors, Treg express high CD39 levels (Gourdin et al. submitted). Moreover, TLR-7-activated pDC selectively induce the upregulation of CD39 levels on Treg (unpublished data). However, CD73 is observed on a subset of non-Treg CD4+ T cells able to proliferate and secrete high IFN-γ levels upon activation. Our recent data show that CD39+ TATreg cooperate with CD73+CD4+ T cell effectors to mediate Ado production and their local suppressive function (Gourdin et al. submitted) highlighting CD39/CD73 axis as an important Treg-mediated suppression mechanism.
- *Targeting DC:* Interaction of CTLA-4, constitutively expressed on Treg surface with its ligands CD80 and CD86 on DC is an important pathway by which Treg could mediate their suppressive function. Indeed, Treg downregulate or prevent CD80 and CD86 upregulation on murine as well as human DC in vitro blocking their co-stimulatory function and subsequent T cell activation and function [87, 88]. Lag-3 expressed on Treg may play a role in Treg-induced suppression of DC function as Lag-3, by binding MHC class-II molecules expressed by immature DC, induces an ITAM-mediated inhibitory signal that blocks their maturation and reduces their allo-stimulatory functions [89].

8.3 Therapeutic Strategies

8.3.1 Strategies to Neutralize Treg Function Based on pDC/Treg Interaction

We developed a clinically relevant murine tumor model in which the HER2/neu+ NEU-15 cell line growing in WT hosts escapes from immunosurveillance through pDC and Treg-mediated immunosubversion [63], thus closely mimicking our observations in human breast cancer (Fig. 8.6) [32, 33, 35, 42, 62].

8.3.1.1 TApDC Reactivation

Therapeutic strategies aimed at reactivating TApDC and inducing their IFN-I production through TLR agonists stimulation could reduce Treg expansion.

Despite their negative impact on tumor progression, TApDC could be reactivated in vivo

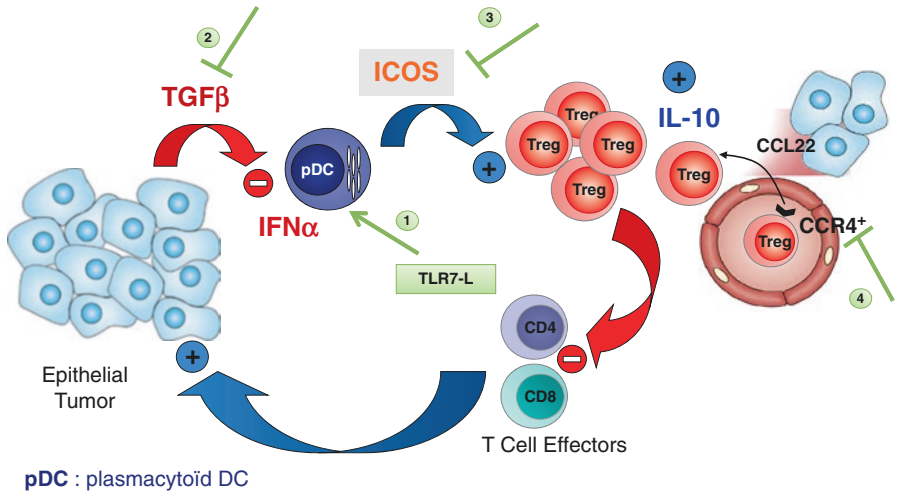

Fig. 8.6 Therapeutic strategies through targeting pDC/Treg crosstalk. Based on the observation of pDC-mediated Treg expansion we are proposing several complementary approaches to reactivate anti-tumor immunity. (*1*) pDC reactivation for type-I IFN production through TLR-7 ligands that are currently under clinical trials; (*2*) TGF-β neutralization to restore pDC function, several mAbs against TGF-β receptor or TGF-β or TGF-β receptor kinase domain inhibitors are also under clinical trials; (*3*) ICOS neutralization though ICOS/ICOSL neutralizing mAbs to block Treg proliferation; (*4*) mAbs neutralizing CCL22/CCR4 to block Treg recruitment within the tumor, neutralizing anti-CCR4 mAbs or small inhibitors are under clinical trials and/or development

via intratumoral injection of TLR-7L, thus mediating a Th1 signature, and subsequent tumor regression. TLR-7L induced long-term protective memory response as 100% of cured mice were protected against subsequent tumor challenge [63].

In vivo depletion of pDC abrogated the therapeutic activity of TLR-7L, demonstrating the central role for TApDC in TLR-7L-mediated anti-tumor response. Importantly, we demonstrated that this therapeutic activity is mediated by locally induced and not systemic type-I IFN. This points towards the importance of intratumoral TApDC reactivation that will lead to type-I IFN production and subsequent additional functions such as antigen cross-presentation and Treg neutralization. We indeed observed that type-I IFN neutralization led to the inhibition of the intratumoral Th1 signature. In this context, the treatment of skin cancers with Imiquimod (TLR-7L) resulted in TApDC recruitment and IFN-α production that correlate to local immune reaction and destruction of tumor lesions [90]. This anti-tumor activity of Imiquimod is dependent on direct tumor killing activity of TLR7-activated TApDC mediated by granzyme B and/or TRAIL leading to subsequent capture and antigen cross-presentation [90, 91].

Based on results of the literature, ongoing phase I/II clinical trials are currently evaluating the therapeutic potential of TLR agonists for the treatment of various types of cancer [92]. In the light of our observation that TApDC remain responsive to TLR-7L while lacking response to CpG ODN (TLR-9L), the therapeutic potential of TLR-7 agonists in human breast tumors should be considered.

8.3.1.2 TGF-β Neutralization

We detected phosphorylated Smad2 in the nuclei of BDCA2 TApDC by immunofluorescence (IF) on BT sections. These observations demonstrate that TApDC are exposed to TGF-β released in BT environment and suggest a role for Smad signaling in TGF-β-mediated TApDC inhibition, as already demonstrated for Smad3 in mice [93].

Our study identifies TGF-β as a novel target for restoring IFN-I production by TApDC. As (1)

TLR-9 (CpG ODN) and TLR-7 (Imiquimod) ligands that specifically target human pDC are currently evaluated in clinical trials for immune stimulation in tumor patients [94], (2) drugs that target TGF-β or its receptors' signaling are currently in development [95], and (3) pDCs are mandatory for the anti-tumor response mediated by Imiquimod in a murine model of melanoma through IFN-α production and acquisition of cytotoxic properties [91], our results suggest that restoring TApDC-derived IFN-I using TGF-β antagonists combined to TLR7/9 activation represent a promising new therapeutic strategy in localized BT.

Furthermore, as TGF-β is one of the elements driving the development of FoxP3$^+$ iTreg from naïve CD4$^+$ T cells in the periphery, targeting TGF-β pathway would help in reducing Treg development.

8.3.1.3 ICOS Neutralization on Treg

ICOS is a member of the CD28 superfamily of molecules that includes CTLA-4, PD-1, or BTLA, all playing critical roles in immune regulation. Based on our observations showing Treg amplification through ICOS/ICOS-L interaction in BT and Follicular Lymphoma [62, 69], confirmed in ovarian tumors [38, 61], we are pursuing ICOS as a therapeutic target to neutralize Treg. However, it has been shown that some of the effects of anti-CTLA-4 antagonist antibodies are directly due to perturbation of ICOS-ICOS-L interactions [71]. Furthermore, ICOS is expressed on other T-cell subsets such as Tfh cells [96] suggesting a central role for ICOS on their biology. Tfh cells constitute a subset of helper T cells specialized in the regulation of humoral immunity by providing germinal center B cells with survival and differentiation signals within B cell follicles [97]. The importance of ICOS in Treg and Tfh biology and pathophysiology will need further exploration for considering ICOS as a potential target in immunotherapy using neutralizing anti-ICOS antibodies. Moreover, different anti-ICOS agonists mAbs (JTX-2011 (#NCT-02904226), GSK-3359609 (#NCT-02723955)) are currently evaluated in phase I-IIa clinical trials in advanced solid tumors, alone or in combination with anti-PD-1 treatment, to reactivate immune T cell effectors.

8.3.1.4 CD39/CD73 Axis

CD73 cooperates with CD39 to generate Ado from ATP [75]. The critical role of Ado/CD73 in preventing anti-tumor immunity and promoting tumor development has been revealed by several teams using mouse tumor models [84, 85].

In particular, it was demonstrated that inhibition of CD73 using three separate techniques: (1) shRNAs, (2) a small chemical inhibitor (APCP), or (3) an anti-CD73 mAb (TY-23) blocks the growth of mammary (AT-3) and ovarian (ID8) murine tumor models though induction of an adaptive immune response [85, 98]. Responses include increased tumor-specific T cell immunity, and improved trafficking of anti-tumor T cells to the tumor microenvironment. It was also demonstrated in mouse tumor models that part of the immunosuppressive function mediated by Treg relies on CD73 enzymatic activity [85, 99]. In this regard, CD73-deficient Treg were less able to hinder tumor-specific immunity and promote tumor growth in mouse models of ovarian cancer and lymphoma. Moreover, Ado acts through A2A receptor to suppress endogenous tumor immunosurveillance potently as demonstrated [81] in A2A receptor-deficient mice mounting spontaneous anti-tumor T-cell responses able to induce T cell-dependent tumor rejection. Finally, constitutive (in CD73$^{-/-}$ mice) or transient (shRNA, antibodies, APCP) neutralization of CD73 was not associated with any overt immune disorders [100].

Altogether, these studies suggest that interfering with CD39/CD73/AdoR represents a potential strategy to reverse Treg-mediated suppression. In this regard, 2 companies (BMS, MedImmune) are currently in phase-I clinical trial with anti-hCD73 mAbs (BMS-986179 #NCT02754141, MEDI-9447 #NCT02503774) neutralizing the enzymatic activity in combination with anti PD-1 [101, 102].

8.3.2 Other Strategies Based on Treg Targeting/Depletion

8.3.2.1 Blockade of Treg Cell Induction and Recruitment

We contribute to show that CCL22 mediates Treg migration into human ovarian and breast tumors [31, 33, 103] and blockade of CCL22 significantly decreases Treg migration into ovarian tumors in an immune-deficient murine xenograft model, leading to immune rejection in the presence of anti-tumor effector T cells [103]. Small-molecule chemokine receptor antagonists (AF-399) [104] or mAb (Mogalizumab) were able to block in vitro CCL22-mediated recruitment of human Treg and Th2 cells and have gone into phase I clinical trials in advanced solid cancer patients (lung, gastric, esophageal, and renal cell carcinoma) either alone [105, 106] or in combination with anti-PD-1 (#NCT02946671).

8.3.2.2 Treg Depletion

- *Chemotherapy regimen*: Metronomic doses of cyclophosphamide or paclitaxel can selectively reduce Treg numbers [107–109].
- *Depletion of CD25 expressing cells*: CD25 expression remains the master target for Treg depleting strategies. In murine models administration of depleting anti-CD25 mAb (PC61) allowed a strong Treg reduction in peripheral lymphoid tissue and induced regression of established tumors [110]. Several drugs targeting CD25 are in clinical trial: (1) Denileukin diftitox (Ontak®, DAB389IL-2) is a recombinant protein fusing the active domain of diphtheria toxin to human IL-2; (2) Daclizumab (zenapex®) and basiliximab (simulect®) are anti-human CD25 mAbs. However, because of the non-specific expression of CD25, the major risk of such strategy is to eradicate activated T cells present within the tumor that could help for development/initiation of T cell anti-tumor immunity.
- *anti-CTLA-4 depleting mAbs:* Treg targeting could be achieved with other Treg receptors. Among them, the major work has been performed with anti-CTLA-4 antagonist mAb. Two fully humanized mAbs developed by BMS (MDX-100: ipilimumab®/yervov®) and Pfizer/Astrazeneca (CP675206: tremelimumab®) have been tested in clinical trials in cancer patients with various tumor types. Based on mouse studies [111] it is proposed that part of the therapeutic activity of the ipilimumab of IgG1 isotype (ADCC competent) is mediated through the depletion of activated Treg upon cell surface CTLA4 translocation. This has been confirmed in vitro in human melanoma patients [112].

8.3.2.3 Blockade of Treg Function

- *TNFR family*: Although several members of the TNFR family (OX40, GITR, CD137, etc.) have been targeted to modulate Treg function, it is unclear so far how the Treg function can be blocked without impairing T effector function. Similarly to anti-CTLA-4, ADCC-competent agonist anti-GITR mAbs may induce Treg depletion while inducing activation of T effectors through its agonist activity [113].
- *Blockade of Treg suppressive function through TLR agonist stimulation*: TLR-2 signaling (PAM_2CSK_4, PAM_3CSK_4, FSL-1) reduced Treg suppressive function in vitro [114] and in vivo in tumors models [115]. Treg-mediated suppression can be inhibited in vitro by TLR-8-derived signals [116] and treatment of melanoma patients with CpG (TLR-9 agonist) in vivo results in a reduced frequency of Treg [117].

Conclusion

Our work highlights the importance of Treg/pDC interaction in the breast and ovarian tumor microenvironment. Treg are recruited through CCR4 and CCL22 produced by tumor cells under inflammatory cytokine secretion following NK and macrophages activation. Recruited TATreg are then expanded by TApDC defective for their capacity to produced type IFN as a result of TGFb exposure and smad2 phosphorylation in the tumor environment. Expanded Treg expressed upregulated level of CD39 that with CD73 expressed on effectors will mediate immune suppression through Ado production. This suppressive

pathway may be particularly relevant during chemotherapy treatment leading to tumor cell death and ATP release. This pathway of pDC/Treg interaction identifies several potential targets to restore anti-tumor immunity: (1) mAbs neutralizing CCL22/CCR4 to block Treg recruitment within the tumor; (2) pDC reactivation for type-I IFN production through TLR-7 ligands; (3) TGF-β neutralization to restore pDC function; and (4) ICOS neutralization though ICOS/ICOSL neutralizing mAbs to block Treg proliferation.

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Cancer Immunosurveillance by Natural Killer Cells and Other Innate Lymphoid Cells

9

Camille Guillerey and Mark J. Smyth

Contents

9.1 Introduction

T cells screen the body for antigens presented on Major Histocompatibility Complex (MHC) molecules and have gained a central position in cancer immunotherapy for their ability to mount a response specifically directed against the tumor [1]. Indeed, tumor-infiltrating cytotoxic $CD8^+$ T cells and Th1-polarized $CD4^+$ T cells are usually associated with better prognosis [2]. However, conventional T cells are not the sole mediators of anti-cancer immunity. Several immune cells are able to detect the earliest signs of malignant transformation despite their lack of receptors specific for antigenic peptides. Innate immune cells provide immediate protection and are therefore likely to play a crucial role in the early stages of tumorigenesis [3, 4]. Innate immune surveillance of cancers involves myeloid cells such as macrophages [5] and neutrophils [6], unconventional T cells bearing invariant or semi-invariant T cell receptors (TCR) such as Natural Killer T (NKT) cells [7] and γδ T cells [8], and a growing family of lymphocytes called innate lymphoid cells (ILCs) [9].

Innate lymphoid cells are characterized by a lymphoid morphology, the lack of RAG-rearranged antigen-specific receptors, and the absence of surface markers of the dendritic or myeloid lineages [10]. ILCs constitute a heterogeneous population of cells that share a common origin [11]. They have been divided between killer and helper-like ILCs [12] (Fig. 9.1). Natural

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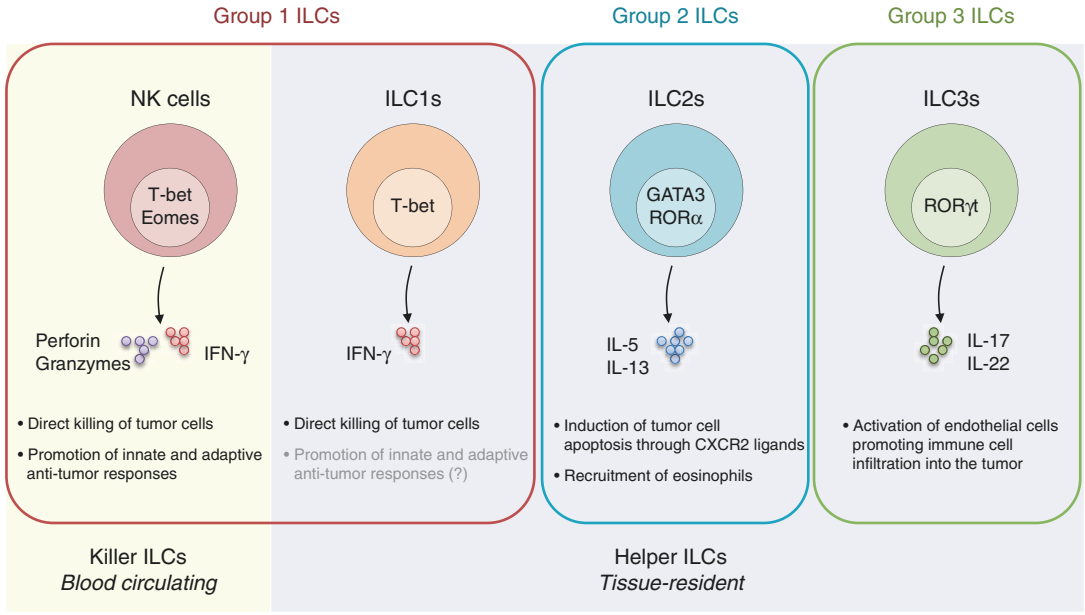

Fig. 9.1 The ILC family members and their putative role in cancer. The ILC family comprises killer blood-circulating ILCs and helper-like tissue-resident ILCs. ILCs are further divided into three groups according their signature cytokines and their transcription factor requirement. The main characteristics of the three classes of ILCs are represented here, as well as their anti-tumor activity. *Question marks* (?) indicate possible roles that have not been demonstrated yet. Direct killing of tumor cells is well established for NK cells but only few studies have described ILC1-mediated cytotoxicity through the lytic granule pathway [13] or through TRAIL in the case of liver ILC1s [14]. Of note, only the protective roles of ILCs against tumors are depicted here

Killer (NK) cells are the best-known cytotoxic members of the ILC family. These killer ILCs may be seen as the innate equivalents of $CD8^+$ cytotoxic T cells, housing similar cytotoxic granules and contents, whereas helper-like ILCs mirror the activity of $CD4^+$ helper T cells. Moreover, ILCs have been split into 3 categories based on their cytokine expression profiles and their specific transcription factor requirement. Group 1 ILCs are defined by their ability to produce IFNγ and depend on the Th1 cell-associated transcription factor T-bet. This group encompasses NK cells and helper-like type 1 ILCs (ILC1s). NK cells and ILC1s are developmentally distinct [15]. However, these two innate cell populations are not always easy to discriminate because they share many phenotypic markers [16]. Importantly, expression of the transcription factor Eomes is considered a hallmark of NK cells that distinguishes them from other group 1 ILCs [17], with the exception of salivary gland ILC1s [18]. Group 2 ILCs rely on the transcription factors GATA3 and RORα for their development and function and produce type 2 cytokines, mainly IL-5 and IL-13. Finally, group 3 ILCs are defined by their ability to produce IL-17 and/or IL-22 and their dependency on the transcription factor RORγt for their development and function.

NK cells constitute the first ILC subset to be discovered and were initially identified for their spontaneous cytotoxic activity [19]. NK cells are often characterized as CD3 negative cells expressing CD56 in humans, NK1.1 in mice, and NKp46 in both species [20]. Of note, such phenotypic definition may include other ILC subsets. Moreover, NK cells are not a homogeneous population and can be divided into different subtypes [21]. In humans, the two main NK cell subsets are $CD56^{bright}CD16^-$ NK cells which are important cytokine producers and are abundant in lymph nodes, and $CD56^{dim}CD16^+$ NK cells which represent the main NK cell population in the blood and are highly cytotoxic [22]. NK cell-depleting antibodies as well as mouse models displaying NK

Table 9.1 Mouse models and depleting antibodies used to investigate NK cell functions

Model	Characteristics	References
Anti-NK1.1 Abs	These Abs deplete NK cells and NKT cells in C57BL/6 mice but do not deplete NK cells in mouse strains that do not express NK1.1 such as BALB/c mice	[23]
Anti-AsialoGM1 Abs	These Abs deplete NK cells but not NKT cells. Anti-AsialoGM1 Abs may affect other cell populations and have been found to deplete basophils	[24]
Beige mice	Beige mice have a defect in granulation and exhibit severe NK cell deficiency but also display defects in other granulocytes, cytotoxic T cell responses, and antibody responses	[25]
$Rag^{-/-}\gamma_c^{-/-}$ mice	Compared with $Rag^{-/-}$ mice that lack T and B lymphocytes, $Rag^{-/-}\gamma_c^{-/-}$ mice lack both innate and adaptive lymphocytes. Indeed, $Rag^{-/-}\gamma_c^{-/-}$ mice lack the common gamma chain (γ_c or *Il2rg*) that is required for IL-7 and IL-15 signaling and thus essential for ILC development	[26]
NKDTR/EGFP transgenic mice	These mice express the diphtheria toxin receptors under the NKp46 promoter. Diphtheria toxin injection in these mice leads to NK cell depletion and may also deplete other $NKp46^+$ ILCs	[20]
$Mcl1^{fl/fl}$Ncr1-Cre mice	These mice lack NK cells and other $NKp46^+$ ILCs	[27]
$Ncr1^{greenCre}Il2rg^{fl/fl}$	These mice lack all $NKp46^+$ ILCs	[28]

cell deficiencies have been fundamental for the demonstration of NK cell anti-cancer activity (Table 9.1). NK cells harbor many surface receptors allowing them to distinguish malignant-transformed cells from healthy cells. Moreover, NK cells are endowed with potent cytotoxic functions and are a major source of the anti-tumor cytokine, IFN-γ. These characteristics make NK cells key protagonists of innate immunosurveillance of cancers [29]. In neat opposition to the numerous reports on NK cells in cancer, the possible pro- or anti-tumor functions of helper-like ILCs remain largely unexplored [30]. Nonetheless, recent work suggested an involvement of tissue-resident type 1-like ILCs in the immune surveillance of spontaneous tumors [13]. Furthermore, various reports described tumor-suppressive activities of ILC2s [31, 32] and ILC3s [33, 34].

In this chapter, we will review the different mechanisms by which ILCs detect malignant cells and prevent cancer development. Most of our current knowledge is restricted to NK cells which constitute the prototypical anti-cancer ILC subset. Therefore, we will predominantly focus on NK cells while introducing emerging data on helper-like ILCs. It should be noted that both pro- and anti-tumor activities of helper-like ILCs have been described [35] but herein we will only discuss their potential host protective functions.

9.2 Surface Receptors Involved in Tumor Recognition by ILCs

Innate cells express a fixed set of germline-encoded receptors that allow the recognition of foreign, aged and damaged cells [36]. Activating and inhibitory surface receptors are crucial for the regulation of NK cell functions and some of these receptors are also expressed on helper-like ILCs subsets (Fig. 9.2). Furthermore, interactions with accessory cells, generally monocytes or dendritic cells (DCs), also stimulate NK cells to produce pro-inflammatory cytokines and potentiate their killing functions [37]. Historically, NK cells were described for their ability to kill tumor cells having down-regulated MHC class I molecules (MHC-I), a concept termed "missing-self" recognition [38]. Most cell types express self-peptide-MHC-I complexes on their surface but partial or complete loss of MHC-I expression is a common feature of cancer cells [39]. This phenomenon is often caused by $CD8^+$ T cell-mediated immune pressure and renders tumor cells susceptible to NK cell-mediated cytotoxicity. Conversely, MHC-I molecules expressed at the surface of healthy autologous cells bind to inhibitory NK cell receptors and deliver negative signals thereby avoiding NK cell autoreactivity.

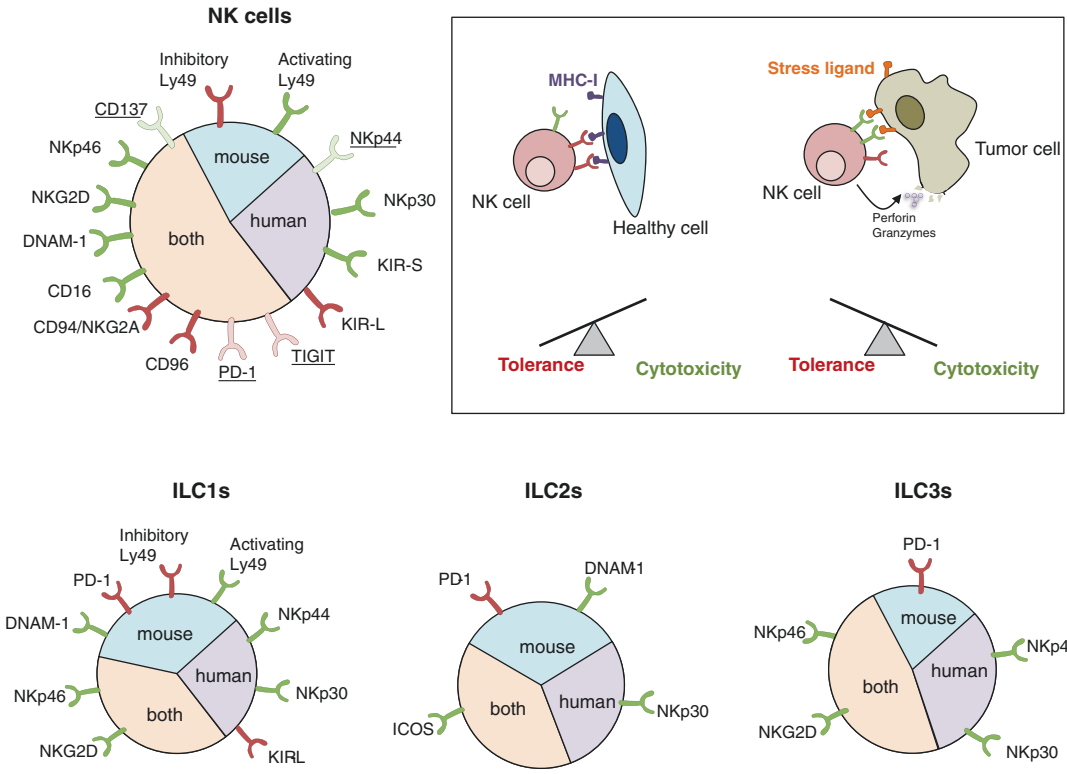

Fig. 9.2 Cell surface receptors involved in tumor cell recognition by ILCs. NK cells express an array of activating (*green*) or inhibitory (*red*) cell surface receptors. Some receptors are only expressed on activated NK cells (*underline*). The outcome of NK cell interactions with a target cell is determined by the balance between activating and inhibitory signals. Healthy cells express MHC-I molecules that engage NK cell inhibitory receptors whereas cancer cells down-regulate MHC-I molecules and/or express stress ligands recognized by NK cell activating receptors. An excess of activating signals over inhibitory signals leads to NK cell activation and the cytotoxicity of the target cell. Helper-like ILCs also express receptors that may regulate the sensing of tumor cells and some of these receptors have been found to regulate cytokine production by helper-like ILCs. DNAM-1 and PD-1 have recently been observed on mouse ILCs but their expression on human ILCs has not been investigated yet

Besides the "missing-self" recognition, the detection of stress-induced self-ligands expressed at the surface of damaged cells also promotes NK cell killing capacities. In fact, the outcome of NK cell interaction with a target cell is determined by the balance between inhibitory signals transmitted by NK cell receptor binding to self MHC-I and activating signals transmitted upon recognition of stress ligands at the surface of the target cell [40]. Activating NK cell receptors involved in the immunosurveillance of cancers include the natural cytotoxicity receptors (NCRs), NKG2D (also known as CD314 and KLRK1) and DNAM-1 (also known as CD226) [36]. Other receptors such as the low-affinity activating receptor FcγRIIIa (CD16) or the co-stimulatory molecules CD137, OX40, and GITR are promising clinical targets for their ability to mediate potent NK cell activation [41]. Nonetheless, these receptors have not been reported to play any role in the early detection of nascent tumor and therefore will not be discussed here.

There are three members of the NCR family: NKp46 (NCR1; CD335) is expressed in both mice and humans, whereas NKp44 (NCR2, CD336) and NKp30 (NCR3; CD337) are restricted to human NK cells [42]. Unlike NKp46 and NKp30, NKp44 is not detected on resting NK cells but is up-regulated after activation. NCR engagement triggers NK cell-mediated

cytotoxicity and secretion of IFNγ [36]. Importantly, NCR expression is not restricted to NK cells and is shared with helper ILC1s [16], a subset of human ILC2s [43] as well as a subset of ILC3s named NCR$^+$ ILC3s [10]. Engagement of NKp44 by tumor cells and tumor-associated fibroblast stimulates NCR$^+$ ILC3 to release IL-8 and TNF [33]. Similarly, NKp30-mediated recognition of human tumor cell lines induces the NF-κB signaling pathway in ILC2s, leading to the production of IL-13 and other type 2 cytokines [43]. Whether NCRs also govern helper-like ILC1 recognition of malignant cells is yet to be demonstrated. NCR ligands on tumor cells have only been partially defined and those reported include NKp44L, HLA-B associated transcript 3 (BAT3), B7-H6, heparan sulfates, and proliferating cell nuclear antigen (PCNA) [42]. Curiously, PCNA differs from other NCR ligands as it does not stimulate but rather inhibits NK cell functions [44]. Interestingly, NCR genes encode different splice variants, some of them being immunosuppressive. A recent report suggested that the cytokine-defined microenvironment may influence NKp30 and NKp44 isoform expression profile in NK cells and that alternative splicing gives rise to inhibitory isoforms that dampen NK cell functions [45]. Furthermore, in gastrointestinal stromal tumors, predominant expression of the immunosuppressive NKp30c isoform over the immunostimulatory NKp30a and NKp30b isoforms is associated with reduced survival [46]. Remarkably, mice lacking NKp46 have been useful to define NKp46 involvement in the control of initial tumor growth [47] and the prevention of tumor metastasis [48] in vivo. Unfortunately, in vivo investigation of the other NCRs in tumor immunosurveillance is limited by the lack of mouse orthologs for NKp30 and NKp44.

NKG2D is a major NK cell activating receptor also expressed by some T cell subsets [36]. NKG2D recognizes several MHC-related ligands that are poorly expressed at the surface of healthy cells but are frequently up-regulated with the process of malignant transformation [49]. For instance, in non-transformed mouse or human cells, activation of the DNA-damage response induces the up-regulation of NKG2D ligands and enhances cellular sensitivity to NK cell killing [50]. NKG2D ligands are RAE-1α-ε, MULT1 and H60a-c in mice; and MICA-B and ULBP1–6 in humans. NKG2D engagement stimulates signaling cascades leading to cell activation, killing, and cytokine production. A pioneer study demonstrated that mouse tumor cell lines engineered to express high levels of NKG2D ligands and injected subcutaneously into syngeneic mice are rapidly rejected by conventional NK cells without a requirement for T and B cells [51]. Notably, the pivotal role of NKG2D in tumor immunosurveillance has been evidenced in mouse models of de novo tumorigenesis [52] and carcinogenesis [53]. In a model where expression of the Epstein–Barr virus transforming protein LPM1 in mouse B cells led to the development of B cell lymphomas, the arising lymphoma cells expressed ligands for NKG2D and were killed in vitro by NK cells [54]. However, in this model, T cells were the major effectors of immunosurveillance. It is possible that arising lymphomas may have developed escape mechanisms to circumvent NK cell antitumor activity in vivo. In fact, tumor progression is usually associated with an immunoediting process resulting in the emergence of malignant clones that are resistant to NK cell activity [55]. This has been illustrated in multiple myeloma where the transition from a pre-malignant to a malignant stage of the disease is associated with shedding of MICA from the surface of the tumor cells [56]. In addition to reducing NKG2D ligand surface density on tumor cells, this shedding process generates soluble ligands that down-regulate NKG2D expression on immune cells and promote tumor immune evasion [57]. Actually, chronic exposure to low-affinity surface-attached NKG2D ligands also leads to NK cell desensitization to both NKG2D-dependent and -independent pathways [58]. Surprisingly, instead of blocking tumor cell recognition, shedding of the high affinity NKG2D ligand MULT1 promotes tumor rejection by boosting NK cell effector functions [59]. It was suggested that MULT1 prevents immunosuppressive interaction with low-affinity ligands such as RAE-1 expressed in the

tumor microenvironment and restores NK cell responsiveness.

DNAM-1 is an adhesion molecule expressed on NK cells and T cells that associates with the integrin LFA-1 and participates to the stabilization of the cytolytic synapse [60]. DNAM-1 recognizes a the family of nectin and nectin-like molecules initially identified for their role in cell-cell adhesion [61]. Nectin and nectin-like molecules have been involved in a wide range of biological processes and they notably regulate the immune functions of T cells, NK cells, and antigen presenting cells. In addition to enhancing NK cell adhesion and cytotoxicity, DNAM-1 promotes the secretion of IFN-γ. Moreover, DNAM-1 expression distinguishes two functional NK cell subsets in mouse [62]. Compared with DNAM-1$^-$ NK cells, DNAM-1$^+$ NK cells have enhanced IL-15 signaling and produce higher levels of pro-inflammatory cytokines. Intriguingly, DNAM-1 is expressed at high levels on mouse liver ILC1s [17] and is also detected on early ILC precursors as well as ILC2 progenitors in the mouse bone marrow [63]. Still, the putative role of DNAM-1 in the regulation of helper-like ILC function remains to be investigated. DNAM-1 ligands, CD155 (also known as PVR) and CD112 (also known as nectin-2, PRR2, or PVRL2) are often over-expressed by solid and hematological malignancies [60]. Similarly to what has been described for NKG2D ligands, DNA-damage resulting from replication stress seems responsible for tumor cell expression of the DNAM-1 ligand CD155, and this pathway is dependent on ATM, an enzyme that senses double strand DNA breaks [64]. An interesting study performed in the Eμ-Myc mouse lymphoma model established that the DNA-damage response induces CD155 expression on early stage transformed B cells and thereby leads to spontaneous tumor regression that is partially DNAM-1-dependent [65]. Further strong evidence of DNAM-1 contribution to immunosurveillance comes from the observation of enhanced development of carcinogen-induced fibrosarcomas [66] as well as accelerated growth of spontaneous and transplantable tumors in DNAM-1-deficient mice [67, 68].

The variety of activating receptors expressed by NK cells is complemented by numerous inhibitory receptors that prevent the killing of healthy tissue. Receptors binding to self-MHC-I are responsible for the "missing-self" recognition. NK cell receptors of the KIR family in humans and of the Ly49 family in mice directly recognize MHC-Ia molecules [36]. Moreover, the CD94/NKG2A heterodimeric receptor is expressed in both species and binds to a peptide presented by the non-classical MHC molecule HLA-E in humans and Qa-1 in mice. Successful engagement of MHC-I by these receptors transmits an inhibitory signal that disrupts activating pathways. For instance, engagement of the inhibitory receptor KIR2DL2 blocks activating receptor clustering and induces actin remodeling and concomitant retraction from the target cell [69]. The importance of the missing-self recognition in NK cell-mediated immunosurveillance has been highlighted by the report of accelerated onset of carcinogen-induced sarcomas and spontaneous B cell lymphomas in mice expressing reduced levels of Ly49 inhibitory receptors [70]. So far, NK cells are considered the sole ILC subset mediator of the "missing-self" recognition. Notwithstanding, Ly49 receptors have been detected on the surface of other ILCs subsets [13, 16] but their function needs to be assessed.

Additional receptors may regulate ILC functions in the tumor microenvironment. The immune checkpoint molecules CTLA-4 and PD-1 have been found to hinder NK cell activity and constitute important clinical targets [41] but little is known about their role in innate immunosurveillance of cancer. Interestingly, mouse precursors for helper-like ILCs have been characterized by high expression levels of PD-1 [63]. Furthermore, few mature ILCs express PD-1, but they up-regulate this immune checkpoint molecule upon activation [71]. More investigation would be required to assess the role of PD-1 on ILCs within tumors. Besides, two receptors interacting with the nectin and nectin-like molecule family, TIGIT and CD96 (also known as TACTILE), have recently gained clinical interest for their potent inhibition of NK cell- and T cell-functions [72]. TIGIT and CD96 bind to CD155 and counterbalance DNAM-1-mediated

activation of NK cells [60]. TIGIT inhibits mouse and human NK cell-mediated cytotoxicity [73, 74] and CD96 was shown to reduce mouse NK cell production of IFN-γ [75]. CD96-deficient mice display robust resistance to experimental lung metastasis and carcinogenesis [75], but the role of TIGIT in NK cell-mediated surveillance of cancers remains to be established.

9.3 Cytokines and Soluble Factors that Activate ILCs

Besides cell-to-cell interactions, ILCs integrate multiple signals provided by soluble mediators such as cytokines, alarmins, lipids, or hormones produced by epithelial, stromal, or myeloid cells [76] (Fig. 9.3). Tumor growth is likely to disturb the homeostasis of the surrounding tissue, leading to the release of cytokines and danger signals that might shape ILC-mediated immunosurveillance. Both NK cells and helper-like ILC1s are responsive to IL-12, IL-15, and IL-18, while ILC2s mainly respond to IL-25, IL-33, and TSLP; and ILC3s are activated by IL-23 and IL-1β. There is little direct evidence of the role of physiologically secreted cytokines on ILCs in cancer and most studies have rather investigated the effect of exogenous cytokine administration or used genetically manipulated tumors or mice. For example, a report described the resistance of transgenic mice over-expressing IL-15 to subcutaneously injected B16 melanoma cells lacking MHC-I [77]. In this study, the anti-tumor activity was maintained in the absence of $CD4^+$ or

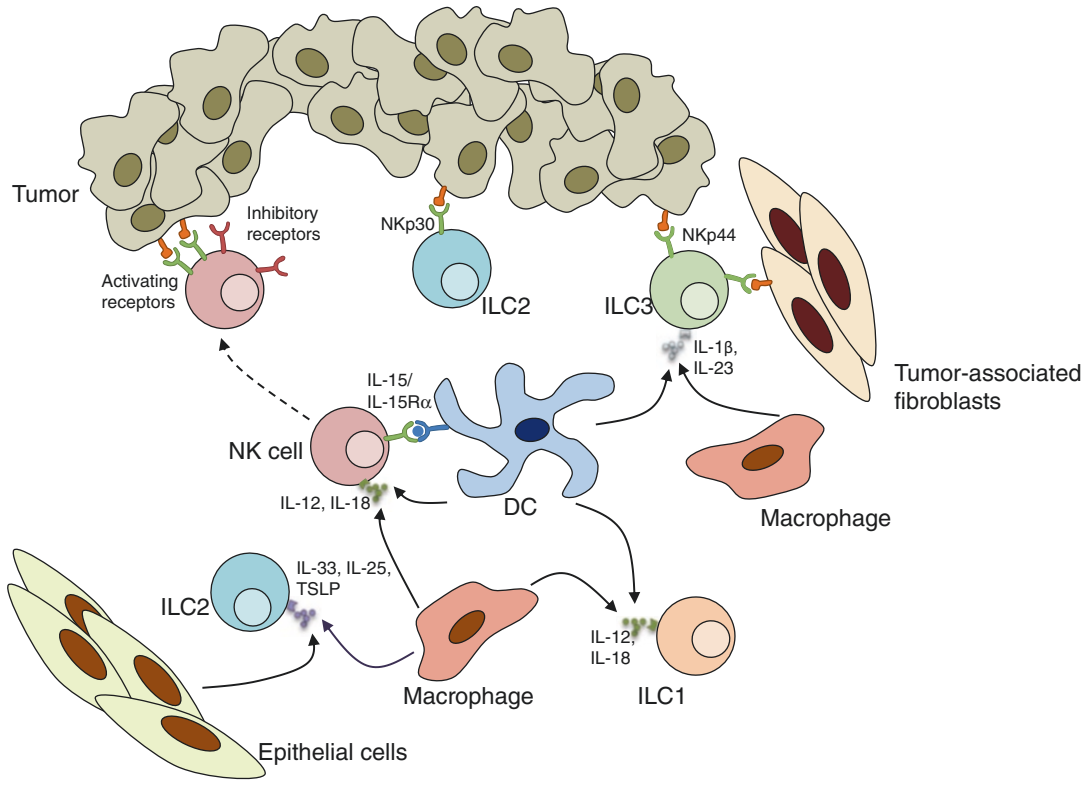

Fig. 9.3 Cytokines and membrane-bound ligands contribute to ILC activation. Cytokines such as IL-12, IL-15, and IL-18 produced by DCs and macrophages prime NK cells increase their capacities upon engagement of activating receptors. IL-12 and IL-18 also stimulate IFN-γ production by ILC1s. ILC2s respond to IL-25, IL-33, and TSLP. In addition, human ILC2s are activated upon NKp30 engagement, while human ILC3s respond to NKp44 stimulation. Moreover, ILC3s are activated by the cytokines IL-1β and IL-23

$CD8^+$ T cells, but protection was lost upon NK cell depletion with anti-asialo-GM1 antibodies. Moreover, IL-2, IL-12, and IL-18 promote NK cell-mediated control of experimental metastasis in mice [78, 79] and NK cells pre-activated with IL-12, IL-15, and IL-18 display sustained effector functions and delay the growth of MHC-I-deficient tumors in mice [80]. Intriguingly, a more recent study established that the IL-12-induced suppression of subcutaneous B16 melanoma tumors was mediated by a $NKp46^+NK1.1^-$ ILC3 population identified using a fate-mapping reporter mouse strain for the transcription factor RORγt [34]. Finally, the expression of Toll-like receptors by NK cells and ILC3s [81, 82] might enable these cells to detect danger-associated molecular patterns (DAMPs) present in the tumor microenvironment. DAMP may induce NK cell activation either directly, or indirectly through the stimulation of accessory cells [37]. Interestingly, a recent study established that NK cells from TLR3-deficient mice are hyporesponsive to cytokine stimulation, a defect that is associated with increased experimental lung metastasis in these mice upon challenge with B16F10 melanoma cells [83].

Notably, helper-like ILCs are mainly activated via soluble mediators while NK cell receptors represent a predominant pathway for NK cell activation. Nevertheless, cytokine-activated NK cells are more responsive to NK cell receptor signaling than resting NK cells. NK cell-activating cytokines are generally secreted by myeloid cells or dendritic cells (DCs) [84]. In fact, it has been suggested that naïve NK cells only acquire their full killing capacity following an interaction with DCs or macrophages termed "NK cell priming" [85], a process that is influenced by the commensal microbiota [86]. Of particular interest, similar synergy between cytokine-mediated activation and NCR signaling has recently been described for NCR^+ ILC3s [87].

9.4 Direct Clearance of Cancer Cells by ILCs

The release of cytotoxic granules containing perforin and granzymes constitutes the main pathway by which NK cells exert their killing activity [88]. Perforin is a pore-forming protein that allows granzymes to enter into target cells, thereby triggering apoptosis [89]. Perforin protects mice against spontaneous lymphomas [90] and mouse studies have established the major contribution of perforin to NK cell-mediated rejection of tumor lacking MHC-I [91, 92], as well as NK cell-mediated control of metastasis [23] and protection against carcinogen-induced fibrosarcomas [93]. Granule-dependent cytotoxicity was originally thought to be a characteristic of NK cells distinguishing them from ILC1s [12]. However, it was recently demonstrated that a population of ILC1-like cells clearly distinct from conventional NK cells could also kill tumor cells in a perforin-dependent fashion [13]. Notably, this study used a spontaneous mouse mammary cancer model to suggest that ILC1-like cells, but not conventional NK cells, contribute to reduce tumor growth.

Fas ligand (FasL or CD95L) and TNF-related apoptosis inducing ligand (TRAIL) belong to the death receptor pathway and represent alternative mechanisms by which NK cells eliminate target cells [14, 94, 95]. The binding of Fas or TRAIL to their receptors (Fas and DR5 or DR4, respectively) triggers the activation of the common death signaling molecules FADD, caspase 8, and caspase 3, and leads to apoptosis [96]. The relevance of targeting Fas- and TRAIL-death receptor pathways to bypass the refractory nature of cancer stem cells to conventional therapy has been demonstrated in mice [97]. Initially, TRAIL-positive NK cells were described in the liver of naïve mice; and in vitro killing activity of hepatic but not splenic NK cells was found to be TRAIL-dependent [14]. Accordingly, TRAIL appears necessary for the control of experimental liver metastasis in mice [14]. Interestingly, TRAIL is largely expressed by immature NK cells in newborn mice and is required for fetal NK cell killing activity of TRAIL-sensitive targets in vitro [98]. Similarly, TRAIL is required for the in vitro killing activity of human cord blood NK cells whereas the cytotoxicity of mature human NK cells mostly relies on the perforin and FasL pathways [99]. While the origin of $TRAIL^+$ fetal NK cells remains unclear, mouse liver $TRAIL^+$

NKp46$^+$ cells have now been assigned to the ILC1 lineage [100]. In the healthy human liver, NK cells do not express TRAIL but its expression can be induced by pro-inflammatory cytokines [101]. It was recently shown that TRAIL up-regulation is confined to a specific population of human intra-hepatic NK cells that express CXCR6 and are absent from the periphery [102]. Thus, in the absence of inflammatory stimuli, TRAIL expression might be restricted to tissue-resident subsets of the group 1 ILCs. As a matter of fact, in mice, TRAIL has also been detected on salivary gland ILC1s [18] and tumor-infiltrating ILC1-like cells [13]. However, the observation that cytokine stimulation induces TRAIL expression on CD3$^-$NK1.1$^+$ cells in the murine spleen [103] and CD3$^-$CD56$^+$ cells in the human blood [104] suggests that TRAIL also contributes to conventional NK cell functions under some circumstances. In fact, membrane-bound TRAIL supplements perforin-mediated killing of neuroblastoma and multiple myeloma cell lines by activated NK cells isolated from human peripheral blood [105, 106]. Additional investigation should shed light on the respective roles played by blood-circulating conventional NK cells and tissue-resident ILC1s in the TRAIL-mediated control of nascent tumors. In opposition to TRAIL, there is very limiting data supporting a role of FasL in NK cell-mediated-control of tumors in vivo [107]. Noteworthy, a recent study elegantly demonstrated that IL-18 induces a rapid expression of FasL on the surface of mouse NK cells and NK cell-mediated FasL-dependent cytotoxicity was found to control MC38 liver metastases in mice [108].

An important characteristic of group 1 ILCs is the secretion of IFN-γ and TNF [109]. These two cytokines play a major role in tumorigenesis. Not only do they modulate immune responses, but they also directly impact on tumor cell biology. Actually, enhanced in vivo growth of various mouse cell lines has been observed upon ablation of tumor-responsiveness to IFN-γ [110]. Responses to IFN-γ are induced by the JAK-STAT signaling pathway. In cancer cells, this pathway has been shown to inhibit cellular proliferation and to promote apoptosis [110]. The central role played by endogenously produced IFN-γ in promoting the immune-mediated elimination of nascent tumor cells has been demonstrated by Schreiber and colleagues [111]. An early study indicated that the combination of perforin and IFN-γ pathways fully accounts for NK cell anti-metastatic activity in mice [112]. Nonetheless, since IFN-γ can be produced by many different innate and adaptive immune cell types, the formal proof of ILC contribution to IFN-γ-mediated immunosurveillance is still lacking. As for TNF, the role of this cytokine in cancer biology is rather ambiguous [113]. The two receptors to TNF are TNFR1, which is expressed on all cell types and TNFR2, which expression is restricted to immune and endothelial cells. Paradoxically, TNFR1 can transmit both pro-survival and pro-apoptosis signals. As a result, some reports described cytostatic or cytotoxic effects on tumor cells while others observed an enhancement of malignant cell proliferation (for a review, see [113]). The observation of reduced in vitro killing capacity of NK1.1$^+$ splenocytes from TNF-deficient mice against YAC-1 suggested that TNF contributes to NK cell-mediated killing [114]. Furthermore, NK cell cytotoxicity against chemotherapeutic-sensitized mouse MC38 tumors was found to be TNF-dependent [115]. But overall, to date there is no convincing evidence of a major contribution of TNF to innate cell-mediated cytotoxic activity. In fact, spleen cells from TNF-deficient mice are perfectly able to lyse MHC-I deficient RMA-S tumor cells in vitro [116]. However, defective elimination of RMA-S cells was observed following intraperitoneal injection in TNF-deficient mice [116]. This phenomenon was explained by reduced NK cell accumulation in the peritoneum in the absence of TNF. Moreover, TNF-neutralization inhibits NK cell activation and thus reduces human NK cell-mediated cytotoxic activity against myeloma cells in the presence of anti-CD319 mAbs (Elotuzumab) [117]. Collectively, these data indicate that albeit not directly cytotoxic, TNF secreted by NK cells might act to regulate the tumor microenvironment.

Of note, although direct cytotoxic activity toward tumor cells is considered as a

specificity of group 1 ILCs, ILC2s were recently found to induce tumor cell apoptosis via the CXCR2 pathway [32].

9.5 Cross-Talk between ILCs and Other Immune Cells Resulting in Anti-Cancer Immunity

NK cell functions expend far beyond the simple killing of cancer cells [118]. In addition to IFN-γ and TNF, activated NK cells release a broad range of cytokines, including GM-CSF, IL-6, and IL-10, and they may facilitate the recruitment of other immune cells by secreting chemokines such as MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), IL-8 (CXCL8), and IP-10 (CXCL10) [119]. IFN-γ production by NK cells has been shown to promote macrophage-mediated immunoediting of carcinogen-induced tumors in mice [120]. Moreover, NK cells influence the outcome of developing T cell responses in many different ways [121]. For instance, NK cells enhance the priming cytotoxic $CD8^+$ T cell responses by eliminating myeloid-derived suppressor cells [122] and activating DCs [123, 124]. Tumor cell killing by NK cells and the subsequent release of antigen further contributes to T cell priming [125]. Besides, NK cells recruited to inflamed lymph nodes provide an early source of IFN-γ necessary for Th1 polarization of $CD4^+$ T cells [126]. The direct cytotoxicity of activated regulatory T cells (Tregs) represents another mechanism by which NK cell could potentiate effector T cell responses [127]. Importantly, in some mouse tumor models, NK cells were found to contribute to the priming of tumor-specific memory T cells required for the long-term control of cancer [123, 128].

Our understanding of helper-like ILC mediated-regulation of anti-tumor responses is still in its infancy. Given the important role of IFN-γ in NK cell-mediated tuning of adaptive immune responses, it is tempting to hypothesize that IFN-γ production by ILC1s would contribute in a similar manner. However, there is currently no study supporting this statement. In fact, the evaluation of the relative contribution of NK cells and ILC1s to anti-tumor immunity is complicated by the high phenotypic resemblance of these cells. Of note, $TRAIL^+$ members of the group 1 ILC might also dampen T cell responses [129]. Regarding ILC2s, the type 2 cytokines produced by these cells usually inhibit type 1 anti-tumor responses and foster tumor progression [9]. Nevertheless, in the mouse B16F10 model of experimental metastasis, IL-5 production by lung ILC2s cells has been shown to promote eosinophil recruitment and clearance of lung tumors [31]. A positive role of ILC3s in anti-tumor immunity was suggested in a study where combined treatment of chemotherapy with tumor-targeting antibodies resulted in delayed growth of B16 subcutaneous tumors [130]. In this setting, tumor clearance was found to be dependent on $CD90^+NK1.1^-ROR\gamma t^+$ innate lymphocytes and was associated with increased infiltration of macrophages within the tumor tissue. Another report demonstrated that $NKp46^+$ ILC3s suppress the growth of subcutaneously injected B16F10 tumor cells engineered to secrete IL-12 [34]. IL-12-secreting tumors were still repressed in the absence of T cells or of conventional NK cells. It was suggested that $NKp46^+$ ILC3s mediated their anti-tumor functions by up-regulating adhesion molecules on the tumor endothelium. Similarly, the production of soluble factors by NCR^+ ILC3s present in human non-small cell lung cancer (NSCLC) tissues was found to activate mesenchymal stem cells and endothelial cells [33]. In this study, ILC3 numbers within the tumor tissue were found to correlate with the density of TLS tertiary lymphoid structures (TLS) which are ectopic lymphoid organs associated with a favorable prognosis in NSCLC patients [131]. Very interestingly, lower frequencies of tumor-infiltrating NCR^+ ILC3s were observed in advanced tumors, suggesting that NCR^+ ILC3s might be associated with a better prognosis for NSCLC patients [33].

Conclusions

ILCs act as sentinels that react promptly upon disturbance of host homeostasis [76]. Their rapid and robust response allows the temporary control of the danger and alerts other immune

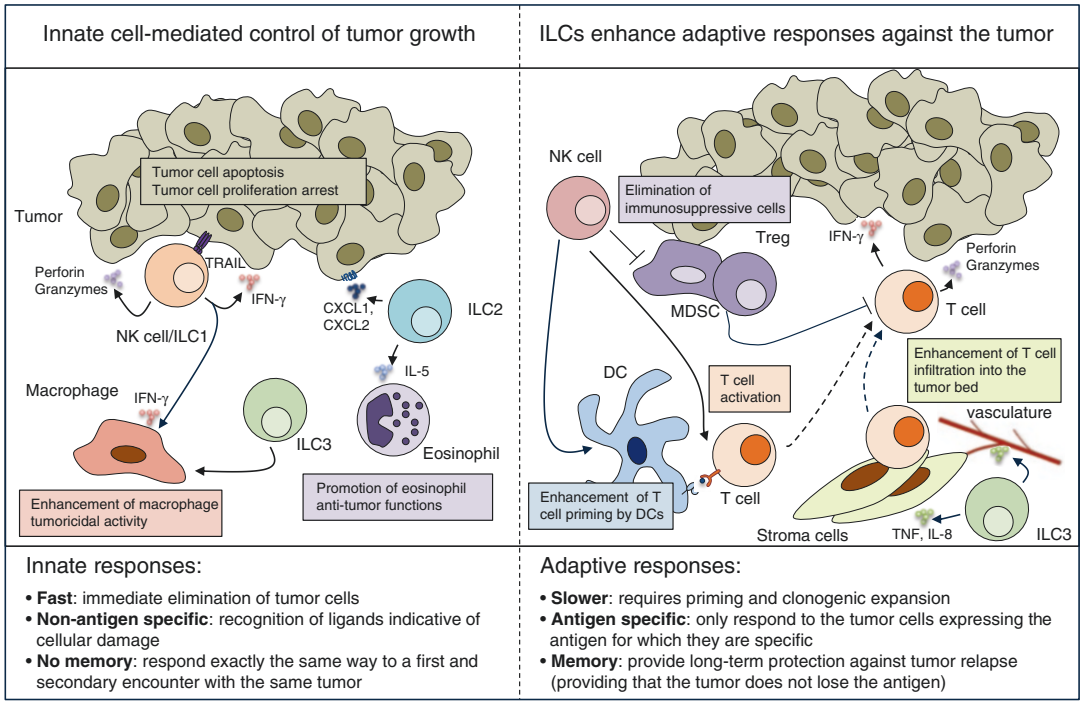

Fig. 9.4 Innate and adaptive lymphocytes contribute to cancer immunosurveillance. *Left panel*: ILCs directly induce tumor cell apoptosis and/or growth arrest and they also stimulate other innate cells to ensure the fast clearance of cancer cells. Group 1 ILCs exert a direct cytotoxic activity against tumor cells either via the cytotoxic granule pathway (perforin/granzymes) or the death ligand pathway (TRAIL). Of note, such cytotoxic activity has been described for both conventional NK cells and ILC1s but NK cells are currently considered more potent killers. IFN-γ production by group 1 ILCs also directly inhibits tumor growth and may stimulate macrophage tumoricidal activity. ILC2s might directly induce the apoptosis of CXCR2-expressing tumor cells via the secretion of CXCL1 and CXCL2. ILC2s also secrete IL-5 and thereby recruit eosinophils. ILC3s may increase macrophage infiltration into the tumor. *Right panel*: ILCs stimulate T cell responses and thereby ensure long-term protection against cancer cells. NK cells activate DCs, enhance anti-tumor T cell responses, and also kill immunosuppressive cells such as Tregs and myeloid-derived suppressor cells (MDSC). ILC3s activate stroma and endothelial cells and thereby facilitate immune cell infiltration into the tumor bed

cells to provide long-term protection (Fig. 9.4). NK cells have long been known as the most powerful innate guardians against cancer development. However, the recent expansion of the ILC family may challenge this idea and raises the question of the relative contribution of the different ILC subsets, in particular NK cells and ILC1s. Noteworthy, despite considerable transcriptomic overlap with ILC1s, NK cells express higher transcripts encoding proteins of the cytotoxic machinery as well as cell surface receptors involved in the detection of transformed cells [100]. Moreover, helper-like ILCs are tissue-resident cells [132] that would only sense alterations of the specific organ where they are located whereas conventional NK cells circulate in the blood and can scan the whole body for the presence of damaged cells. Thus, NK cells are probably the most efficient ILC subset in tumor clearance.

The importance of ILCs in the presence of functional adaptive immunity was recently questioned as ILC deficiency occurring in a cohort of SCID patients appeared to have no major clinical consequences [133]. This study provided a 7–39 year follow-up of 18 patients with mutation of IL2RG or JAK3 treated with hematopoietic stem cell transplantation in the absence of myeloablation. However, this cohort was too small to address the role of ILCs in

tumor immunosurveillance and such investigation may also require a longer follow-up. Perhaps the most convincing evidence of the importance of cytotoxic ILCs for human cancer immunosurveillance comes a prospective study demonstrating that individuals with high spontaneous cytotoxic activity of peripheral blood lymphocytes were at significantly lower risk of developing cancers [134]. In gastrointestinal sarcoma patients, NK cell infiltration correlates with the absence of metastasis at diagnosis [46] and in renal cell carcinoma, high densities of NK cells in lung metastases are associated with prolonged survival [135]. Conversely, a study in NSCLC reported no correlation between NK cell numbers and clinical outcome [136]. An absence of NK cell activity against advanced cancers may be caused by (1) tumor escape from NK-cell immunosurveillance due the selection of resistant variant clones through the immunoediting process [120] and/or (2) the exhaustion of NK cells within the tumor microenvironment where a variety of mechanisms contribute to hinder NK cell functions [55]. Adenosine and TGF-β are two examples of soluble factors proven to affect NK cell activity against tumors [137, 138]. Furthermore, poor NK cell infiltration in human cancer tissues could explain their limited impact on solid tumor progression. However, NK cells seem particularly efficient against metastatic disease [27] and hematological malignancies [139].

Evidence for helper-like ILC subsets involvement in human cancers is limited. ILC3 infiltration has been observed in human colorectal cancer [140], primary tumors of breast cancer patients [141], and NSCLC tissues [33]. Importantly, the function of helper-like ILCs in malignant diseases remains unclear and may depend on the cancer type and stage. This chapter is focused on the protective role of ILCs in the detection and elimination of nascent tumors and the reader is invited to refer to other reviews for a complete discussion of the opposing abilities of helper-like ILCs to either promote or repress tumor growth [9, 30, 35]. It is possible that the tumor microenvironment hijacks ILCs, either by dampening ILC anti-tumor activity such as IFN-γ release, or by influencing ILC plasticity. Indeed, some ILC subsets are not stable and depending on the cytokine microenvironment, NK cells can acquire an ILC1 phenotype [18], ILC2s can convert into ILC1s [142] or ILC3s [143], and ILC3s can convert into ILC1s [144]. The hypothesis that tumor may escape immunosurveillance by modulating ILCs is supported by the report that both ILC functions and subtype composition are dysregulated in the blood of acute myeloid leukemia patients [145].

ILCs represent an interesting clinical target since they react immediately to stimulation and their responses are not antigen-driven. Their production of large amounts of cytokines could shift the tumor microenvironment and awaken the anti-cancer capacities of myeloid cells and adaptive lymphocytes. However, a better understanding of helper-like ILC functions, biology, and plasticity is definitely needed before these cells could be efficiently exploited in the clinic. On the other hand, manipulation of NK cells has emerged as a very promising therapeutic option for cancer patients. The multiple strategies employed to take advantage of NK cell anti-tumor activity have been reviewed elsewhere [41] and are the subject of another chapter of this volume. Importantly, some strategies currently developed to target T cells or NK cells, such as anti-PD1 mAbs [146], NKG2D-bispecific engagers [147], or cytokine infusions [148] might also influence ILC functions. As our knowledge of the ILC family increases, the interest of the cancer immunotherapy field in these cells is likely to rise.

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Conflict of Interest M.J. S. has contract research agreements with Bristol Myers Squibb, Aduro Biotech, Corvus Pharmaceuticals, and Medimmune. M. J. S. is an advisory board member for Arcus Biosciences, Astrazeneca, Kymab, and F-star. C.G. declares no conflict of interest.

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Biology of Myeloid-Derived Suppressor Cells

10

Kevin Alicea-Torres and Dmitry I. Gabrilovich

Contents

10.1 Introduction

Tumor progression is associated with the altered myelopoiesis. Myeloid-derived suppressor cells (MDSCs) are now established as a critical factor that modulates immune responses in cancer. MDSCs represent an expanded heterogeneous group of immature myeloid cells that are associated with poor prognosis and survival of cancer patients [1, 2]. MDSCs are characterized by potent immune-suppressive activity, which contributes to tumor growth and progression. In addition, MDSCs are also implicated in the promotion of angiogenesis and metastasis [3, 4]. It is clear that MDSCs have many roles in cancer; however, immunosuppression of T cell responses within the tumor microenvironment is their hallmark and the focus of many studies [5]. Therefore, by understanding the biology of MDSCs, therapeutic strategies can be developed in hopes of targeting these cells in cancer patients.

10.2 Characterization of MDSCs in Mouse and Humans

MDSCs were initially characterized and described in tumor-bearing mice as heterogeneous cells of myeloid origin that express both myeloid lineage differentiation markers, Gr-1 (glutathione reductase) and CD11b (αM-integrin) markers [6] (Fig. 10.1). MDSC subsets have been identified based on the intensity of GR1 expression (GR1^{low},

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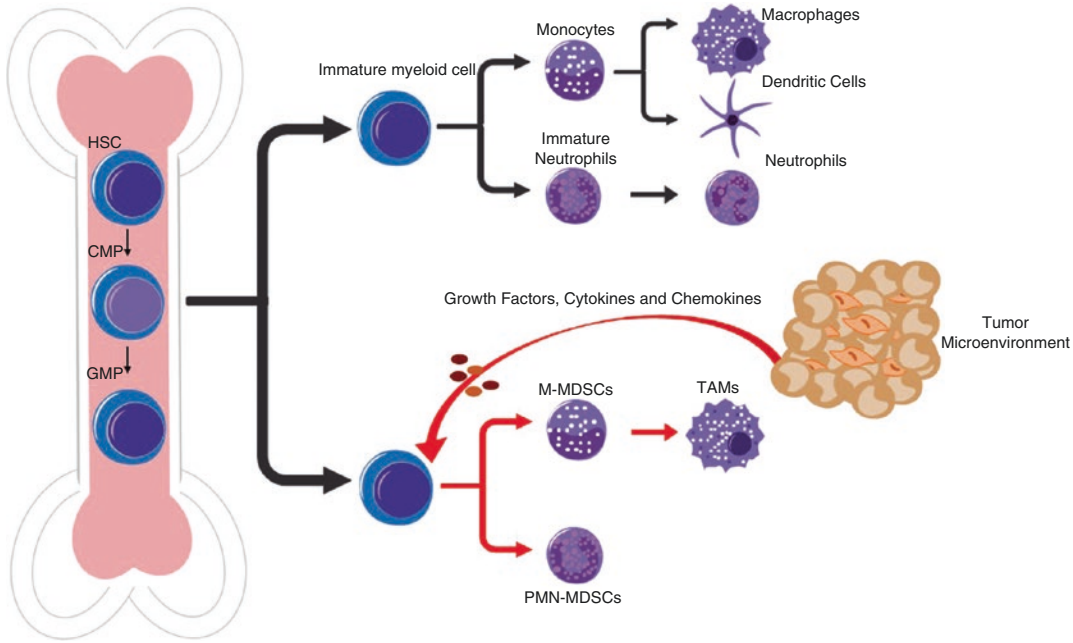

Fig. 10.1 Origin of MDSCs. In the bone marrow, hematopoietic stem cells (HSC) differentiate into common myeloid progenitor (CMP) followed by granulocyte-macrophage progenitor (GMP), which give rise to immature myeloid cells mainly comprised of precursors of neutrophils and monocytes. In normal condition, immature myeloid cells differentiate into macrophages, dendritic cells, and neutrophils (*black arrows*). However, during cancer progression the tumor microenvironment produces growth factors, cytokines, and chemokines that altered the myelopoiesis in the bone marrow. These tumor-derived factors block the differentiation of immature myeloid cell to fully mature cells creating an accumulation of pathological activated immature monocytes and neutrophils known as M-MDSCs and PMN-MDSCs, respectively (*red arrows*). Besides the expansion of MDSCs during tumor progressions, these cells acquire potent immunosuppressive activity. Within the tumor microenvironment, M-MDSCs differentiate to TAMs with similar ability to suppress antitumor immune responses

GR1^{int}, and GR1^{bright}) with other phenotypic markers, such as F4/F80 (marker for macrophages) or MHCII (major histocompatibility complex class II) [7]. However, GR1 consists of two epitopes, Ly6C and Ly6G, which can be detected by different antibodies. This led to the identification of two different MDSC subsets: granulocytic or polymorphonuclear (G- or PMN-MDSCs, CD11b^{+}Ly6G^{+}Ly6C^{low}) and monocytic (M-MDSC, CD11b^{+}Ly6G^{-}Ly6C^{high}) cells; these cells are morphologically and phenotypically similar to neutrophils and monocytes, respectively [8] (Fig. 10.1). In the majority of cancer types, PMN-MDSCs are the most abundant population representing more than 70% of all MDSCs, whereas M-MDSCs represent the majority of the remaining cells [9]. In addition, within these MDSC subsets, there is a small population representing less than 5% that consists of a mixture of progenitors and precursors that are currently under investigation. Murine MDSCs are generated in the bone marrow upon exposure to tumor-derived factors and predominantly accumulate in the peripheral blood, spleen, liver, lungs, and tumor.

In humans, MDSCs have been identified and studied primarily in the peripheral blood mononuclear cell (PBMC) fraction. However, humans do not have Gr-1 antigens, which make direct comparison between mice and human MDSCs difficult. PMN-MDSCs are defined as CD11b^{+}CD14^{-}CD15^{+} or CD11b^{+}CD14^{-}CD66b^{+}, whereas M-MDSCs are defined as CD11b^{+}CD14^{+}HLA-DR$^{-/lo}$CD15^{-} [10]. There is a small population that includes a mixed group of MDSCs characterized as Lin^{-} (including CD3, CD14, CD15, CD19, and CD56) HLA-DR^{-}CD33^{+} cells. This population that comprised a more immature phenotype has been defined as "early-stage MDSCs (e-MDSCs). At the moment,

the minimum requirement to define and characterize human MDSCs, including PMN-MDSCs, M-MDSCs, and e-MDSCs, should follow the phenotypic criteria [10]. Using these criteria, M-MDSCs are separated from monocytes based on the expression of HLA-DR (MHC class II molecule). On the other hand, the only method to separate PMN-MDSCs from normal neutrophils is by gradient centrifugation using a standard Ficoll gradient. PMN-MDSCs are found enriched in the low-density fraction (PBMCs), whereas neutrophils are found within the high-density fraction [11, 12]. Recently, lectin-type oxidized LDL receptor 1 (LOX-1) has been identified as a potential marker of PMN-MDSCs in humans [12]. LOX-1 expression on neutrophils could be used for direct identification of PMN-MDSCs found in blood and tumor tissue. Furthermore, LOX-1$^+$ neutrophils isolated from the blood of cancer patients were shown to suppress T cell proliferation, whereas LOX-1$^-$ neutrophils were not suppressive.

10.3 Mechanism of MDSC-Mediated Immune Suppression

To fully identify myeloid cells as MDSCs, their functional activity needs to be tested; this is achieved usually by an in vitro suppression assay, which measures their ability to suppress the function of immune cells in tumor-bearing hosts. Activated MDSCs are implicated in the direct suppression of NK and B cells along with their primary target, T cells [13–15]. Immune suppression by MDSCs involves several mechanisms such as the increase of arginase 1 (Arg1) and inducible nitric oxide synthase (*i*NOS) production, the increased production of reactive oxygen and nitrogen species (ROS and peroxynitrite, PNT), and other immune-suppressive factors. Upregulation of Arg1 in MDSCs leads to the depletion and conversion of L-arginine, an essential amino acid needed for T cell proliferation, to urea and L-ornithine [16]. Under limiting amounts of L-arginine, upregulation of *i*NOS in MDSCs leads to the production of nitric oxide (NO) which reacts with superoxide and generates PNT [17]. Production of PNT by MDSCs causes the nitration and nitrosylation of the T cell receptor (TCR), thus disrupting potential CD8$^+$ T cell-antigen interactions leading to T cell tolerance [18]. Also, PNT reduces the binding of antigenic peptides to MHC molecules on tumor cells and blocks T cell migration by nitrating T cell-specific chemokines, such as CCL2 [19, 20]. Another mechanism for suppression of T cell responses is the production of ROS by MDSCs. It has been shown that tumor-derived factors generate MDSCs that produced high levels of ROS which contribute to the suppressive activity of these cells [21]. Although these are the major mechanisms of immunosuppression by MDSCs, there are several other factors involved including transforming growth factor-β (TGF-β), interleukin-10 (IL-10), cyclooxygenase-2 (COX-2), indoleamine 2,3-dioxygenase (IDO), and many others. The main immunosuppressive cytokines produced by MDSCs are TGF-β and IL-10. Although TGF-β has shown to be produced by tumor cells, the production of this cytokine by MDSCs inhibits cytotoxic T cell responses in tumor-bearing mice [22]. MDSCs produced high amounts of IL-10 which impairs antitumor responses by inhibiting T cell activation [23]. In addition, MDSCs expressed several enzymes that are involved in the depletion of essential nutrition factors for T cell function. Upon stimulation by pro-inflammatory molecules, COX-2 is activated and induces Arg1 activity in MDSCs [16]. Other studies had implicated IDO expression in MDSCs which inhibit T cell function by depleting L-tryptophan and inducing T cell apoptosis within the tumor microenvironment [24]. MDSCs also block T cell function by depleting cysteine and impairing T cell activation in tumor [25]. In addition, MDSCs have the ability to induce the expansion of regulatory T cells (Treg) [26, 27]. Most of the mechanisms of suppression found to be implicated in MDSC function do not act simultaneously and are dependent on type of MDSC, type of tumors, and location of the cells.

Several recent studies have provided evidence that the ratio of PMN-MDSCs to M-MDSCs is really important because these cells utilize different mechanisms to suppress T cell responses [28]. For instance, M-MDSCs have the ability to suppress T cell activation in an antigen-specific and nonspecific manner. This suppression of T cell responses by M-MDSCs is associated with the

increased expression of *i*NOS and production of NO [28, 29]. On the other hand, PMN-MDSCs are capable of suppressing immune responses primarily in an antigen-specific manner, which induces $CD8^+$ T cell tolerance in the host. Immune suppression by PMN-MDSCs is associated with the increased expression of Arg1 along with high levels of ROS and PNT [8, 18]. Although PMN-MDSCs are more abundant than M-MDSCs in a tumor-bearing host, they are less immunosuppressive than M-MDSCs when compared on a per cell basis [30]. Location was also determined to be important in dictating the strength of suppression by MDSCs. Recent years have provided ample evidence indicating that MDSCs in the tumor microenvironment are more suppressive than MDSCs in peripheral lymphoid organs and peripheral blood [31, 32]. There is clear evidence now suggesting that the increased suppressive activity of MDSCs is regulated by the low levels of oxygen (hypoxia) found in tumor tissues [33]. However, more studies are needed to better understand what other tumor-associated factors and mechanisms are implicated in the potent suppressive activity by MDSCs within the tumor microenvironment (Fig. 10.2).

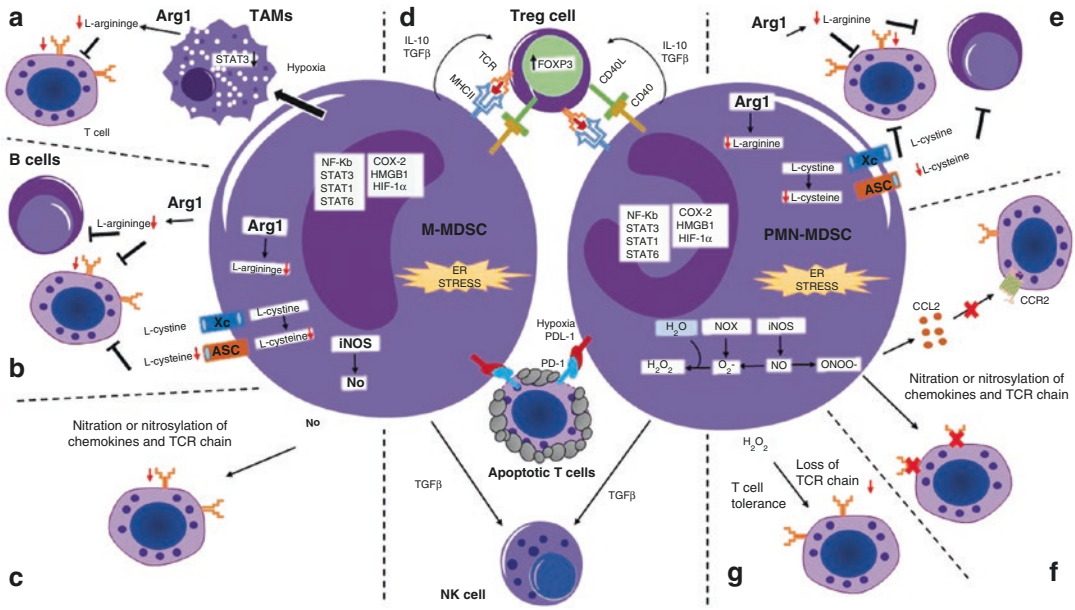

Fig. 10.2 Mechanisms of immunosuppression by MDSCs in cancer. In cancer, both M-MDSCs and PMN-MDSCs can suppress antitumor T cell responses through different mechanisms. During tumor progression, these mechanisms are regulated by several transcription factors and enzymes and by the activation of the ER stress response. (**a**) In hypoxia conditions, upregulation of HIF-1α induces the differentiation of M-MDSCs into TAMs in a STAT3-dependent manner. In the tumor microenvironment, TAMs suppress T cell responses by sequestering arginine through activation of Arg1. (**b**) M-MDSCs deprive T and B cells of essential amino acids including arginine and cysteine. (**c**) The main mechanism of immunosuppression by M-MDSCs is the expression of *i*NOS and release of NO. The release of NO causes the nitration or nitrosylation of T cell receptors as wells as chemokines that attract T cells. (**d**) Both M-MDSCs and PMN-MDSCs release IL-10 and TGFβ which induces the development and expansion of antigen-specific Tregs that may require CD40-CD40L interactions. Furthermore, the release of TGFβ is implicated in the suppression of NK cells by MDSCs. Within the tumor microenvironment, MDSC subsets upregulate PDL-1 expression which interact with PD-1 on T cells resulting in cell death. (**e**) Similarly to M-MDSCs, PMN-MDSCs suppress T cell responses by depleting arginine and cysteine from the tumor microenvironment. (**f, g**) PMN-MDSCs are characterized by their release of reactive nitrogen or oxygen species. (**f**) The production of PNT by PMN-MDSCs causes the nitration or nitrosylation of the TCR chain inhibiting T cell proliferation. Another important function of peroxynitrite release by MDSCs is the modification of CCL2, chemoattractant, which affects T cell migration. (**g**) Also, activation of NOX is responsible for the increased production of reactive oxygen species by tumor-associated PMN-MDSCs

10.4 Mechanisms Regulating MDSC Accumulation and Function

One of the main questions in the MDSC field is how is their expansion, accumulation, and activation regulated? Several years ago we proposed a two-signal model describing that MDSC accumulation requires two distinct types of signals [34]. This model is divided into two phases: (1) the expansion phase associated with the inhibition of their terminal differentiation and (2) the activation phase that is responsible in the conversion of immature myeloid cells into immunosuppressive MDSCs. However, we assert that these two phases partially overlap but are governed by different sets of transcription factors and intermediates (Fig. 10.3).

The first phase is mostly driven by tumor-derived growth factors along with STAT3, IRF8, C/EBPβ, RB1, notch, adenosine receptor A2b, and NLRP3.

STAT3 Activation of signal transducer and transcription activator 3 (STAT3) in MDSCs requires myeloid-specific growth factors such as GM-CSF, G-CSF, M-CSD, IL-6, VEGF, and several other factors [35]. STAT3 plays a major role in the regulation of MDSCs. For example, tumor-bearing mice treated with different STAT3 inhibitors have shown a decrease in MDSC accumulation [36]. Interestingly, STAT3 is not only involved in the expansion of MDSCs but also in their function and differentiation; for instance, M-MDSCs are able to differentiate into tumor-associated macrophages (TAMs) within the tumor microenvironment through STAT3 regulation [37, 38].

IRF8 IFN regulatory factor 8 (IRF8) has been described as a negative regulator of MDSC differentiation. In the absence of IRF8, there is an increase in MDSC accumulation in both spleen and tumor tissues; however, overexpression of IRF8

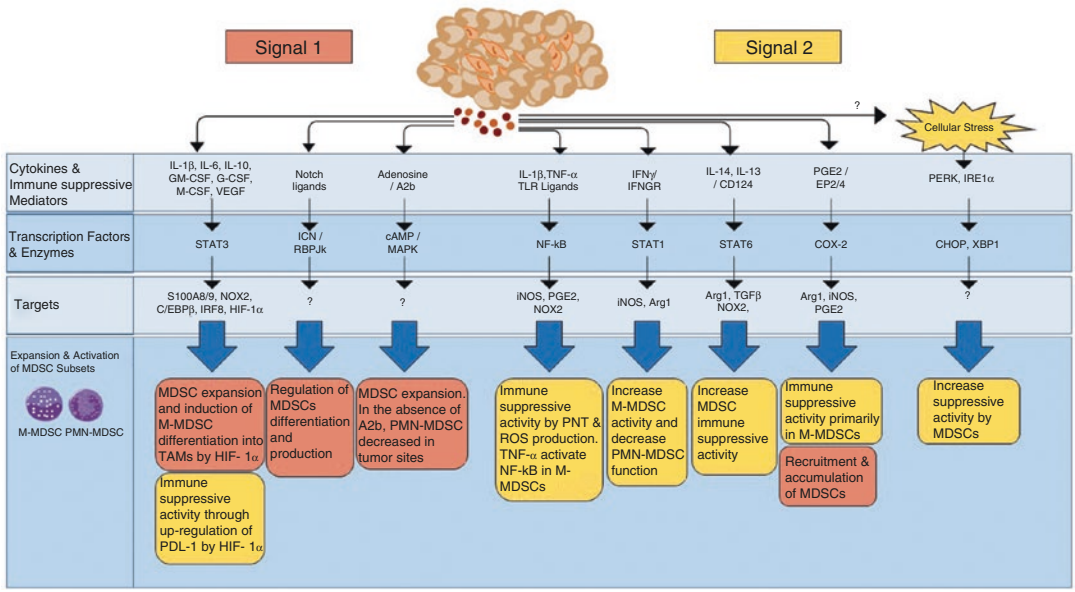

Fig. 10.3 Tumor-associated factors and molecular pathways regulating MDSC expansion and function in cancer. The tumor microenvironment produced a large number of cytokines and immune-suppressive mediators that regulate MDSC expansion and immune-suppressive activity, signals 1 and 2, respectively. Exposure of MDSCs to tumor-secreted growth factors (signal 1) and other mediators regulates several signaling pathways involved, especially STAT3, in M-MDSC or PMN-MDSC expansion and accumulation. However, for the acquisition of immune-suppressive activity by immature myeloid cells, signal 2 is required giving rise to both M-MDSCs and PMN-MDSCs. These signaling pathways are important for both PMN-MDSCs and M-MDSCs (NF-κB, STAT3, STAT6, ER stress response) or may have opposite roles in MDSC subsets (STAT1). COX-2 has been described to be more specific to M-MDSCs

resulted in a decreased accumulation of MDSCs in a spontaneous mouse tumor model [39, 40].

C/EBPβ CAAT-enhancer-binding protein beta (C/EBPβ) is the only member of its family that has been implicated in MDSC expansion. Mice lacking C/EBPβ specifically in hematopoietic cells had lower frequencies of MDSCs—especially M-MDSCs—suggesting its role in MDSC differentiation [41].

Rb1 The retinoblastoma protein 1 (Rb1) has been implicated in MDSCs found in both mice and humans [42]. In tumor-bearing mice, Rb1 was shown to regulate the differentiation of M-MDSCs to PMN-MDSCs. M-MDSCs with high levels of Rb1 mainly give rise to macrophages and DCs, whereas the vast majority of M-MDSCs with low levels of Rb1 differentiate toward PMN-MDSCs. Recently, the accumulation of $Rb1^{lo}$ $Ly6G^{+}$ PMN-MDSC was confirmed in a transgenic model of breast cancer in mice [43].

Notch Pathway Another pathway involved in the accumulation and differentiation of MDSCs is notch signaling pathway. It has been shown that notch is downregulated upon activation of casein kinase 2 (CKII) in MDSCs. Therefore, tumor-bearing mice treated with CKII inhibitor showed an improvement in notch signaling and DC differentiation [44] These results suggests that the downregulation of notch signaling skewed the differentiation of the hematopoietic progenitors toward MDSCs instead of DCs. Recently, it has been shown that the inhibition of notch signaling enhances the generation of PMN-MDSCs but decreases the production of M-MDSCs [45].

Adenosine Receptor A2B In the tumor microenvironment, there is an increased level of extracellular adenosine that is associated with MDSC accumulation via engagement of the adenosine receptor A2b expressed on MDSCs [46]. Tumor-bearing mice lacking A2b had lower number of MDSCs compared to wild type, suggesting that the A2b receptor is critically involved in the expansion of MDSCs, especially PMN-MDSCs [47]. This correlates with the most recent study using a melanoma model treated with an agonist and an antagonist of A2b, in which treatment with the agonist increased both tumor growth and MDSC accumulation whereas treatment with the antagonist resulted in a decreased of tumor progression with a reduction of MDSC numbers within tumor tissues [48].

NLRP3 NLR family, pyrin domain containing 3 (NLRP3) is an intracellular sensor associated with the inflammasome, which, upon activation, induces the generation of interleukin (IL)-1β and IL-18. Tumor-associated MDSCs express NLRP3, and $Nlrp3^{-/-}$ mice had lower levels of MDSCs within tumor tissues compared to wild-type mice, suggesting that NLRP3 may play a role in the expansion and accumulation of MDSCs [49]. However, more studies are needed to determine the exact role of NLRP3 in MDSC function. Clearly, these signaling pathways regulate MDSC expansion and are involved in blocking normal immature myeloid cell differentiation. However, the majority of these factors are implicated in the first phase of MDSC regulation but are not sufficient to promote their activation into immunosuppressive cells (Fig. 10.3).

As part of our two-signal model, the acquisition of the suppressive activity found in MDSCs is mediated by factors mostly produced by the tumor stroma including pro-inflammatory molecules such as IFNγ, IL-1β, IL-13, toll-like receptor (TLR) ligands, and others. Each of these molecules is involved in the activation of several signaling pathways associated with many factors such as NF-κB, STAT1, STAT6, prostaglandin E_2 (PGE_2) and cyclooxygenase (COX-2), high-mobility group box 1 (HMGB1), and hypoxia-inducible factor-1α (HIF-1α), all of which are implicated in the suppressive activity of MDSCs. Recently, the endoplasmic reticulum (ER) stress pathway has been linked to the suppressive activity of MDSCs found in cancer.

NF-κB In MDSCs, the activation of the NF-κB pathway is predominately mediated by the TLR ligands: IL-1β or TNF-α—both of which have been shown to increase suppressive activity [34].

The role of TLR ligands in MDSC function is still not clear. Some studies have shown that TLR ligands drive the suppressive activity of MDSCs, whereas others suggest that these ligands inhibit their function [50–52]. During inflammation and cancer, IL-1β produced by tumors cells leads to the activation of MDSCs through the NF-κB pathway resulting in the increased production of PNT [14, 19, 53, 54]. Another well-known activator of the NF-κB pathway is tumor necrosis factor-α (TNF-α), which is implicated in the maturation and function of MDSCs [55]. It was shown that the transmembrane (tm) form of TNF-α could increase the suppressive activity of MDSCs via its receptor and regulate *i*NOS expression in a NF-κB-dependent manner [56]. One study using an inflammatory mouse model showed that the role of TNF-α is restricted to M-MDSCs [57]. Also, activation of NF-κB through TNF-α receptor could promote survival of MDSCs [58]. At this moment, NF-κB can be involved in MDSC expansion, but its main role is to signal activation of these cells leading to acquisition of a suppressive phenotype, especially in M-MDSCs.

STAT1 Several studies had shown that activation of STAT1 is linked to the suppressive activity of MDSCs [17, 29, 59]. STAT1 is activated upon IFNγ stimulation and is involved in the upregulation of *i*NOS and Arg1 expression. Sinha et al. demonstrated that activation of STAT1 through IFNγ did not regulate MDSC accumulation or function [60]. Subsequent studies focused on the role of the IFNγ-STAT1 pathway in the suppressive activity of MDSC subsets. A recent study showed that STAT1 plays a major role by increasing the suppressive function of M-MDSCs [61]. However, activation of STAT1 through IFNγ receptors was shown to decrease PMN-MDSC function and survival [62]. These studies suggest that the IFNγ-STAT1 signaling pathway may have opposite roles in M-MDSCs and PMN-MDSCs. Indeed, this could explain why the initial study did not observe the involvement of IFNγ and/or STAT1 in the suppressive activity of MDSCs since the study of Sinha et al. analyzed the function of total MDSCs ($CD11b^{+}Gr\text{-}1^{+}$) rather than the subsets separately. It is possible that high PMN-MDSC numbers, and not M-MDSCs, compensate with the suppressive activity observed in the absence of the STAT1 signaling pathway.

STAT6 Another transcription factor involved in the function of MDSCs is STAT6. In MDSCS, the STAT6 signaling pathway is activated upon stimulation with IL-4 and IL-13 through its receptor (CD124, IL-4Rα) leading to the upregulation of Arg1 expression and production of TGF-β, a potent inhibitor of T cell proliferation [22, 63, 64]. In addition, STAT6 has been shown to be involved in the survival and accumulation of MDSCs [65].

PGE2 Prostaglandins, especially PGE2, play a major role in the suppressive activity of MDSCs in cancer. Rodriguez and colleagues found that the signaling through the PGE2 receptor E-prostanoid (EP) 4, which is expressed in MDSCs, induces Arg1 expression and its activity [16]. Furthermore, expression of the main regulator of PGE2 production, COX-2, was directly correlated with the induction of Arg1 and *i*NOS in tumor-infiltrating MDSCs [66]. These results were confirmed by Obermajer et al. in which they described a positive feedback loop between PGE2 and COX-2 that led to the conversion of monocytes into M-MDSCs [67]. Based on this, ex vivo MDSCs were generated using PGE2 which induce the production of suppressive factors [68]. In mouse models of mesothelioma and glioma, treatment with a COX-2 inhibitor blocked the accumulation and function of MDSCs [69, 70]. Moreover, the involvement of PGE2 in MDSC function was confirmed in melanoma patients that were treated with a COX-2 inhibitor and had MDSC-like cells with less suppressive activity [71]. Primarily, PGE2 has shown a role in the regulation of immune suppression by MDSCs, but has been implicated also in their recruitment and accumulation [72].

HGMB1 HGMB1 is a DNA-binding protein that present at high levels within the tumor microenvironment [73]. HGMB1 affects MDSC via

binding to TLR4 and receptor for advanced glycation endproducts (RAGE). Signaling through both receptors activates the NF-κB pathway. Parker et al. demonstrated that HGMB1 drives the accumulation and suppressive activity of MDSCs and also promotes the survival of MDSCs by inducing autophagy in cells within the tumor microenvironment [74]. Recently, Su et al. demonstrated that in vivo HGMB1 blockade with a monoclonal antibody against HGMB1 B box decreased the accumulation of M-MDSCs in both spleen and tumor tissues [75]. These studies demonstrate that HGMB1 may play a key role in the accumulation and function of MDSCs.

HIF1α The absence of oxygen (hypoxia) is one of the biggest differences between the tumor microenvironment and peripheral lymphoid organs. It has been shown that within the tumor microenvironment and in response to hypoxia, HIF-1α can regulate MDSC function and the differentiation of M-MDSCs to TAMs in a STAT3-dependent manner [37, 38]. More recently, Norman et al. demonstrated that HIF-1α selectively upregulate programmed death-ligand 1 (PD-L1), an immune checkpoint ligand on MDSCs [76] (Fig. 10.3).

Recent studies have suggested that ER stress responses are involved in the suppressive behavior of MDSCs in tumor-bearing hosts. The ER stress response pathway, also known as the unfolded protein response (UPR), is responsible for preserving ER homeostasis during extrinsic or intrinsic stress [77, 78]. ER stress response is comprised of three ER-localized transmembrane protein sensors: inositol-requiring enzyme 1α (IRE1α), activating transcription factor 6α (ATF6α), and double-stranded RNA-dependent kinase (PKR)-like ER-related kinase (PERK). Activation of these ER sensors leads to the upregulation of several transcription factors including the spliced X-box protein 1 (sXBP1), CCAAT-enhancer-binding protein homologous protein (CHOP), and others. Both MDSCs isolated from tumor-bearing mice and cancer patients overexpress several markers of ER stress, including sXBP1 and CHOP, and display an enlarged ER—a hallmark of ER stress [79]. Tumor-bearing mice treated with thapsigargin, an ER stress inducer, have an increased number of MDSCs with potent suppressive activity [80]. We showed that induction of ER stress with thapsigargin converts normal human neutrophils to a suppressive PMN-MDSC phenotype in an IRE1α-/XBP1-dependent manner [12]. Besides the role of IRE1α/XBP1 in MDSC function, recent findings have implicated CHOP in the suppressive activity of tumor-associated MDSCs. For example, CHOP-deficient MDSCs lose their suppressive function and acquire an immune stimulatory phenotype [81, 82]. Although CHOP-deficient MDSCs did not have the ability to suppress T cells that were stimulated in an antigen-nonspecific manner, they retained their ability to block antigen-specific T cells [83]. At this moment, only the IRE1α/XBP1 and PERK/CHOP signaling pathways have been implicated in the function of MDSCs. However, more studies are needed to understand the specific mechanisms of ER stress responses that regulate the suppressive activity of MDSCs.

10.5 Relationship of MDSCs with Other Myeloid cells

Over the years, our understanding of myeloid cells in cancer has shed light to their important role in tumor development, progression, and metastasis. The main three groups of terminally differentiated myeloid cells are macrophages, DCs, and PMNs. It is now clear that the tumor microenvironment alters myeloid cell differentiation by arresting these cells in an immature stage with potent immunosuppressive activity. Another myeloid cell involved in immune suppression is the tumor-associated macrophage (TAM) that arises from a tumor-infiltrating monocyte or M-MDSC within the tumor microenvironment. However, the relationship of MDSCs with other myeloid cells is still not clear. The main concern is the lack of specific markers for MDSCs making it difficult to discreetly identify these cells separate from monocytes and neutrophils. This is why in many previous studies, MDSC-like cells with suppressive activity were called monocytes

and neutrophils. Recent data has provided evidence about the specific nature of MDSCs in cancer [1]. Immunosuppressive activity is an intrinsic feature of MDSCs. For instance, MDSCs could be generated in vitro from bone marrow progenitor cells in the presence of tumor-secreted factors. Unlike MDSCs, mature neutrophils or monocytes in the presence of these tumor-associated factors cannot suppress T cell responses in vitro [2]. Several studies, involving genomic, proteomic, and transcriptomic analysis, had provided evidence that supports the differences between PMN-MDSCs and neutrophils in tumor-bearing mice [84–88]. Moreover, a recent study provided information that suggests that PMN-MDSCs are different to neutrophils from healthy donors or cancer patients based on their gene profile [12]. In humans, PMN-MDSCs can be distinguished from neutrophils by LOX-1 expression. In mice the role of LOX-1 is not clear [3]. Within the hypoxic tumor microenvironment, M-MDSCs differentiate into TAMs, and this is regulated by HIF-1α and STAT3 activation [9, 33, 37, 38]. Both myeloid cells have potent immune-suppressive function, especially within the tumor sites; however, M-MDSCs can be distinguished from TAMs by changes in monocyte-/macrophage-associated markers. The main changes observed during M-MDSC differentiation into TAMs include an increased in F4/F80 and CD115, intermediate expression of Ly6C, and low expression of IRF8 and S100A9 protein [4, 89, 90]. MDSCs possess several biochemical features that are not observed in neutrophils or monocytes including high Arg1 and *i*NOS expression and activity as well as high levels of ROS and PNT production. In addition, recent studies have shown that MDSCs have an increase in the ER stress response that is linked to their suppressive function.

Among the different types of myeloid cells in cancer, there is a better understanding of the relationship between M-MDSCs and TAMs. Recent data from several groups have shown that M-MDSCs are TAM precursors within the tumor microenvironment. In addition, there are two polarized phenotypes of macrophages: the classically activated (M1 macrophages) with antitumor properties and the alternatively activated (M2 macrophages) with pro-tumor properties. The latter is more related to TAMs, but it is unclear if these two phenotypes are that of the same macrophage and what the molecular mechanism is that regulates them within the tumor microenvironment [91]. In addition, it is unknown if the polarization of macrophages is dictated by M-MDSCs before TAM differentiation. On the other hand, the nature of PMN-MDSCs and their relationship with neutrophils is a subject of discussion in different studies and an ongoing debate. Although PMN-MDSCs are widely accepted in the field, the concept of neutrophil polarization in cancer raises questions of whether these cells are pathologically similar to PMN-MDSCs [92]. Similarly to macrophages, the concept of tumor-associated neutrophils (TANs) consists of neutrophils with antitumor (N1 cells) or pro-tumor (N2 cells) properties within the tumor microenvironment [93]. Studies have implicated TGF-β and type 1 interferons (IFNs) as cytokines in the regulation of TAN plasticity in cancer [93–95]. Neutrophils by definition are short-lived and terminally differentiated cells, whereas PMN-MDSCs are immature cells. For this reason, it is hard to imagine that TANs could be polarized in the tumor microenvironment. Indeed, several studies have shown that these cells have the ability to suppress T cell responses in tumor-bearing mice, which supports these cells as PMN-MDSCs [96, 97]. However, the antitumor role of TANs has been described in cancer patients. Recently, Eruslanov et al. observed that TANs from early-stage lung cancer patients were not immunosuppressive, but rather stimulate T cell responses [98]. This study provides evidence for the role of TANs during early stages of tumor initiation. Only few studies have addressed the role of MDSCs in tumor initiation and how it regulates the immune responses at early stages. For instance, Ortiz et al. showed that the exposure of mice to cigarette smoke resulted in an accumulation of non-suppressive MDSC-like cells in various organs [99]. However, when cigarette smoke was combined with a carcinogen to promote the development of lung cancer, MDSCs presented potent immunosuppressive activity. These data suggest that MDSC function

is controlled by the tumor microenvironment. Our understanding of the nature of MDSCs and their relationship with other myeloid cells during tumor development would open new therapeutic opportunities.

10.6 Therapeutic Strategies to Target MDSCs in Cancer

There is ample evidence that MDSC accumulation in peripheral lymphoid organs and tumor tissues correlates with a poor prognosis and clinical outcome in cancer patients [100]. In addition, MDSCs are implicated in resistance to anticancer therapies including the receptor tyrosine kinase inhibitor sunitinib as well as several chemotherapeutic drugs for lung cancer and multiple myeloma [101–103]. Since these cells have the ability to suppress antitumor immune responses, the success of cancer therapies, including immunotherapy, would depend on the immunosuppressive effect by MDSCs. Indeed, several clinical studies had shown an association with MDSC levels and response to immune checkpoint inhibitors, ipilimumab (anti-CTLA-4) and nivolumab (anti-PD-1) [104–107]. Today, the increased knowledge of the molecular mechanisms responsible for accumulation and function of MDSCs in cancer has allowed for the development of therapeutic strategies targeting this cell population. Some strategies for targeting MDSCs include (a) elimination of MDSCs, (b) blocking the accumulation of MDSCs, and (c) inactivation of MDSCs [108] (Fig. 10.4). Several of these strategies have been developed and are currently being tested in the clinic.

Elimination of MDSCs has shown to enhance antitumor efficacy of cancer immunotherapy including adoptive T cell transfer [109]. Conventional anticancer agents, in addition to their direct effect on cancer cells, have demon-

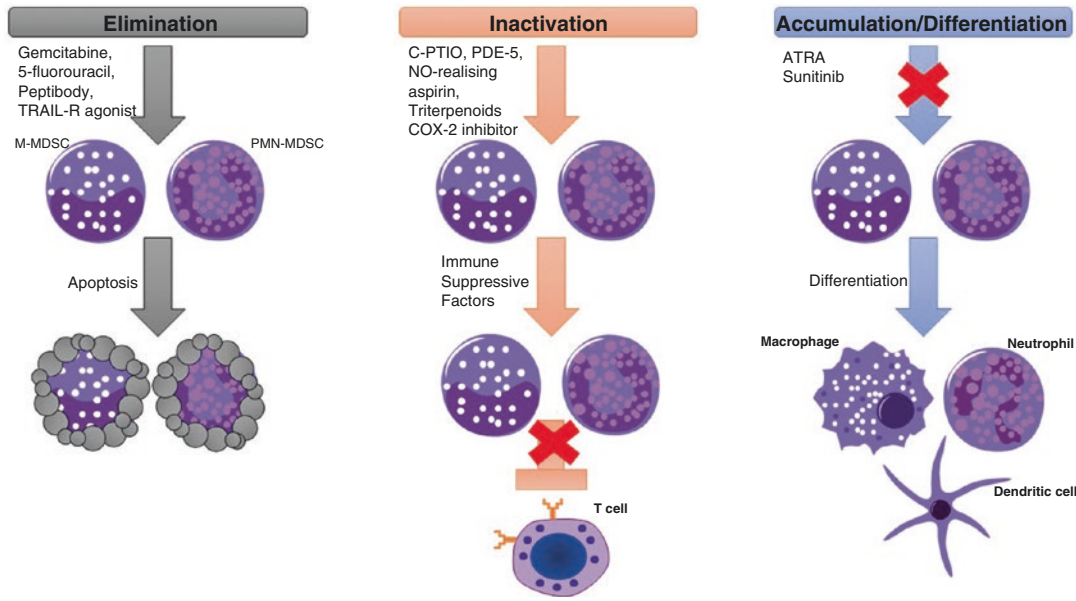

Fig. 10.4 Therapeutic strategies to target MDSCs. The main strategies to target MDSCs involved the elimination, inactivation, or blocking the accumulation and inducing the differentiation of MDSCs. First, preclinical and clinical studies have shown that MDSCs can be eliminated with low doses of chemotherapeutic drugs and with TRAIL agonist resulting in MDSC cell death. Second, in vivo inactivation of MDSCs by targeting their immunosuppressive machinery has been demonstrated in several studies. PDE5, NO-realizing aspirin, triterpenoids, and COX-2 inhibitors had shown to inhibit MDSC function by reducing ROS, RNS, and arginase levels. Finally, MDSC accumulation can be inhibited by inducing their differentiation into terminally differentiated cells with immunostimulatory activity

strated the depletion of MDSCs in tumor-bearing hosts. Gemcitabine was the first chemotherapy agent reported to be capable of eliminating MDSCs in tumor models [110]. This study demonstrated that elimination of MDSCs by gemcitabine improves antitumor responses and enhances the effects of immune therapy resulting in tumor regression. Other chemotherapeutic drugs showing elimination of MDSCs include 5-fluorouracil, cisplatin, doxorubicin, and others [111–113]. Another strategy to eliminate MDSCs is by targeting TNF-related apoptosis-induced ligand-receptors (TRAIL-R) [79]. It was shown that upregulation of TRAIL-R, especially DR5, regulates MDSC survival in tumor-bearing host, and by using a TRAIL-R agonist, MDSCs were selectively eliminated leading to a decrease in tumor growth in a $CD8^+$ T cell-dependent manner. Results from a phase 1 trial showed promising data that supports the use of TRAIL-R agonist in cancer patients [114]. Recently, a novel therapeutic approach to target MDSCs was developed by genetically fusing S100A9-derived peptides with the antibody Fc portion to generate a peptibody [115]. These peptibodies successfully depleted MDSCs from blood, spleen, and tumors of mouse models. However, future studies are needed to further elucidate the mechanisms of action that leads to MDSC elimination (Fig. 10.4).

MDSCs use several mechanisms to suppress antitumor immune responses, and targeting the suppressive machinery of MDSCs has been tested in cancer patients. Increased production of ROS and NO by MDSCs plays a major role in the suppression of $CD8^+$ T cell responses. Inhibitors for the production of ROS using ROS scavengers N-acetylcysteine (NAC) and catalase are commonly used to test the function of mouse and human MDSCs ex vivo. NF erythroid 2-related factor 2 (NRF2) is a transcription factor involved in the activation of the antioxidant response and in protecting cells against damage caused by ROS. Upregulation of NRF2 by a synthetic triterpenoid was able to neutralize human MDSC activity by reducing production of ROS and dampening their suppressive function ex vivo [116]. However, a recent study from Beury et al. showed that $Nrf2^{-/-}$ MDSCs had a decrease in their suppressive activity compared to $Nrf2^{+/+}$ MDSCs in two mouse models [117]. Scavengers of NO, such as carboxy-PTIO (C-PTIO), have been tested recently for MDSCs. Treatment with C-PTIO decreased the function of MDSCs and improved the efficacy of adoptive T cell transfer in tumor-bearing mice [118]. Besides blocking ROS and NO, another approach to inactivate MDSC function is to inhibit the catabolic enzymes upregulated by MDSCs including Arg1 and *i*NOS which are implicated in the suppression of T cell activation and proliferation. [63]. Inhibitors for Arg1 and *i*NOS have been used extensively in functional assays for MDSCs. Early studies by Rodriguez et al. demonstrated that a specific inhibitor for Arg1, *N*-hydroxyl-nor-L-Arg (nor-NOHA), could inhibit tumor growth and decrease Arg1 levels in MDSCs [63, 119]. Arg1 inhibitor, as well as *i*NOS inhibitor L-NG-monomethyl-L-arginine (L-NMMA), had shown to reduce MDSC function and improve T cell proliferation in vitro. Moreover, both Arg1 and *i*NOS levels in PBMCs of cancer patients have been shown to decrease in response to phosphodiesterase-5 (PDE5). Several PDE5 inhibitors had been used in the clinic to treat cancer and other conditions showing a decrease in MDSC numbers [120, 121]. Recently, a clinical report indicated that head and neck cancer patients treated with the PDE5 inhibitor tadalafil had a reduction in MDSCs and the expression of both Arg1 and *i*NOS, associated with an increase of T cells [122]. A parallel study demonstrated that treatment with tadalafil decreased not only MDSCs but also Tregs in blood and tumors of patients with head and neck squamous carcinoma [123]. Also, these patients treated with PDE5 inhibitor had an increase in tumor-specific $CD8^+$ T cells. Nitroaspirin, a NO-releasing aspirin, has been shown to induce downregulation of both Arg1 and *i*NOS and PNT in MDSCs from a colon carcinoma model [124]. Since nitroaspirin was poorly effective as an adjuvant for adoptive T cell transfer, Molon et al. developed AT38 ([3-(aminocarbonyl)furoxan-4-yl]methyl salicylate) to inhibit reactive nitrogen species in MDSCs [20]. In this study, AT38 was shown to inhibit Arg1, *i*NOS, and PNT in MDSCs as well as inhibition

of CCL2 nitration leading to an increase in $CD8^+$ T cells within the tumor microenvironment. Another major mechanism of immunosuppression by MDSCs is the production of PGE2 through activation of COX-2 [125]. Several COX-2 inhibitors, including celecoxib, have demonstrated a reduction in PGE2 production and inhibition of Arg1 and *i*NOS in MDSCs from different mouse models [16, 17, 125]. Selective COX-2 inhibition by celecoxib reduces MDSC numbers and production of ROS and NO and improved a dendritic cell-based immunotherapy in a mesothelioma model [69] (Fig. 10.4).

In addition to eliminating MDSCs or targeting their immunosuppressive machinery, blocking expansion and inducing differentiation of MDSCs are another therapeutic strategy with potential. It is well established that tumor-secreted factors play a critical role in preventing the differentiation of MDSCs into dendritic cells or macrophages [126, 127]. The differentiation of MDSCs into mature myeloid cell was initially shown by in vitro and in vivo treatment with *all-trans* retinoic acid (ATRA), a natural oxidative metabolite of vitamin A [128, 129]. Cancer patients treated with ATRA had an improvement in their myeloid/lymphoid dendritic cell ratio and immune responses [130]. The mechanism involved in MDSC differentiation by ATRA involves the activation of ERK1/ERK2 MAPK kinase that upregulates the expression of glutathione synthase increasing the synthesis of glutathione [131]. Accumulation of glutathione in MDSCs by ATRA reduces the levels of ROS leading to MDSC differentiation into mature myeloid cells. Depletion of MDSCs by ATRA enhanced the effects of a cancer vaccine in patients with extensive stage small cell lung carcinoma [132]. Moreover, treatment with ATRA demonstrated a reduction of MDSC levels and function and improved chimeric antigen receptor (CAR) therapy responses in a sarcoma model [133].

Conclusions

The field of MDSC research has dramatically gained more attention over the past 10 years. MDSCs are critical for the regulation of immune responses in cancer and other pathological conditions. These cells may serve as a powerful biomarker for the selection of immunotherapies or anticancer therapies for cancer patients. In addition, eradicating MDSCs from cancer patients could improve the effects of recent and upcoming cancer immunotherapies.

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11 Effect of Pharmaceutical Compounds on Myeloid-Derived Suppressor Cells

Mélanie Bruchard and Francois Ghiringhelli

Contents

11.1 Introduction

Myeloid-derived suppressor cells (MDSCs) are a population of myeloid origin that exert immunosuppressive functions. MDSCs are distinct from terminally differentiated myeloid cells such as macrophages, dendritic cells (DCs), or neutrophils. MDSCs are hematopoietic cells generated in the bone marrow that can be divided into two subtypes. Monocytic MDSCs come from the macrophages/DC progenitor, while polymorphonuclear (PMN) MDSCs arise from the granulocytic arm of myeloid differentiation. Both subtypes can be found in humans and mice. In humans, M-MDSCs are characterized by their expression of CD14, CD11b, and CD33 and their lack of lineage marker as well as a low expression for HLA-DR. PMN-MDSCs express CD15, CD11b, and CD33 and are negative for lineage markers and HLA-DR. PMN-MDSC could be distinguished from PMN thanks to their difference in density. Although additional markers have been studied to further identify MDSCs, none of them is yet considered as a specific MDSC marker. However, recently, Dmitry Gabrilovich's group demonstrated that the LOX1 marker could differentiate PMN-MDSC from PMN in human [1]. In mice, different molecules are used to delineate MDSCs populations. CD11b and Gr-1 expression identify both subsets of MDSC. Gr-1 is a combination of two markers, Ly6C and Ly6G. Using these, MDSC populations can be separated more accurately,

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M-MDSCs being Ly6C+ and Ly6G, while PMN-MDSCs are found out to be Ly6G+ and Ly6C.

In healthy individuals, MDSCs are almost undetectable. However, under certain circumstances such as acute infection (sepsis choc), chronic infection (tuberculosis), or cancer, MDSCs accumulate. MDSC expansion involves multiple factors like GM-CSF, G-CSF, SCF, or S100A8 and S100A9 that can be secreted among others by tumor cells [2]. The uprising of MDSCs in cancer patients can be seen in the tumor bed and the secondary lymphoid organs but also in the bone marrow where accumulation of MDSCs has been observed in several studies [3, 4]. In both humans and mouse, PMN-MDSCs represent the majority of MDSCs in most type of cancer. MDSCs support tumor growth and metastasis in various ways. MDSCs help establish a microenvironment favorable to tumor growth, thanks to their production of proangiogenic mediators like VEGF, bFGF, Mmp9, or PDGF, critical molecules for the development of new vessels that are essential to maintain tumor growth. MDSCs also play an important role in the initiation of metastasis process. A study showed an increase in MDSCs in the lung of mice bearing mammary adenocarcinoma up to 2 weeks before the arrival of tumor cells [5], an increase in MDSCs that was dependent on the production of Mmp9. The proinflammatory proteins S100A8 and S100A9 whose organ expression is induced by the primary tumors can attract MDSCs to a premetastasized niche. As MDSCs accumulate in a new organ, their production of S100A8 and S100A9 can then amplify the mechanism and favor tumor cell migration and metastasis [6, 7]. Recent studies also revealed MDSC potential to enhance stemness of cancer cells, thus facilitating their epithelial-to-mesenchymal transition (EMT), supporting metastasis [8].

Aside from promoting tumor cell growth and metastasis, MDSCs also support the tumor thanks to their immunosuppressive properties. MDSCs exert immunosuppression through multiple mechanisms. MDSCs can produce IL-10 and TGF-beta [9] and induce regulatory T cells [10]; they also produce reactive oxygen species like O_2^- and H_2O_2 [11] and NO [12]. MDSCs lower TCR formation and induce T cell cycle arrest by depleting the milieu of L-arginine, thanks to their expression of Arg1 [13] or by reducing the levels of available tryptophan because of their indoleamine 2,3 dioxygenase (IDO) activity [14]. M-MDSCs and PMN-MDSCs do not possess exactly the same immunosuppressive mechanisms. M-MDSCs, which have a higher immunosuppressive activity than PMN-MDSCs, express Arg1 and produce IL-10, TGF-beta, and NO when PMN-MDSCs tend to produce more ROS that are short-lived molecules, explaining the lesser suppressive potential of PMN-MDSCs compared to M-MDSCs [15]. The ratio between PMN-MDSCs and M-MDSCs, in favor of PMN-MDSCs at the periphery in most cancers, has been shown to notably vary inside tumor bed depending on the type of cancer, especially in humans.

MDSCs are one of the major immunosuppressive components in tumor-bearing animals and patients. Consequently, their elimination or their differentiation into effective dendritic cells and macrophages is a major issue in immunooncology. Many data show that such a strategy can enhance antitumor immunity, allowing T cells to attack tumor cells and reduce the tumor burden. We will here focus on the various ways existing to reduce MDSCs, using chemotherapies and upcoming immunotherapies.

11.2 Impact of Cytotoxic Chemotherapies on MDSCs

Some chemotherapies have been shown to directly kill MDSCs. Gemcitabine is a chemotherapy consisting in a nucleoside analog of the cytidine that acts as an antimetabolite agent. Gemcitabine is used to treat various cancers like ovarian, pancreatic, lung, and breast cancer and cholangiocarcinoma. In 2005, Suzuki made the first demonstration that gemcitabine-based chemotherapy could specifically target MDSC [16]. Using a dose equivalent to classical dose used to treat human patients, it was shown that gemcitabine could, in the spleen of tumor-bearing mice, decrease in a selective manner the number

of MDSCs without impacting number and function of CD4 cells, CD8 cells, NK cell macrophages, or B cells. The antitumor activity of CD8+ T cells and NK cells after gemcitabine was increased, and less immunosuppression could be observed in the spleen of gemcitabine-treated mice [16]. However, no major impact of gemcitabine alone was observed on the tumor growth. These results were confirmed in 2009 by another team using a different tumor model. In addition to seeing a drop in the percentage of MDSCs in the spleen, they also observed a drop in MDSC both in the bone marrow and peripheral blood. The kinetic of treatment administration is of particular importance [17]. Only an early treatment with gemcitabine could delay tumor growth, suggesting that compensatory mechanisms limit the antitumor effect of MDSC depletion in established tumors.

Docetaxel and paclitaxel are the drugs of the taxane family used in clinic for cancer treatment. These two drugs both target tubulin, preventing the depolymerization of microtubules and thereby blocking mitosis. Docetaxel is a commonly used anticancer drug and was primarily developed for use against breast cancer in the 1990s. Now taxanes are used to treat various types of cancer including lung cancer, digestive cancer, and ovarian cancer. Docetaxel was demonstrated to have an effect on MDSCs. Mice bearing the mammary tumor model 4T1 and treated with docetaxel had significantly less MDSCs in their spleen and displayed an increased CTL response [18]. The decrease in MDSCs was partly due to the cytotoxic effect of docetaxel on PMN-MDSCs, while M-MDSCs differentiated toward an M1-like phenotype. M-MDSCs were later found to be resistant to docetaxel, thanks to their expression of secretory/cytoplasmic clusterin (sCLU) which expression prevented the induction of the apoptotic cascade by taxanes [19]. The analog of docetaxel, paclitaxel, has a cytotoxic activity weaker than its analog, but this drug is also largely used for the treatment of lung, breast, and ovarian cancers. Tumor-bearing mice, treated with paclitaxel at a low dose with non-cytotoxic effect, showed a decrease of MDSCs compared with non-treated mice. This decrease in MDSC was the consequence of their differentiation into DCs, and no MDSC cell death could be detected [20]. Using a model of spontaneous melanoma, the same team showed that low non-cytotoxic dose of paclitaxel could decrease the accumulation of MDSCs as well as their immunosuppressive activities (with less TNF-alpha and less S100A9 produced by the remaining MDSCs) [21].

Doxorubicin is a chemotherapy belonging to the anthracycline family of drugs. It interacts with DNA by intercalation and inhibits the progression of the topoisomerase II, thus blocking DNA replication. Doxorubicin is commonly used to treat sarcomas, breast cancers, leukemias, and non-Hodgkin's lymphomas. Doxorubicin has been shown to selectively deplete MDSCs in the spleen, blood, and tumor bed of 4T1 mammary cancer-bearing mice. The residual MDSCs showed impaired suppressive functions, with a lesser production of ROS, arginase-1, and IDO, while a higher proportion of CD4 and CD8 lymphocytes and NK cells were observed [22]. However, it was recently shown that doxorubicin could also induce the secretion of prostaglandin E2 (PGE2) by cancer cells like 4T1 cells. PGE2 stimulates MDSC expansion and accumulation reestablishing a subsequent MDSC population and immunosuppression in the tumor-bearing host [23]. Such data underline that anthracyclines may have a contrasting effect on MDSCs.

Trabectedin is a cytotoxic agent that binds to the minor groove of DNA inducing a perturbation of the cell cycle. Trabectedin caused selective depletion of monocytes/macrophages in blood, spleens, and tumors, with an associated reduction of angiogenesis in different experimental models. Trabectedin activates caspase-8-dependent apoptosis selectivity in monocytic myeloid cells and not neutrophilic ones because of a differential expression of signaling and decoy TRAIL receptors. Such data underline the possibility to use trabectedin to target tumor-infiltrated M-MDSCs [24].

Local irradiation could change the tumor microenvironment and remove immunosuppressive cells. In the CT26 and MC38 mouse colon carcinoma models, high-dose radiation transformed the

immunosuppressive tumor microenvironment resulting in an intense CD8(+) T cell tumor infiltrate and a loss of MDSC accumulation [25]. In a mechanistic point of view, $CD8^+$ T cell production of IFN-γ, induced by radiotherapy, controlled the survival and infiltration of MDSCs in the tumor and reversed the immunosuppressive environment. Furthermore, antitumor immune $CD8^+$ T cells can kill MDSCs via their production of TNF-α, IFN-γ, or expression of FasL and thereby reduce MDSC infiltration in tumor. In contrast, low dosage of radiotherapy did not positively affect MDSC number.

5-Fluorouracil (5-FU) is a thymidylate synthase inhibitor, preventing the synthesis of thymidine, a nucleoside essential for DNA replication. This antimetabolite drug is used to treat most digestive cancers and is a major drug for colon cancer. 5-FU can selectively deplete MDSCs, both PMN and monocytic, in several mouse cancer models (Fig. 11.1). MDSC depletion is due to the triggering of apoptosis after a 5-FU treatment. 5-FU selectively kills MDSCs because of their weak expression of the target enzyme of 5-FU, the thymidylate synthase. 5-FU is a competitive inhibitor of thymidylate synthase. Cells with a low expression of thymidylate synthase are very sensitive to cell death induced by 5-FU. In tumor-bearing mice, a 5-FU treatment significantly delayed tumor growth and induced a specific CD8 T cell activation in tumor bed [26]. A closer look at the impact of 5-FU on immune populations in tumor-bearing mice showed an increase in the number of Th17 cells 10 days after a 5-FU treatment accompanied by a return of the tumor growth. This increase in Th17 cells was due to the production of IL-1beta by dying MDSCs. Indeed, 5-FU induced BAX activation and lysosome permeabilization in MDSCs. The protein cathepsin B was released from the lysosomes into the cytoplasm where it interacted with NLRP3 and triggered the formation and activation

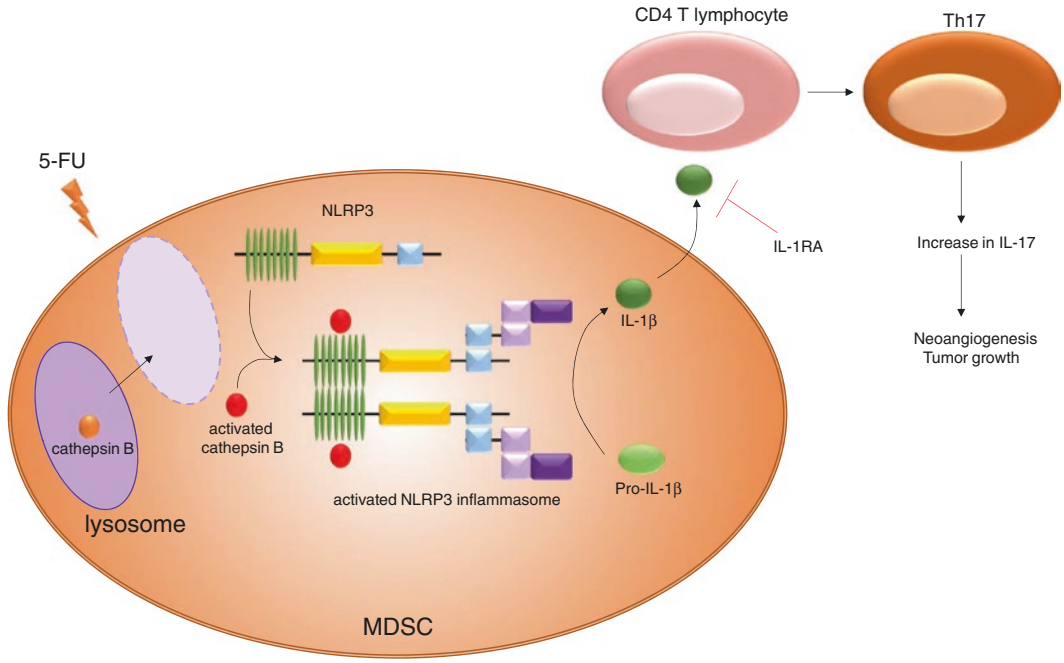

Fig. 11.1 5-FU-dependent depletion of MDSCs. 5-FU specifically targets and depletes MDSCs, reducing the overall immunosuppression. However, 5-FU treatment also induces a permeabilization of the lysosome in MDSCs. Cathepsin B can then escape the lysosome and enter the cytoplasm where it interacts with NLRP3, inducing the formation and activation of the NLRP3 inflammasome. The activated NLRP3 inflammasome cleaves the pro-IL-1β into active IL-1β that activates CD4 T cells to produce more IL-17, enhancing neoangiogenesis and tumor growth

of the NLRP3 inflammasome, leading to the production of cleaved and bioactive IL-1beta. The IL-1beta would then promote Th17 polarization of CD4 T cells. In vitro stimulation of CD4 T cells with 5-FU-treated MDSC promoted their capacity to produce IL-17. In vivo, 5-FU induced accumulation of Th17 cells in tumor-bearing mice in a NLRP3-dependent manner. Interestingly IL-17 promoted angiogenesis, and this neoangiogenesis seemed to be an essential effector of the deleterious effect of 5-FU [27]. The use of IL-1RA, a soluble receptor of IL-1, along with 5-FU, could block the action of IL-1beta. Such therapy reduced the generation of Th17 cells and neoangiogenesis and dramatically improved the efficacy of 5-FU on the tumor growth.

The impact of chemotherapies on MDSCs is often a double-edged sword, tumor cells and immunosuppressive immune cells being masters at finding a loophole allowing for the return of an immunosuppressive tumor environment as seen here. We should be careful not to make any precocious conclusion before the full picture is before our eyes.

11.3 Effect of Chemotherapies on MDSCs in Human

Studies in humans on the effect of chemotherapies on MSDCs have sometimes shown contradictory data. In patients with a pancreatic cancer, 5-FU and gemcitabine were first shown to reduce MDSC percentage in about 40% of patients and in most of patients when associated with the GV101 vaccine using GM-CSF as an adjuvant. MDSC number did however come up in some patients, and this was correlated with an increase of pro-inflammatory cytokines [28]. These results match with a more recent study where treatment with gemcitabine or 5-FU was associated with an upregulation of GM-CSF secreted by tumor cells inducing the differentiation of monocytes in MDSCs [29]. On the contrary, a positive effect of gemcitabine and 5-FU on MDSCs was observed when associated with an immunotherapy consisting in cytokine-induced killer cells [30]. In metastatic renal cell carcinoma and pancreatic cancer, the use of this chemotherapy in association with an immunotherapy successfully reduced the number of MDSCs in the peripheral blood of patients and increased the survival time. Interestingly, in colorectal cancer patients, the positive or negative outcome of a 5-FU treatment is dependent on the type of combination used in association with 5-FU. Indeed, 5-FU is not used alone in colorectal cancer. The use of 5-FU with folic acid and oxaliplatin (FOLFOX) was proven beneficial with a decrease of the overall immunosuppression and MDSC percentage, whereas the association of 5-FU with folic acid and CPT11 (FOLFIRI) was detrimental and even increased the number of MDSCs in patients [31]. In another study testing the effect of FOLFOX associated with bevacizumab, an anti-VEGF-A antibody, on MDSCs in patients treated in first line of metastatic colorectal cancer, authors observed a drop of PMN-MDSCs in 15 out of 25 patients [32]. As cancer chemotherapies always associate several agents, it is crucial to study the effects of these associations.

11.4 Tyrosine Kinase Inhibitors

Aside from cytotoxic chemotherapies, several other classes of anticancer agents have been studied for their capability to block MDSC proliferation or to enhance their differentiation. Tyrosine kinase inhibitors (TKIs) are a group of molecules aiming to target tyrosine kinases in various pathways. Targeted pathways, the RAS-RAF/MAPK pathway, the PI3K/AKT/mTOR pathway, and the EGFR pathway, are involved in the regulation of cell survival, proliferation, differentiation, migration, and angiogenesis [33, 34]. Mutations in these pathways are often found in cancer, explaining the rapid and ongoing development of TKI these past few years [35, 36]. The potential effects of TKIs on MDSCs have raised a growing interest.

Sunitinib is a TKI targeting multiple receptor tyrosine kinases including VEGF-R1 and VEGF-R2, PDGF-Rs, but also c-KIT. It was approved by the FDA for the treatment of advanced renal cell carcinoma (RCC) in 2007

and is currently used in the frontline treatment for RCC. In RCC, sunitinib reversed MDSC accumulation by affecting their viability and proliferation. The decrease in MDSCs was linked to an increase in IFN-gamma production by CD3 cells [37]. Sunitinib decreased the number of MDSCs and Tregs as well as the production of the immunosuppressive cytokines IL-10, TGF-beta. Interestingly, the expression of the negative costimulatory molecules CTLA-4 and PD-1 on CD4 and CD8 T cells was decreased after a sunitinib treatment [38]. Sunitinib may reduce the expansion of monocytic MDSC while inducing apoptosis in the granulocytic MDSC subset [39]. However, intratumoral MDSC number and function were not affected by sunitinib, as the high quantity of GM-CSF produced in the tumor bed was protecting MDSCs in a STAT5-dependent pathway [40, 41]. Sunitinib was reported to also affect other cell types than MDSCs as reduction in the percentage of neutrophils and monocytes and an increase in lymphoid cells can be observed [37] (Fig. 11.2).

A study using another VEGF pathway inhibitor, bevacizumab, an anti-VEGF-A mAb, showed that MDSCs were responsible for the refractoriness to clinical effect of anti-VEGF therapy [42], but no effect of bevacizumab on MDSC viability or differentiation was observed. This was later confirmed by another study showing that bevacizumab treatment did not decrease the percentage of MDSCs nor change their level of arginase-1 expression [43]. However, in patients with non-small cell lung carcinoma, three cycles of bevacizumab associated with chemotherapy regimens could reduce PMN-MDSC numbers in a bevacizumab-dependent way [44]. The impact of bevacizumab on MDSCs remains to be confirmed, and observed difference may be consequences of additional drugs used to treat cancer or due to the tumor types.

Sorafenib is an inhibitor directed against several kinases, among which are C-RAF, BRAF, and VEGF-R2 and VEGF-R3. Sorafenib was first demonstrated to have the capability to reduce Tregs and MDSCs in a murine liver cancer model, along with a slower tumor growth [45]. In addition, sorafenib was able to decrease the suppressive activity of MDSCs on CD8 T cells, while sunitinib, another inhibitor, could not [41]. Different protocols of administration with various doses were tested, and repetitive low doses of sorafenib appeared to enhance the efficacy of adoptive T cell therapy by decreasing MDSCs and Tregs but also by decreasing the expression of immunosuppressive molecules like IL-10 or TGF-beta [46]. Along with the selected dosage, the kinetic of treatment should also be considered as sorafenib could reduce the percentage of MDSC derived from monocytes but did not affect

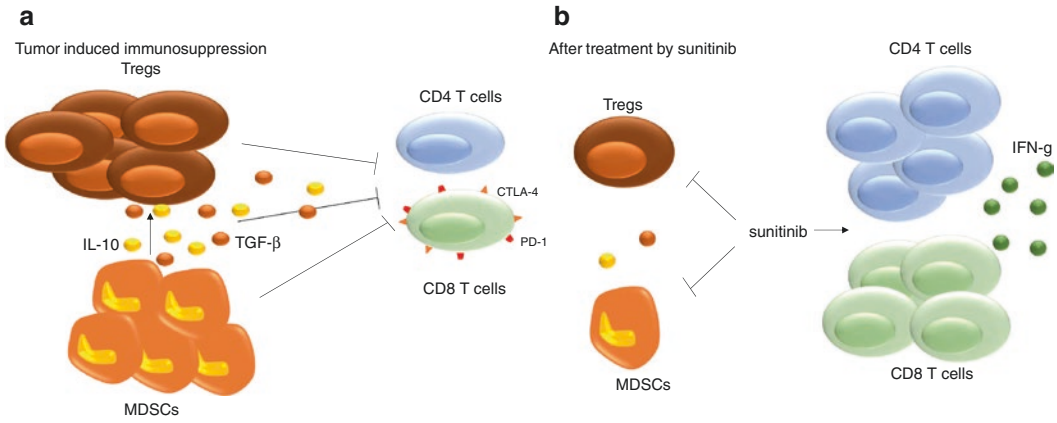

Fig. 11.2 Sunitinib immune effects. Sunitinib, a TKI targeting VEGF-Rs and PDGF-Rs, blocks MDSC accumulation by reducing their viability and proliferation. Sunitinib also targets Tregs and decreases IL-10 and TGF-β production while enhancing the proliferation of CD4 and CD8 T cells along with increasing the production of IFN-γ

already differentiated MDSCs. Sorafenib effects might however not be restricted to MDSCs. Indeed, sorafenib decreased STAT1 and STAT5 phosphorylation in T cells, B cells, NK cells, Tregs, and MDSCs after stimulation with IL-2 or IFN-alpha [47]. Such data suggest that sorafenib could have deleterious effect on effector cells of the adaptive immune response. We probably should keep in mind these data when using sorafenib to deplete MDSCs.

Specific for c-kit and BCR-ABL, imatinib was the first TKI approved for chronic myeloid leukemia [48]. Imatinib efficiently reduced MDSC expansion and arginase-1 expression [49]. However, various reports also mention contradictory results in regard to its effects on other immune cell populations. Imatinib was shown to impair Tregs immunosuppressive functions [50], restore plasmacytoid dendritic cell function, and suppress tumor-induced CD4+ T cell tolerance [51, 52]. On the other hand, imatinib treatment was also shown to block the expansion of antigen-experienced CD8 T cells while leaving primary T and B cell responses unaffected [53]. Dasatinib is a second-generation compound used in patients with chronic myeloid leukemia who fail to respond to imatinib. Like imatinib, dasatinib blocked MDSC expansion [41, 49] and could trigger the development of a broad repertoire of tumor-associated CD8+ tumor-infiltrating lymphocytes when associated with a DC-based vaccine in a melanoma model [54]. However, in several studies dasatinib also inhibited CD4+ and CD8+ T cell activation and proliferation in a dose-dependent manner [55]. The beneficial use of imatinib and dasatinib against MDSCs is unambiguous, but the consequences of their use on the T cell compartment remain unclear.

Many other TKIs have been shown to deeply affect MDSCs, their proliferation, differentiation, as well as their suppressive functions. Vemurafenib was approved for the treatment of unresectable or metastatic melanoma with the BRAF V600-activating mutation by the FDA in 2011. Vemurafenib could decrease the proportion and absolute number of M- and PMN-MDSCs as well as Tregs in melanoma both in mice models and human. Following a vemurafenib treatment, an increase in tumor-infiltrating CD8 T cells was observed and was correlated with a reduction in tumor size [56–58]. Approved by the FDA in 2013 to use against some B cell lymphoma, the Bruton tyrosine kinase (BTK) inhibitor ibrutinib could reduce MDSC accumulation in the tumor bed and reduce the expression of IDO. These effects are likely to be a direct consequence of the inhibition of BTK in MDSCs [59, 60].

Recently a growing interest regarding the effects of the PI3K/AKT/mTOR pathway on MDSCs has arisen. The mTOR pathway activation in both tumor cells and MDSCs seems favorable to MDSCs. Indeed, rapamycin, an inhibitor of mTOR, has been shown to significantly decrease MDSC number as well as the immunosuppressive functions of M-MDSCs in tumor-bearing mice. mTOR appears to be an intrinsic factor involved in the differentiation and suppressive functions of MDSCs [61, 62]. Moreover, activation of the mTOR pathway in cancer cells could also favor the recruitment and accumulation of MDSCs in a G-CSF-dependent fashion in human breast cancer [63]. However, we have to keep in mind that mTOR activation is also essential for T cell activation and mTOR inhibitor could have some deleterious effect on CD8 antitumor immune response. So far only rapamycin derivatives are used in clinic to block the PI3K/AKT/mTOR pathway, but other inhibitors targeting this pathway are in development. Such drugs should also be tested to address their capacity to inhibit MDSCs or reduce their number.

11.5 Other FDA-Approved Molecules with Impact on MDSCs

Molecules from different categories approved by the FDA are found to display activity against MDSCs. One of them is a phosphodiesterase 5 inhibitor named tadalafil. Tadalafil inhibited MDSC immunosuppressive functions via down-regulation of iNOS and Arg-1, two key immunosuppressive enzymes of MDSCs [64]. In head and neck squamous cell carcinoma, tadalafil could reduce MDSCs and Tregs in both blood and tumor

bed while increasing the concentration of CD8 T cells specific for tumor antigens in the blood [65, 66]. The dose at which tadalafil is used seems of importance as an important dose triggered off-target effects on PDE11 which may affect antitumor immunity by different ways [65]. A case report on a man with end-stage multiple myeloma showed that tadalafil reduced MDSC functions (Arg-1 and iNOS expressions downregulated) and established a durable anti-myeloma immune and clinical response although not complete [67].

CTL antigen-4 (CTLA-4) is a negative immune checkpoint expressed by T cells. Ipilimumab is a human antibody directed against CTLA-4, and it has been shown that in patients with metastatic melanoma, the frequency of PMN-MDSCs significantly decreased 3 weeks after a first treatment with ipilimumab [68]. In contrast, no impact on M-MDSCs was observed. Another study on melanoma patients showed a decrease in circulating MDSCs after an ipilimumab treatment and a positive association between decreased in MDSCs and a better PFS [69]. Complementary studies are required to understand the mechanisms by which ipilimumab affects MDSCs.

ATRA, for all-trans retinoic acid, is used to treat promyelocytic acute myeloid leukemia. This drug is also capable of inducing the maturation of MDSCs into DCs, macrophages, and granulocytes. As expected, the decrease in MDSCs by ATRA treatment improved CD4 and CD8 T lymphocyte tumor-specific response first in two mouse tumor models, DA3-HA adenocarcinoma and C3 fibrosarcoma [70], and then in patients with RCC [71] and small cell lung cancer [72].

11.6 Drugs in Developments

A broad spectrum of molecules from various origins have displayed an activity against MDSCs; they can either block the immunosuppressive functions of MDSCs, inducing their differentiation in dendritic cells or in M1-like macrophages, or deplete them.

In the MC38 colon carcinoma, the Lewis lung carcinoma and the EL-4 thymoma mouse models and in patients with RCC or soft tissue sarcoma, the triterpenoid CDDO-Me did not affect the size of the MDSC population but abrogated their immunosuppressive activities and improved immune responses [73]. Nitroaspirin also reduces MDSC functions by inhibiting NOS and Arg-1 activity, resulting in increased number and functions of tumor antigen-specific T lymphocytes [74]. The inhibitor of the ubiquitin receptor RPN13/ADRM1 RA190 was recently shown to lower the level of Arg-1, iNOS, and IL-10 in MDSCs. MDSCs treated by RA190 lost their capacity to suppress CD8+ T cells, thus enhancing antitumor immune response, in an ovarian mouse model [75]. TLR9 activation of MDSCs by CpG treatment was shown to block their suppressive activity on T cells in two mouse models of cancer and to induce MDSC differentiation [76]. The impact of CpG on MDSCs was confirmed in the Renca renal mouse tumor model [77]. On the opposite side, it is interesting to note that CpG emulsified in incomplete Freund's adjuvant treatment expanded MDSCs and increased their expression of Arg-1 in aged mice free of tumor, suggesting contrasting effect of CPG on MDSCs [78].

Curcumin can differentiate MDSCs. Curcumin suppresses PMN-MDSC function and favors M-MDSC differentiation toward an M1-like phenotype [79] in a clusterin-dependent fashion [19]. Other molecules induce a depletion of MDSCs as the use of an antibody-targeting DR5, a death receptor present at the surface of MDSCs, can effectively eliminate them without affecting other myeloid populations and resulted in an enhanced antitumor immune response [80]. Beta-glucan-like curdlan can promote the differentiation of M-MDSCs into a mature CD11c+ F4/80+ population. That differentiation happens via a NF-κB-dependent dectin-1 pathway. A beta-glucan treatment diminished MDSCs in the tumor bed and increased infiltrated DCs and macrophages, leading to an enhanced CD8 and CD4 T cell responses and delayed tumor growth [81]. Two cationic polymers, cationic dextran (C-dextran) and polyethyleneimine (PEI), can differentiate MDSCs into an M1-like phenotype, decreasing IL-10 and TGF-beta production as well as suppressing Arg-1 expression while increasing M1-type cytokines production. This decrease in MDSC restored antitumor immunity and slowed tumor growth in the 4T1 mouse mammary carcinoma [82].

Depletion of MDSC can be achieved, thanks to a various set of molecule ever-expanding. However, the mechanisms behind that depletion are not always yet defined. A new therapeutic peptide, developed after identification and characterization of MDSC-binding peptides, depleted both monocytic and PMN-MDSCs in the blood and spleen of EL4 or EG7 thymoma-bearing mice and successfully delayed tumor growth [83]. The S100 protein family is a candidate target for this peptide, but a more thorough study may be needed in order to fully understand the mechanism of action of this peptide (Fig. 11.3).

CSF-1, also known as M-CSF, is overexpressed in many tumors and is a growth factor for M-MDSCs and macrophages. Several CSF-1 receptor inhibitors have been developed and, when tested in tumor-bearing mice, displayed the potency to deplete M-MDSC in tumor bed and spleen. Blockade of CSF1R increases antigen-specific T cell activity at the tumor site, delaying tumor growth in B16 melanoma [84, 85], RM-1, RM-9, and Myc-CaP prostate cancer-bearing mice [86].

Contradictory data can be found about histone deacetylase (HDAC) on MDSCs. It was first published that HDAC inhibition by TSA, a naturally occurring antifungal metabolite that potently inhibits HDAC, enhanced the expansion of MDSCs in a GM-CSF-dependent manner [87]. This was confirmed by another study showing that HDAC11 is a negative regulator of MDSC expansion and function and that EL4 tumor-bearing HDAC11 KO mice possessed a more suppressive MDSC population compared to wild-type mice [88]. However, in 2016, a team demonstrated that HDAC inhibitors depleted MDSCs induced by 4T1 mammary tumor both in vitro and in vivo in the spleen, blood, and tumor bed and increased the population of CD8 T lymphocytes. Interestingly, HDAC inhibitors also increased the apoptosis of MDSC precursor in the bone marrow, GR1+ cells [89]. Our understanding of the role of HDAC on MDSCs remains to be completed. Here the difference in models and inhibitors might be responsible for the difference in results, proving the complex interplay between MDSCs and the immune system state in tumor-bearing individuals.

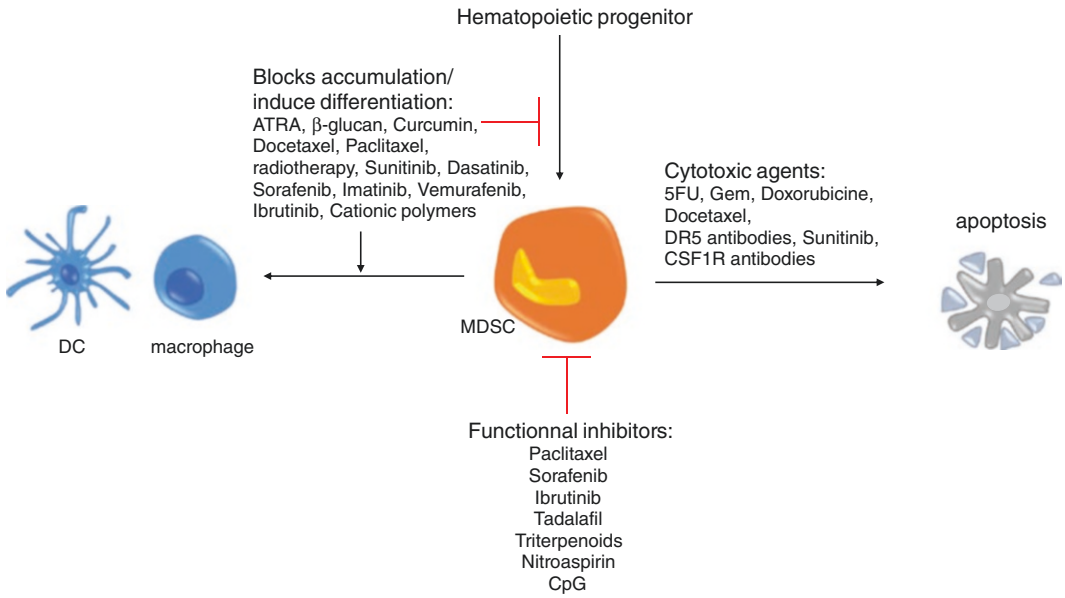

Fig. 11.3 Therapeutic approaches targeting MDSCs. There are three different ways to aim at MDSC. Molecules targeting MDSCs can directly kill them, like 5-FU or DR5 antibodies, or inhibit MDSC immunosuppressive functions as tadalafil does or also block MDSC accumulation or induce their differentiation like ATRA or curcumin does

11.7 Combination with Checkpoint Inhibitors

CTLA-4 and PD-1 (programmed death 1) are negative immune checkpoints regulating lymphocyte functions. CTLA-4 can be found in the cytoplasm of naïve T cells and is exported to the membrane after activation. Quantities of CTLA-4 found at the surface of activated T cells increase with their activation, allowing the creation of a negative feedback loop to avoid an overactivation of lymphocytes. CTLA-4 is a CD28 homologue binding CD80 and CD86 with a greater affinity than CD28. The ratio of CTLA-4/CD28 bound to costimulatory molecules will determine if the lymphocyte is activated or inhibited. Tregs express constitutively CTLA-4 which might play a part in the Tregs immunosuppressive functions. Blocking CTLA-4 results in an enhanced immune response explaining the interest it receives in oncology. PD-1 is found on highly activated T cells, NK cells, B cells, and monocytes. The binding of PD-1, with its ligands PD-L1 or PD-L2, found at the surface of tumor cells, and various tumor-infiltrated immune cells like MDSC and dendritic cells reduces T cell functions to avoid excessive immune responses [90]. PD-L1 plays a major role in the immunosuppression established in a tumor environment because of its expression on tumor cells and MDSCs; this is why the interaction PD-1/PD-L1 has led to the development of several antibodies aiming to block this interaction to restore potent antitumor immune responses [91]. Removing MDSC-dependent immunosuppression along with suppressing immune checkpoint blockade should induce a massive T cell response in tumor-bearing hosts. This is why several preclinical studies have tried to associate MDSC depletion or differentiation with antibody directed against negative checkpoint inhibitors (Fig. 11.4).

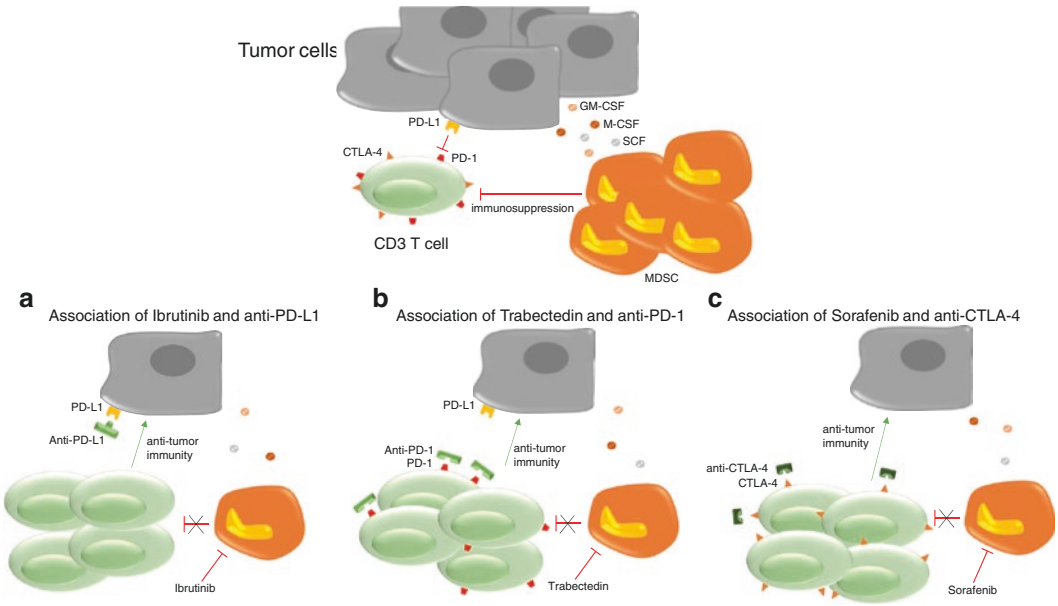

Fig. 11.4 Combination with checkpoint inhibitors. Tumor-induced immunosuppression is dependent on three aspects. The direct immunosuppression mediated by tumor cells expressing PD-L1 on PD-1+ T cells, the induction of MDSCs by tumor cells and MDSC-dependent immunosuppression. By associating a molecule depleting MDSCs with a checkpoint inhibitor, it is possible to restore a potent antitumor immunity. (**a**) Ibrutinib depletes MDSCs, while the anti-PD-L1 blocks the PD-1/PD-L1 immunosuppression. This allows T cells to proliferate and establish an antitumor immunity, preventing tumor growth. (**b**) Trabectedin can directly suppress MDSCs and, when associated with an anti-PD-1, restores a proper T cell-mediated antitumor immunity. (**c**) The action of sorafenib against MDSCs in association with an anti-CTLA-4 allows T cells to develop an antitumor immune response leading to a reduced tumor growth

Ibrutinib, an inhibitor of BTK and ITK (interleukin-2-inducible T cell kinase) known to reduce MDSC accumulation in the tumor bed [59, 60], can enhance therapeutic antitumor immunity when associated with a PD-L1 treatment in the A20 lymphoma but also the CT26 colon carcinoma and 4T1 breast carcinoma models [59]. This was later confirmed by another study where the association of an anti-PD-L1 with ibrutinib almost completely abrogated tumor growth of 4T1 breast cancer [60]. In a model of ovarian cancer, trabectedin was associated with an anti-PD-1. This association cured about half of the mice and induced a strong tumor-specific immunity from CD4 and CD8 T cells. CD8 T cells exhibited tumor antigen-specific responses, and an increase in IFN-gamma was observed along with a reduction in immunosuppressive populations. Interestingly, in vivo trabectedin might be responsible for a rise in PD-L1 expression within tumor explaining the improved efficacy of the association over single therapies [92].

High-dose ionizing irradiation (IR) results in direct tumor death and is used in many cancers. In the TUBO breast cancer and MC38 colon cancer models, IR also decreased the population of MDSCs but increased PD-L1 expression inside tumors. To overcome this issue, IR was used along with an anti-PD-L1. This association reduced MDSC population to close to zero percent in the tumor bed while enhancing cytotoxic CD8 T cells in a synergistic manner, delaying tumor growth [93]. In HPV-related oropharyngeal cancer, radiotherapy is often being associated with chemotherapy to treat patients. In a clinical trial, authors observed HPV-specific T cell responses in 13/18 patients prior to treatment. This immune response was lost in 10/13 patients within 3 months after chemoradiotherapy (CRT). CRT decreased circulating T cells and increased the MDSC population. PD-1 expression on CD4 T cells was also enhanced after CRT. The use of a PD-1 blocking antibody in ex vivo culture restored the HPV CD4 T cell-specific response, further encouraging the study of such association to help improve patient treatments [94].

As previously seen, sorafenib can effectively deplete MDSCs. It was associated with an anti-CTLA-4 in a RENCA mouse model and whereas the monotherapies did reduce tumor growth, the combination displayed a synergistic effect with the highest rate of tumor rejection and a strong increase in infiltrating CD4 and CD8 T lymphocytes in the tumor bed [95]. Unfortunately, the number of MDSCs was not assessed. There is an ongoing phase I study about the combination of sorafenib and ipilimumab (anti-CTLA-4) in patients with advanced hepatocellular cancer with a stable disease that should give us more information.

Ipilimumab is also often associated with an anti-PD-1 named nivolumab, and this combination is now used as standard treatment of metastatic melanoma in patients. While ipilimumab could decrease MDSC number, no study thoroughly examined the impact of this association on MDSCs. However, an increase of CD4 and CD8 T lymphocytes to MDSC ratio was observed in the mouse melanoma model B16 using a combination of anti-PD-1 and anti-CTLA-4 [96].

Sunitinib, a multi-target TKI that affects the viability and proliferation of MDSCs and Tregs, was used associated with IL-12, an activating cytokine and 4-1BB, a positive immune checkpoint expressed by T cells. In the MCA26 colon carcinoma mouse model, the combination of sunitinib, IL-12, and 4-1BB significantly improved long-term survival and had an efficacy superior to that of IL-12 and 4-1BB in association [38].

Conclusion

Targeting immunosuppression and particularly MDSCs in the setting of cancer is a major issue to improve the efficacy of immune therapy aimed at targeting CD8 T cell response. Many preclinical and clinical data underline that some cytotoxic chemotherapies and tyrosine kinase inhibitors could eliminate or decrease immunosuppressive functions of MDSCs leading to the rational that combination of such drugs with checkpoint inhibitor could improve their efficacy. However, a careful analysis of such data must be performed

before moving to clinical trial because the type of tumor and the association of drugs frequently impact their effects on MDSCs. In addition, a large analysis of immune responses must be performed on many of these drugs as their positive effect on MDSCs could be accompanied by negative impact on other components of the immune system, resulting in a null effect.

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12 Immunogenic Stress and Death of Cancer Cells in Natural and Therapy-Induced Immunosurveillance

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Contents

12.1 Introduction

Before the renaissance of the immunosurveillance theory that accompanied the approval of immune checkpoint blockers [1], cancer was generally viewed as a cell-autonomous disease that is solely caused by genetic and epigenetic alterations of the malignant cells [2]. Therapeutic interventions hence were conceived to take advantage of the cancer cell-intrinsic vulnerabilities (making them particularly susceptible to antiproliferative and cytotoxic insults) or to tar-

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get pathways that would be specifically activated in malignant cells yet absent in their normal counterparts (much like antibiotics that affect bacterial enzymes but not those of their host). Based on this paradigm, cytotoxic and targeted therapies have been developed following a workflow in which anticancer agents were first identified on cultured human cell lines, then tested on immunodeficient mice carrying human cancers, and, finally, introduced into the clinics [2, 3]. Seemingly supporting this strategy, several successful chemotherapeutics have been developed. In particular, combination therapies involving several distinct cytotoxic agents have been highly successful in reducing the risk of relapse after adjuvant chemotherapy in breast and colorectal cancers [4–6]. Moreover, the success of the first targeted anticancer agent, imatinib mesylate, which targets several oncogenic tyrosine kinases (such as the BCR-ABL kinase activated in chronic myeloid leukemia and KIT activated in gastrointestinal stromal tumors) [7–12] apparently comforted the idea that cancer can be treated with specific agents (although it turned out later that the therapeutic efficacy of imatinib relies on NK and T lymphocytes) [13].

Our laboratory has been adhering to this cell-autonomous vision of cancer therapy until 2004 when we performed a stunningly simple experiment. We subcutaneously implanted a mouse colorectal cancer cell line, CT26, either in immunocompetent BALB/c mice (the strain from which CT26 was originally derived from) or in immunodeficient *nu/nu* mice (which are athymic and hence lack thymus-derived T lymphocytes) and treated the emerging tumors with chemotherapy based on the anthracycline doxorubicin. To our dismay, the growth of CT26 cancers was only reduced if they evolved in an immunocompetent setting (in BALB/c mice), not if they grew on *nu/nu* mice [14]. Hence, the efficacy of chemotherapy turned out to depend on a cellular immune response.

The next surprise came when we analyzed the cell death modality induced by doxorubicin in CT26 cells. At that time, only two major cell death pathways were known, namely, apoptosis and necrosis [15]. Apoptosis was conceived to constitute a physiological pathway accounting for cellular demise in developmental cell death and adult tissue homeostasis [16]. Necrosis was conceived as a purely pathological pathway resulting in pro-inflammatory tissue reaction due to the uncontrolled spilling of the cellular content through the permeabilized plasma membrane [17]. In CT26 cells, doxorubicin induced two hallmarks of the apoptotic pathway, namely, an early loss of the mitochondrial inner transmembrane potential as well as the activation of caspases [14]. Addition of a pharmacological caspase inhibitor prevented the cells to adopt an apoptotic morphology with nuclear condensation and fragmentation and led to a more necrotic phenotype. When doxorubicin-treated apoptotic CT26 cells were injected subcutaneously into BALB/c mice, they induced an immune response that protected the mice against a subsequent challenge with live CT26 cells that were injected 1 week later into the opposite flank. In contrast, doxorubicin-treated necrotic CT26 cells (that were killed in the presence of Z-VAD-fmk) failed to stimulate such an immune response [14]. These results pleaded in favor of a novel caspase-dependent modality of apoptosis that could stimulate anticancer immunosurveillance and that we dubbed "immunogenic cell death" (ICD) [14]. Later, it turned out that CT26 cells lack the expression of receptor-interacting serine/threonine-protein kinase 3 (RIPK3), a protein required for necroptosis (which is a regulated version of necrosis) and that other mouse cancer cell lines that possess the entire molecular machinery required for necroptosis can undergo ICD in response to necroptotic stimuli [18–20] including anthracyclines. Hence, different forms of regulated cell death (apoptosis and necroptosis) can contribute to ICD.

Based on the aforementioned results that were replicated in multiple different cancer cell types and mouse strains [21–26], we have been postulating that ICD would constitute an important mechanism to convert the cell-autonomous chemotherapeutic response, leading to focal apoptosis and necroptosis within the tumor, into a systemic immune-mediated response that can amplify and prolong the anticancer effects of

chemotherapy [19, 27–29]. In other words, ICD would convert cancer into its own vaccine. We also found that not all chemotherapeutic agents are equally potent in causing ICD (and hence in provoking an antitumor immune response) observing that anthracyclines and oxaliplatin are particularly efficient in doing so while many other cytotoxicants are unable to do so [27–29]. In subsequent studies, we observed that ICD inducers are able to trigger *premortem* stress responses such as autophagy and endoplasmic reticulum (ER) stress that lead to the release and exposure of DAMPs required for ICD [28, 30]. Hence, it is not only cell death as such but a constellation of stress pathways and lethal events that yields ICD. These pathways and their connection to the exposure or release of DAMPs, as well as their clinical implications, will be discussed in this chapter.

12.2 Annexin A1

Annexin A1 (ANXA1) is a relatively abundant and ubiquitously expressed cytoplasmic protein [31] that is released from dying cancer cells responding to chemotherapy with anthracyclines or oxaliplatin in vitro [32]. The exact mode of release is not known, although a relative of ANXA1, annexin A2 (ANXA2), has been shown to be secreted by an unconventional pathway [33]. Alternatively, ANXA1 may be released passively, via the permeabilized plasma membrane as cells die. Mouse cancer cell lines from which ANXA1 was removed by CRISPR/Cas9 technology failed to undergo ICD in vitro (meaning that, if they were cultured with anthracyclines and then injected in vivo, they would fail to induce a protective anticancer immune response). Cancers arising from such ANXA1-deficient cancer cell also failed to reduce their growth in vivo, in response to systemic injections of anthracyclines or oxaliplatin [32].

ANXA1 can bind to formyl peptide receptor-1 (FPR1), a seven transmembrane G protein-coupled receptor mostly expressed by myeloid cells [34]. Knockout of FPR1 in the host immune system (as well as transfer of FPR1-deficient hematopoietic stem cells into FPR1-sufficient irradiated hosts) led to the incapacity of the host to mount an anticancer immune response against dying cancer cells. Moreover, the absence of FPR1 from the immune system led to a failure to control the growth of cancers treated with anthracyclines or oxaliplatin in vivo [32]. These results underscore the importance of the interaction between ANXA1 and FPR1 for the chemotherapy-triggered dialogue between cancer cells and the immune system. Mechanistically, it turned out that FPR1 guides differentiating dendritic cells within the tumor into the proximity of dying cancer cells, allowing the dendritic cell-mediated uptake of tumor-associated antigens and their subsequent presentation to T cells (Fig. 12.1) [32]. As a result, FPR1-deficient hosts are unable to mount an immune response mediated by $CD8^+$ T cells against tumor antigens.

The aforementioned findings, which have been obtained in mice, are supported by epidemiological studies in cancer patients. A loss-of-function mutation in FPR1 (A299G), which affects the intracellular domain of the protein within its N-terminus abolishing the dimerization of the receptor required for its activation [35], had negative prognostic features in two types of cancer. Breast cancer patients bearing one loss-of-function allele of FPR1 exhibited a shorter progression-free and overall survival upon adjuvant anthracycline-based chemotherapy than patients bearing two normal alleles of FPR1. This finding was obtained

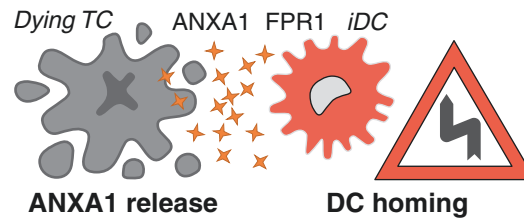

Fig. 12.1 Annexin A1-mediated homing of dendritic cells. Annexin A1 (ANXA1) is released from cancer cells in response to certain therapeutic approaches including the anthracycline- and oxaliplatin-based chemotherapeutic induction of immunogenic cell death. Driven by chemotaxis, immature dendritic cells (iDCs) are homed in on their target in a FPR1-dependent fashion, finally leading to a close proximity of dying cancer cells and antigen-presenting cells. FPR1, formyl peptide receptor 1

for two independent cohorts of breast cancer patients [32]. Moreover, colorectal cancer patients bearing two loss-of-function alleles of FPR1 had a statistically shorter survival upon adjuvant oxaliplatin-based chemotherapy than patients bearing one or two normal alleles of FPR1 [32]. The mechanistic bases for these differences are not understood yet. In addition, it appears that mammary carcinoma cells express lower ANXA1 levels than their normal epithelial counterparts [32], perhaps reflecting immunoselection in favor of cancers that lack the DAMP ANXA1.

12.3 ATP

In response to treatment with chemotherapeutics in vitro, cancer cells release adenosine triphosphate (ATP) into the culture supernatant, an event that can be visualized by a reduction in quinacrine-labeled, ATP-containing lysosomal compartments [36]. The accompanying increase in extracellular levels of ATP can be measured by means of a firefly luciferase construct that is tethered to the cancer cell surface and that detects pericellular ATP upon addition of D-luciferin [37, 38]. This latter system is suitable for measuring extracellular ATP in vivo in tumor-bearing mice, in which the luminescence signal strongly increases 2 days post-chemotherapy [25]. The mechanism of ATP release has not been entirely elucidated yet appears to involve a lysosomal secretion mechanism that depends on at least two processes, namely, a *premortem* autophagy response and caspase activation. Autophagy must occur to allow ATP to redistribute from lysosomes to autolysosomes and to be secreted by a mechanism that requires the lysosomal-associated membrane protein 1 (LAMP1), which translocates to the plasma membrane in a caspase-dependent manner. The release of ATP additionally involves the caspase-mediated activation of the Rho-associated coiled-coil-containing protein kinase (ROCK1) resulting in myosin II-dependent membrane blebbing as well as the opening of pannexin 1 (PANX1) channels, subsequent to their cleavage by caspases. While autophagy and LAMP1 do not affect PANX1 channel opening, PANX1 is required for the ICD-associated translocation of LAMP1 to the plasma membrane [39]. Hence, apoptosis-associated ATP release is a complex process that is abolished in autophagy-deficient tumors, knowing that inactivation of autophagy occurs rather frequently, especially during early oncogenesis [25, 40–43]. Necroptotic signaling via RIPK3 and the mixed lineage kinase domain-like (MLKL) pseudokinase may also contribute to ATP release [18], although it is not known whether this process also requires *premortem* autophagy to be induced. In any case, it appears that cancer cells manipulated to suppress the autophagic process fail to undergo ICD and do not reduce their growth upon treatment with anthracyclines or oxaliplatin in vivo [25]. A similar abolition of ICD and chemotherapeutic responses can be obtained by overexpressing the ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1, also known as the ectoATPase CD39) on the cancer cells [25, 44].

Extracellular ATP acts on two classes of purinergic receptors, namely, the metabotropic P2Y2 and the ionotropic P2X7 receptors. P2Y2 receptors facilitate the ATP-mediated chemotaxis of myeloid cells (dendritic cell precursors, neutrophils and macrophages) into the tumor bed post-chemotherapy (Fig. 12.2). Both autophagy-deficient

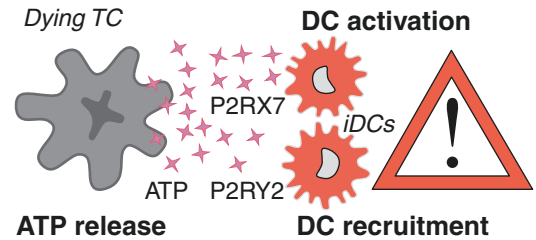

Fig. 12.2 ATP-dependent recruitment and activation of dendritic cells. The autophagy-dependent lysosomal secretion of ATP from cancer cells that undergo immunogenic cell death leads to the recruitment and activation of immature dendritic cells (iDCs). Extracellular ATP acts on purinergic receptors of the metabotropic P2Y2 and the ionotropic P2X7 type. Most prominently P2Y2 receptors drive the ATP-mediated chemotaxis of myeloid cells including immature dendritic cells (iDCs) into the tumor bed post-chemotherapy. In summary, ATP release from the dying cancer cells leads to an enrichment of the tumor bed with immune cells. P2RX7, purinergic receptor P2X7; P2RY2, purinergic receptor P2Y2

and CD39-overexpressing cancers fail to accumulate myeloid cells post-chemotherapy in the tumor bed [25], and a similar effect can be obtained upon pharmacological inhibition of P2Y2 [44]. P2X7 receptors facilitate the ATP-stimulated activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome in dendritic cells, which then triggers the secretion of interleukin-1β (IL1β) and the IL1β-dependent priming of tumor antigen-specific $CD8^+$ T cells [45]. Indeed, neutralization of P2Y2 or IL1 receptors and knockout of P2X7, NLRP3, or caspase-1 abolish the capacity of the immune system to mount a protective immune response against cancer cells that succumb to ICD [45].

The aforementioned interaction between extracellular ATP and purinergic receptors again appears clinically relevant. Indeed, in breast cancer patients treated with adjuvant chemotherapy, the absence of autophagy has a negative impact on the local immune response with an unfavorable ratio of $CD8^+$ T lymphocytes over forkhead box $P3^+$ ($FOXP3^+$) regulatory T cells. Such observation correlates with poor patient survival [46]. Similarly, high expression of ATP-degrading ectoenzymes such as CD39 and the ecto-5′ nucleotidase NT5E (best known as CD73) indicates poor prognosis in multiple distinct cancers including breast and ovarian cancers [47, 48]. Finally, a loss-of-function mutation in P2X7 has been linked to poor prognosis in a segment of breast cancer patients that are treated with anthracycline-based adjuvant chemotherapy [45].

Experimentally, it is possible to stimulate autophagy, ATP release, and consequent myeloid cell recruitment and anticancer immune responses by fasting or by non-immunosuppressive autophagy inducers that fall into the class of "caloric restriction mimetics" (CRMs) [49–52]. Several CRMs including hydroxycitrate can be used in mouse models to improve anticancer immunosurveillance and to boost the anticancer immune responses elicited by ICD-inducing chemotherapeutics [49]. Whether this strategy is applicable to cancer patients awaits urgent clarification.

12.4 Calreticulin

Calreticulin (CALR) is the most abundant protein in the lumen of the endoplasmic reticulum (ER). In the context of ICD, a fraction of CALR translocates to the surface of the plasma membrane (and it is possible that another fraction of CALR is secreted as well) [23, 26, 53, 54]. The complex mechanisms that underlie CALR exposure are linked to the apical phosphorylation of eukaryotic initiation factor-2α (eIF2α) in the context of an ER stress response that culminates in the activation of an eIF2α kinase (EIF2K) such as EIF2K2 (best known as PKR) and EIF2K3 (best known as PERK) and/or in the inhibition of the corresponding phosphatase (composed by the catalytic subunit PP1 and the regulatory subunit GADD34) [26, 55, 56]. Downstream of eIF2α phosphorylation, caspases (and in particular caspase-8, CASP8) are activated, and calreticulin is transported to the cell surface following anterograde ER-Golgi traffic and soluble *N*-ethylmaleimide-sensitive factor attachment receptors (SNARE)-dependent exocytosis that involves the vesicle-associated membrane protein 1 (VAMP1) and the synaptosomal-associated protein 23 (SNAP23) [55].

Once on the cell surface, CALR acts as an "eat-me" signal to facilitate the transfer of tumor-associated antigens to dendritic cells [57], which

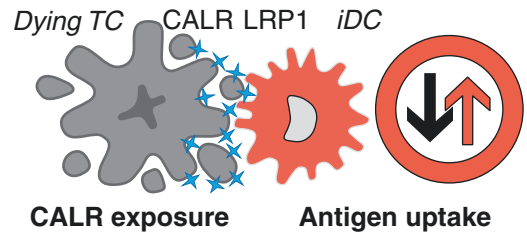

Fig. 12.3 Calreticulin as de novo uptake signal for dendritic cells. The endoplasmic reticulum (ER) stress-mediated exposure (or release) of the ER chaperone calreticulin (CALR) to the surface of the plasma membrane in the course of immunogenic cell death (ICD) serves as de novo uptake signal for dendritic cells. The binding of CALR to the low-density lipoprotein receptor-related protein 1 (LRP1) receptor expressed on dendritic cells (DC) serves the transfer of tumor-associated antigens to DC

express the CALR receptor low-density lipoprotein receptor-related protein 1 (LRP1, best known as CD91; Fig. 12.3) [58]. CALR can be locally antagonized by CD47, which is constitutively expressed on cancer cells and can function as a "don't eat me" signal [59]. Knockdown of PERK, CASP8, CALR, or SNAP23 as well as pharmacological inhibition of caspases and anterograde ER-Golgi transport is sufficient to abolish ICD in vitro and in vivo [55]. Conversely, stimulation of eIF2α phosphorylation by thapsigargin (which activates PERK) or inhibitors of PP1 can stimulate CALR exposure and enhance anticancer immune responses in vivo, in the context of chemotherapy [55, 60]. Coating of cancer cells that are deficient in the CALR exposure pathway with recombinant CALR protein (which binds to the plasma membrane surface, presumably via interaction between its lectin domain and the glycocalyx) can restore deficient ICD [26, 61]. Similarly, intratumoral injection of CALR can enhance the chemotherapy-elicited immune response and improve tumor growth inhibition in vivo, in mouse models [62].

There is widespread evidence that CALR expression and exposure contribute to anticancer immunosurveillance in vivo, in cancer patients. Low intracellular CALR expression levels have been correlated with a low presence of CALR on the cell surface, both in acute myeloid leukemia (AML) and in non-small cell lung cancer (NSCLC), as well as reduced phosphorylation of eIF2α [59, 63, 64]. In AML, reduced expression of CALR protein has a negative impact on progression-free and overall survival post-chemotherapy, correlating with poor T cell-mediated immune responses against AML-associated tumor antigens [59, 64]. In NSCLC, approximately 15% of the patients have barely detectable CALR protein in cancer cells, correlating with dismal prognosis, reduced infiltration by DC-LAMP$^+$ dendritic cells and CD8$^+$ T lymphocytes [63]. Of note, low CALR expression supersedes in importance the TNM classification of NSCLC with respect to prognosis, meaning that patients with CALR^{low} stage 1 NSCLC exhibit a poorer survival than stage 3 and stage 4 patients bearing CALR^{high} cancers [63]. These results have been confirmed for two distinct NSCLC cohorts by detecting CALR protein with immunohistochemistry [64], as well as for an additional NSCLC cohort by measuring CALR mRNA levels and its correlations with metagenes reflecting the presence of CTL and dendritic cells [65]. Similarly, in ovarian cancer, high levels of CALR mRNA expression have a favorable impact on patient survival, if combined with the analysis of activated dendritic cells [65]. Conversely, high CD47 expression has a negative impact on the prognosis of multiple distinct cancers [66–68]. Mutations in the CALR gene have been described in myeloproliferative neoplasms [69–71], causing mislocalization of the corresponding gene product [72], although the exact impact of these mutations on tumor immunosurveillance remains elusive. Regardless, the clinical data validate the importance of the CALR exposure pathway for tumor biology.

12.5 HMGB1

High molecular group B1 protein (HMGB1) is the most abundant nonhistone chromatin-binding protein [73–75]. HMGB1 is usually found in an exclusively nuclear location yet can translocate to the cytoplasm, for instance, after inhibition of histone deacetylases [76]. Moreover, HMGB1 is usually released from cells that undergo necroptosis or secondary necrosis [77, 78]. Mouse cancer cells in which either RIPK3 or MLKL have been knocked out release lower amounts of HMGB1 in response to anthracyclines than their necroptosis-competent controls [18].

Experiments on tumors implanted in mice revealed that HMGB1 is released from cancer cells upon chemotherapy in vivo [79–83]. Cancer cells from which HMGB1 has been depleted by RNA interference are unable to undergo ICD and become resistant to chemotherapy in vivo. Similarly, injection of neutralizing anti-HMGB1 antibodies abolished the anticancer immune response elicited by ICD-inducing chemotherapy in vivo and hence compromised tumor growth reduction [81]. These results support the importance of extracellular HMGB1 as a DAMP in tumor immunology.

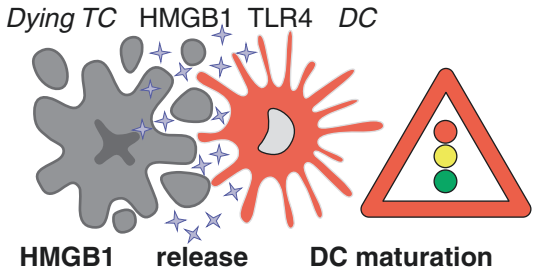

Fig. 12.4 HMGB1 facilitates antigen presentation by dendritic cells. High-mobility group box 1 (HMGB1), normally secluded in the nucleus, is released at later stages of immunogenic cell death in response to treatments such as anthracycline-based chemotherapy or ionizing irradiation. Extracellular HMGB1 serves as a ligand for TLR4 on dendritic cells (DCs) and triggers a MYD88-dependent signaling that stimulates DC maturation and antigen presentation to cytotoxic T cells (CTLs). TLR4, toll-like receptor 4

Once present in the extracellular space, HMGB1 can interact with multiple additional factors including nucleic acids and bacterial polysaccharides [84–88]. HMGB1 also binds to several receptors including toll-like receptor 4 (TLR4), which is expressed on multiple immune cell types including dendritic cells, in which it stimulates maturation and antigen presentation (Fig. 12.4) [81, 89, 90]. Knockout of TLR4, or that of its adaptor MYD88, from the host immune system abolishes the perception of ICD as well as tumor growth reduction by anthracyclines or oxaliplatin [22, 80, 81, 91]. This defect has been linked to a reduced antigen presentation by dendritic cells and can be partially rescued by treatment with the lysosomal inhibitor chloroquine [81].

In human breast cancer, reduced HMGB1 expression has been linked to the advancement of the disease and increased tumor size [46]. Reduced HMGB1 expression is a negative prognostic feature in breast cancer and correlates with an intratumoral infiltration by fewer $CD8^+$ cytotoxic T lymphocytes and more immunosuppressive populations of $FOXP3^+$ regulatory T cells and $CD68^+$ tumor-associated macrophages [46]. Moreover, there are at least two cancer types in which a loss-of-function allele of TLR4 compromises patient prognosis, namely, (1) breast cancer and (2) colorectal cancer treated with adjuvant chemotherapy based on anthracyclines and oxaliplatin, respectively [92]. These findings underscore the likely importance of the HMGB1/TLR4 interaction for the fate of cancer patients.

In the case of HMGB1-negative cancers, artificial supply of a synthetic TLR4 ligand, dendrophilin, can compensate for the HMGB1 defect and restore anticancer immune responses elicited by chemotherapy in mouse models. Whether such a strategy might also work in cancer patients bearing HMGB1-negative neoplasia remains to be investigated.

12.6 Type-1 Interferons and Chemokines

In response to chemotherapeutics, tumor cells liberate nucleic acids including DNA and double-stranded RNA that may activate intracellular or extracellular sensors for ectopic molecules of this kind. One example for such nucleic acid sensor is the toll-like receptor-3 (TLR3) [93], although other sensors including the GAS/STING pathway might be involved as well [94]. In response to these stimuli that resemble those induced by a viral infection and hence can be referred to as "viral mimicry," cancer cells transcriptionally activate one or several type-1 interferon genes, secrete the corresponding gene products, and then stimulate their type-1 interferon receptor (IFNAR) to induce a multipronged type-1 interferon response consisting in the activation of multiple antiviral and immunostimulatory gene products (Fig. 12.5) [95]. One quintessential antiviral gene product is myxovirus resistance 1 (MX1), and one well-known immunostimulatory gene product is the C-X-C motif chemokine ligand 10 (CXCL10), which acts on the C-X-C motif chemokine receptor 3 (CXCR3) to attract T lymphocytes into the tumor bed [95]. Cancer cells that lack TLR3, IFNAR, or CXCL10 are unable to elicit anticancer immune responses upon chemotherapy and hence become refractory to the treatment [95]. Local injection of recombinant type-1 interferons and CXCL10 can overcome this defect [95],

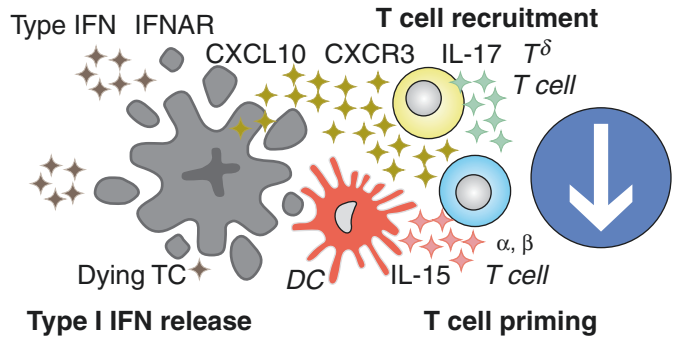

Fig. 12.5 Type I interferon-dependent chemokine release triggers T cell priming. In response to chemotherapeutics that mimic a viral infection, with regard to the liberation of nucleic acids from the dying cells, sensors for such molecules like the toll-like receptor-3 (TLR3), transcriptionally activate one or several type-1 interferon genes. Once secreted, the interferon stimulates the type-1 interferon receptor (IFNAR) to induce a multipronged type-1 interferon response including the production of C-X-C motif chemokine ligand 10 (CXCL10), which acts on the C-X-C motif chemokine receptor 3 (CXCR3) to recruit T lymphocytes into the tumor bed finally leading to a γδ T cell-mediated priming of αβ T cells. CXCR3, CXC-chemokine receptor 3; IFNAR1; interferon α/β-receptor subunit 1; IL, interleukin

underscoring the importance of the type-1 interferon response for therapeutic outcome in mouse tumor models.

At least in breast cancer patients, the aforementioned pathway seems to be therapeutically relevant. Thus, MX1 expression is induced by chemotherapy in vivo. The absence of signaling through IFNAR, indicated by the lack of signal transducer and activator of transcription 1 (STAT1) phosphorylation [96] or low MX1 expression, constitutes a poor prognostic feature, in particular in the context of anthracycline-based adjuvant chemotherapy [95]. Moreover, a polymorphism that affects the function of TLR3 reportedly influences the fate of breast cancer patients [97]. These results have to be interpreted in the context of mounting clinical evidences that type-1 interferons can be injected into patients to stimulate anticancer immune responses in the context of renal cancer and chronic myeloid leukemia (CML) [98].

12.7 Concluding Remarks and Perspective

As mentioned above, there are multiple DAMPs (such as ANXA1, ATP, CALR, HMGB1, and type-1 interferons) that function as adjuvant signals in the context of immunogenic chemotherapies (Fig. 12.6). It is important to note that these DAMPs do not act in a redundant fashion (in which case they would be able to replace each other) but in a non-redundant way, meaning that removal of one single DAMP (or its receptor) from the system is sufficient to undermine anticancer immunosurveillance elicited by immunogenic chemotherapies. One possibility to look at this problem is to postulate that each of the DAMPs must come into action following a defined spatiotemporary sequence, perhaps within a narrow range of intensity, following the "key-lock principle" [99]. Only if the DAMPs are expressed in the correct order, at the correct intensity, they are able to form the "key" that opens the vault that normally precludes an immune response [99]. Speculatively, this particular design of the system may reduce the probability of unwarranted autoinflammatory and autoimmune reactions in normal tissues [100]. On the other hand, this means that suppression of one single DAMP due to mutation (perhaps driven by immunoselection) or inhibition of one single DAMP receptor is sufficient to subvert anticancer immunosurveillance and to reduce the chance of cancer patients to control their disease upon chemotherapy.

Irrespective of these speculations, it is possible to measure all known DAMPs in cultured

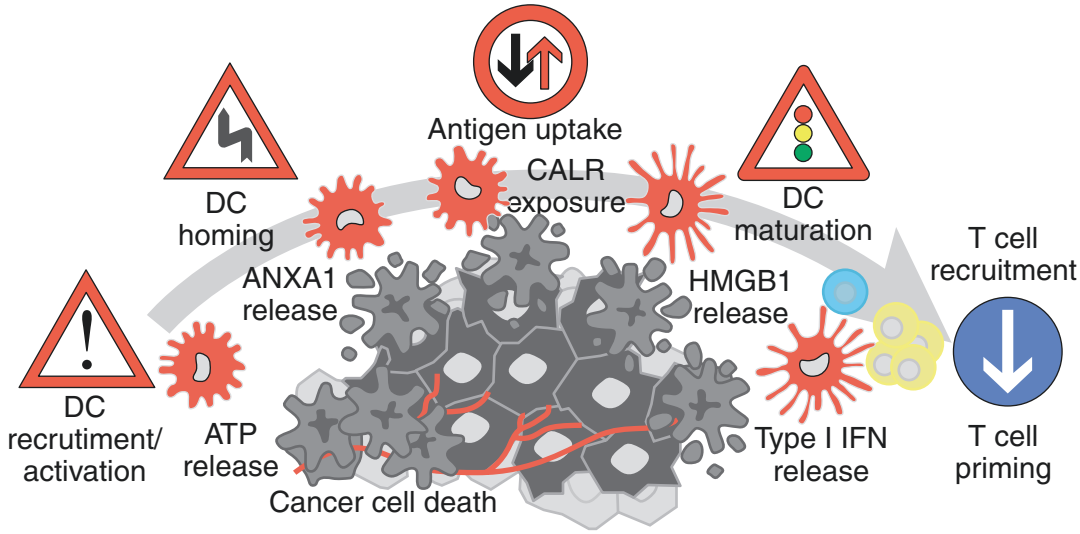

Fig. 12.6 Mechanisms of immunogenic cell death in therapy-induced immunosurveillance. Cancer cells undergoing immunogenic cell death (ICD) in response to chemotherapeutic treatments, such as doxorubicin or oxaliplatin, exhibit or release certain danger-associated molecular patterns (DAMPs) such as calreticulin (CALR) ATP, type I interferon (IFN), high-mobility group box 1 (HMGB1), and annexin A1 (ANXA1). Ligation of cognate receptors on the surface of myeloid or lymphoid cells facilitates the recruitment and activation of dendritic cells, their homing to the dying cancer cells, subsequent tumor antigen uptake, and final presentation (upon maturation of the dendritic cells). The production of immunostimulatory cytokines eventually leads to the onset of an adaptive immune response involving αβ and γδ T cells that reestablishes cancer immunosurveillance

cells exposed to libraries of anticancer agents to identify ICD inducers. In practical terms, this is achieved by generating biosensor cell lines that express fluorescent versions of ANXA1, CALR, or HMGB1 that have been fused to green fluorescent protein (GFP) or its derivatives. ATP release can be measured upon staining with chloroquine. The activation of the type-1 interferon response can be determined by placing GFP under the control of the MX1 promoter. Using this battery of biosensors, it is hence possible to select anticancer agents that stimulate all aspects of ICD. We have successfully used this approach to identify ICD inducers that are effective in stimulating anticancer immune responses in vivo, in mouse models [101–103].

It is tempting to speculate that such an approach may become even more useful in selecting successful anticancer drugs based on their ICD-stimulatory potential. Obviously, this approach would require additional in vivo experimentations in preclinical models while carefully avoiding the use of immunodeficient mice carrying xenotransplants like it was done in the past (see Sect. 12.1). Rather, anticancer drug candidates should always be evaluated in immunocompetent rodent models, including humanized mouse models. It is tempting to predict that this kind of approach will greatly reduce the attrition rate that has been characterizing the traditional drug development pipeline.

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13 Genetics and Immunology: Tumor-Specific Genetic Alterations as a Target for Immune Modulating Therapies

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Contents

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13.1 Introduction

Targeting the immune system therapeutically has been a long-standing approach in oncology treatment. The interaction between the immune system and cancer was already discovered in 1863 by Virchow, who hypothesized that sites of chronic inflammation are likely to be the origin of cancer [1]. More recently, immune evasion was announced a hallmark of cancer [2]. In the early phase of tumor induction, the immune system is still able to eliminate most of the cancer-initiating cells. However, the selection pressure for cells circumventing immune responses results in an equilibrium between immune attack and the growing tumor. Eventually, the cancer cells manage to evade the immune response via several immunosuppressive and escape mechanisms [2]. Therefore, overcoming the cancer-mediated immune escape using immune modulating therapies has become an appealing therapeutic option and the focus of oncology research. Currently, a wide range of immune modulating therapies for several tumor types are clinically available. These include interleukins, interferon, vaccination, dendritic cell therapy, adoptive T cell transfer as well as recently CAR T cell therapy, and immune checkpoint inhibitors. All mentioned therapeutic approaches improve different parts of the so-called cancer-immune cycle, whose proper function is required in order to generate an effective immune response. In

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more detail, an effective T cell-mediated antitumor response can only be mediated if antigens are released from the tumor and uptaken by antigen-presenting cells like dendritic cells. Next, the dendritic cells have to travel to the regional lymph nodes in order to present the antigens to naïve T cells and induce T cell activation. The affinity of the presented tumor antigens to major histocompatibility complexes (MHCs; defined by the human leukocyte antigen (HLA) haplotype) is critical in this step of immune response initiation in order to generate sufficient T cell activation. Furthermore, several regulatory T cell coreceptors have enhancing as well as suppressive regulating properties and prevent an overwhelming self-directed immune response. The activated T cells then enter circulation and travel to the tumor site, where they have to face the immunosuppressive properties of the local tumor microenvironment. Only if all steps of this cancer-immune cycle interact properly, an effective tumor-specific cytotoxic T cell immune response arises [3, 4]. Consequently, the cancer interferes with the cancer-immune cycle at several levels and thereby prevents the generation of an effective immune response. The understanding and selective targeting of improperly functioning steps in the cancer-immune cycle are therefore essential to overcome the immune escaping properties and generate a clinically meaningful response.

Interestingly, genetically similar tumors in regard to their active driver mutations and mutation signature respond very differently to immune modulating therapies. Consequently, durable responses after immune modulating therapies can be observed only in a fraction of an otherwise very homogenous patient population [5]. A deeper insight on the involved genetic alterations and their specific immunogenicity might facilitate highly personalized immunotherapy approaches. Factors such as genetic alterations like mutational load, presence of a mismatch repair deficiency, neo-antigen signatures, and mutations in certain immunological driver pathways were identified to correlate with the likelihood of response to immune checkpoint inhibitors [6–9]. However, besides their predictive potential for response to immune checkpoint inhibitors, genetic alterations can also serve as a treatment target for immune modulation therapies like mutation-specific vaccination with peptides or RNA/DNA and dendritic cells or tumor-specific adoptive T cell transfer, particularly chimeric antigen receptor (CAR) T cell therapy. Much less investigated in this matter, however certainly of high interest, are the genetic host characteristics like the individual immune status, the T cell receptor repertoire, or the HLA haplotype influencing the cancer-immune cycle and potentially supporting the cancer immune escape. Certainly, a deeper insight into the host genetics in addition to the specific tumor genetics is needed to formulate effective personalized immunotherapy approaches. The following chapter will concentrate on specific genetic characteristics of the tumor as well the host immune system associated with immunity and take vaccination as an example to introduce personalized immunotherapy.

13.2 Hot and Cold: How to Measure the Tumor Immunity

The immunogenicity of cancers can be measured by analyzing characteristics of the inflammatory tumor microenvironment. A very standardized approach is to conduct histological analyses of immune cell infiltration in the tumor tissue. Thereby, three typical patterns of immune response can be identified: immunologically "cold" (no active immune response) or "hot" (indicating a potential active immune response) tumors and tumors with a pronounced immune cell rim at the invasive margin but lack of infiltration in the tumor core (Fig. 13.1, I–III). These three phenotypes represent situations where the tumor has evaded the host immune system and has successfully grown to a detectable degree [2]. The theoretically fourth pattern of immune elimination of tumor cells by the host immune system is certainly never observed as only occurring in not clinically evident situations

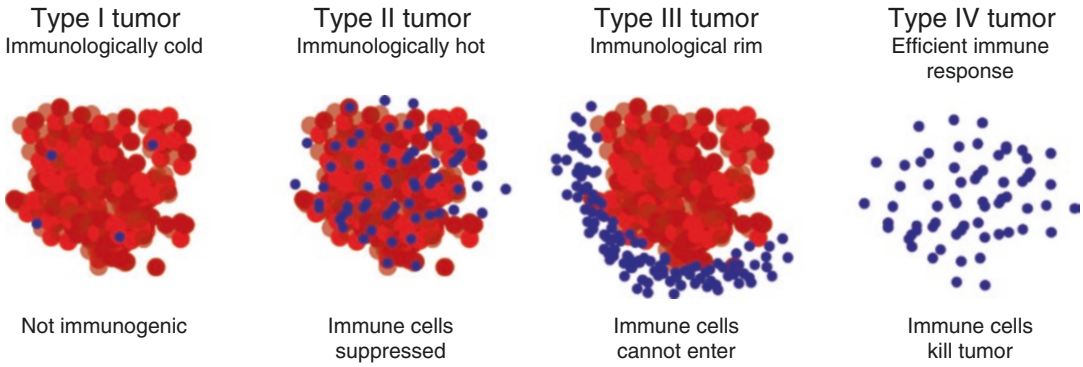

Fig. 13.1 Four phenotypes (I–IV) of host response to a tumor (adapted from [10])

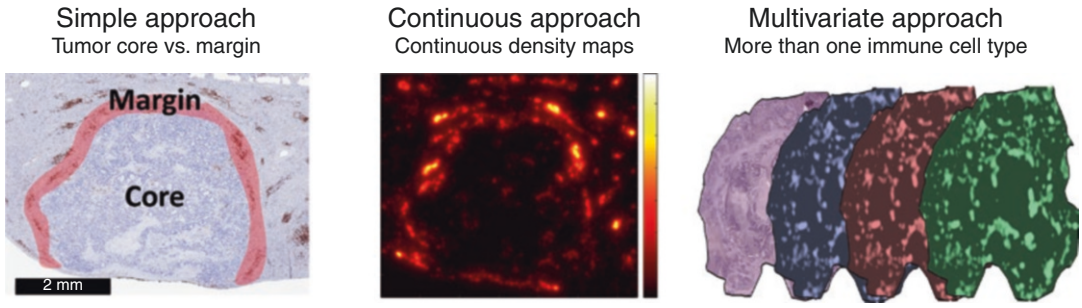

Fig. 13.2 Methodical approaches to quantification of immune cell infiltrates. *Left*: a standard method is to delineate a "core" region and a "margin" region and to count cells separately in each of these regions. *Middle*: cells can also be analyzed in a continuous way, enabling detailed analysis of spatial clustering. *Right*: Integrating multiple spatially aligned staining can yield information about co-localization of different immune cell subtypes

(Fig. 13.1, IV). It should be noted that these four types of immune infiltrates represent idealized situations, whereas in reality, these patterns can vary spatially and temporally in a given tumor.

Using immunohistochemistry, the cell types of these immune infiltrates can be defined: quantitatively, the most abundant immune cells are CD3+ lymphocytes (mostly composed of CD4+ T-helper cells, CD8+ cytotoxic T cells, and FOXP3+ regulatory T cells). Also, CD68+ and/or CD163+ macrophages are extremely abundant in many solid tumors. Enumeration of these cells, for example, by means of digital pathology, has proven to be effective in predicting survival, chemotherapy response, and immunotherapy response [11–13]. Additional to enumeration of cells in the invasive margin and the tumor core, other spatial analysis methods enable the definition of higher-order spatial features of immune cell infiltrates: continuous approaches for density analysis (Fig. 13.2, center) do not rely on predefined static regions, but enable a more fine-grained analysis of spatial patterns, including spatial clustering. Multivariate approaches (Fig. 13.2, right) enable the analysis of co-localization of different markers, thereby allowing to generate hypotheses about mechanistic interaction between different immune cell subtypes.

Interestingly, spatial patterns of immune infiltrates within or close to the tumor tissue only reflect one part of the host response to the tumor. It has been shown that host response can also be detected at a distance, most notably in "tertiary lymphoid structures" that emerge at a distance of up to several millimeters around solid tumors. These features are most notable in lung cancer and have also been detected in other entities such as colorectal cancer [14–16].

13.3 Genetic Characteristics of the Tumor Influencing the Host's Immune Response

Cancer is a genetic disease as the accumulation of several driver mutations eventually transforms a cell in a cancer cells. In general, the immune system identifies most cells with genetic aberrations and eliminates them in order to prevent the occurrence of malignant transformation. However, certain cancer-initiating cells manage to overcome this initial immune elimination and enter a process of equilibrium. Consequently, the selection pressure for cells able to evade the immune response rises, and eventually they escape the immune regulatory mechanisms [2]. Although these cancer cells have theoretically evaded the immune system, they remain an immunogenic target as the infiltration with immune cells in established cancer samples proofs [11, 14, 17].

Molecular cancer subgroups based on the presence of certain driver mutations were shown to correlate with characteristics of the inflammatory tumor microenvironment. Interestingly, several known targetable driver mutations, like EGFR mutation, estrogen overexpression, or presence of IDH mutation, are rather associated with a so-called "cold" inflammatory tumor microenvironment and subsequently rather low density of tumor-infiltrating lymphocytes and absence of programmed cell death ligand 1 (PD-L1) expression [18, 19]. Further, loss of *PTEN* was shown to enhance immune escape as increased expression of immunosuppressive cytokines results in reduced T cell-mediated tumor killing and T cell trafficking into the tumor. In line, application of a PI3Kβ Inhibitor reduced these immunosuppressive properties in preclinical models and as a consequence the efficacy of anti-PD1 and anti-CTLA4 treatments [20].

The genetic aberrations of a tumor are further the most important immunological target, as the immune system can detect peptides generated from aberrantly expressed genes or neo-antigens expressed as a direct consequence of somatic mutations. The resulting proteins are divergent from the germ line sequence and therefore entirely absent in the normal human genome. Importantly, while driver mutations directing malignant transformation are similar in a given tumor histology, the specific mutation signature and consequently the (neo-)antigen composure of any particular tumor is largely distinct, resulting in highly diverse (neo-)antigen signatures in tumors of the same histology [21]. These (neo-)antigens are displayed on the major histocompatibility complexes (MHCs; defined by the human leukocyte antigen (HLA) haplotype) on the surface of malignant cells and can thereby be identified by T cells, which are able to generate a tumor-specific cytotoxic immune reaction. However, the presence of (neo-)antigens does not equal a meaningful tumor-specific T cell reactivity [22]. Abnormalities of the MHCs in cancer cells including loss or mutations of genes encoding the MHC heavy chains as well as epigenetic modifications may impair the recognition of tumor cells by the immune system [23]. Furthermore, the presence of (neo-)antigens throughout a given tumor is not a static condition as the genomic landscape of a tumor is heterogeneous in space as well as in time. Spatial genetic heterogeneity is especially observed within a tumor bulk and at least partly explained by cell migration on a small scale [24]. In line, the immunogenicity of a tumor differs locally, resulting in a heterogeneous infiltration with immune cells as observed in various tumor entities. Immunosuppressive factors, like programmed cell death ligand 1 (PD-L1), frequently show a heterogeneous expression throughout a given tumor [25]. PDL1 expression in only 1% of tumor cells might already substantially impact the immunogenicity and is associated with survival prognosis in entities like breast cancer and lung cancer [26, 27]. In conclusion this data suggests that the spatially heterogeneous genetic landscape of tumors shapes the also spatially heterogeneous immune response.

Besides this spatial heterogeneity, the genetic characteristics of a given tumor change over time. Here, matched primary tumor and metastatic samples revealed marked genetic differences in a process called branched

evolution from the common ancestor cell to the primary tumor and the metastasis [28]. As a result, also the (neo-)antigen signature is not a static condition over time: first, due to the genetic evolution of the tumor itself and second due to immune editing as response to survival advantage of non-immunogenic cancer cell clones [2, 29].

13.4 Host-Specific Genetic Characteristics Determining the Tumor-Specific Immune Response

Importantly, besides the above outlined genetic characteristics of the tumor, also the characteristics of the host's immune system determine the efficacy of the resulting immune response. Density of tumor-infiltrating lymphocytes and more precisely preexisting CD8+ T cells in the invasive tumor margin are predictive markers for the response to PD1 checkpoint inhibitors as well as chemotherapy [13, 30]. However, the magnitude of T cell infiltration and the ratio of T cell subsets vary substantially between patients, despite tumor histology and location being similar. In line, identical to the genomic evolution of the tumor cells, also the T cell infiltration phenotype might underline a host-specific evolution generating the observed heterogeneity in tumor cell genotype and immune cell infiltration (Fig. 13.3).

The host factors' impact on the tumor-specific T cell response has been only partly understood and so far less intensively investigated compared to tumor-specific characteristics. One important factor here might be T cell receptor (TCR) repertoire, which is unique in every person as the TCR-β alleles are subjected to strict allelic exclusion. The TCR repertoire expressed in tumor-infiltrating T cells determines the degree of tumor reactivity and (neo-)antigen specificity, and a further insight could potentially function as predictive signature for immune modulating therapies [31]. The TCR clonality of the host is an important predictive factor for the response to PD1 checkpoint inhibitors [32].

Furthermore, adaptive immune responses within a certain organ might impact the host's immune cell phenotype. Organs with a comparably strict immune regulation, such as the brain, might actively restrict the generation of an active immune response. In line, the density of tumor-infiltrating lymphocytes in primary brain tumors is rather low and matched brain metastases present with a less active inflammatory tumor microenvironment compared to their matched primary lung cancer samples [33, 34].

The HLA haplotype is a further host-specific factor determining the anticancer immune response, and it defines the binding capacity with a certain (neo-)antigen and as a consequence the resulting T cell-mediated immune response. In line, vaccination strategies using specific, single neo-antigens are frequently restricted to patients with a certain HLA haplotype as only this certain combination has the potential to induce a specific immune response [35, 36]. A recently upcoming host factor influ-

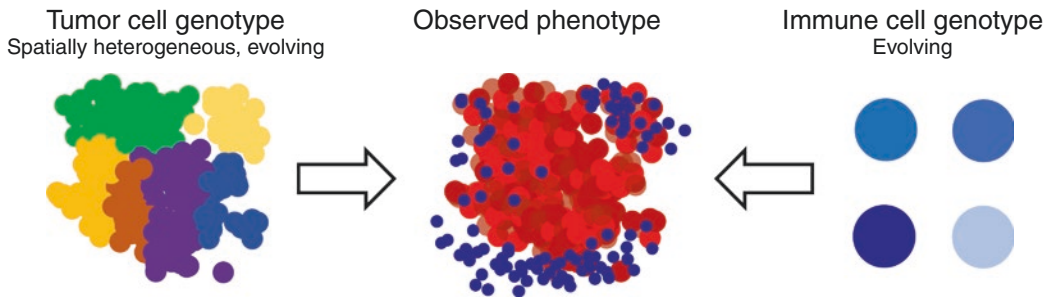

Fig. 13.3 Immune infiltrates in solid tumors typically show a spatial and temporal heterogeneity. This heterogeneity is driven by tumor factors (e.g., genetic heterogeneity) and host factors (e.g., adaptive immune response)

encing the cancer-specific immune response is the gut microbiome, and further studies concentrate on their interference as well as the therapeutic implications [37].

Further systemic host characteristics shaping the intensity of the tumor-specific immune response are circulating inflammatory cytokines like interleukin-6 that are associated with a chronic inflammatory state in cancer patients and induce cancer-associated cachexia [38]. In line, blood markers indicating chronic inflammation like increased C-reactive protein (CRP), increased white blood cell counts, or microcytic anemia are associated with impaired survival prognosis in several cancer types [39, 40]. High concentration of tumor necrosis factor alpha, interferon gamma, as well as the chemokine ligands CCL2, CCL4, or CXCL10 in the blood circulation might be associated with an increased tumor-supporting activity of circulating myeloid-derived suppressor cells [41, 42]. Liquid biomarkers like plasma level of soluble PDL1 were shown to be associated with poor prognosis and suppression of antitumor immunity in patients with advanced lung cancer [43]. In conclusion, not only specific factors of the tumor but rather the very personal interaction of a specific tumor and a specific host immune system determine the amplitude of an antitumor immune response.

13.5 Tumor-Specific Genetic Alterations as a Target for Vaccination

In contrast to more general immune activating therapies like immune checkpoint inhibitors or interferons, vaccination induces a more specifically directed immune response to distinct tumor epitopes [44]. Here, several techniques including active vaccination, meaning application of vital, active immune cells like dendritic cells, CAR T cells, or neo-epitope-specific T cells, or passive vaccination with peptides or RNA/DNA constructs either against one or multiple tumor-specific epitope(s) are applied (Table 13.1).

Active immunotherapy approaches use patient-derived cells, activate and expand them ex vivo, and transfer them back in the patient. This personalized immunotherapy requires demanding and complex methods, complicating the commercialization and conduction of large phase III clinical trials [44, 45]. Dendritic cell vaccination is an approved therapeutic approach, which has shown to be safe and generate responses, e.g., in melanoma, glioblastoma, and prostate cancer [46–48]. In brief, peripheral monocyte and dendritic precursor cells are taken from the patients and amplified ex vivo. Next, the monocytic cells are matured using an activation

Table 13.1 Vaccination approaches

Vaccination approach	Principle	Challenges
Mono neo-antigen	(Neo-)antigen vaccination of a frequently expressed peptide or RNA/DNA	Heterogeneity of antigen presence; selection pressure for cells without antigen; HLA haplotypes
Multi-neo-antigen	Vaccination of several frequently expressed (neo-)antigens	Heterogeneity of antigen presence; selection pressure for cells without antigen; HLA haplotypes
Mutanome specific	Identification of patients' specific (neo-) antigens and formulation of specific multi-neo-antigen vaccination	Identification of antigens with immunogenic properties; complex production
Dendritic cell vaccination	Ex vivo activation of patient-derived dendritic cell to increase antigen presentation	Activation with mono (neo-)antigen or tumor-specific lysate; complex production
Adoptive transfer of neo-epitope-specific T cells	Ex vivo expansion of tumor-infiltrating lymphocytes specifically reactive a certain (neo-)antigen	Complex production
CAR T cells	Genetically engineering of patient-derived T cells in order to express an antigen-specific T cell receptor	Heterogeneity of (neo-antigen) presence; selection pressure for cells without (neo-) antigen; complex production

cocktail containing pro-inflammatory cytokines, CD40 ligand, or TLR agonist. The matured dendritic cells are then loaded with tumor antigen either by inoculation with peptides, proteins or known tumor neo-antigens or with patients' specific tumor lysates [44, 49]. These activated, matured dendritic cells are reinfused in the patients and can induce a specific immune response by antigen presentation to T cells as well as support of B cell memory maintenance and activation of natural killer cells [47, 49]. So far, dendritic cell vaccination therapy has only showed a survival benefit in one prospective phase III study in metastatic castration-resistant prostate cancer [48].

Adoptive transfer of neo-antigen-specific T cells is another immunotherapy approach currently investigated in clinical trials and was shown to generate immune response also in tumor entities that are generally not prone to immune modulating therapies, like colorectal cancer and cholangiocarcinoma [50, 51]. Here, next-generation whole genome sequencing is used to reveal non-synonymous mutations potentially functioning as neo-epitopes. Dendritic antigen-presenting cells, retrieved from the specific patient, are then loaded in vitro to present these neo-epitopes to T cells isolated from the tumor tissue (tumor-infiltrating lymphocytes). T cells responding specifically to the presented neo-antigen are then expanded ex vivo and subsequently reinfused into the patient. Here, the neo-antigen-specific T cell can mount a tumor-specific immune response, resulting in the persisting presence of these antigen-specific T cells and potential tumor control [50, 51].

Another immunotherapy approach is the application of T cells with a chimeric antigen receptor (CAR) [44]. To generate an antigen-specific CAR T cell type, peripheral T cells are harvested from a patient. Next, the desired antigen-specific CAR is introduced in the T cells trough viral or nonviral methods resulting in T cells with genetically engineered, antigen-specific T cell receptor. The CAR T cells are expanded ex vivo and reintroduced in the patient [52, 53]. Here, CAR T cells can target cells expressing the antigen and enhance T cell effector function in an MHC-independent manner. Therefore, the HLA haplotype potentially does not affect the efficacy of CAR T cell therapy [52]. Neo-antigens like human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), mesothelin, and carcinoembryonic antigen (CEA) among others are currently tested as potential targets for CAR T cells in solid cancers [53–56]. Besides the already substantial clinical efficacy in hematologic malignancies, the first case reports also indicate efficacy in solid cancers and several studies are currently recruiting [54, 56].

Besides treatment approaches using ex vivo cultured patient-specific cells, several peptide- or RNA/DNA-based vaccination approaches have been postulated in order to increase the host's immune response by increasing the availability of (neo-)antigen(s). Mono neo-epitope vaccinations encompass one specific antigen of a given tumor. The targeted epitope has to be expressed in the majority of cancer cells to ensure that the initiated T cell response is effective. Clinical trials investigated, e.g., a HER2-specific vaccination in HER2 overexpressing breast cancer or gp100 vaccination in melanoma [57, 58]. However, the mono epitope-directed immune response results in a selection pressure of the antigen-positive cancer cells and provides a survival advantage to negative cells, potentially resulting in an immune escape. Furthermore, the affinity of the peptides to the MHC varies according to the HLA haplotype, restricting the efficacy of certain peptide vaccines to patients expressing a specific HLA haplotype [59].

A multi-epitope vaccination using several frequently present epitopes of a tumor can potentially generate a broader immune response and prevent immune escape more efficiently compared to mono epitope vaccination. A set of predefined epitopes, which have been identified to be frequently expressed in a certain tumor type, are included in the vaccination [60, 61]. However, as outlined before, the specific antigen signature of a certain patient is very unique although histology and location of the given tumors might be comparable [62]. Mutanome-specific vaccination provides an even more personalized vaccination approach: specific multiple

neo-epitopes can be identified in a given patient and facilitate the manufacturing of an individual vaccine [63]. These neo-epitopes can be identified either via genome sequencing and in silico antigen prediction or directly via mass spectrometry [63, 64]. Hereby, tumor heterogeneity is more adequately addressed, and clinical application is more broadly possible, as limitations like the presence of a certain antigen or HLA haplotype are reduced [64]. However, not all genetic alterations result in transcribed proteins and second in the generation of immunogenic epitopes. Only about 20–40% of (neo-)antigens are recognized by the immune system and result in the generation of a tumor-specific T cell response [64]. Therefore, a key question is how immunogenic cancer mutations of relevance can be identified and therapeutically explored in a personalized manner. Here, the numerical difference NetMHC score between the wild-type peptide and the mutated neo-antigen and the conformational stability of the MHC I and peptide interaction are essential to define the immunogenicity of a given neo-antigen [65].

Currently, several clinical trials are testing various neo-epitope vaccine approaches, either in monotherapy or combination. An important matter to discuss, as for the application for immune checkpoint inhibitors, is to identify which patients will most likely benefit from these specific vaccination approaches. Here, the extend of the disease should be taken into account. Vaccination, although generating a tumor-specific immune response, does likely not result in tumor shrinkage but rather control of existing tumor. Therefore, patients with minimal residual disease are likely to benefit most. Therefore, careful trial design is essential to identify patients benefiting most from vaccination-based treatment approaches.

13.6 Genetic Alterations of the Tumor as Predictive Biomarkers for Immune Response to Immune Checkpoint Inhibitors

Besides the high response rates observed in patients treated with CTLA4 and PD1 axis targeting immune checkpoint inhibitors, a significant fraction of patients does not respond. Therefore, accurate and precise predictive biomarkers are urgently needed to adequately select patients with the highest likelihood of response (Table 13.2).

Indeed, rather the total mutational load than the presence of specific alterations was shown to correlate with an immune response and increased likelihood of response to immune checkpoint inhibitors (Fig. 13.4) [7, 68]. In line, cancer types with high mutational load like melanoma or lung adenocarcinoma are more sensitive to immune modulating therapies in comparison to tumors with less frequent mutations like, e.g., breast cancer [63]. Cancer types induced by a high carcinogenic exposure like melanoma trough UV radiation or lung cancer in smokers, or virally induced cancer types such as head and neck squamous cell carcinoma present with a particularly high mutational burden [69]. Interestingly, the mutational load differs greatly between the individual cancers, although other characteristics like histology, location, and presence of driver mutations are very similar [8].

Table 13.2 Genetic alterations of the tumor as predictive biomarkers for immune checkpoint inhibitor based therapy

Predictive marker	Immune modulating therapy	Entity	References
Number of somatic mutations	CTLA4 and PD1 blockade	Melanoma, non-small cell lung cancer	[7, 8]
Mismatch repair deficiency	PD1 blockade	Colorectal cancer	[66]
Neo-antigen signature/load	PD1 and CTLA4 blockade	Lung adenocarcinoma, melanoma	[6, 9, 67]
Copy number loss	CTLA4 blockade	Melanoma	[32]

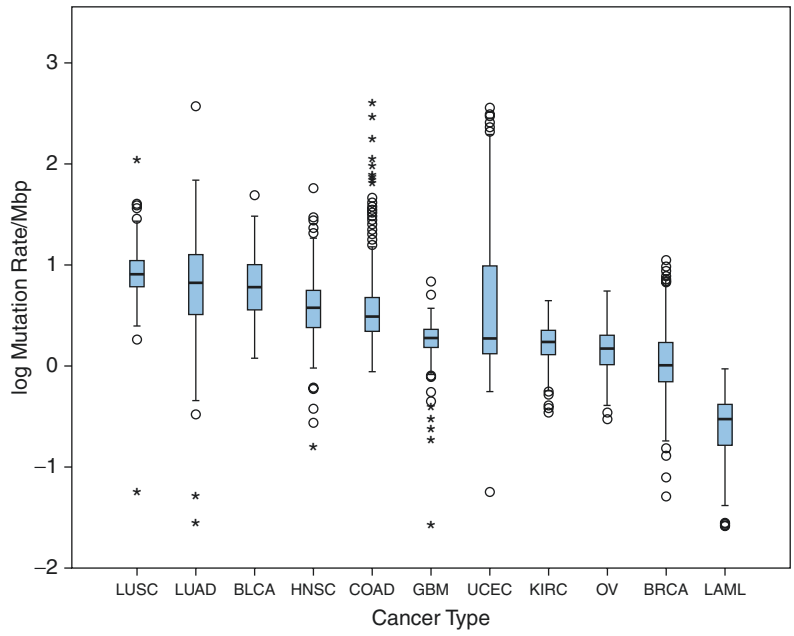

Fig. 13.4 Mutational load of the different primary tumor types, adapted from [69]; *LUSC* lung squamous cell carcinoma, *LUAD* lung adenocarcinoma, *BLCA* bladder urothelial carcinoma, *HNSC* head and neck squamous cell carcinoma, *COAD* colon adenocarcinoma, *GBM* glioblastoma, *UCEC* uterine corpus endometrial carcinoma, *KIRC* kidney renal clear cell carcinoma, *OV* ovarian serous cystadenocarcinoma, *BRCA* breast invasive carcinoma, *LAML* acute myeloid leukemia

Some cancer types with a rather low mutational burden, like colorectal cancer or glioblastoma, might respond impressively in the presence of certain specific genetic subtypes like the microsatellite instability or mismatch repair deficiency [66, 70, 71]. However, also cancer types with a lower median number of somatic mutations have shown impressive and durable responses like renal cell carcinoma, arguing that besides the raw number of mutations, the immunological quality of the mutations might influence the cancer immunosurveillance. More precisely, the mutational load correlates with immunogenicity of a given tumor as each additional mutation increases the odds that a relevant patient-specific cancer neo-antigen is created.

Indeed, a high number of mutations and a high load of neo-antigens are associated with increased infiltration of CD8+ tumor-infiltrating lymphocytes and improved prognosis [62, 72]. This neo-antigen signature can vary in its immunogenicity depending on the affinity to the MHC complex, the difference to wild-type peptides, and the heterogeneity of the neo-antigen within the tumor. The majority of mutations found in melanoma and other tumors with a high mutational load are "passenger mutations" that are unrelated to the cellular transformation process. As such, the vast majority of potential neo-epitopes in these cancers are patient-specific. In melanoma patients responding to CTLA4 inhibitors, a specific neo-antigen signature of neo-epitopes homologous to many viral and bacterial antigens was discovered [9]. Whether this particular neo-antigen signature also has predictive potential in other entities as well as the definitive cutoff values defining a tumor with high mutational load remains unknown [8, 9].

Furthermore, the burden of copy number loss was shown to correlate with likelihood of response to CTLA4 inhibition. Here, copy number loss is associated with melanoma progression and might therefore be one cause of immune escape as a correlation of copy number loss and downregulation of immune-related gene expression was found [32]. Interestingly, effects of low copy number loss and high mutational load had nonredundant effects on the clinical response, indicating that the combination of several predictive markers could potentially provide a more

precise prediction of response [32]. Some truncating genetic alterations encoding the interferon-receptor-associated Janus kinases 1 (JAK1) and 2 (JAK2) were shown to correlate with acquired resistance to anti-PD1 immune checkpoint inhibitors [29].

13.7 Personalized Immunotherapy: Combination of Immune Modulation Therapies

Although encouraging response rates have been observed with immune modulating therapies, the majority of patients still will not benefit in the long term from currently available treatments. This might be due to several simultaneously active immune escaping factors of the tumor in addition to the host-specific factors. Combination of immune modulation therapies with each other or with other established treatment options can enhance the interaction of host and cancer by improving different steps of the cancer-immune cycle [4].

A lack of available antigen can be overcome by vaccination with tumor-specific antigens. Indeed, preclinical studies suggest a synergistic effect of vaccination with checkpoint inhibition, although a clinical study investigating the combination of CTLA4 inhibition and gp100 vaccination did not show a clinically meaningful additional efficacy [57, 73]. However, the recent insights on peptide vaccination therapies including the establishment of mutanome-specific vaccinations might provide a better combination partner for immune checkpoint inhibitors.

The so-called abscopal effect argues for the reasonable combination of radiotherapy and immune checkpoint inhibitors [74]. In theory, radiotherapy first increases the availability of antigens by apoptosis of tumor cells inducing tumor-specific T cell responses. In addition, radiotherapy has several local effects on the inflammatory microenvironment and induces the infiltration of the tumor with antigen-presenting cells, macrophages, and cytotoxic T cells [10, 75]. Therefore, vaccination and radiation but also other therapies resulting in increased antigen presentation can overcome insufficient antigen transport, and presentation and combination with immune checkpoint inhibitors might be synergistically [76, 77]. However, today little is known about how to identify patients with insufficient antigen presentation, as no reliable markers to quantify efficacy of antigen presentation have been proven.

As outlined above, a tumor-specific immune response is highly regulated as several TCR co-receptors control the resulting T cell response with either enhancing or suppressive signals [78]. The PD1 and CTLA4 axis are both T cell response suppressing pathways, actually reducing the activation of T cells. In line, the inhibition results in an "unleashing" of the tumor-specific T cell response [78]. However, PD1 checkpoint inhibition results in upregulation of other immunosuppressive immune checkpoint pathways like TIM3 [59]. Therefore, combination of two checkpoint inhibitors might overcome this immune escape mechanism. The combination of CTLA4 and PD1 blockade was shown to be more clinically effective compared to either checkpoint inhibitor alone, although in light of a higher toxicity rate [79, 80]. Further, CTLA4 therapy was shown to increase TCR clonality present in the tumor and thereby enhance the likelihood of response to PD1-directed therapies [32]. Several studies are currently investigating the clinical efficacy of second-generation checkpoint inhibitors targeting, e.g., TIM3, LAG3, OX40, and others in combination with first-generation checkpoint inhibitors.

Further combinations aim to increase the intra-tumoral T cell density in addition to an immune checkpoint inhibitor. The PI3K and MEK pathways were shown to influence the density of T cell within the tumor tissue [20, 81]. Addition of MEK tyrosine kinase inhibitors to immune checkpoint inhibitors might therefore result in increased density to tumor-infiltrating lymphocytes, thereby converting "cold" tumors into "hot" ones, which are then more susceptible for immune checkpoint inhibitor therapy [82].

A further promising combination is the addition of immune checkpoint inhibitors to standard

chemotherapy regimes, as many standard chemotherapies have been shown to have immune modulating function [44]. The response rate as well as the progression-free survival was increased significantly by the combination of standard platinum-based chemotherapy and a PD1 inhibitor [83, 84].

Lastly, in order to generate a durable adaptive immune response, the innate immune system has to be considered as well. It has long been known that macrophages and neutrophils modulate T cell activity in a clinically relevant way [85]. Recently, it was shown that macrophage repolarization can lead to an increased lymphocyte response against the tumor in otherwise non-immunogenic tumors [86, 87]. Currently, clinical trials exploiting these regulatory axes are ongoing.

In summary, cancer immunotherapy is rapidly changing from single-agent approaches toward combination therapies addressing the patient-specific interactions between the immune system and the given cancer [88]. The combination of immune modulating therapies certainly creates another layer of complexity that has to be addressed by better preclinical understanding of the underlying biology and resulting reasonable biomarkers. Here, several genetic properties of the tumor itself as outlined above but also host-specific factors and dynamic factors caused by the interaction of specific host and tumor have to be incorporated in potential predictive algorithms. In depth characterization of the inflammatory tumor microenvironment, genetic characteristics of the tumor and the host have to be assessed and further analyzed to develop predictive biomarker signatures. We have to get used to monitor immediate treatment effects in sequential tumor biopsies to better understand the mechanisms of response, even more important to understand the mechanisms of resistance in the individual patient. Such data will be very important to feed our prediction algorithms. Ultimately, we can envision clinical trial strategies where every patient is receiving a different drug combination that might even change in sequence. Real-time assessment of treatment effects on the tumor will help to constantly adopt and modify the treatment strategy in the individual patient.

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Part II

Breakthrough Status

Peptide-Based Therapeutic Cancer Vaccines

14

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Contents

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14.1 Introduction

Peptides have attracted much interest as a platform for the induction of therapeutic T cell responses, ever since the discovery of MHC-presented protein fragments (peptides) as the antigenic basis for T cell recognition [1–4]. CD8+ cytotoxic T lymphocytes (Tc) recognize short peptides of 8–11 amino acids in length, presented by MHC class I molecules (HLA class I in human beings), expressed on all nucleated cell types. CD4+ T helper cells (Th) recognize the combination of slightly longer peptides of 12–15 amino acids in length in the context of HLA class II molecules, expressed mainly on cells of the immune system such as dendritic cells (DC), macrophages, and B cells [5]. Since T cells recognize these antigen fragments (epitopes) only when expressed on cell surfaces by HLA molecules tethered to cell membranes, T cells are not distracted by free antigen in body fluids, but are specialized in the recognition and destruction of cells presenting abnormal (microbial, nonself) fragments by T cell receptors on T cells that have not been deleted in the thymus by central tolerance. Importantly T cell can recognize fragments of proteins expressed at any cellular location, including intracellular ones, providing an ideal surveillance system covering sampling and display from even the remotest cellular locations, such as the nucleus. As such T cells are the specific adaptive cell-mediated immunity arm to combat intracellular pathogens

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and cancer cells. Because these tolerance-inducing mechanisms are not foolproof, therapeutic T cells can also be directed to antigenic fragments that are derived from overexpressed self-antigens or differentiation antigens, but blunting of the T cell repertoire for these antigens by tolerance may be a problem (reviewed in [6]).

14.2 Mode of Action of Therapeutic Cancer Vaccines

Therapeutic cancer vaccines need to induce powerful tumor-killing T cell responses, which can be achieved by subcutaneous, intradermal, or intramuscular injection of different vaccine modalities: DNA, RNA, synthetic long peptides (SLP), or recombinant viruses containing the sequences of tumor-associated antigens of choice. The vaccine antigens need to gain efficient access to DC (e.g., epidermal or dermal DC). These DC then migrate to vaccine-draining lymph nodes through afferent lymphatic vessels and settle into T cell areas of these lymph nodes to initiate T cell responses by appropriate contact with CD4 and CD8 T cells. Properly activated T cells leave the lymph nodes through efferent lymphatic vessels and eventually reach the blood stream through the thoracic duct. From the blood they may or may not infiltrate into antigen-bearing tumor sites. Usually the T cells there have to cope with a hostile microenvironment, including regulatory T cells, T cell checkpoint ligands such as PD-L1, myeloid suppressor cells, and factors released by T cells such as immunosuppressive cytokines and chemokines and indoleamine 2,3-dioxygenase (IDO) (reviewed in [6]. Together these conditions impair T cell migration, function, and proliferation (Fig. 14.1).

14.3 What Makes a Good Therapeutic Cancer Vaccine?

Recently we have reviewed the factors that determine success or failure of cancer vaccines [6, 7]. A successful cancer vaccine has to meet each of four requirements: (1) selection of the right tumor antigens, (2) choice of an efficient vaccine platform, (3) use of the proper vaccine adjuvant(s), and (4) use in combination with the proper cotreatment. The critical success factors and the corresponding choices to be made for success are listed in Table 14.1.

With respect to the choice of the right antigens, cancer-associated antigens that have not been subject to central thymic tolerance-driving mechanisms are favored, in particular the antigens encoded by cancer viruses such as human papillomavirus (HPV), Epstein-Barr virus, human T-lymphotropic virus I (HTLV-I), hepatitis B virus (HBV), hepatitis C virus (HCV), Kaposi sarcoma virus (KSV), and Merkel cell carcinoma virus. The most important antigens encoded by these viruses have been reviewed in [6]. Together cancer viruses are the cause of approximately 20% of all cancer worldwide. An emerging and very important category of antigens expressed by an even larger proportion of human cancers are neo-antigens caused by mutations in cancer cells induced by, e.g., chemical carcinogens in cigarette smoke (lung cancers, H&N squamous cell cancers, bladder cancers), UV light (melanomas and basal cell carcinomas of the skin), or DNA repair deficiencies (e.g., microsatellite instability colorectal cancers). The importance of neo-antigens as highly effective targets for cancer-specific T cells follows from the marked correlation observed between clinical efficacy of checkpoint blocking monoclonal antibodies against CTLA-4 or PD-1 and the number of mutations in cancers of individual patients [8–10]. In one patient successful therapy with anti-CTLA-4 antibody was indeed associated with the expansion of a major tumoricidal Tc population directed against a UV-induced mutation [11]. In mouse models it was possible to predict and validate mutation-derived neo-antigens and vaccinate successfully with neo-antigen-containing SLP [12] [13, 14] or an RNA-based vaccine [15]. Similar efforts have been applied in a first proof of concept of immunogenicity of neo-antigens in the clinic with peptide-loaded DC [16]. Although the non-

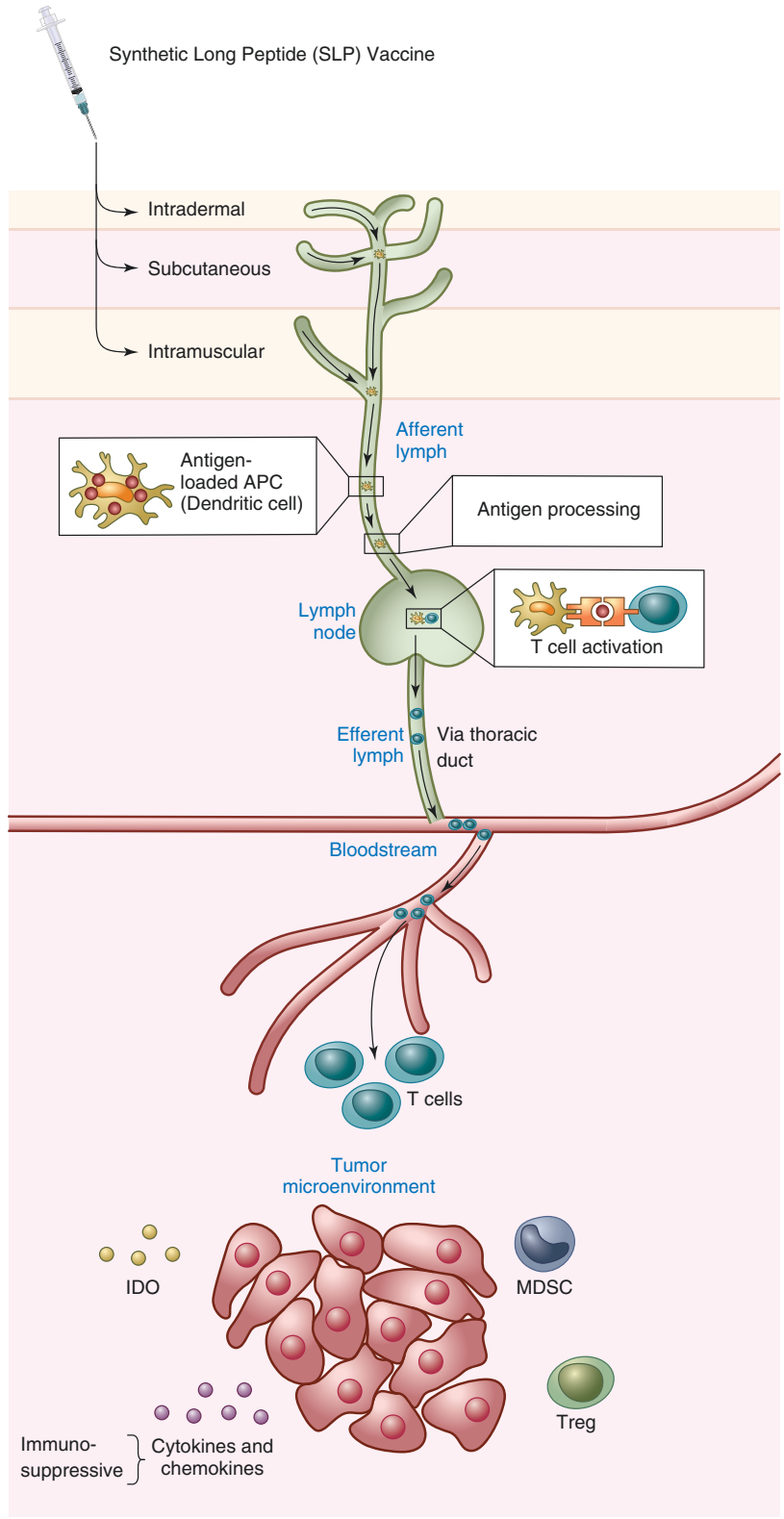

Fig. 14.1 Mode of action of synthetic long peptide vaccination. The tumor-associated antigens are efficiently delivered by SLP easily obtaining access to DC in the vaccine injection area. Antigen-loaded DC migrate via afferent lymphatics to vaccine-draining lymph nodes to settle into *para*-cortical T cell areas, where antigen processing from ingested SLP continues, after which they engage in antigen presentation to CD4+ and CD8+ T cells. Activated T cells leave the lymph nodes via efferent lymphatic vessels ideally migrating to sites of tumor antigen expression in tumors. There a hostile environment is encountered, including hostile cellular elements such as myeloid-derived suppressor cells (MDSC) and Tregs as well as immunosuppressive chemokines and cytokines, expression of the enzyme IDO, and ligands for inhibitory T cell checkpoints like PD-1 and LAG-3. Together these conditions impair T cell migration, function, and expansion. (Adapted and updated from Melief et al. [6]

Table 14.1 Critical success factors in development of therapeutic cancer vaccines

Selection of the right tumor-associated antigens	E.g., viral targets, neo-antigens
	Avoid (central) tolerance
Choice of an efficient vaccine platform	Avoid antigenic competition: concentrated , defined and quantified pure cancer antigen source (DNA, RNA, SLP)
	Avoid inefficiency and undefined nature of protein/RNA cancer cell extracts
	Exploit excellent processing by dendritic cells in vivo: durable CD4 and CD8 responses
Use of the proper built-in or added adjuvant(s)	Robust DC activating agents that strengthen and broaden T-cell response
	Th1 polarization
Use with proper combination treatment	Drive tumoricidal T cells and overcome hostile cancer micro-environment
	Chemotherapy, Checkpoint blockers, TNFR family agonists, gammaC cytokines, TGFβ antagonists

self-antigens of viruses and mutation-derived neo-epitopes are favored cancer vaccine ingredients, the T cell repertoire against many over-expressed antigens of the cancer-testis variety, against differentiation antigens, and against antigens such as survivin, Wilms tumor 1 (WT-1), wild-type or mutant p53, and mesothelin may be sufficient to induce robust T cell responses with antitumor activity (reviewed in [6, 7].

14.4 T Cell Epitope-Based Vaccination

Classical preventive vaccines consist of heat-killed or attenuated microorganisms that express properly folded proteins to induce protective antibodies against conformational antigens. Therapeutic vaccines, however, need to induce CD4 and CD8 T cell responses against defined T cell epitopes, presented by HLA class II or I molecules. The epitopes only assume the right conformation when bound to the HLA molecules. Peptide vaccines for induction of T cell responses thus consist of the exact length peptide constituting an epitope, or of a longer peptide (synthetic long peptide, SLP) that contains one or more epitopes, but needs further processing by antigen-presenting dendritic cells (DC) for proper binding of the precise epitope(s) into HLA molecules. Both exact fitting peptides (short peptides) and SLP (long peptides) have been used in therapeutic vaccines, but SLP are preferred for reasons outlined in the next paragraph. An advantage of epitope-based vaccines is that only amino acid sequences of epitope-rich regions of proteins need to be represented, and those segments of proteins that do not contain appreciable numbers of T cell epitopes can be omitted. Quite apart from containing immunologically silent sequences, proteins are also poorly processed by DC for presentation (so-called cross-presentation) to T cells, in particular CD8 T cells, whereas SLP are efficiently processed for presentation by HLA class II and class I, respectively, to CD4+ and CD8+ T cells [17]. In composing SLP vaccines, it is important to include epitopes capable of binding to the most common HLA class I and II molecules. To this end enough length of amino acid sequence needs to be represented in the peptide vaccine to harbor binding motifs for the most common HLA class I and II molecules, associated with the capacity to become efficiently processed by either the proteasome in the cytosol, followed by transport through the transporter of antigen processing (TAP) (HLA class I processing pathway) or cathepsins in the endosomes (HLA class II processing pathway) of DC [5].

14.5 Short Peptides Versus Long Peptides for Cancer Vaccines

Initial attempts with peptide vaccines involved single MHC class I binding epitopes + adjuvant in mice. Although such vaccinations showed antiviral or antitumor activity in several instances [18–20], reviewed in [21], such short peptide vaccines (<15 amino acids) are clearly suboptimal for a number of reasons. First, short peptides

can directly bind exogenously to HLA class I molecules of all nucleated cells without antigen processing. Consequently, short vaccine peptides will end up at high concentrations in HLA class I molecules of nonprofessional antigen-presenting cells (APC) in the absence of co-stimulatory molecules, causing either short-lived Tc responses or even Tc tolerance [22–24]. A second disadvantage of exact class I binding short peptide vaccines is the absence of class II epitopes for CD4+ Th cell activation. Such Th cells are needed for optimal induction and maintenance of Tc effector responses ([25–29] and for generation of Tc memory cells [30–32]. Also, CD4+ T cells can themselves exert important antiviral or antitumor effector functions [26] [33]. Vaccination with short peptides formulated in incomplete Freund adjuvant (IFA) leads to weak or abortive T cell responses [22, 33–35], in part because of the previous problems, compounded by the fact that T cells elicited by short peptides in IFA travel to the vaccine site rather than to the tumor [34]. Vaccination with SLP (>20 amino acids long), but not short peptides in IFA or the related adjuvant Montanide ISA-51, in contrast, leads to sustained CD8+ T cell responses and robust antitumor immunity [22, 34, 36, 37]. Indeed vaccination with SLP in IFA or Montanide supports robust and sustained effector and memory T cell responses also in patients with premalignant lesions or cancer [38–43]. To some extent tolerance induction by short peptides can be overcome by vaccination with mature DC, loaded ex vivo with short peptides prior to injection [44, 45] or replacement of the Th signal with agonist antibody against CD40, with or without TLR3 ligand poly I:C [46, 47]. Upon subcutaneous injection of SLP, or in vitro, DC, but not other types of cells, are capable of effectively processing the SLP for effective MHC class I and II presentation [17, 48, 49]. In addition processing of SLP for MHC class I or II presentation is much more efficient than that of protein [17, 49]. SLP are capable of induction of both potent CD4+ and CD8+ responses in preclinical and clinical settings [36, 38, 39, 42, 50–52], whereas vaccination with intact proteins is associated with demonstrable CD4+ but not CD8+ responses [53, 54].

14.6 Alternative Vaccination Platforms

Delivery of concentrated antigen in a platform that allows efficient access to DC for HLA class I and class II presentation is important for any successful therapeutic vaccine aiming at an effective level of therapeutic T cells [6, 55]. Next to SLP, other powerful platforms are DNA or RNA vaccines. DNA vaccination is usually delivered with electroporation, as in two types of therapeutic vaccines against lesions caused by high-risk HPV16 [56, 57], and a successful RNA platform is delivery of tumor antigen-encoding RNA in cationic liposomes [15]. Viral vectors have also been used for therapeutic cancer vaccination. Viral vectors contain vector sequences that show antigenic competition with the inserted tumor antigens [58, 59], a problem that can be overcome to some degree by priming and boosting with two different viral vectors, the immune responses against which cross-react at the boost level against the inserted tumor antigen, but not at the vector level as in the PROSTVAC therapeutic vaccine directed against prostate-specific antigen (PSA) [60]. Interestingly, SLP can also be used to effectively boost CD4 and CD8 T cell immune responses in *Rhesus macaques* against conserved HIV antigens after priming with a replication competent poxvirus (NYVAC-C-KC) encoding conserved HIV antigen sequences [61].

14.7 Efficacy of SLP Vaccination Depends on Addition of a Strong Adjuvant

Although SLP enter easily into DC for processing into the HLA class I and II pathways, these DC need to be activated by a powerful adjuvant into mature DC that express not only the proper HLA-peptide complexes but also the necessary co-stimulatory molecules for induction of proper effector and memory CD4+ and CD8+ T cell responses [36]. Adjuvants that serve this purpose are poly I:CLC (TLR3 ligand), CpG (TLR9 ligand), incomplete Freund's adjuvant-like Montanide

ISA-51, or stimulator of IFN genes (STING) agonists (reviewed in [6]). The action of SLP vaccines can be further enhanced by attaching a TLR ligand such as CpG or Pam3Cys, a TLR 1/2 ligand, to the SLP by covalent coupling [62–64].

14.8 Clinical Efficacy of SLP Vaccines in Patients with Premalignant Disease

SLP vaccination appears to be very efficient to eradicate premalignant lesions caused by high-risk human papillomavirus type 16 (HPV16). HPV16 causes approximately 50% of cervical cancers and the large majority of HPV-positive head and neck cancers. Robust CD4+ and CD8+ T cell responses were induced in our studies of vulvar intraepithelial neoplasia, a premalignant disease of the vulva, caused by HPV16. In this case a vaccine containing 13 overlapping long peptides (25–35 amino acids long, together covering the entire amino acid sequence of 257 amino acids of the combined HPV16 E6 and E7 oncogenic proteins), was delivered subcutaneously as an emulsion in the mineral oil adjuvant Montanide ISA-51 at a dose of 300 μg per peptide. More than 50% of the patients benefited from partial or complete regression of lesions [38, 42]. Moreover we observed a highly significant correlation between the strength of the vaccine-induced T cell response and the clinical response [38, 42]. Recently, we reported similar results in twice the number of VIN patients [41]. Again a strong correlation between vaccine-induced T cell responses and clinical responses was observed, and those patients with histologically complete lesion regression had also lost demonstrable papillomavirus in the original skin lesions sites [41]. In patients with cervical cancer, the same vaccine was not as immunogenic, and clinical benefit could not be demonstrated in late-stage (recurrent, metastatic) cervical cancer patients [65], pointing toward an immunologically compromised situation in these patients and the need for additional combination therapy (see below).

14.9 Clinical Efficacy of Therapeutic SLP Vaccines in Cancer Patients Requires Combination Treatment That Overcomes the Hostility Toward T Cells Within Cancers

If the T cells that have responded to tumor-associated antigens do not succeed in eradicating cancer cells right away, a chronic situation may arise which has been well described by Schreiber and colleagues as an equilibrium between tumor cells and the immune system, manifesting itself as latency of tumors due to an ongoing T cell immune response [66]. Eventually tumors may escape from control by T cells through a variety of mechanisms (reviewed by [7, 66]). One of the least surprising conditions that allows tumors to escape is that the microenvironment in which tumors arise characteristically does not set the stage for robust effector T cell and memory T cell responses of the type seen in acute viral infections. Acute viruses incite a spate of immediate powerful danger signals generated by the virus, which strongly activate the innate and adaptive immune systems. Interestingly many cancers caused by oncogenic viruses start with long-term (many years!) persistent infection with a cancer virus such as human papillomavirus (HPV) or hepatitis B virus (HBV). Chronic persistent viruses employ many immune evasion and stealth mechanisms that undermine both innate and adaptive immunity to establish persistent infection (reviewed for HPV in [67]. Also the T cell host defense machinery adapts to chronic persistent infection by avoiding immunopathology through T cell checkpoint regulation [68–70] [7]. This of course has dire consequences if the persistent virus happens to have oncogenic properties. T cell immunogenic tumors with many mutations employ very similar mechanisms, also engaging in T cell checkpoint regulation and antigen loss [13, 71, 72]. Usually escape through toning down of the immune response is brought about by interaction between an ongoing T cell immune response and

the cancer cells. For example, PD-L1 expression is induced on cancer cells by interferon γ released from antigen-specific tumor-infiltrating T cells (reviewed by [73]. Some of the most dangerous escape mechanisms from T cell immunity are complete loss of HLA class I expression from cancer cells by deletion of β2 microglobulin, an essential molecule for expression of HLA class I at the cell surface or loss of responsiveness of tumor cells to interferon γ [74]. Fortunately these instances are relatively rare. In fact in most cases countermeasures against immune escape are possible. For example, T cell–intrinsic inhibition of function can involve other mechanisms than PD-1-PD-L1 inhibition such as egr2-driven expression and inhibition by LAG3 and 4-1BB (CD137). If this is the case, rescue of tumoricidal T cell activity is possible by inhibition of LAG3 and application of agonistic antibody against 4–1 BB [75]. Also, in case of loss of an immuno-dominant neo-antigen, second in line neo-antigens in the hierarchy of immuno-dominance can take over, particularly following vaccination against these subdominant neo-antigens or following PD-1 checkpoint blocking [13]. This last example illustrates that spontaneous generation of sufficient T cells to tumor-associated neo-antigens may be lacking in many potentially immunogenic tumors that are not spontaneously rejected. Likewise an HPV16 E6/E7-positive tumor in mice does not spontaneously generate TIL that infiltrate the tumor and eradicate it, even though the viral antigens are expressed by the tumor. However a combination of cisplatin or carboplatin and paclitaxel chemotherapy and HPV-specific SLP vaccination is able to generate tumor-infiltrating HPV-specific TIL that completely eradicate established tumors [43, 76]. The mechanisms by which this type of chemo-immunotherapy operates are manifold and include immunogenic cell death causing DC activation (reviewed in [77]), synergistic apoptosis by TNF-α, released in the cancer microenvironment from vaccine-induced TIL in combination with cisplatin [76, 78], and depletion of immunosuppressive mononuclear myeloid cells that are present in excessive numbers in the blood and probably the cancers of many late-stage cancer patients [43]. An attractive choice for combination with therapeutic vaccines is the combination with checkpoint blockers, in particular anti-PD-1. Synergy between therapeutic vaccines and anti-PD-1 checkpoint blocking has been noted in a number of preclinical models [52, 79, 80]. Vaccination with autologous DC loaded with melanoma-associated antigens may act synergistically with CTLA-4 blocker ipilimumab in mediating clinical responses in late-stage melanoma patients [81], but this observation needs to be confirmed in a randomized study. Synergy has also been reported between therapeutic vaccines and one of the following agonistic monoclonal antibodies: anti-CD27, anti-CD40, anti-CD134 (anti-OX40), or anti-CD-137 (anti4-1BB) (reviewed in [6]). In addition inhibitory monoclonal antibodies have been used against the cytokines IL-10, TGFβ, and IL-6 (reviewed in [6]). An overview of the different attractive combination therapies with therapeutic vaccines is shown in Fig. 14.2.

Recently we showed in a pilot trial in patients with late-stage (recurrent or metastatic) cervical cancer that much better HPV16-specific T cell responses could be induced by SLP vaccination in Montanide adjuvant by combination with standard of care chemotherapy consisting of carboplatin and paclitaxel [43]. The chemotherapy acted as an immunomodulator in the sense that T cell numbers and function were not affected by this chemotherapy combination, but the suppressive mononuclear myeloid cell levels found in all patients declined to those of healthy donors onward from 2 weeks into the second of six cycles of chemotherapy. A single vaccine dose given at that time point led to a much higher and broader T cell response against HPV16 E6/E7 [43] than observed in the previous trial without timed chemotherapy [65]. Based on these observations, we have now concluded a much larger clinical trial in 70 patients with late-stage (recurrent or metastatic) HPV16+ cervical cancer. Interestingly, in this trial the first of three vaccine doses with the HPV16 SLP vaccine,

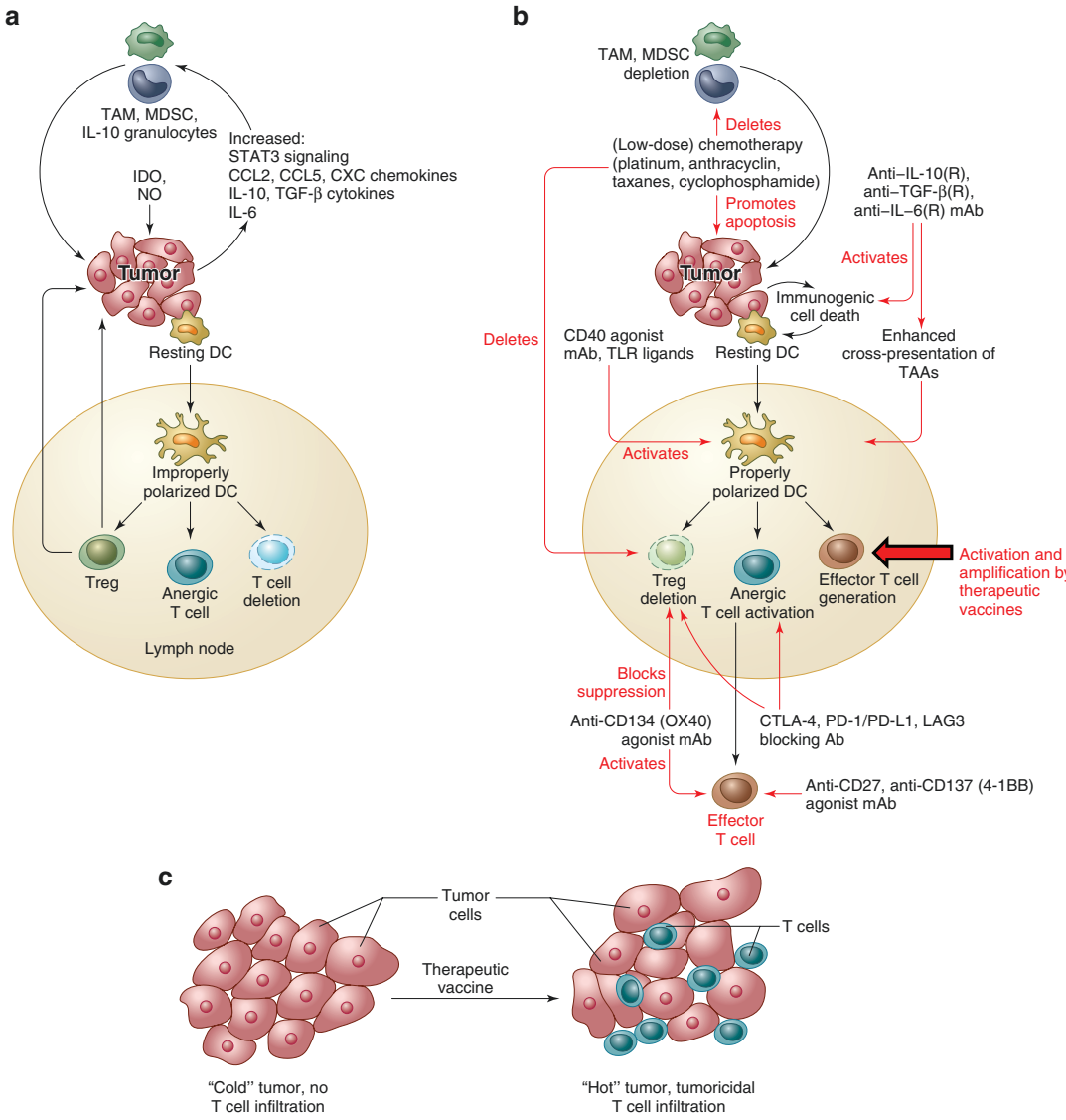

Fig. 14.2 Vaccines synergize with immunomodulators to yield an attractive combination treatment of cancer. (**a**) Hostile elements for T cells in the cancer microenvironment include tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), and IL-10-producing granulocytes. In addition a number of immunosuppressive cytokines and chemokines are released in this milieu. Nitric oxide and the enzyme IDO may further compound the immunosuppressive situation. (**b**) Vaccines will expand the low numbers of spontaneous anticancer T cells and generate de novo T cells against new antigens, increasing the chances of success of the therapy compared to monotherapy with immunomodulators. (**c**) SLP vaccines can thereby turn “cold” tumors (no T cell infiltrate) into “hot” tumors (T cell infiltrate). The immunomodulators (shown in *red* in (**b**)) can repeal the immunosuppressive cancer microenvironment in a number of ways, according to each cancer patient’s needs. Thereby vaccines and immunomodulators synergize for effective cancer therapies

called ISA101, was given again at the nadir of the mononuclear myeloid cell count in PBMC (i.e., 2 weeks after the second cycle of chemotherapy), and a highly significant correlation was subsequently observed between the strength of the vaccine-induced HPV16-specific T cell response and overall survival of the patients (unpublished observations).

14.10 Perspectives

Cancer vaccines have only recently shown clinical efficacy for the treatment of premalignant disease [38, 41, 42, 56, 57] and prostate cancer ([82]) as monotherapy. Combination treatments with various checkpoint blocking or agonistic monoclonal antibodies are highly promising for the treatment of cancer, provided the right cancer target antigens are chosen in combination with efficient vaccine platforms and proper adjuvants. The best target antigens are clearly viral antigens on virus-induced cancers or neo-antigens on mutagen-induced cancers, such as those associated with smoking or UV light exposure. Overexpressed antigens or differentiation antigens may also be chosen, provided an appreciable repertoire of CD4+ and CD8+ T cells against the antigen of choice is demonstrable. Cancer vaccines are highly attractive in patients in whom the natural T cell response has fallen short, resulting in so-called "cold" tumors, which lack spontaneous infiltration with T cells. In many of these patients, therapeutic vaccination may cause sufficient numbers of vaccine-induced tumor-specific T cells to infiltrate into the tumors and turn them into "hot" tumors. This alone is unlikely to cause robust clinical responses, but co-treatment with a number of other cancer drugs, which each by themselves are also insufficiently effective, may reap a rich harvest of more effective combination treatments with relatively few side effects. One of the most attractive of these combinations is combination of therapeutic SLP vaccination with chemotherapy or with anti-PD-1 and/or CTLA-4 checkpoint blockers. SLP vaccines are particularly attractive for vaccination against neo-antigens created by point mutations in cancers, because of the relative ease of production under GMP conditions.

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Cancer Vaccines for HPV Malignancies

15

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15.1 Introduction

On a global scale, approximately one in six new cancer diagnoses is attributable to a specific infectious pathogen [1, 2] (Fig. 15.1). Human papillomaviruses (HPVs) cause approximately 30% of infection-associated cancers, including cancers of the cervix, vagina, vulva, anus, and oropharynx [1, 2], and nearly 5% of all cancers worldwide. In women, the majority of HPV-induced cancers are cervical cancers; in men, most HPV-associated malignancies are oropharyngeal [3] (Fig. 15.2). Despite the availability of preventive HPV vaccines, and screening methods to detect HPV in the lower genital tract, cervical cancer is the second most common cause of cancer death in women in low-resource settings.

Exposure to HPV occurs with sexual debut and is essentially endemic [4–7]. Infections are asymptomatic, and although most are transient, resolution can take 1–2 years [8–10]. Older women take longer to clear infections, as do smokers, and women with underlying immunosuppression [8]. The clinically silent nature of these infections facilitates maintenance of a large herd burden of transmissible HPV. Moreover, rates of preventive vaccination in eligible US cohorts, young people aged 9–26, have been suboptimal. In 2015, 43% of eligible girls and 28% of eligible boys had completed all three vaccinations in the three-vaccination regimen [11]. Existing prophylactic vaccines, Gardasil© and Cervarix©, are approved for prevention of

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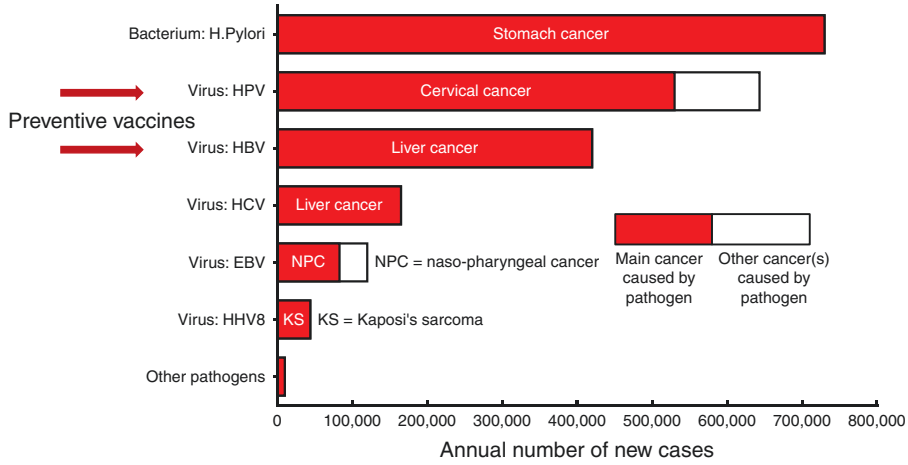

Fig. 15.1 Global incidence of pathogen-induced human malignancies in 2012 [96]

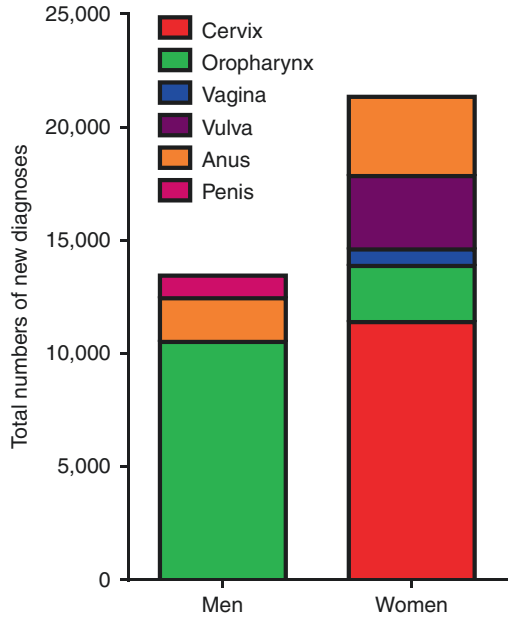

Fig. 15.2 Number of new HPV-associated cancers in 2009 [3]

cervical, vulvar, vaginal, and anal cancers. Because HPV is the underlying cause of a subset of oropharyngeal squamous cell carcinomas (OSCC), which are restricted to the epithelium of the palatine tonsil and base of the tongue in mostly young, nonsmoking, and non-heavy drinking men, their usage as prevention for oropharyngeal cancers is currently being investigated [12, 13].

15.1.1 Biology of HPV Infection

HPVs are non-enveloped, double-stranded DNA viruses which are tropic for mucosal tissues. The genome consists of three functionally divided regions: (a) a noncoding regulatory region; (b) an early proteins region, which encodes for six early proteins (E1, E2, E4–E7); and (c) a late region which encodes for the viral capsid proteins L1 and L2 [14, 15]. HPVs infect basal epithelial (skin or mucosal) cells. Over 200 HPV genotypes have been identified. Oncogenic genotypes include HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66 [16].

The development of squamous cervical cancer, other anogenital cancers, and head and neck cancers occurs in the setting of a persistent infection with an oncogenic HPV. Virtually all squamous cell carcinoma of the cervix (SCCx) and its precursor, high-grade squamous intraepithelial lesions/cervical intraepithelial neoplasia 2/3 (HGSIL/CIN2/CIN3), are caused by HPV, most commonly, HPV 16 (Fig. 15.3) [17]. Most cervical cancers are attributable to HPVs 16 and 18.

The development of both cervical cancer and HGSIL/CIN2/CIN3 is associated with integration of the HPV genome into the host genome and subsequent expression of two HPV early gene products, E6 and E7, which inactivate p53 and pRb, respectively [18, 19]. Viral integration

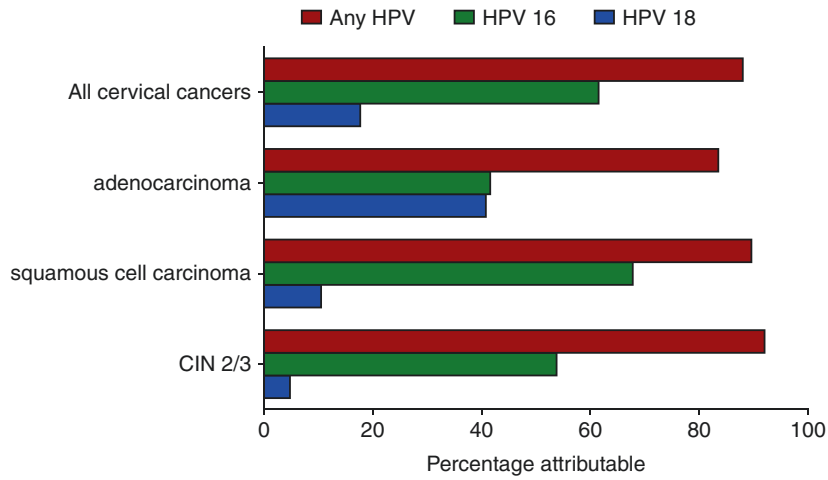

Fig. 15.3 HPV genotypes most commonly associated with cervical disease [17]

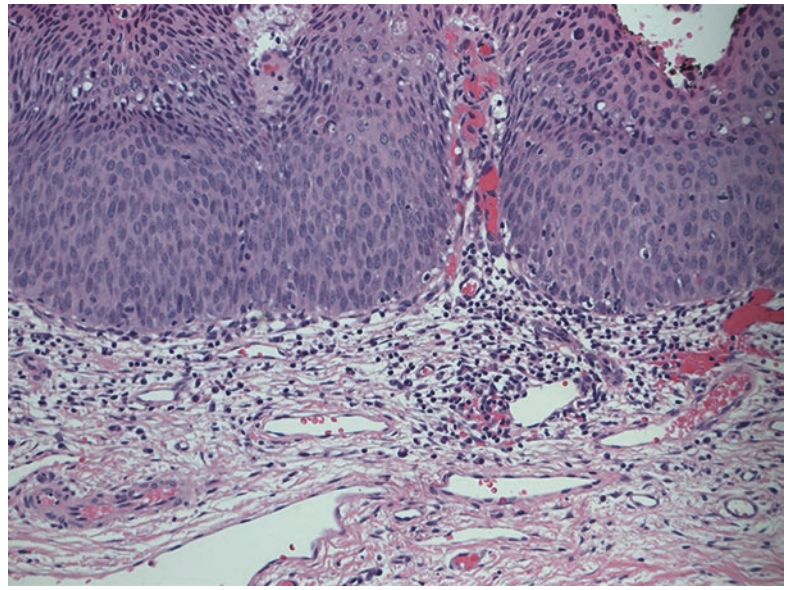

Fig. 15.4 Hematoxylin and eosin staining of CIN 2/CIN3 tissue taken with 20× magnification

sites, although randomly distributed within the human genome [20], occur principally at sites where human DNA is prone to breakage (e.g., fragile sites) and appear to affect only the expression of the HPV genome itself. Specifically, E1 and E2 are most frequently disrupted in integration, while the E6 and E7 viral oncogenes are retained, resulting in constitutive expression. Expression of both E6 and E7 is functionally required to initiate and maintain neoplastic transformation [21, 22]. Morphologically, at the cellular level, high-grade intraepithelial lesions are characterized by a high nuclear to cytoplasmic ratio. Histologically, high-grade lesions display full thickness, lack cell maturation, and are mitotically active (Fig. 15.4).

15.1.2 Systemic Immune Responses in Natural Infection

In the setting of a natural HPV infection, both humoral and adaptive systemic endogenous immune responses to HPV antigens required for disease initiation and persistence are weak. After viral clearance, antibody titers are detectable in

50–70% of persons [23, 24]. Most memory B cells elicited by natural infection generate antibodies that have low avidity, are nonneutralizing, and do not necessarily protect against reinfection with the same HPV genotype [25, 26]. Similarly, T cell responses to viral antigens are marginal, require ex vivo stimulation to be detectable, and do not reliably distinguish persons whose lesions will regress from those whose lesions will not [27–30]. In point of fact, persistent HPV infections and preinvasive HPV lesions are limited to the squamous mucosa and are presented in a non-inflammatory context without systemic viremia. Infections are anatomically restricted to the mucosal epithelium and do not elicit systemic symptoms [5, 8].

Using ex vivo stimulation, HPV 16 E7-specific cytotoxic T cells have been detected in the peripheral blood of persons who have HPV 16+ CIN2/CIN3 [27, 29] and cervical cancer [31, 32]. However, to date, no clear association has been made between the magnitude of response to E7 in the blood and clinical outcomes. In contrast, peripheral blood cytotoxic T cell responses to HPV 16 E6 have been linked to clinical outcomes. In particular, a CD4+ T cell response to E6 is has been associated with better clinical outcomes [33]. Conversely, functionally impaired E6-specific CD4+ T cell responses have been associated with cervical cancers [34].

Despite the rarity of HPV-specific memory T cells in the blood, a subset of persons with CIN2/CIN3 do mount an effective response; not all CIN2/CIN3 lesions progress to invasive cancer. We and others have reported that in a timeframe of 4–6 months, ~35% of CIN2/CIN3 lesions undergo spontaneous regression [35–37]. Similarly, in a Phase II clinical trial testing VGX-3100, a therapeutic synthetic DNA vaccine targeting HPV 16/18 E6 and E7, in persons with HPV 16 or 18+ CIN2/CIN3 prior to planned standard therapeutic resection, 30% of subjects who received placebo experienced histopathological regression [38]. Lesions resulting from mono-infection with HPV 16 were less likely to undergo regression than lesions caused by other HPV genotypes: in this timeframe, ~20–25% of HPV 16-associated CIN2/CIN3 lesions regressed [38].

15.1.3 Tissue-Localized Immune Responses to Natural Infection

The fact that neither the magnitude nor the breadth of naturally occurring T cell responses detected in the blood is robust predictors of regression of pre-invasive HPV disease of the cervix raises the question of whether it is possible to identify factors in the target lesion that could predict disease outcome or characteristics of the immune response that eliminate either infection, incipient malignancy, or cancer. Tissue-based studies of HPV lesions are identifying factors associated with clinical outcomes. In CIN2/CIN3 lesions that do not regress, although CD8+ T cell infiltrates in the mucosa are robust, they are largely restricted to the stroma, failing to access the lesional epithelium [39]. The presence of intraepithelial CD8+ T cells, on the other hand, is associated with subsequent regression. Similarly, in HPV-associated cancers of the cervix and oropharynx, the presence of intra-tumoral CD8+ T cells is associated with better prognosis [31, 40, 41]. The fact that despite the presence of tumoral T cells, a persistent HPV infection can lead to progression to cancer is likely to be the result of immunological tolerance within the tumor microenvironment, caused by various factors, including the presence of regulatory T cells, expression of CTLA-4 and PD-1, or downregulation of MHC class I on the cell surface [42]. Quantitative methods, including image analysis-directed rapid immuno-laser capture microdissection, will make it possible to analyze specific cell subsets isolated from specific histologic contexts [10] and to correlate them with the clinical outcome. In the end, systemic immune responses to viral proteins required for disease are weak, and do not reliably predict clinical behavior, underscoring the need for a better understanding of the mucosal microenvironment.

15.1.4 Preventive Vaccines

Current strategies for preventing HPV disease include screening, using either cytology or HPV testing, or a combination of both. Prophylactic

Table 15.1 Currently existing preventive HPV vaccines [26, 45, 97–99]

Vaccine	Manufacturer	HPV specificity	Adjuvant	Immune response
Cervarix	GlaxoSmithKline	16, 18	AS04 (TLR4 agonist + aluminum salt	• Predominantly IgG1
				• Cross-protection against HPVs 31, 33, 45, 51, and 52
Gardasil4	Merck	6, 11, 16, 18	Aluminum salt	• Cross-protection against HPV 31
Gardasil9	Merck	6, 11, 16, 18, 31, 33, 45, 52, 58	Aluminum salt	• Not fully tested to date

vaccines that protect against infection with oncogenic HPV genotypes are comprised of noninfective recombinant virus-like particles (VLPs) of L1, one of the two HPV capsid proteins. These VLPs do not contain viral DNA and thus are completely noninfectious and non-oncogenic. Currently, three constructs are available: Cervarix©, which targets HPV types 16 and 18 (bHPV) and is administered with adjuvant ASO4; Gardasil4©, which targets HPV types 6, 11, 16, and 18 (qHPV); and Gardasil9©, which targets HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58 (nHPV) (Table 15.1). All formulations are highly effective against HPV infections, not only in the cervix but also in other anatomical sites, in both sexes [43, 44]. These vaccines elicit robust antibody responses that are 1–2 logs greater than those elicited by natural infection [25]. In persons known to have been previously exposed to HPV, a single vaccination with qHPV drastically enhanced both the magnitude and the quality of the antibody response. In contrast to the nonneutralizing antibodies generated by natural infections, antibodies elicited by vaccination were neutralizing [25]. While both vaccines bHPV and qHPV have demonstrated cross-protection to other HPV types [43, 45], Cervarix© provided cross-protection to HPVs 31, 33, 45, 51, and 52, while Gardasil4© has been shown to be cross-protective only to HPV 31. The broad cross-reactivity of Cervarix© is most likely attributable to its adjuvant, ASO4, which is a TLR4 agonist. The existing prophylactic vaccines, Gardasil© and Cervarix©, have no therapeutic effect. Nevertheless, emerging evidence from the study using quadrivalent HPV vaccine suggests that vaccination with a preventive vaccine after excisional treatment of CIN2/CIN3 significantly decreases the likelihood of disease recurrence [46, 47]. The mode of action of this protective effect is not known and is a subject of active investigation.

15.1.5 Therapeutic Vaccines

In contrast to the prophylactic vaccines, the development of new therapeutic vaccines is focused on targeting E6 and E7. Effector T cell responses to these viral, non-self-oncoproteins, which are constitutively expressed by transformed cells, are likely to play a role in mediating lesion regression. Persons with preinvasive disease present an unparalleled opportunity to determine proof of principle for immunotherapeutic strategies. These lesions are directly accessible and clinically indolent, providing an opportunity to assess the relevant tissue before and after intervention. Moreover, a subset of lesions do regress, thereby making it possible to determine either pretreatment characteristics that predict therapeutic effect or characteristics of induced immune responses that predict therapeutic benefit. Tissue studies will also afford the ability to determine mechanisms of immune suppression mediated by different stages of HPV disease.

Several vaccine platforms have been evaluated, including naked DNA administered intramuscularly (IM) [23, 48], DNA administered IM with electroporation [38, 49, 50], viral vectors including Modified Vaccinia Ankara (MVA) virus administered subcutaneously [51], vaccinia virus (TA-HPV) administered by scarification and by IM injection [52], peptides administered subcutaneously with adjuvant [53–55], and bacterial constructs such as *Listeria monocytogenes*

[56]. Despite promising data derived from preclinical models, translation to humans has been modest. To date, vaccine-induced immune responses in humans with any stage of HPV disease have been minimal. In retrospect, this apparent discrepancy may be in part a consequence of trial design. Most trials administered immunotherapeutics peripherally [23, 38, 52], and most trials were designed to evaluate conventional, relatively binary endpoints, such as the magnitude of virus-specific CD8+ T cell immune responses in the blood and lesion regression versus persistence. In fact, in a Phase I clinical trial evaluation of heterologous DNA prime, recombinant vaccinia (TA-HPV) boost vaccination in persons with HPV 16+ CIN2/CIN3, prior to planned standard therapeutic resection, in persons who did have residual dysplasia at the tissue endpoint, post-vaccination tissue resection specimens showed drastic immunologic changes in the target lesions [57]. These included robust immune cell infiltrates in both the stromal and epithelial compartments, which were restricted to residual CIN2/CIN3, and did not involve immediately adjacent normal mucosa. These infiltrates were comprised of clonally expanded populations of T cell receptors, were in many cases organized into tertiary lymphoid structures or outright germinal centers, and had a T_H-1 phenotype. This study established two critical points: that it was possible to elicit an effector response to antigens that had been present in a chronic fashion and that T cells generated by peripheral, intramuscular vaccination could traffic to the relevant immunologic target. In resections that had residual CIN2/CIN3, these lesions were heavily infiltrated with $CD8^+$ T cells that were co-localized with apoptotic lesional squamous epithelial cells. This finding suggested that a planned resection proximate to vaccination essentially censored the tissue endpoint. By conventional measures, i.e., peripheral blood T cell responses to vaccine antigens, and complete histologic regression, this regimen was a failure. However, there was no way to conclude that vaccination had "failed," given the findings in the target tissue. This insight has informed the design of subsequent clinical trials in persons with preinvasive HPV disease—tissue endpoints are obtained at a longer interval after therapeutic interventions. Although peripheral blood T cell responses are measured, quantitative measures in the lesion microenvironment are included.

A subsequent randomized placebo-controlled Phase II trial testing therapeutic vaccination for HPV 16 or 18+ CIN2/CIN3, using a synthetic DNA vaccine VGX-3100 administered IM followed by electroporation, reported a 49% rate of histologic regression in vaccinated subjects [38]. Although a subset of subjects who received placebo also had histologic regression, the rate of viral clearance was different; HPV became undetectable in 80% of vaccinated subjects who had histologic regression, in contrast to 30% of spontaneous regressions. The concomitant clearance of detectable virus in vaccinated subjects suggests that rates of recurrence may be lower in vaccinated subjects compared to those who received placebo; persistent HPV infection after resection of a preinvasive lesion is the most predictive risk for recurrence [58].

15.1.6 Adoptive T Cell Therapy

To date, no viable therapies have been identified for either metastatic or recurrent cancers. One approach that shows promise in patients with metastatic HPV disease involves identifying tumor-specific T cells from the endogenous response, namely, from tumor infiltrating lymphocytes (TILs). While some of the T cells in the tumor bed may be nonspecific, recruited by a chemokine gradient, in some solid tumors, many of the tumor T cells express activation markers that are upregulated when the cell is activated by engagement of the T cell receptor (TCR) with its cognate antigen. Two markers that have been investigated include PD-1 and CD137. Several groups have reported that activated T cells in the tumor microenvironment are enriched for clonally expanded populations of tumor-reactive T cells [59, 60]. The ability to isolate autologous HPV-specific TILs will provide the opportunity to assess the TCR repertoire and the functional polarization of relevant cells. A recent report of

HPV-targeted TILs in persons with pretreated, metastatic cervical cancer describes tumor responses in three out of nine women, two complete responses and one partial response [61]. This outcome is significant because there are no effective therapies for either recurrent or metastatic cervical cancer or for any other metastatic HPV-attributable cancers. There is currently an ongoing clinical trial for treatment of metastatic HPV-associated cancers using tumor infiltrating lymphocytes (NCT01585428).

Another approach involves ex vivo stimulation of peripheral blood lymphocytes and infusion of antigen-specific T cells to the patient. Identification of TCRs with high avidity for tumor epitopes could pave the way to generate TCR libraries across HLA phenotypes. Analysis of the magnitude, breadth, and the quality of tissue TCRs is likely to yield insights to the successful cancer treatment modalities including adoptive T cell transfer. Autologous T cells could be genetically engineered to express HLA-matched HPV-specific TCRs and used as individualized treatment for HPV disease [60]. Currently ongoing clinical trials are investigating adoptive T cell therapies, including T cell receptor immunotherapy targeting HPV 16 E6 for HPV-associated cancers (NCT02280811) [62] and the use of HPV 16/18 E6-/E7-specific T lymphocytes, in relapsed HPV-associated cancers (NCT02379520).

15.1.6.1 Mechanisms of Immune Evasion in the Tumor Microenvironment

Like many other solid tumors, HPV-associated malignancies establish an immune-suppressive local microenvironment. The HPV life cycle is not cytolytic. Viral replication and assembly are temporally linked with cellular differentiation of squamous epithelial cells, and proinflammatory signals are absent [63]. Virus is shed in terminally differentiated squamous cells; thus, the initial exposure to HPV antigens is minimal and may not prompt robust activation of an immune response. Additionally, HPV downregulates cell surface MHC class I expression [64] and inhibits the production of proinflammatory cytokines [65–67]. E6 and E7 inhibit both interferon receptor signaling and activation of the interferon response genes [68]. E7 also further impairs the innate response by downregulating TLR9 transcription. Even incipient HPV disease recruits and functionally polarizes circulating monocytes, which migrate to the tumor site, where they differentiate into macrophages and dendritic cells [69].

Macrophage infiltration in both the stromal and epithelial compartments increases with the severity of HPV lesions, from HPV-infected cells to CIN2/CIN3 to squamous cancers [70, 71]. Their functional polarization to an immune-suppressive phenotype is mediated by tumor-secreted TGF-β and IL-10 [72]. As early in disease development as CIN2, the intensity of macrophage infiltrates correlates directly with the number of lymphatic vessels [71]. Epithelial expression of COX-2 increases with disease severity, which in turn inhibits dendritic cell (DC) maturation, reduces the ability of DCs to stimulate T cell proliferation, and increases production of IL-10 [73]. HPVs have also been shown to suppress maturation and function of Langerhans cells, which are epithelial-resident antigen-presenting cells [74–76]. However, this suppressive phenotype can be reversed in the presence of TLR3 agonists [77]. Finally, the tissue microenvironment induces and maintains tissue-specific gene expression and function of resident and recruited macrophages [78–80]. A growing body of evidence demonstrates functional plasticity in tissue macrophages; an induced suppressive or tolerizing phenotype can be reeducated by CD4+ T_H1 T cells, to an activated, effector phenotype, with cell surface expression of costimulatory molecules [81].

Finally, individual cell subsets in HPV-associated malignancies, including tumor epithelium, tumor-associated macrophages, and CD8+ T cells, frequently express PD-L1, which is involved in normal immune homeostasis. Binding to its ligands, PD-1 and CD80, reduces CD8 T cell responses [82, 83]. While the presence of PD-L1 expression is associated with impaired cell-mediated immunity in HPV disease [83], tumor expression in and of itself is not a reliable biomarker for likelihood of response to PD-1

blockade [84]. The presence of a gene signature of interferon-γ-inducible genes, however, is associated with response to PD-1 blockade [85]. This finding is consistent with what has been observed in other solid tumors, namely, that clinical benefit from PD-1 blockade is more likely to occur in the setting of a preexisting host tumor-specific immune response [86]. Therefore, it is possible that an indirect measure such as Ki67, which is upregulated after activation via engagement of cognate antigen with the T cell receptor, may be more predictive of response.

15.1.7 Immune Checkpoint Inhibitors

One of the mechanisms by which tumors often suppress effector responses is by hijacking normal mechanisms of immune homeostasis. Under normal physiologic conditions, these mechanisms regulate the nature, quality, and duration of adaptive immune responses. Many malignancies essentially co-opt these homeostatic mechanisms to suppress effector T cell responses recruited to the tumor microenvironment. Therapies targeting ligand-receptor interactions of immune checkpoint pathways have been shown to be effective in enabling the function of immune effector responses in a subset of human cancers. One of the first immune checkpoint inhibitors tested in cancer immunotherapies was antibodies targeting CTLA-4, which is expressed on activated T cells. Blockade results in the broad enhancement of immune responses [87]. Clinical trials testing anti-CTLA-4 antibodies (ipilimumab), following standard of care chemoradiation in HPV-associated cancers, are ongoing in patients with advanced cervical cancer and head and neck cancers. The PD-1/PD-L1 pathway is another immune checkpoint that can be targeted by immunotherapeutic antibodies. PD-1 expression is upregulated on T cells after activation via recognition of cognate ligand. Expression is also upregulated by chronically stimulated T cells, in which it can reflect functional "exhaustion." It is also present on B cells and NK cells [87]. Expression of one of its ligands, PD-L1, is induced by IFN-γ in the tumor microenvironment. Cell surface PD-L1 expression can be seen on the leading edge of tumor cells. Cytoplasmic expression has also been observed in both tumor cells and in tumor-associated macrophages [87]. Side effects associated with PD-1/PD-L1 blockade pathways have been considerably less severe than what has been observed with CTLA-4 blockade. Many anti-PD-1 (pembrolizumab and nivolumab) and anti-PD-L1 antibodies (durvalumab) are now being tested in clinical applications, either alone or in combination with conventional cancer treatment modalities, in patients with HPV-associated cancers. Similarly, interference with other immune checkpoint pathways, including OX40, 4-1BB, GITR, LAG-3, and HLA-E, is also being evaluated [62]. To date, across the board, approximately 20% of tumors respond to checkpoint blockade. The development of strategies to distinguish tumors that are likely to respond to blockade, versus tumors that are not, is an area of active investigation. Two clinical settings that are a focus of intense interest include tumors in which a preexisting antitumor response exists and tumors in which the mutational or neoantigen burden is high [42, 88].

15.1.8 Immunomodulatory Effects of Conventional Cancer Treatment Modalities

Conventional anticancer treatment modalities such as chemotherapy and radiation have immunomodulatory effects. Metronomic cyclophosphamide, for example, depletes circulating regulatory T cells (Tregs) [89]. The treatment options for inoperable primary tumors, and for recurrent and metastatic disease, include a combination of platinum-based chemotherapy and radiation, both of which are known to enhance susceptibility of cervical cancers to cytotoxic T cells, in addition to their direct cytotoxic effect [90]. Alkylating agents, including cisplatin, induce a high rate of genomic mutations [91]. Some of the immunomodulatory effects of radiation include upregulated expression of tumor-associated antigens [92] and upregulation of

MHC class I expression on tumor cells. Ionizing radiation enhances epitope spreading [93]. An example of how these different attributes could be leveraged in persons with metastatic or recurrent disease might be to deplete Tregs with low-dose cyclophosphamide and prime initially with a therapeutic vaccine or infusion of tumor-specific CTL, followed by chemoradiation. In a recent report of the effect of a combination of carboplatin and paclitaxel (CarboTaxol) in patients with advanced cervical cancers, this regimen was followed by a significant decrease in the frequency of circulating myeloid cells, which reached nadir at 1–2 weeks after two cycles [94]. A second cohort then received a therapeutic vaccination 2 weeks after the last cycle of CarboTaxol. None of the patients had an endogenous, preexisting response to HPV 16. These responses were of greater magnitude than those observed in a previous trial in which patients were vaccinated 1 month after chemotherapy [95]. However, no clinical responses were reported. To date, no combinatorial immunotherapeutic trials have been carried out for cervical disease. However, in a recent report of pembrolizumab, an anti-PD-1 antibody, in patients with PD-L1-positive recurrent or metastatic squamous cancers of the head and neck, persons with HPV-associated disease had a 25% response rate. The expression of PD-L1 alone did not identify tumors likely to respond to PD-1 blockade. Rather, using a composite of PD-L1 expression on tumor cells and a gene expression signature of genes induced by IFN-γ identified a significant association with good response [84]. This finding is congruent with the observation that a preexisting CTL response to tumor was associated with better response to PD-1 blockade.

In sum, although much is known about the immunobiology of HPV-associated malignancies, there is much to learn about the timing and sequence of anticancer treatment modalities. HPV-associated lesions are relatively accessible, and viral antigens provide targets for both immunotherapy and for monitoring, and so it becomes possible to dissect out mechanisms of action in the lesion microenvironment, as well as identify proof of principle of therapeutic approaches.

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NK Cell-Based Therapies

16

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Contents

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After many years of research, recent research advances have shed new light on the role the immune system plays in advanced-stage cancer. Immunotherapies have terribly increased in numbers and in diversity. Various types of immune cells may provide useful therapeutic resources, along with chemical molecules and engineered monoclonal antibodies. Among immune effectors suitable for manipulation prior to adoptive transfer or for drug targeting *in vivo*, natural killer (NK) cells are of particular interest. They are a population of innate lymphoid cells (ILCs) that can induce the death of allogeneic and autologous cells undergoing malignant transformation and microbial infection [1]. They represent 5–15% of the cells in the total peripheral blood mononuclear cell (PBMC) in humans, and because of their ability to recirculate between peripheral organs they contribute to tumor immunosurveillance. Early studies showing the importance of NK cells in the antitumor response were performed in mice [2, 3]. In humans, cases of selective NK cell deficiency are rare, and it is difficult to assess the role of NK cells on the incidence of cancer. However, several studies have shown a link between low NK cell activity in peripheral blood and an increased risk of cancer [4, 5]. In addition, tumor infiltration by NK cells has been shown to be a favorable prog-

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nostic factor in non-small cell lung cancer (NSCLC), clear cell renal cell cancer, and colorectal cancer [6–8].

NK cells express a repertoire of activating and inhibitory receptors that allow them to detect target cells while sparing normal cells. It is the integration of all these signals that will determine the activation status of the NK cells [9]. They can detect the lack of major histocompatibility complex (MHC) class I ("missing self") [2] thanks to the expression of KIRs (killer cell immunoglobulin-like receptors) in humans and Ly49 receptors in mice. The KIR family has been extensively characterized, revealing the existence of different genes and alleles giving rise to distinct haplotypes. Every receptor recognizes a group of classical HLA class I allotypes sharing particular features at the α1 domain. KIR inhibitory receptors signal through their immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domain. When inhibitory KIR receptors bind to a ligand, their ITIMs are tyrosine phosphorylated, and the SHP-1 protein tyrosine phosphatase is activated, leading to the inhibition of the NK cell activation. The engagement of NK cell receptors by MHC-I molecules is required during NK cell maturation for the generation of functional effector cells adapted to the host-specific MHC-I environment [10]. A related family of receptors that recognize MHC class I molecules, termed Ig-like transcripts (ILT) or leucocyte Ig-like receptors (LIR), can be detected on subsets of NK cells. In particular, ILT2 (LIR-1) and ILT4 (LIR-2) contain cytoplasmic ITIMs that recruit SHP-1 and can also contribute to the control of NK cell activation [11]. NK cells also possess another inhibitory receptor, CD94/NKG2A, which is expressed as a heterodimer in humans and mice. It recognizes the non-classical MHC class I molecules corresponding to HLA-E in humans and Qa-1b in mice. Unlike classical HLA-A, HLA-B, and HLA-C molecules that bind and present self-peptides, HLA-E binds leader peptides derived from the signal sequence of certain HLA-A, HLA-B, HLA-C, and HLA-G molecules. Therefore, the interaction between the CD94/NKG2A complexes and HLA-E molecules allows NK cells to indirectly monitor the expression of other MHC-I molecules [12].

During tumor transformation, often the cells decrease the expression of MHC-I molecules, and this makes them potential targets for NK cells [13]. However, NK cells must also be stimulated by activating receptors which recognize their ligands on the tumor cell membrane. These activating receptors include NKp46, NKG2D, and DNAM-1 in both humans and mice, while NKp30 and NKp44 are only expressed by human NK cells [14]. NKp30 and NKG2D detect molecules that are not present in the basal state but whose expression increases under the effect of stress or infection by a pathogen. Other triggering surface molecules including 2B4 and NKp80 appear to function as co-receptors. Indeed, they can induce natural cytotoxicity only when co-engaged with a triggering receptor. The majority of mature NK cells also express CD16 (FcγRIIIA) which is a low-affinity receptor for the Fc region of G-type immunoglobulins (IgG) and is responsible for the antibody-dependent cell-mediated cytotoxicity (ADCC) [15].

The recognition of the target leads to NK cell activation and degranulation, which corresponds to the exocytosis of lytic granules containing perforin and granzymes. In addition to the pathway dependent on exocytosis of the granules, another pathway involving the interactions between the TNF family death receptors and their ligands (such as TRAIL and FasL) leads to apoptosis of the target cell [16]. NK cells are also capable of secreting pro-inflammatory cytokines, including IFNγ and TNF, which have a direct antitumor effect; many chemokines, including MCP-1, MIP1-α, MIP1-β, RANTES, lymphotactin, and IL-8; and growth factors such as GM-CSF, which contribute to the orientation of the adaptive immune response [17].

16.1 NK Cell Manipulations in Therapeutic Approaches

The discovery that NK cell can recognize and lyse tumor cells translated into hope that NK cells might find a place as therapeutic tools. Many efforts have been made to exploit NK cells into clinics, and more than 200 (see on clinicaltri-

als.gov) therapeutic trials have been carried out with the aim of potentiating their effector capacities *in vivo* [13, 18, 19].

16.1.1 Infusion of Purified Activated NK Cells

This strategy involves their purification from a healthy donor and culture with immune-stimulating cytokines (IL-2 or IL-15) before injecting them into patients (Fig. 16.1a). This approach has proven to be effective and safe [20], but its limiting factor remains the scarce ability of infused NK cells to persist and proliferate in the patient. In view of the recent knowledge on ILC complexity, in the next future, it would also be possible to design new therapeutic strategies targeting different ILC populations. For example, in patients undergoing hematopoietic cell transplantation due to hematologic malignancies, there are evidences suggesting that ILCs expressing activating NK cell receptors may have a protective effect against acute graft versus host disease (GVHD), likely through their ability to enhance tissue repair [21, 22]. These data need further confirmation, but they may open the way to ILC infusion or, more likely, administration of ILC-derived molecules (such as IL-22) involved in mucosal healing.

16.1.2 Gene Modification of NK Cells to Improve the Efficacy of Adoptive Immunotherapy

New gene therapy methods to enhance NK cell function against tumor cells have been developed and are under investigation in preclinical and clinical trials. A promising approach aims to enhance NK cell tumor specificity using chimeric antigen receptors (CARs) (Fig. 16.1b). Various preclinical

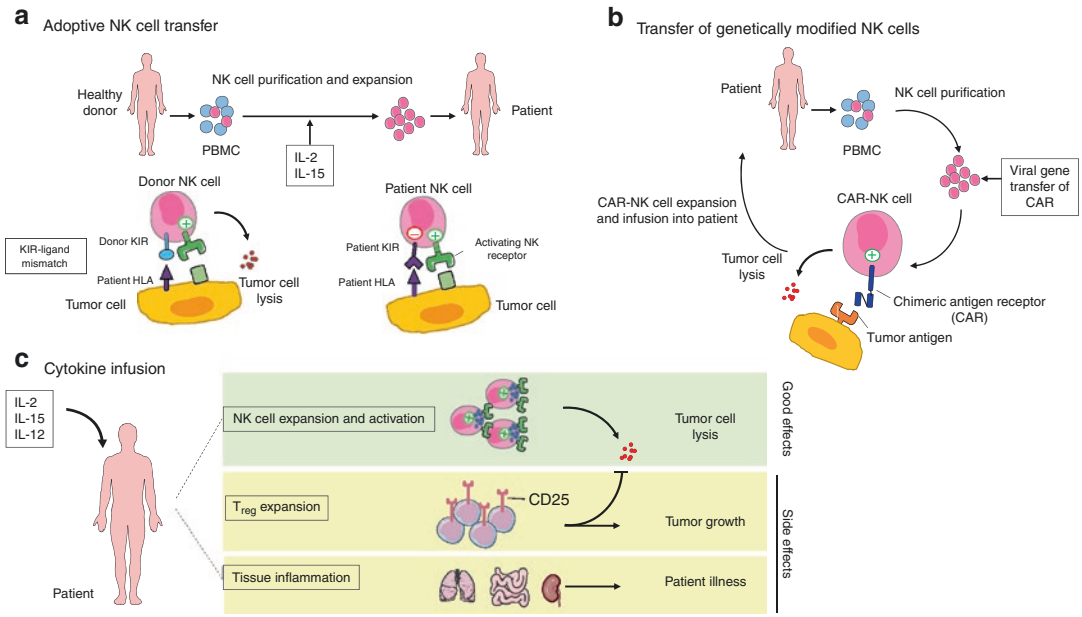

Fig. 16.1 NK cell-based therapies. (**a**). NK cells are purified from the peripheral blood of a healthy donor and activated *in vitro* with cytokines (IL-2 or IL-15) before being injected into the patient. The best responses are obtained when the donor does not express KIRs that recognize the patient's HLA molecules, so that infused NK cells do not receive inhibitory signals from cancer cells. (**b**). NK cells are purified from the peripheral blood of the patient and genetically modified to express a chimeric receptor specific for a tumor antigen (CAR) or other molecules able to direct NK cells more efficiently against their targets. (**c**). Immune stimulatory cytokines are administered to patients to induce the activation and expansion of autologous NK cells. The cytokine that induces the strongest antitumor response is IL-2 but also causes significant side effects, such as regulatory T-cell (Treg) expansion and tissue inflammation that make it difficult to use

studies are underway to explore the use of CAR-NK cells expressing a receptor specific for CD19 or CD20 in B-cell pathologies [23, 24]. Alternatively, NK cells retrovirally transduced to express NKG2D or TRAIL revealed improved tumor recognition and killing in preclinical studies [25, 26]. Another strategy is to protect NK cells from the immunosuppressive effect of TGF-β present in the tumor microenvironment using a dominant negative receptor II for TGF-β [27] or to improve the survival of NK cells *in vivo* by forcing the expression of IL-2 or IL-15 [28–30].

16.1.3 Infusion of Cytokines

Clinical studies are currently underway to test the efficacy and side effects of cytokines such as IL-2, IL-15, and IL-12 in several types of cancer (Fig. 16.1c). In an early study by Rosenberg et al., the combination of lymphokine-activated killer cells (LAK cells) and IL-2 resulted in a 20% response rate in patients with melanoma or metastatic renal cell carcinoma [31]. However, the major problem of this approach is the toxicity determined by an exacerbated activation of the immune system in a nonspecific way that leads to neuropathy, capillary leak syndrome, and renal failure. IL-15 represents an attractive alternative to IL-2 and has already been investigated in patients with metastatic melanoma and metastatic renal cell cancer [32].

16.1.4 Infusion of Tumor Antigen-Specific Antibodies to Induce NK Cell ADCC

A number of clinically approved therapeutic antibodies targeting tumor-associated antigens (such as rituximab or cetuximab) function at least partially by the initiation of NK-mediated ADCC [33, 34] (Fig. 16.2a). In order to improve the affinity of the activating receptor FcγRIIIA for the Fc region of IgG, different approaches have

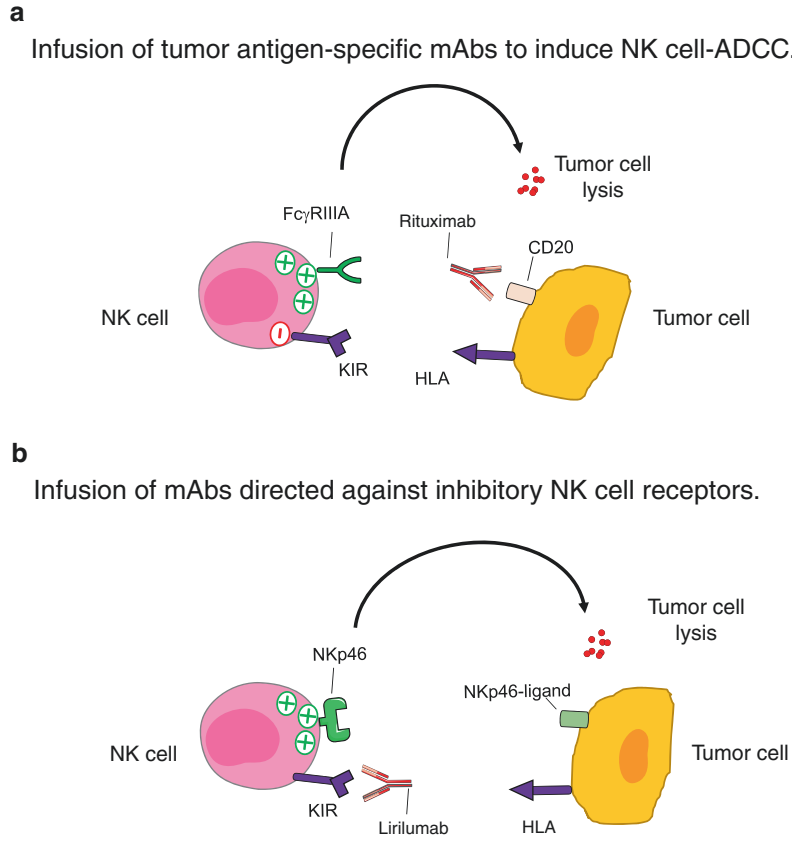

Fig. 16.2 Infusion of NK cell-specific mAbs. (**a**). Monoclonal antibodies directed against tumor antigens can also bind to the FcγRIIIA receptor expressed by NK cells and induce NK-mediated ADCC. (**b**). Infusion of mAbs directed against inhibitory NK cell receptors blocks the interactions with their ligands and allows NK cells to efficiently kill target cells

been tried, including insertion of mutations or glycosylation. This resulted recently in the approval of mogamulizumab [35], a low-fucose anti-CCR4 mAb for the treatment of T-cell lymphomas, and obinutuzumab [36], an anti-CD20 mAb for the treatment of chronic lymphocytic leukemia (CLL).

16.1.5 Infusion of Antibodies Directed Against Inhibitory NK Cell Receptors

The aim of this approach is to increase the reactivity of autologous NK cells by treating patients with mAbs that block the engagement of the inhibitory NK receptors (Fig. 16.2b). In the case of unmodified mAbs (such as rituximab), cytotoxicity mediated by NK cells is still under the control of inhibitory receptors [37]. By using antibodies that block the interaction of certain inhibitory receptors with their ligands, it is possible to potentiate the antitumor NK cell response *in vivo*. Although there is no truly specific molecule of NK cells, some surface receptors are mainly expressed by NK cells and play a major role in regulating their function [38]. These molecules activate negative signaling pathways and function as immune checkpoints in the control of cytotoxicity. In the next paragraph, we focus our attention on this approach as it represents a real revolution in cancer therapy.

16.2 Immune Checkpoint Inhibitors (ICI)

The role of the immune system in cancer patients has not been appreciated for several decades because tumors effectively suppress immune responses by activating pathways that normally regulate immune homeostasis. Immune checkpoint inhibitors (ICI), such as blocking antibodies, have been shown to be effective and to have manageable safety profiles for several cancer types (Fig. 16.3). Their use has resulted in long-term survival, for more than a decade, in some patients with cancers for which significant therapeutic advances have been made, such as metastatic melanoma and NSCLC. The identification of inhibitory pathways of immune responses has thus been a seminal series of discoveries leading to a tremendous increase in our understanding and the manipulation of immunity.

The majority of the inhibitory receptors contain one or more ITIM in their cytoplasmic domain which are phosphorylated and recruit protein tyrosine phosphatase (SHP-1/2 or SHIP). A bioinformatics research across the entire genome revealed the existence of more than 300 type I and type II integral membrane proteins that

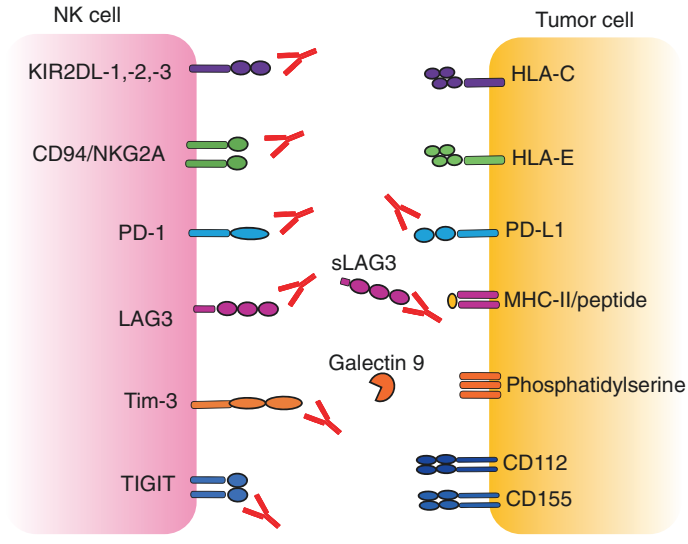

Fig. 16.3 Receptor/ligand inhibiting interactions at the NK/target cell interface. Several inhibitory receptors and co-receptors cooperate in the regulation of NK cell function. Combinations of blocking mAbs, defined on the basis of patient- and tumor-specific features, may allow to release the brake on NK cell activation

Table 16.1 NK cell receptor/ligand targeting drugs in clinical development

Lymphocyte receptor	Tumor ligand	Drug	Status
KIR2DL1/2/3	HLA-C	Lirilumab	Phase II
NKG2A	HLA-E	Monalizumab	Phase II
PD-1	PD-L1/−L2	BMS/ONO: nivolumab	Marketed (Opdivo)
		Merck: pembrolizumab	Marketed (Keytruda)
		Regeneron/Sanofi: REGN2810	Phase II
		Novartis: PDR001	Phase II
		AZN: MEDI0680	Phase I/II
		Pfizer: PF-06801591	Phase I
		Agenus/Incyte: INCSHR1210	Phase I
		Janssen: JNJ-63723283	Phase I
PD-L1	PD-1	Roche: atezolizumab	Marketed (Tecentriq)
		AZN: durvalumab	Approved (Imfinzi)
		Pfizer/Merck KGa: avelumab	Approved (Bavencio)
LAG3	HLA-II	BMS: BMS-986016	Phase II
		Novartis: LAG525	Phase I
		Anaptys/Tesaro	Preclinical
		Merck	Preclinical
		Regeneron/Sanofi	Preclinical
		Macrogenics (PD-1 bispecific)	Preclinical
		IMP321	Phase I/II
Tim-3	Gal-9	Novartis: MBG453	Phase I
	CEACAM1	Anaptys/Tesaro: TS-022	Phase I
	Phosphatidylserine	Jounce/Celgene	Preclinical
	LILRB2	Roche	Preclinical
TIGIT	CD155, CD112	Roche	Phase I
		Merck	Preclinical
		BMS	Preclinical
VISTA	Unknown	Janssen: JNJ-61610588	Phase I
		Igenica: IGN381	Preclinical
Unknown	B7-H3	Enoblituzumab	Phase I

contain at least one ITIM domain [39]. Of these receptors, only a few are targeted in therapeutic approaches (Table 16.1).

16.2.1 KIRs

KIR2DL1, KIR2DL2, and KIR2DL3 are the inhibitory receptors that, upon binding to HLA-C on target cells, are involved in the control of NK cell cytotoxicity and cytokine production [40]. Evidence of the role of KIR receptors in the NK cell antitumor response was formally demonstrated in patients with acute myeloid leukemia (AML) undergoing haploidentical bone marrow transplantation [41]. Indeed, the subgroup of patients that received KIR/HLA-C mismatched bone marrow transplant had significantly lower rate of relapse without GVHD, which translated in follow-up studies into significantly better survival [42], suggesting that donor-derived alloreactive NK cells were capable of mediating safe and durable antitumor immunity. In order to mimic this strategy with a drug, a therapeutic mAb blocking the three inhibitory KIR receptors for HLA-C was generated: 1-7F9 is a fully human IgG4 that increases NK cell cytotoxicity against HLA-C-expressing tumor cells. This was demonstrated *in vitro*, by using patient-derived AML blasts targeted by autologous or heterologous NK cells and confirmed in a humanized AML mouse model [43]. The efficacy of this approach was

also validated in a syngeneic tumor model where the blocking of Ly49C/I (the homologous receptor for KIR in the mouse) with a specific mAb could induce NK cells to selectively reject tumor cells while sparing normal tissues [44]. This translated in the clinical development of a hybridoma-derived product, IPH2101. It was tested in several phase I clinical trials [45–48] showing that KIR blockade is safe, with minimal side effects, deserving of further clinical investigations. Then, lirilumab (IPH2102, BMS-986015), a stabilized recombinant IgG4, with the same antigen specificity, was developed to increase stability and manufacturability of the mAb. It is currently tested in multiple phase I and II trials: as maintenance monotherapy in elderly AML patients (NCT01687387) or in combination with tumor-targeting mAb elotuzumab in multiple myeloma (NCT02252263) or DNA hypomethylating agent 5-azacytidine in AML (NCT02399917) and myelodysplastic syndromes (NCT02599649) or rituximab in CLL (NCT02481297). Interestingly, *in vitro* and *in vivo* studies showed that lirilumab increases the rituximab-induced ADCC [49].

Recently, a phase II clinical study that tested the efficacy of lirilumab as monotherapy in patients with multiple myeloma was prematurely discontinued due to lack of efficacy [50]. According to the authors, this could be due to a reduction of NK responsiveness that selectively affected KIR2D$^+$ cells, accompanied by a loss of KIR2D expression on the cell surface. However, this contraction has not been detected in other clinical trials, and this data needs to be confirmed. These results may suggest the requirement, at least in some malignancies, of adapted therapeutic regimens (intermittent blocking) or combined therapy in order to induce an effective antitumor response. For example, given the clinical success of anti-PD-1 blocking mAbs and their ability to induce immune-stimulating cytokines like IFNγ that may boost NK cells, lirilumab is also explored in association with nivolumab in order to combine the functional restoration of both NK and T cells, in solid tumors (NCT01714739) and hematological malignancies (NCT01592370). In two phase I clinical trials in patients with advanced solid tumors, this combination showed no additional toxicity compared to nivolumab monotherapy (Segal N.H. et al., unpublished). More recently, a phase I/II trial has shown the efficacy of the treatment with lirilumab in combination with nivolumab in patients with advanced and chemically resistant head and neck carcinoma (Leidner R. et al., unpublished). This was a single-arm study, so there was no control population, but there are well-established historical control data for head and neck cancers to judge the outcomes. The study revealed that lirilumab was well tolerated for the head and neck cancer population. Although the follow-up and maturation of the data are ongoing, what was observed from an efficacy point of view was encouraging, but the results have to be understood in the context of a single-arm, fairly small study. The overall survival was 90 and 60% at 6 and 12 months, respectively, for patients treated with the lirilumab and nivolumab combination therapy as compared to 55.6 and 36% for nivolumab alone which has been recently FDA approved for this indication. These preliminary clinical data suggest a very exciting tail of the curve for head and neck cancer patients in a way analogous to what has been reported in other cancer indications, such as melanoma treated with a PD-1 or CTLA-4 immune checkpoint inhibitors. An in-depth evaluation of the immuno-correlative biomarkers would be very useful.

The results of a randomized, double-blind, placebo-controlled phase II clinical trial (EffiKIR) evaluating the efficacy of lirilumab as a single agent in elderly patients with AML have been recently released (NCT01687387). Although the study did not meet the primary efficacy endpoint, it confirmed the safety profile of lirilumab as a monotherapy. In more details, two arms of the trial tested single agent lirilumab at different doses and treatment intervals (0.1 mg/kg every 3 months or 1 mg/kg every 4 weeks), whereas in the third arm, patients received placebo. There was no statistically significant difference between either lirilumab arms and the placebo arm on the leukemia-free survival (LFS) or on other efficacy endpoints. The 1 mg/kg q 1month arm of the trial was discontinued as the

objective of achieving a superior LFS in this arm compared to placebo could not be reached. There was no concern with tolerance. Data analyses are ongoing and will complete the understanding of the results of this trial.

Lirilumab is still being investigated in six trials, across a range of solid and hematological cancer indications in combination with other agents, including nivolumab (see on clinicaltrials.gov).

16.2.2 NKG2A

NKG2A is an inhibitory receptor expressed by the majority of NK cells and part of cytotoxic T lymphocytes which recognizes HLA-E molecules in humans and Qa-1 in mice. Unlike classical HLA class I molecules, HLA-E expression is retained, or even increased, in 50–80% of patients with solid tumors or leukemias/lymphomas [6, 47, 51, 52]. A study in NSCLC showed that infiltrated NK cells had low expression of activating receptors and KIRs, while the expression of NKG2A was not affected by tumor microenvironment [6]. At the same time, HLA-E was highly expressed on tumor cells and negative on surrounding epithelial cells. The expression of NKG2A on tumor-infiltrated NK and T cells has also been confirmed in breast and cervical cancer and appears to be related to IL-15 and TGF-β secreted by tumor cells [51, 53]. A study in melanoma patients demonstrated that the effector functions of tumor antigen-specific $CD8^+$ T cells that expressed NKG2A were inhibited by this receptor [54]. Accordingly, tumor-infiltrating $CD8^+$ T cells were a favorable prognostic factor in NSCLC only in patients whose tumors maintain the expression of classical HLA class I molecules and do not express HLA-E [52]. Moreover, increased HLA-E expression (documented in 20% of patients with colorectal carcinoma) was associated with a poor clinical outcome in the presence of massive $CD8^+$ T-cell infiltration [55].

All of these results suggest that NKG2A is an important checkpoint to block in order to increase the antitumor immune response, acting directly on the infiltrated lymphocytes. In a preclinical trial, a humanized mAb specific for NKG2A (Z270) showed its efficacy in releasing the cytotoxicity of NK cells against leukemia and lymphoma cells, both *in vitro* and *in vivo* [56]. This antibody was then developed for its clinical use under the name monalizumab (IPH). It is currently used in phase II clinical trials as a single agent for the therapy of gynecologic malignancies (NCT02459301) and in the preoperative setting in squamous cell carcinoma of the oral cavity (NCT02331875) or in combination therapy with cetuximab (an anti-EGFR mAb) in head and neck cancer (NCT02643550), with ibrutinib (a BTK inhibitor) in CLL (NCT02557516), and with durvalumab (an anti-PD-L1 mAb) in various solid tumors (NCT02671435).

16.2.3 PD1

PD-1 is an inhibitory receptor that binds PD-L1 and PD-L2, its specific ligands expressed on several tumor or infected cells but also by professional antigen-presenting cells (APC) present in inflammatory foci. Initially described on T, B, and myeloid cells [57], more recent papers reported the expression of PD-1 on NK cells from patients affected by multiple myeloma and ovarian carcinoma [58–61]. Studies *in vitro* demonstrated that the engagement of PD-L1 expressed by tumor cells can inhibit their lysis by NK cells, while PD-1 blocking mAbs restore NK cell function and favor the migration of NK cells toward the tumor site. During the last few years, several antibodies blocking PD1/PD-L1 interactions have been developed (Table 16.1) and are currently employed for treatment of advanced solid tumors. Among them, nivolumab has been shown to induce in T lymphocytes the expression of genes typically involved in cytotoxicity and NK cell function, such as IFNγ and granzyme B [62]. These data suggest that blocking this checkpoint can rescue the antitumor immune response by acting on the same molecular pathway in both T and NK cells. Although the blocking of PD-1 has long been known to reestablish an antitumor response in

mice [63, 64], clinical trials now provide important results in cancer patients. Clinical responses to monotherapy have been observed in a wide range of solid and hematologic cancers [65, 66]. Importantly, the responses are often durable and without serious toxicity in most people. Nevertheless, only a minority of people treated with antibodies specific for PD-1 or PD-L1 have a strong response, with a rapid rate of reduction of the tumor ranging from 10 to 40%, depending on the patient [66]. In order to explain this variability, various tumor biopsies collected from patients prior to treatment were examined from a histological point of view, and their composition was related to the grade of response observed in patients. The results showed that it is possible to distinguish three immune profiles that are correlated with the efficacy of anti-PD-L1/PD-1 therapy [65]. The first profile, the "inflamed" phenotype, is characterized by the presence in the tumor parenchyma of numerous immune cells positioned in proximity to the tumor cells. In these samples, the infiltrated immune cells may exhibit staining for PD-L1. This profile suggests the presence of a preexisting antitumor immune response, which may have been stopped by immunosuppression in the tumor. Indeed, clinical responses to anti-PD-L1/PD-1 therapy occur most often in patients with inflamed tumors. The second profile is the "immune-excluded" phenotype, which is also characterized by the presence of immune cells, but they are retained in the stroma that surrounds the tumor cells and do not penetrate the parenchyma. The third profile, the "immune-desert" phenotype, is characterized by a shortage of hematopoietic cells in either the parenchyma or the stroma of the tumor. This phenotype probably reflects the absence of preexisting antitumor immunity, and it is not surprising that these tumors rarely respond to anti-PD-L1/PD-1 therapy [67]. In contrast, not all tumors characterized by an immune-inflamed profile respond to anti-PD-L1/PD-1 treatment. In these cases, probably other immune checkpoints are responsible for inhibiting the antitumor response [68].

16.2.4 LAG3

Lymphocyte-activation gene 3, also known as LAG3, is a cell surface molecule that belongs to immunoglobulin superfamily, expressed by activated T lymphocytes, NK cells, B cells, and plasmacytoid dendritic cells, with diverse biologic effects. LAG3 is a ligand for MHC class II molecules, to which it binds to very high affinity. It is an immune checkpoint receptor that negatively regulates cellular proliferation, activation, and homeostasis of T cells and has been reported to play a role in Treg suppressive function [69]. Early after its discovery, experiments in LAG3-deficient mice have suggested that LAG3 was crucial for NK cell function [70]. But, since LAG3 was also found to be expressed on tumor-infiltrating $CD8^+$ T cells and to inhibit their proliferation and IFNγ production [71], thereafter, studies have been mostly focused on its role in regulating adaptive immunity. LAG3 is now the target of various drug development programs by pharmaceutical companies seeking to develop new treatments for cancer and autoimmune disorders. The predicted mechanism of action for LAG3-specific mAbs is to release the negative regulation of NK and T cells. MHC class II molecules are generally expressed only by APCs, but some cancer cells have been reported to express them as well. LAG3 interaction with MHC class II molecules expressed by melanoma cells has been shown to protect them from FAS-mediated apoptosis [72]. LAG3 also encodes an alternative splice variant that is translated to a soluble form of LAG3 (sLAG3), which exhibits immune adjuvant activity [73]. Although sLAG3 is not the intended target of clinical trials using LAG3-specific mAbs, it may be informative to bear in mind its role. Interestingly, sLAG3 is thought to bind only to MHC class II molecules present in lipid raft micro-domains on a minor subset of APC. Interestingly, a clinical-grade soluble form of LAG3 protein (LAG3-Ig fusion protein, IMP321), a physiological high-affinity MHC class II binder, has been shown to induce IFNγ and TNF production by NK cells and $CD8^+$ T cells in short-term *ex vivo* assays [74]. IMP321 is currently employed as immune-stimulating drug

in phase I and phase II clinical trials, as adjunctive to standard chemotherapy or in combination to anti-PD-1 therapy, respectively. BMS-986016, an anti-LAG3 mAb, is currently in phase II clinical testing. A number of additional LAG3 antibodies are in preclinical development. LAG3 may be a better checkpoint inhibitor target than PD-1 since it can both activate T and NK effector cells and inhibit Treg suppressive activity [75]. A therapy targeting LAG3 in combination with nivolumab (NCT01968109) is currently investigated in subjects with select advanced (metastatic and/or unresectable) solid tumors.

16.2.5 Tim-3

Tim-3 is a type I glycoprotein expressed by innate and adaptive immune cells. It was first identified as a molecule selectively expressed on IFNγ–producing $CD4^+$ T helper 1 (Th1) and $CD8^+$ cytotoxic T cells. Tim-3 is also constitutively expressed by functional and mature NK cells. Upon activation, it functions as an inhibitory receptor on NK and T cells by reducing cytotoxicity and cytokine production [76, 77]. Tim-3 is also found on T cells in patients with advanced melanoma, NSCLC, and follicular B-cell non-Hodgkin lymphoma. In these three cancers, all Tim-3^+ T cells co-expressed PD-1 and exhibited defects in proliferation and cytokine production [78]. A recent study showed that Tim-3 expression was increased on circulating NK cells in advanced melanoma patients and that this correlated with their exhausted phenotype [79]. Accordingly, blockade of Tim-3 signaling with mAbs resulted in the increased cytotoxicity and IFNγ production of peripheral NK cells from patients with lung adenocarcinoma [80]. Thus, Tim-3 expression in NK cells can function as a prognostic biomarker in human malignancies. Anti-Tim-3 blocking mAbs are now tested in phase I clinical trials, and upcoming evidences suggested their use in combination with anti-PD-1 therapy. Indeed, in the setting of anti-PD-1 therapy, treatment failure is associated with upregulation of alternative immune checkpoints (most notably Tim-3), while resistance to anti-PD-1 therapy was prevented when an anti-Tim-3 mAb was administered together with an anti-PD-1 agent [68].

16.2.6 TIGIT

TIGIT (T-cell immunoglobulin and ITIM domain) is a receptor of the Ig superfamily that is expressed on NK cells, effector and memory T cells, and Treg cells, where it functions as a co-inhibitory receptor [81–83]. It binds two ligands, CD155 (PVR) and CD112 (PVRL2, nectin-2), which are expressed on APCs, T cells, and a variety of non-hematopoietic cell types including tumor cells. CD226 (DNAM-1) binds to the same ligands but with lower affinity. Importantly, TIGIT can inhibit the interaction between CD226 and CD155. Engagement of TIGIT induces its phosphorylation and the recruitment of SHIP1 (SH2 domain-containing inositol-5-phosphatase 1) and results in the inhibition of NK cell cytotoxicity, granule polarization, and cytokine secretion [82, 83]. Upon interaction with CD155 and CD112 that are highly expressed on tumor cells, TIGIT negatively regulates antitumor responses. Indeed, TIGIT-deficient mice show significantly delayed tumor growth in two different tumor models [84]. TIGIT is highly expressed on tumor-infiltrating lymphocytes across a broad range of tumors. In $CD8^+$ T cells from melanoma patients, co-blockade of TIGIT with PD-1 additively improved proliferation, cytokine production, and degranulation [85]. Combined treatment resulted in complete tumor rejection in preclinical models [86]. Interestingly, TIGIT synergizes not only with PD-1 but also with Tim-3 in impairing protective antitumor responses [84]. Therefore, co-blockade of either TIGIT with PD-1 or TIGIT with Tim-3 promotes antitumor immunity and induces tumor regression. A fully human anti-TIGIT mAb (MTIG7192A, RG6058) is now in phase I clinical trial in combination with anti-PD-1 therapy in various solid tumors.

16.3 Perspectives

Recent breakthroughs in treatment reflect decades of research focused on understanding the biological basis of cancer and unlocking the power of the immune system. The therapeutic targeting of immune checkpoints led to outstanding results in patients with different types of cancer. However, in spite of the clinical success of mAbs against PD-L1/PD-1 and the expectations that accompany the clinical development of antibodies blocking other immune checkpoints (Table 16.1), only a subset of patients have long-lasting responses. Moreover, the immune profile of an individual reflects the contribution of an array of factors, including the intrinsic properties of the tumor and environmental factors such as infectious agents. This suggests that a broader view of cancer immunity is required. Further studies are needed to identify all the receptors involved in the regulation of NK cell cytotoxicity, in order to develop mAbs blocking the interactions with their ligands and being hence capable to enhance the antitumor NK cell response. By histologic examinations of tumor biopsies, it will be important to identify the patients most likely to respond to treatments blocking the different receptors in order to decide a targeted tumor- and patient-specific therapy.

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IDO/TDO Inhibition in Cancer

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Contents

Abbreviations

1MT	1-Methyl-tryptophan, a racemic mixture of L,D isoforms termed IDO pathway inhibitors, the latter of which (D-1MT) is in phase II/III clinical development now known as indoximod
AhR	Aryl hydrocarbon receptor (kynurenine receptor)
BID	Twice-daily dosing
CCL-2	Myeloid attraction cytokine (also known as MCP-1) which binds to receptors CCR2 and CCR4 and causes basophils and mast cells to release their granules
CR	Complete response

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eIF-2α	Master regulatory eukaryotic translation initiation factor
Gcn2	Starvation-induced kinase that phosphorylates and suppresses eIF-2α
GLK1	A kinase that responds to amino acid sufficiency by activating mTORC1
IDO	Indoleamine 2,3-dioxygenase enzyme; in older literature refers to the IDO1 gene product; in more recent literature may refer to IDO1 and IDO2 gene products which are structurally related
IDO1	Indoleamine 2,3-dioxygenase-1 enzyme (gene nomenclature IDO1)
IDO2	Indoleamine 2,3-dioxygenase-2 enzyme (gene nomenclature IDO2) distinct IDO-related gene product (gene nomenclature IDO2, located immediately downstream of IDO1 in the murine and human genomes)
IFN-γ	Interferon-γ
INCB024360	A lead small-molecule inhibitor of IDO1 enzymatic activity presently in phase II/III clinical development now known as epacadostat
Indoximod	D racemer of 1-methyl-tryptophan (D-1MT), an IDO pathway inhibitor in clinical trials that may act at clinical dose levels by relieving IDO1-mediated suppression of the mTORC1 pathway (also known as NLG-8189)
MDSC	Myeloid-derived suppressor cells
mTORC1	Mammalian target of rapamycin complex-1 (master cell growth regulatory kinase)
NLG919	A small-molecule inhibitor of IDO1 enzymatic activity in clinical trials also known as navoximod
NK	Natural killer immune cells
ORR	Overall response rate
PFS	Progression-free survival
PGE-2	Pro-inflammatory prostaglandin produced by activation of COX-2 which may rely on IDO function for its pro-cancerous activity
PKC-θ	Protein kinase C variant that phosphorylates and limits the function of the T-cell receptor
PR	Partial response
SD	Stable disease
TDLN	Tumor-draining lymph node
TDO	Tryptophan dioxygenase enzyme; structurally unrelated to IDO enzymes (gene nomenclature TDO2)
TDO2	Tryptophanase dioxygenase gene nomenclature
TLR	Toll-like receptor (infection/inflammation-associated PAMP receptor)
TGF-ß	Transforming growth factor-ß
TPA	12-O-tetradecanoylphorbol-13-acetate (pro-inflammatory chemical also known as PMA)
Treg	T regulatory cells

17.1 Introduction

One widespread feature of advanced cancers is elevated tryptophan catabolism, a phenomenon that tracks with tumor burden noticed initially at least several decades ago [1]. Tryptophan is the rarest amino acid and it is essential in the diet. Thus, its levels are tightly controlled, in part by catabolism along the serotonin and kynurenine pathways which handle this role in the body. The serotonin pathway is better understood and medicinally important in controlling affect (mood) and gut peristalsis. However, only 5% of total tryptophan catabolism occurs through this pathway. The kynurenine pathway is relatively less understood despite its dominant role in tryptophan catabolism. This pathway has been studied mainly in biochemistry and neurology,

in the latter case as a source of catabolites contributing to psychogenic disease [2]. Biochemically, IDO and TDO control the rate-limiting first step in tryptophan catabolism leading to generation of the key enzyme cofactor nicotinamide adenine dinucleotide (NAD). However, NAD is scavenged from the diet to satisfy metabolic needs such that the physiological need of the kynurenine pathway seemed incomplete. TDO encoded by the TDO2 gene has long been known as the predominant liver enzyme-mediating catabolism of dietary tryptophan. In contrast, IDO is an inducible enzyme that is more widely expressed. The IDO1 gene-encoding IDO was identified in the 1960s as the first interferon-activated gene to be described [3], but despite some study in the context of infectious disease, a fuller impact of this association was not appreciated.

A pivotal conceptual breakthrough with regard to the physiological meaning of tryptophan catabolism occurred in 1998 with the seminal work of Munn, Mellor, and their colleagues who implicated IDO in T-cell-directed immunosuppression during pregnancy [4]. Briefly, they proposed that tryptophan deprivation would impair antigen-dependent T-cell activation in microenvironments where IDO was active. Initial evidence supporting this concept was offered by studies of how immune tolerance to "foreign" paternal antigens in pregnant mice could be reversed by the IDO pathway inhibitor 1-methyl-D,L-tryptophan (1MT), the administration of which elicited MHC-restricted, T-cell-mediated rejection of allogeneic concepti [4, 5].

Several reports founded the concept of IDO as a mediator of immune tolerance in cancer. First, overexpression of IDO1 occurs commonly in human tumors [6, 7]. Normally IDO1 is under the control of the tumor suppressor Bin1 [8, 9], one of the more commonly attenuated genes in human tumors [10–14]. Thus, IDO elevation in cancer cells can be ascribed directly to disruption of a tumor suppressor function for which a powerful selection appears to exist during malignant progression [14]. In immunocompetent mouse models of cancer, 1MT doses that elicited conceptus rejection displayed some limited antitumor effect [6, 15]. However, the same doses dramatically empowered the efficacy of co-administered immunogenic chemotherapy through a mechanism relying upon $CD4^+/CD8^+$ T cells [8, 16], offering a more promising perspective on therapeutic utility. As discussed below, the D and L racemers of 1MT act by complex mechanisms of action in vivo that are in large part distinct from systemic IDO1 enzyme inhibition (especially in the case of D-1MT, taken to clinical trials with the nomenclature indoximod). Thus, the discovery of a bioactive IDO1 enzyme inhibitor by Muller, Prendergast, and colleagues that could empower chemotherapy similarly to 1MT offered the first therapeutic proof of concept [8, 17–19]. Preclinical pharmacological validation was achieved through the study of other structurally distinct bioactive IDO1 enzyme inhibitors [20–22], including most notably the phenylimidazole and hydroxyamidine chemotypes from which the clinical leads NLG919/navoximod and INCB024360/epacadostat were developed, respectively [21, 23]. Genetic studies in mice deficient in IDO1 strengthened its preclinical validation as a cancer therapeutic target [24, 25]. Overall, these efforts helped establish IDO1 as a pivotal mediator of immune escape that is a critical trait of cancer [26]. At the present time, clinical lead agents D-1MT/indoximod, INCB024360/epacadostat, and NLG919 have advanced furthest in human trials. In this chapter, we summarize evidence supporting the concept of IDO/TDO enzymes as inflammatory modifiers involved not only in adaptive tolerance but also in tumor neovascularization and metastasis; the discovery and development of indoximod, epacadostat, and NLG919 as lead clinical compounds in the field; and the rationale for and ongoing exploration of TDO inhibitors and mixed IDO/TDO inhibitors based on a broader rationale involving TDO and IDO2 in driving cancer progression and their potential roles in IDO1 bypass (inherent resistance) and acquired resistance to IDO1 selective inhibitors currently at the vanguard of clinical development as a unique class of immunometabolic modifiers of cancer-associated inflammation and adaptive immunity. Figure 17.1

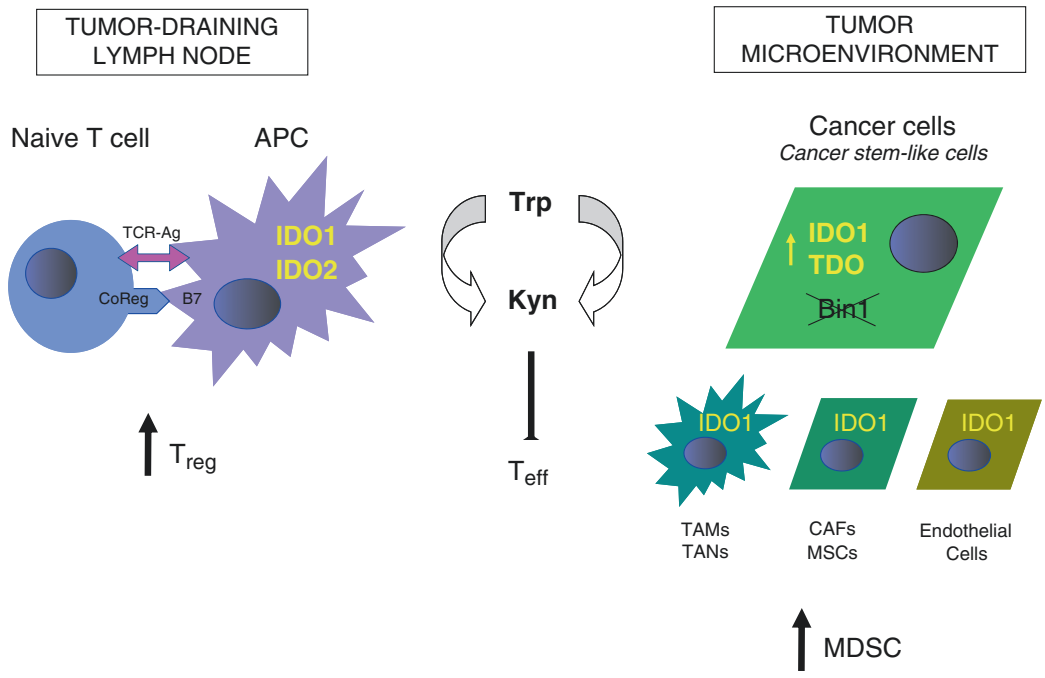

Fig. 17.1 Sites of IDO/TDO expression and action in cancer. Expression of IDO1, IDO2, and TDO documented in various cells in the tumor microenvironment (including metastatic sites) and in tumor-draining lymph nodes (TDLN) is indicated, including in tumor, stromal, vascular, and immune cells. Both tryptophan deprivation and kynurenine production mediated by IDO1 and TDO have been implicated in inflammatory processes and immune escape (antigenic tolerance). Effects of IDO/TDO activity on the function of T cells and MDSC are shown. *APC* antigen-presenting cell (e.g., dendritic cell), *MDSC* myeloid-derived suppressor cell, *TAM* tumor-associated macrophage, *TAN* tumor-associated neutrophil, *Teff* T effector cell, *Treg* T regulatory cell

provides a current perspective on the sites of expression and functional reach of IDO/TDO enzymes in cancer as presented below.

17.2 IDO1 in Immune Escape from T-Cell Immunity

The prevailing view among cancer biologists of the determinative importance of intrinsic tumor cell characteristics was encapsulated in a highly influential categorization of the hallmarks of cancer [27]. In this broad conceptualization, even metastasis and angiogenesis, the two recognized hallmarks with clear host dependence, were considered from a tumor-centric perspective, and no consideration was given to possibility that interactions with host immunity might also play an instrumental role in cancer outcomes. The case for including immune escape within the pantheon of critical hallmarks was first promulgated in a 2008 review on IDO1 [20] and eventually gained general acceptance as the hallmark designations were reassessed in light of recognition of the importance of host environmental factors such as immunity and inflammation [28, 29].

IDO1 induction in DC and macrophages promotes immune tolerance by suppressing effector T cells, converting naïve T cells to FoxP3$^+$ Tregs, and elevating the suppressive activity of "natural" Tregs [30]. Extratumoral induction of IDO1 was reported initially in a subset of cancer patients and preclinical tumor graft models [15, 31]. In the mouse B16 melanoma model, IDO1 was not detectable directly in the tumors that formed but rather was elevated in TDLN where it was localized to a specific subset of DC characterized for T-cell suppressive activity [31]. Several different IDO1 inhibitory compounds

have since been identified that can produce highly significant B16 tumor growth suppression that relies both on intact T-cell immunity and host IDO1 function [20, 22, 32], providing pharmacological support for an extratumoral role of IDO1 in limiting antitumor immunity. In like manner, the first genetic validation of IDO1's involvement in driving autochthonous tumor development came from studies in classical two-stage models of skin carcinogenesis, where there was no evidence of IDO1 expression in the developing lesions: similar to the B16 model, IDO1 expression and activity were highly elevated in DC within the TDLN [24]. In this context, where tumor initiation and promotion are distinctly separable, IDO1 was found to be elevated in the tumor-promoting inflammatory environment, even in the absence of tumor initiation, clearly indicating that extratumoral IDO1 elevation is an early event that occurs before initiation in programming a pro-tumorigenic inflammatory microenvironment [33].

17.3 IDO1 in Inflammatory Programming: MDSC Development and Metastasis

Myeloid-derived suppressor cells (MDSC) found to rely upon IDO1 support are another key player in the establishment of an immunosuppressive tumor microenvironment. MDSC are an immature population of bone marrow-derived hematopoietic cells functionally defined by their ability to suppress T-cell activity [34]. In response to inflammatory signals, MDSCs migrate to the lymph node, spleen, and tumor tissue to create local immune suppression. Among the mechanisms utilized by MDSC to exert their T-cell suppressive effects [35–41], there is evidence that IDO1 activity is a critical factor. This connection was first revealed by genetic studies in IDO1$^{-/-}$ mouse models of de novo lung carcinoma and metastases [25]. IDO1$^{-/-}$ mice resisted the outgrowth of lung tumors, and MDSC obtained from tumor-bearing animals were impaired for suppression of CD8^{+} and CD4^{+} T cells. Moreover, IDO1 loss caused an attenuation of IL-6, a major driver of MDSC, and ectopic expression of IL-6 was sufficient to rescue impairment of the T-cell-suppressive activity of MDSC as well as the resistance to pulmonary metastasis in IDO1$^{-/-}$ mice [25]. Thus, IDO1 exerted regulatory control over MDSC suppressive function by its ability to influence the inflammatory milieu. Other studies show that IDO1 is needed for MDSC recruitment to tumors [42, 43]. In light of the pivotal role of IDO1 in supporting MDSC function, it is notable that no compelling evidence exists in mouse models that IDO1 is expressed directly in MDSC. In contrast, human studies have identified populations of IDO1-expressing MDSC and associated the IDO1 expression in those cells with immunosuppressive function [41, 44]. Overall, in addition to regulating MDSC function and recruitment, IDO1 may act through additional mechanisms to support MDSC activity.

17.4 IDO1 in Inflammatory Programming: Pathogenic Neovascularization and Metastasis

The critical importance of neoangiogenesis for supporting tumor outgrowth is well established [45]. Although angiogenesis is sometimes used to refer broadly to all blood vessel development, its specific meaning is the formation of new vessels from the pre-existing vascular network in contrast to vasculogenesis, which refers to vessel formation through recruitment of new cells such as bone marrow-derived endothelial precursor cells. While vasculogenesis has been predominantly associated with embryogenesis and angiogenesis with adult vessel formation, the picture is likely to be more complex, and the distinction between the two processes may not be absolute. A combination of vasculogenesis and angiogenesis has been implicated in the vascularization of organs of both mesodermal and endodermal origin such as the lung, heart, pancreas, and liver, while for organs of ectodermal origin, such as the brain, kidney, thymus, and limb bud, angiogenesis appears to be predominant [46]. Importantly, these observations suggest that the

operative processes for forming new blood vessels may not be the same between different tissue environments, which may be a factor influencing the outgrowth of tumors and metastases at different sites in the body.

Neovascularization refers to the excessive and disorganized growth of blood vessels induced by ischemia in tissues such as the retina and lungs. Neovascularization is also a distinguishing characteristic of growing tumors. In experimental models of ischemia, immune cells have been reported to be important for pruning the excess vasculature and limiting neovascularization (Ishida, 2003) suggesting that immunity might play an important anti-neovascular role in tumors as well. In particular, the inflammatory cytokine IFNγ has been shown to trigger anti-neovascular activity that results in tumor cell killing. In a series of studies, IFNγ-mediated elimination of vessels was implicated as the primary mechanism for both CD4 and CD8 T-cell-dependent tumor rejection (Qin 2000, 2003). However, inflammation is a complex process that can also promote neovascularization. In particular, the inflammatory cytokine IL6 has been shown to be important for ischemia-induced neovascularization [47] and has been demonstrated to promote aberrant angiogenesis through a signaling process that does not require VEGF [48]. IL6 is also generally regarded as pro-tumorigenic as opposed to IFNγ, which is regarded as anti-tumorigenic. This raises the possibility that the cytokine balance in an inflammatory environment may influence tumor outgrowth by how it impacts neovascularization.

In this context, finding that loss of IDO1 resulted in diminished pulmonary vascularization [25] and suggested the hypothesis that the induction of IDO1 by IFNγ might be working in a negative feedback capacity to limit the anti-angiogenic impact of IFNγ and that this might be an important factor accounting for the ability of IDO1 to counteract immune-based restriction of tumor outgrowth. IDO1 loss was also associated with attenuated induction of the inflammatory cytokine IL6, and it was demonstrated in a pulmonary metastasis model that ectopic expression of IL6 could overcome the resistance to metastatic tumor outgrowth exhibited by IDO1$^{-/-}$ mice. These findings led to the hypothesis that IDO1 acts downstream of IFNγ and upstream of IL6 from the very onset of tumor initiation to shift the inflammatory environment toward angiogenesis and tumor promotion.

As predicted by this model, pulmonary metastases that developed in IDO1$^{-/-}$ mice exhibited significantly reduced neovascularization relative to their WT counterparts [49]. However, since overall metastatic tumor outgrowth in IDO1$^{-/-}$ mice was also significantly reduced, it was not clear if the reduction of blood vessel formation was a direct effect of IDO1 loss. To test the idea that IDO1 is important for supporting neovascularization outside other possible confounding effects within the tumor microenvironment, studies were conducted in a mouse OIR (oxygen-induced retinopathy) model, a well-established, reproducibly quantifiable surrogate system for studying neovascularization [50, 51]. As predicted, IDO1$^{-/-}$ mice exhibited a significant reduction in OIR-induced retinal neovascularization relative to their WT counterparts [49]. Loss of the related IDO2 isoform had no demonstrable effect on OIR-induced retinal neovascularization, indicating that the effect is specific to IDO1 [49]. No difference in the normal retinal vascularization that develops under normoxic conditions was observed between IDO1$^{-/-}$ and WT groups, and reduction of the avascular region [49], indicative of normal revascularization, was actually higher in the IDO1$^{-/-}$ animals indicative of an improvement in normal vascular regrowth occurring in mice lacking IDO1. The reduction in OIR-induced retinal neovascularization observed in mice lacking IDO1 genetically was recapitulated by siRNA-mediated knockdown of IDO1 expression in the retina [49], demonstrating that the effect of IDO1 loss on neovascularization could be elicited both locally and acutely. Likewise, pharmacologic inhibition of IDO1 with the clinical agent epacadostat reduced OIR-induced retinal neovascularization when delivered systemically to neonates [49]. In parallel studies, epacadostat administration in the pulmonary metastasis model resulted in rapid elimination of the

existing neovasculature [49], validating the potential therapeutic relevance of these findings in the cancer setting.

Having established the importance of IDO1's role in supporting neovascularization, studies were carried out to test the hypothesis that IDO1 produces this effect through its integration at the regulatory interface between the inflammatory cytokines IFNγ and IL6. Consistent with the hypothesis that IDO1 supports neovascularization primarily by counteracting the anti-angiogenic activity of IFNγ, the concurrent elimination of IFNγ in double knockout IFNG$^{-/-}$ IDO1$^{-/-}$ mice reverted the level of neovascularization in both the OIR and pulmonary metastasis models back to wild-type levels [49]. Conversely, IL6$^{-/-}$ mice, as predicted, exhibited a reduction in neovascularization in both the OIR and metastasis models similar to that observed in IDO1$^{-/-}$ mice [49]. The effect of IL6 loss on neovascularization was likewise reversed by the concomitant elimination of IFNγ in double knockout IFNG$^{-/-}$IL6$^{-/-}$ mice [49], consistent with the hypothesis that the upstream potentiation of the pro-angiogenic activity of IL6 may be an important contributing factor in IDO1's ability to support neovascularization. In all cases, neovascularization tracked closely with overall survival in the pulmonary metastasis model [49], indicating that the impact on tumor neovascularization may be a meaningful consequence of treatment IDO1 inhibitors that should be taken into consideration as part of the ongoing clinical development of these agents.

17.5 IDO2 in B-cell Inflamed States and Certain IDO1 Functions: Connections and Questions

Although relatively little studied as yet, IDO2 is a structural relative of IDO1 also implicated in modulating immunity through tryptophan catabolism, particularly autoimmunity [52]. The IDO2 gene is located immediately downstream of IDO1 in the mouse and human genomes, and structural studies suggest a more ancestral function for IDO2 [53]. Deletion of the IDO2 gene does not appreciably affect embryonic development, hematopoiesis, or immune character nor does it affect tryptophan or kynurenine levels in blood [54]. IDO2 enzyme activity clearly relies upon conditions that differ from IDO1, for example, in differing requirements for a physiological co-reductant system [55]. Indeed, earlier characterizations of the "weak" activity of IDO2 simply reflect nonoptimal biochemical conditions which when corrected confer demonstrable activity ([56]; L. Laury-Kleintop, J. DuHadaway and G.C. Prendergast, unpublished data). Thus, the lack of significant effects of IDO2 deletion on systemic blood levels in the mouse may reflect the far narrower normal range of IDO2 expression relative to IDO1 and TDO, which are relatively more broadly and strongly expressed.

Mouse genetic experiments establish a function for IDO2 in immunomodulation [52]. One notable feature of IDO2-deficient mice is a deficiency in their ability to support IDO1-induced T regulatory cells [54]. Parallel evidence of a similar tolerizing function for IDO2 in human dendritic cells has been reported [57]. IDO1-deficient mice have also been found to be mosaic deficient for IDO2 function, strengthening clues of IDO1-IDO2 interaction in immune control [54]. Figure 17.2 summarizes this feature of IDO2 and a model which captures its potential implications in cancer. Interestingly, in a model of autoimmune arthritis, indoximod (D-1MT) administration phenocopied the reduced disease severity associated with IDO2 deletion, and this therapeutic effect was abolished by IDO1 deletion [58], aligning with earlier evidence that indoximod can selectively disrupt IDO2 enzyme activity [59]. However, these connections may be contextual having yet to be extended in other systems (Fatokun, 2013 #4628 [60]), including humans where common genetic variations in IDO2 that reduce tryptophan catabolic activity may be relevant [59].

Recent studies of the reduced susceptibility of IDO2$^{-/-}$ mice to autoimmune arthritis have revealed that IDO2 functions in B cells where it acts to support B-cell inflamed states [58, 61, 62]. These findings are interesting in light of evidence

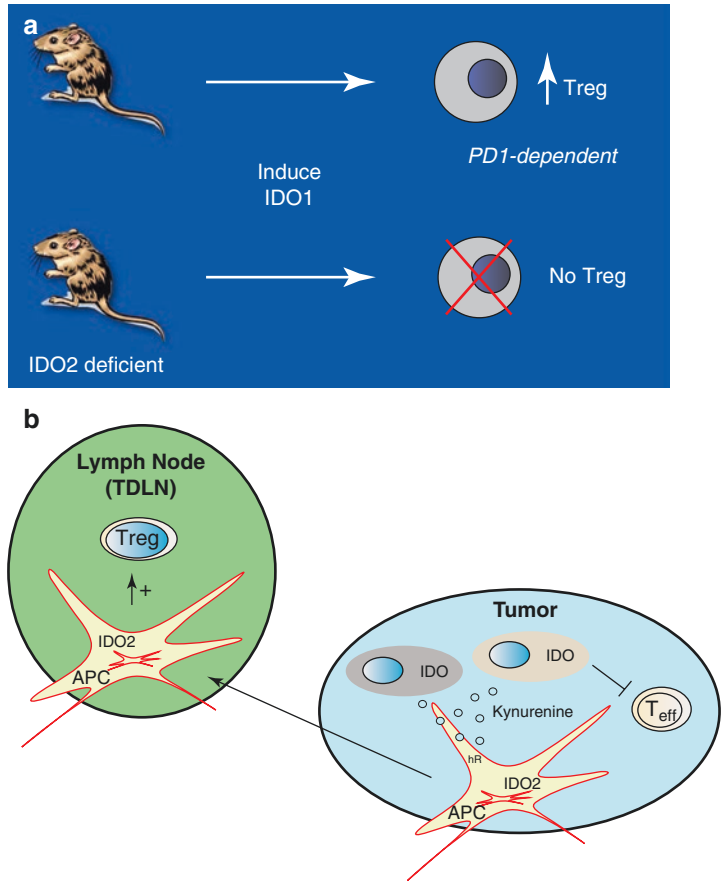

Fig. 17.2 IDO2 as a contributor to IDO1-mediated immune tolerance. (**a**) IDO2-deficient mice are defective in a PD1-dependent mechanism of IDO1-mediated Treg induction, in support of other evidence of IDO1-IDO2 genetic interaction in the mouse [54]. (**b**) Model. IDO2 expression activated by kynurenine/AhR signaling in APC acts to distally propagate tolerance signals produced locally by IDO1 in tumor and tumor-stromal cells (*gray* or *pink* cells in *blue* tumor). Local IDO1 expression blunts antigen-specific T effector cells mediated by kynurenine production and tryptophan deprivation [108]. IDO2 expression is upregulated in roving APC through the IDO1-mediated production of kynurenine, which acts through its receptor AhR to drive IDO2 transcription in APC [109]. IDO2 activity is licensed by IDO1 through transcriptional and posttranscriptional mechanisms [52]. APC are tolerized by kynurenine [110] and IDO2 is evoked as an effector in this model. IDO2 reinforces tolerance in APC by irreversible signals that differ from IDO1 signals which are reversible [59]. APC programmed by IDO2 rove to tumor-draining lymph nodes (*green* TDLN) or other metastatic sites where they reinforce IDO1-dependent Treg formation (IDO1 is also expressed in APC but not shown for clarity). This model is compatible with the latest model for IDO1 function in Treg formation [30], invoking IDO2 as a required intermediate function based on studies in IDO2-deficient mice [54]

that certain cancers rely upon B-cell inflamed states for their development [63, 64]. While IDO2-deficient mice are unchanged with regard to their susceptibility to inflammatory skin carcinogenesis [54], they resist the development of K-Ras-induced pancreatic cancers (G.C. Prendergast and A.J. Muller, unpublished data). IDO2 enzymology differs from IDO1 in requiring different reductant systems, especially for the human enzymes, but recent elucidation of these differences confirms that IDO2 has demonstrable tryptophan catabolic activity [56]. While small-molecule inhibitors of mouse or human IDO2 have been reported [56, 65–68], they are not bioactive or for other reasons have not been studied in vivo as yet. Interestingly, a B-cell-

penetrating bioactive antibody against IDO2 has been reported recently that phenocopies the anti-arthritic effects of IDO2-genetic deficiency in the mouse [62].

In normal tissues IDO2 expression is more narrow than IDO1 or TDO, being confined mainly to liver, kidney, brain, placenta, and antigen-presenting cells (APC) including B cells. Cancers do not tend to overexpress IDO2 although it has been reported in melanoma and gastric, brain, and pancreatic tumors, in the latter case rather widely [69]. IDO2 is regulated by the aryl hydrocarbon receptor (AhR) which binds kynurenine as an endogenous ligand produced by the more active IDO1 enzyme. Thus, given clues of IDO1-IDO2 interaction, it is conceivable that locoregional IDO1 activity may increase levels of IDO2 in roving antigen-presenting cells in the tumor microenvironment, perhaps contributing to a tolerized state that contributes to Treg formation in tumor-draining lymph nodes (TDLN). Figure 17.2 presents a model in which IDO2 functions on the Kyn effector pathway downstream of IDO1/TDO to positively modify decisions made in the TDLN to set tolerance to "altered-self" antigens, along the self-nonself continuum where immune challenges from autoimmunity and cancer arise [70].

17.6 Tryptophan Dioxygenase (TDO) in Inflammatory Programming: Immune Escape, Anoikis Resistance, and Metastasis

TDO expression in liver is responsible for homeostasis of tryptophan levels in the blood. Similar to IDO1, some tumors overexpress TDO as a means of immune escape [71–73]. Thus, there has been growing interest in small-molecule inhibitors of TDO as a parallel immunomodulatory strategy to attack tumors [74–79], the rationale for which has been reviewed in detail recently by pioneers in this area [72, 73]. The initial bioactive lead structure developed in the 1990s termed 68OC91 [74] has been used for mouse studies, but compounds optimized for potency and more favorable pharmacological profiles have been reported [75–78]. Deletion of the TDO-encoding gene TDO2 in the mouse causes higher concentrations of L-tryptophan to accumulate in blood, with some neurologic alterations perhaps attributable to a coordinate elevation in blood/brain levels of serotonin in these mice [80]. Interestingly, mice treated with the TDO2 inhibitor 680C91 will phenocopy $TDO2^{-/-}$ mice in showing an increased sensitivity to endotoxin-induced shock, implicating TDO in inflammatory programming [81]. However, despite this parallel with IDO1, as in the case with IDO2, there are differences in the inflammatory characteristics that appeared to be conferred by TDO, despite the common role of these enzymes in tryptophan catabolism [82]. While enzymological differences may help explain these different roles, it would also seem likely they reflect differences in locoregional control in the production of kynurenine and its metabolite or in the relative availability or efficiency of kynurenine effector mechanisms (AhR, kynurenine pathway catabolic enzymes, etc.). With regard to TDO, while there is evidence of its contribution to tumoral immune escape established preclinically with selective bioactive inhibitors [76, 83], neither a genetic proof in mice nor an understanding of the nature or extent of its expression in tumor cells or the tumor microenvironment has been established as yet. Moreover, TDO inhibitors pose different safety concerns from IDO1 inhibitors, including in the liver and central nervous system, as carefully discussed recently elsewhere [72]. That said, the rationale for developing TDO inhibitors as well as IDO-/TDO-combined inhibitors as next-generation modalities in the field continues to strengthen.

Recent emerging evidence suggests that TDO contributes to cancer-associated inflammatory programming like IDO1. Specifically, upregulation of TDO in cancer cells has been found to contribute to tumor cell survival and metastatic prowess beyond its role in immune escape [84]. Resistance to anoikis—a type of apoptosis triggered by cell adhesion deprival—is a key step in metastatic progression [28]. In a seminal study of aggressive "triple-negative" breast cancer

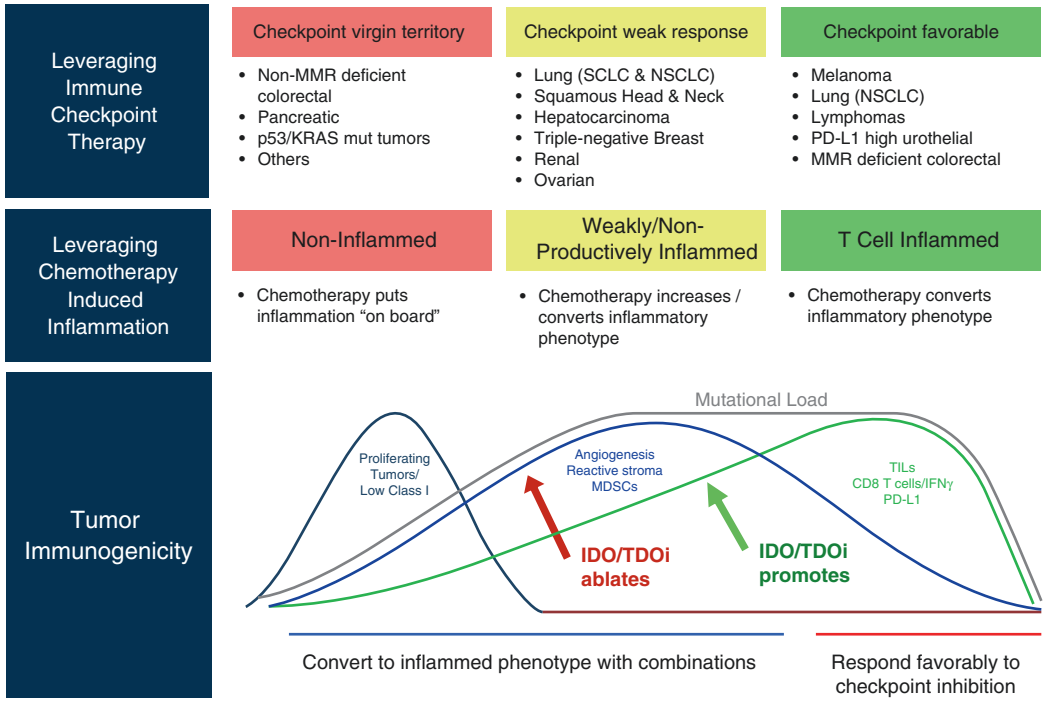

Fig. 17.3 IDO/TDO inhibitors to leverage immune checkpoint therapy and chemotherapy. IDO/TDO inhibitors are effective not only in combination therapeutic regimens, acting as immunomodulators to relieve immune escape and promote adaptive immune escape, but also to ablating or reprogramming inflammatory processes which can leverage the efficacy of chemotherapy as well as immune checkpoint therapy

(TNBC), D'Amato and colleagues showed how TDO upregulation in forced suspension culture was essential for anoikis resistance and metastatic capacity of TNBC cells [84]. Similar to its role in immune escape [83], kynurenine induction resulting from TDO upregulation was sufficient to activate the AhR signaling pathway, and pharmacological inhibition or genetic attenuation of TDO or AhR was each sufficient to restore anoikis sensitivity and reduce the invasive character of TNBC cells. Supporting these observations, tumor-bearing mice treated with the TDO inhibitor 680C91 exhibited reduced pulmonary metastasis. Lastly, elevated expression of TDO in clinical TNBC specimens was associated with increased disease grade, estrogen receptor-negative status, and shorter overall survival [84]. These findings extend the concept that TDO acts like IDO1 to drive a pathogenic inflammatory program(s) in cancer that extends beyond their contributions to enabling adaptive immune tolerance. Figure 17.3 summarizes ways in which IDO/TDO inhibitors may be used to leverage immune checkpoint therapy and chemotherapy through their effects on inflammatory programming and adaptive antitumor immune responses.

17.7 Lead Clinical Agents: Indoximod, GDC-0919, and Epacadostat

17.7.1 Indoximod

We have published previously elsewhere a detailed discussion of the preclinical studies and rationale to embark upon clinical evaluation of this simple 1-methyl derivative of D-tryptophan [85, 86]. By far, the most commonly employed molecular probe to study IDO in the preclinical literature has been the D,L racemic mixture of 1-methyl-tryptophan (1MT). L-1MT is a weak

substrate of IDO rather than a true inhibitor ([52] #4610); D-1MT is neither a substrate nor an inhibitor of IDO, though in multiple model systems, it exhibits relatively greater antitumor properties associated with inhibition of IDO-mediated tryptophan catabolism in human dendritic cells [16]. Thus, neither are selective probes. As the first compound to enter phase I trials, indoximod was found to be well tolerated as a single agent or in combination with chemotherapy in studies which defined a dose of 1200 mg/day for ongoing evaluation in multiple phase II trials [87, 88]. Among this work, three notable trials focus on breast cancer patients in combination with Taxotere (chemotherapy combination), prostate cancer patients in combination with the dendritic cell vaccine sipuleucel-T (vaccine combination), and melanoma patients in combination with anti-PD1 (immune checkpoint combination) (http://clinicaltrials.gov/ct2/results?term=IDO&Search=Search; Vahanian et al., AACR 2017 late-breaking abstract). While the precise mechanism of action of indoximod has not yet been established definitively, striking cell-based experiments reveal that the mTORC1 pathway interprets indoximod at clinically relevant nanomolar concentrations as a mimetic of L-tryptophan [89]. Thus, indoximod may act in part by relieving the inhibitory effects of IDO-/TDO-mediated tryptophan deprivation on mTOR signals needed in T cells for antitumor activity. As further work reveals the precise mechanism of action, the low toxicity of indoximod as a simple D-tryptophan derivative remains an appealing feature of its clinical development, along with the opportunity it affords to leverage IDO1 and IDO/TDO enzymatic inhibitors.

17.7.2 GDC-0919

Prior to 2005, there was little motivation to develop inhibitors of IDO1, an unremarkable tryptophan-catabolizing enzyme. This situation changed with the first preclinical evidence of a role for IDO1 in cancer and of IDO1 inhibitor efficacy when combined with chemotherapy [8, 17–19]. In 2005 the only bioactive IDO inhibitor was 1-methyl-D,L-tryptophan (1-MT) with a reported K_i of 34 μM [90, 91]. One of the few other reported IDO inhibitors at the time was 4-phenyl-imidazole (4-PI) identified in 1989 as a weak noncompetitive inhibitor of IDO1 by Sono and Cady [92]. Interestingly, although 4-PI showed noncompetitive inhibition kinetics through impressive spectroscopic studies, Sono and Cady showed that 4-PI was actually binding to the heme iron at the active site. Subsequently, the first crystal structure of IDO to be reported [93] confirmed this finding by showing 4-PI bound to the heme iron (Fig. 17.4a). This confirmation along with the rich crystal structure information facilitated the first structure-based

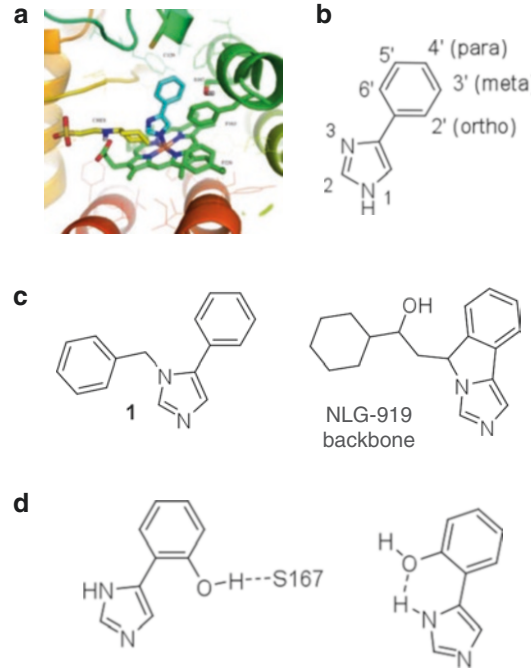

Fig. 17.4 Phenylimidazoles rooted in GDC-0919 development. (**a**) 4-PI bound to heme iron of IDO1. C129 is located above the 4-PI phenyl ring, while S167 resides in the back of the binding site. The buffer molecule CHES (*yellow*) is bound at the entrance of the active site of the IDO crystal structure. Graphics generated with PyMOL 1.0 [http://wwwpymol.org], an open-source molecular graphics system developed, supported, and maintained by DeLano Scientific LLC. http://www.delanoscientific.com. (**b**) Ring numbering of 4-phenylimidazole structures. (**c**) Structure of N-3 benzyl-substituted 4-PI and root structure of NLG-919. (**d**) Two possible benefits of 2′-OH substitution of 4-PI core

drug-design activities of Malachowski and colleagues, seeding work in the phenylimidazole series from which the clinical lead GDC-0919 was later derived [21].

In early foundational work [21], Malachowski and colleagues explored 4-PI analogs to probe the active site of IDO1 with structural modifications that were focused on exploiting three binding interactions within the IDO active site: (1) the *active site entrance region* decorated with the heme 7-propionic acid, (2) the *interior of the active site*, in particular interactions with C129 and S167, and (3) the *heme iron-binding group*. The enhancement of IDO inhibition of 4-PI structures through interactions at the active site entrance focused on the N-1, C-2, and N-3 positions of the imidazole ring (Fig. 17.4b). All three positions were substituted with the goal of appending groups that would occupy the active site entrance. In the crystal structure of 4-PI with IDO, this region contains an *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer molecule whose alkyl portion forms hydrophobic interactions with F163 and F226. In addition, the amino group of the CHES molecule forms an ion pair with the heme 7-propionic acid.

N-1 substituted 4-PI derivatives were completely devoid of inhibitory activity, which, not surprisingly, confirmed the binding of the N-1 nitrogen to the heme iron and, more importantly, demonstrated that the N-3 nitrogen of the imidazole cannot substitute to bind at the heme iron. However, N-3 benzyl-substituted derivatives (Fig. 17.4c1) were unexpectedly found to be roughly equipotent to 4-PI, thereby demonstrating that imidazole ring substitution was tolerable. The N-3 benzyl-substituted compound identified the correct imidazole ring location and spatial tolerance, likely occupying the active site entrance where the CHES buffer molecule sits in the IDO-4-PI crystal structure [93]. This discovery was consistent with the pharmacophore developed in studies of IDO1 inhibition by brassinin derivatives, i.e., a heme iron-binding group flanked by two large aromatic or hydrocarbon structures [94]. This proved prescient in light of subsequent development work by NewLink Genetics Inc. with regard to the backbone structure for the clinical candidate NLG-919 (Fig. 17.4c), renamed GDC-0919 after sublicense to Genentech/Roche. The NLG-919 backbone as shown extended from the same N-3 position to situate a similar hydrocarbon moiety in the active site entrance of IDO1.

Analysis of the crystal structure of 4-PI bound to IDO1 [93] indicated that S167 and C129 were in close proximity to the phenyl ring of 4-PI in the interior of the active site. Systematic evaluation of *ortho*, *meta*, and *para* substitutions of the phenyl ring with oxygen, sulfur, and fluorine was undertaken to ascertain if specific protein-ligand interactions could be exploited. The 2′-hydroxy (*ortho* substituted) modification afforded the most success generating a tenfold increase in potency relative to 4-PI (Fig. 17.4d). Two possibilities existed for this increased activity: intermolecular H-bonding with S167 or intramolecular H-bonding with N-3 to lock the phenyl and imidazole rings. The 2′,6′-dihydroxy-phenyl derivative, which presents a hydroxyl group to S167 or N-3 imidazole in either rotamer, was also synthesized, and it was roughly equipotent to the 2′-hydroxy derivative, thereby demonstrating that there was no additional benefit from both events. Subsequently, NewLink Genetics introduced a hydrocarbon bridge that replicates the H-bond to the N-3 imidazole, i.e., locking the conformation of the benzene and imidazole ring into one plane.

Modification of the critical heme iron-binding imidazole ring and its effect on IDO1 inhibition was also explored by Malachowski and colleagues. To probe the effect of heterocycle binding to the heme iron, alternative aromatic rings were substituted for the imidazole of 4-PI. These changes almost universally led to less potent compounds relative to 4-PI. For instance, pyridine, thiazole, pyrazole, and furan all failed to demonstrate any inhibition. Presumably the thiazole, pyrazole, and furan fail to bind to the heme iron with the same affinity as the imidazole, a well-known iron ligand in nature, e.g., histidine. The replacement of the phenyl group of 4-PI with thiophene was permitted, although there was

approximately a fivefold loss in activity. Only when the hydroxyl groups of the phenyl ring were returned was activity restored or modestly improved over 4-PI. This, again, was consistent with the hydroxyl group forming an intramolecular H-bond with the pyrazole nitrogen and locking the two rings in the same plane, as previously noted. Although these studies demonstrated that the imidazole group was optimal in terms of both iron-binding strength and shape complementarity, subsequent work illustrated that triazoles have related activity [95]. Overall, early studies yielded three critical discoveries about the phenylimidazole series leading to NLG-919/GDC-0919 development: (1) N-3 substitution was permitted and a rather large space existed in the active site to accommodate hydrocarbon moieties in this position; (2) incorporation of an *ortho*-hydroxyl group was beneficial; (3) the imidazole ring was optimal for binding to the heme iron.

Preclinical studies of GDC-0919 illustrate its potency as an IDO1 inhibitor with EC50 = 75 nM in cell-based assays and a 10- to 20-fold selectivity against TDO [96]. GDC-0919 is orally bioavailable and has a favorable pharmacokinetic and toxicity profile. Oral administration was shown to reduce plasma kynurenine levels by approximately 50% in mice. In human IDO1^{+} DCs in an allogeneic mixed lymphocyte reaction, GDC-0919 blocked IDO1-induced T-cell suppression and restored T-cell responses in vitro. In the B16 melanoma mouse model, co-administration of GDC-0919 with pmel-1 T cells and gp100 peptide vaccination reduced relative tumor size ~95% within 4 days of vaccination. Additionally, in the EMT6 syngeneic model, combining GDC-0919 with an anti-PD-L1 antibody improved relative antitumor efficacy [97]. The combination led to an increased CD8^{+} T/Treg ratio and higher plasma levels of interferon-γ. GDC-0919 treatment also resulted in the activation of intratumoral macrophages and DC in the model.

In the clinical setting, GDC-0919 has been studied as monotherapy so far in patients with recurrent/advanced solid tumors, and the safety, pharmacokinetic, and pharmacodynamic results from the phase 1a study were reported [98]. Overall, GDC-0919 was well tolerated up to 800 mg BID on a 21-/28-day cycle. The best response observed was SD in 7/17 patients. Plasma exposures of GDC-0919 increased from 50 to 800 mg in a dose-proportional manner, and plasma kynurenine levels were ~30% decreased transiently 4 h after dosing in a manner consistent with the predicted drug half-life. It was reported that safety, pharmacokinetics, and pharmacodynamics of GDC-0919 are being evaluated on a continuous dosing schedule (BID, 28/28 days). GDC-0919 is also being studied in combination with the anti-PD-L1 antibody atezolizumab (NCT0271846).

17.7.3 Epacadostat (INCB024360)

Epacadostat, developed under the code name INCB24360, is the lead clinical agent from a hydroxylamine series of IDO1-selective inhibitors pioneered by Incyte Corporation which is furthest in clinical development. Details regarding the chemistry effort that led to the development of epacadostat are covered in the recent publication of its identification and structure by the team at Incyte that spearheaded the project [99]. In preclinical studies, epacadostat selectively inhibited the tryptophan catabolic activity of human IDO1 in cell-based assays (IC50 = 10 nM) with little activity against IDO2 and TDO2. In co-cultures of human allogeneic lymphocytes with DC or tumor cells, epacadostat promoted the growth of effector T cells and NK cells, reduced conversion of naïve T cells to Tregs, and increased the number of CD86^{high} DC [100]. Consistent with these effects, administration of epacadostat to tumor-bearing syngeneic mice inhibited kynurenine levels ~90% in both plasma and tumor and reduced tumor growth in immunocompetent but not immunocompromised mice, confirming that drug efficacy relies upon functional immunity. Further, in the B16 melanoma model, epacadostat was found to enhance the antitumor effects of anti-CTLA4 or anti-PDL1 antibodies, where increased IL-2 produc-

tion and $CD8^+$ T-cell proliferation were suggestive of greater pronounced T-cell activity than either agent alone [101].

Clinical evaluation of epacadostat opened with a first-in-human phase I study to investigate safety and maximum-tolerated dose, pharmacokinetics, pharmacodynamics, and antitumor activity [102]. In this study, epacadostat was generally well-tolerated, effectively normalized plasma kynurenine levels and was maximally inhibitory to IDO1 activity at doses of >100 mg BID. While no objective responses were detected, stable disease lasting ≥16 weeks was observed in 7/52 patients [102]. A study co-administering epacadostat in combination with ipilimumab was conducted in patients with advanced melanoma [103]. Doses of epacadostat at 25 mg BID and 50 mg BID were generally well tolerated. Of note was a 31% ORR by Immune-Related Response Criteria (irRC) including 3/32 patients with complete responses. While uncontrolled, the median PFS by irRC was 8.2 months in patients who had not received prior immune therapy. The efficacy endpoints compared favorably with historical controls reported previously for ipilimumab, which demonstrated 11% ORR with a median PFS of 2.86 months [104].

Epacadostat is currently being studied in a total of 14 tumor types as co-administered with anti-PD-1 antibodies (nivolumab or pembrolizumab) or anti-PD-L1 antibodies (atezolizumab and durvalumab). Early pembrolizumab combination data indicated that the combination was well tolerated with promising clinical activity [105]. Among 19 treatment-naïve advanced melanoma patients, 4 CR, 7 PR, and 3 SD were reported resulting in 58% ORR and 74% DCR, with responses in all epacadostat dose cohorts ≥50 mg BID and at all target lesion sites including in liver, lung, and lymph nodes. All responses reported at presentation were confirmed and ongoing, and median PFS had not been reached [105]. These results compare favorably with pembrolizumab monotherapy or nivolumab-ipilimumab combination therapy in melanoma patients [106, 107]. In the epacadostat-pembrolizumab combination study, responses were observed in patients previously treated for advanced melanoma (n = 3; 1 CR, 1 SD) and in patients with NSCLC (n = 12; 5 PRs, 2 SDs), RCC (n = 11; 3 PRs, 5 SDs), endometrial adenocarcinoma (n = 7; 1 CR, 1 PR), TCC (n = 5; 3 PRs), TNBC (n = 3; 2 SDs), and SCCHN (n = 2; 1 PR, 1 SD). Based on these results, a phase III randomized double-blind, placebo-controlled study investigating pembrolizumab in combination with epacadostat or placebo for first-line treatment of patients with advanced or metastatic melanoma was initiated in June 2016 (ECHO-301 [NCT02752074]). Additional studies in lung, renal, head and neck, and bladder cancers are expected to open in 2017.

17.8 Other IDO/TDO Inhibitor Clinical Candidates

Additional IDO1 inhibitory compounds have been reported to be entering clinical testing. PF-06840003 is a tryptophan noncompetitive, nonheme-binding IDO1 inhibitor licensed by iTeos SA to Pfizer for clinical development (Wythes et al., SITC 2016, poster 253). This compound is predicted to have favorable human PK characteristics, a prolonged human half-life that may allow single-dose daily administration and CNS penetration properties that may enable efficient access to brain metastases. In preclinical study, PF-06840003 enhanced the antitumor efficacy of anti-PD1/PDL1 axis blockade. A first-in-patient study was initiated in 2016 in malignant gliomas (NCT02764151).

BMS-986205 is an IDO1 inhibitor licensed by Flexus Inc. to Bristol-Myers Squibb for clinical development. This compound is reported to have improved potency and pharmacokinetics relative to epacadostat and GDC-0919, and in 2015 it entered a phase 1 study in solid tumors both as monotherapy and in combination with nivolumab (NCT02658890). Several other IDO1 inhibitors are reported in late preclinical stages of development with little information disclosed to date. A summary of information on all the compounds mentioned above is listed in Fig. 17.5.

	Incyte	New Link	BMS	Merck	Pfizer	Roche
Partner (Date)	Non-exclusive	None	Flexus (3/15)	IOMet (1/16)	iTeos (12/14)	Curadev (4/15)
Compound Code	INCB24360	NLG 919	BMS-986205 (F001287)	IOM2983	PF-06840003	RG-70099
Lead Structure (patent example)	Published 2015	Markush known; Patent Published	Unknown; Patent Published	Unknown; Patent Published	Published 2016	Unknown; Patent Published
Cell-based Potency (nM)	12	75	2	NA	1100	NA
TDO selectivity (fold)	>133	10-20	>100	>100	>100	Dual Inhibitor 5x
Program Phase	Phase III	Phase II	Phase I	Preclinical	Phase I	Preclinical

Fig. 17.5 Summary of IDO1 inhibitors in clinical trials or reported for near-term entry

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Conflict of Interest G.C.P., W.J.M., and A.J.M. state a conflict of interest as inventors, shareholders, grant recipients, and advisors with NewLink Genetics Corporation, licensee of IDO-related intellectual property from the Lankenau Institute of Medical Research as described in US Patents 7705022, 7714139, 8008281, 8058416, 8383613, 8389568, 8436151, 8476454, and 8586636. The other authors state no conflict of interest. P.A.S. is an employee and shareholder of Incyte Corporation.

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Part III

FDA-EMA Approval of I-O

Tumor-Targeted Antibodies

18

Aurélien Marabelle

Contents

18.1 Introduction

At the end of the nineteenth century in 1894, Dr. Pierre-Paul Emile Roux from the Pasteur Institute in Paris demonstrated the clinical efficacy of serotherapy to treat diphtheria [1]. A century later in 1994, Prof. Ronald Levy from Stanford University, CA, USA, demonstrated that antibodies could also be used to treat cancers [2, 3]. Since then, major improvements have been made in the design and production of antibodies, and they are now part of the conventional therapies of many cancers. More recently, it has been shown that antibodies could also be used to generate adaptive antitumor immunity in patients with cancer, by targeting co-inhibitory receptors expressed at the surface of T cells. The promising results obtained with such immunomodulatory antibodies open many perspectives for synergistic combinatorial strategies between tumor-targeted and immune-targeted antibodies.

18.2 From Polyclonal to Monoclonal Antibodies

Upon their differentiation into plasma cells, B cells can secrete their B-cell receptor (BCR) in a soluble form called antibodies. Antibodies are immunoglobulins made of two heavy chains and two light chains (Fig. 18.1). In humans, there are two types of light chains (κ and λ) and five types

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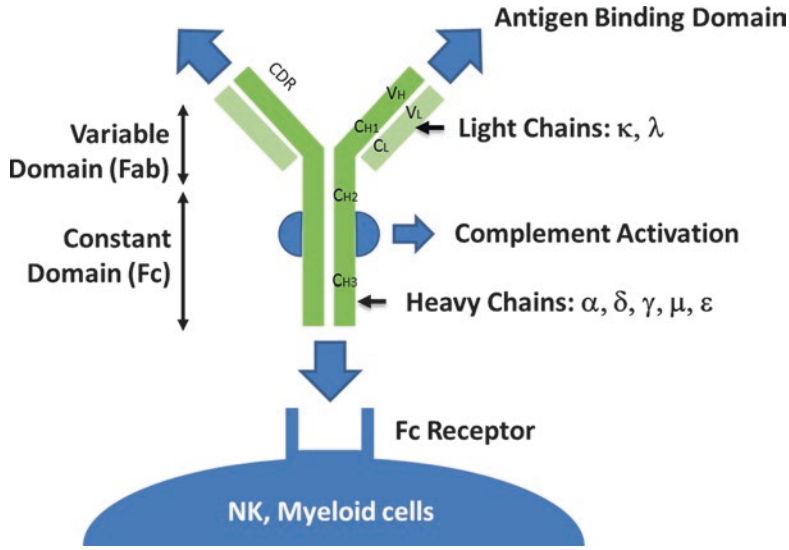

Fig. 18.1 Molecular structure of antibodies. Antibodies of the IgA, IgD, IgE, and IgG have two antigen-binding sites. IgA antibodies can be dimeric and IgM are pentameric. Monomeric antibodies have two antigen-binding sites per molecule. Antigen-binding sites are located in the complementarity determining regions (CDRs) within the Fab portion of the antibody. The Fab part, which stands for "antigen binding," is composed of domains associated with the light chain (VL, CL) and domains associated with the heavy chain (VH, CH1). The Fc portion of the antibody contains the CH2 and CH3 domains of the heavy chains. This region of the antibody can bind to a wide range of cell-associated receptors (e.g., Fc receptors)

of Ig heavy chains (α, δ, ε, γ, and μ). Immunoglobulins are grouped into five classes according to the structure of their heavy chains: IgA, IgD, IgE, IgG, and IgM. Among these, IgG are the predominant class, comprising ~80% of the immunoglobulins in the human serum [4]. In humans, there are four subclasses of IgGs (IgG1, IgG2, IgG3, and IgG4) which have specific biological properties. Immunoglobulins have a molecular weight at around 150 kDa. These large proteins can be artificially cleaved by proteases into two fractions: the Fab domain which contains the target recognition domain of the antibody and the Fc domain which can be of different forms depending on the immunoglobulin isotype (Fig. 18.1).

The first therapeutic antibodies were obtained by immunizing large animal (e.g., horses) against the targeted antigen (e.g., diphtheria toxin), resulting in the production in the serum of large, polyclonal antibodies specifically recognizing multiple epitopes of the antigen. Then the serum of these animals was used to treat patients (e.g., diphtheria). These polyclonal antibodies were responsible for many adverse events in patients, mainly because of their allogeneic nature that could be recognized and rejected by the host immune system.

Köhler and Milstein described in 1975 the hybridoma technique which allows to generate antigen-specific monoclonal antibodies [5]. This technique consisted in vaccinating rodents (mice, hamsters, etc.) with a specific antigen and subsequently collects the B cells from the spleen of the immunized animals. These B cells were then fused with an antibody nonproducing plasma cell cancer cell line (myeloma). These fusions between a primary B-lymphocyte and a myeloma cell are called "hybridoma." The mixture of hybridoma cells, made of multiple different primary B-cell clones, each secreting one specific antibody, could be separated into individual single cell culture wells. Those wells can then be screened to identify the ones which contain the hybridoma secreting the desired antibody. Hybridomas can be expanded in vitro in culture, and antibodies can be concentrated and purified from the culture supernatant.

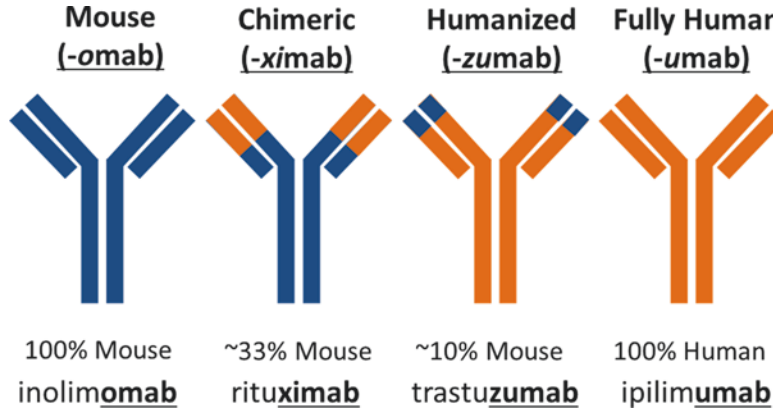

Fig. 18.2 Nomenclature and significance of monoclonal antibody names. As opposed to murine or chimeric monoclonal antibodies, humanized and fully human antibodies are poorly immunogenic and are therefore better tolerated and have better pharmacokinetic profiles

Since the hybridoma technique has been described, numerous other techniques have been developed, generally with the aim of not only improving the yield of monoclonal antibodies production but also reducing the immunogenicity of monoclonal antibodies [6]. Indeed, rodent-derived monoclonal antibodies can be recognized as foreign by the human immune system which can generate human anti-mouse antibodies (HAMA). Patients developing high levels of HAMAs can increase the clearance rate of the monoclonal antibody from their blood-reducing exposure/efficacy. HAMAs can also be responsible for serious anaphylactic or anaphylactoid adverse reactions. Therefore, many strategies have been developed to reduce the immunogenicity of rodent monoclonal antibodies, which have allowed to generate chimeric murine/human monoclonal antibodies or humanized monoclonal antibodies and eventually fully human monoclonal antibodies. The degree of humanization of monoclonal antibodies is specified in their name (Fig. 18.2). Chimeric monoclonal antibodies contain the rodent heavy and light variable chains from the Fab component, thereby retaining the antigen-binding specificity of the initial rodent monoclonal antibody. These variable parts are genetically fused to the human immunoglobulin constant regions (constant Fab and constant Fc regions). This chimerization was a way of preserving the specificity and affinity of the antibody while reducing its immunogenicity in immunocompetent patients. Humanized monoclonal antibodies consist in grafting human framework regions into the variable regions in order to only retain the murine complementarity-determining regions (CDRs) which have the antigen-binding ability. Fully human monoclonal antibodies have been generated, thanks to transgenic mice bearing human immunoglobulin genes. These mice can therefore produce fully human monoclonal antibodies upon immunization. Fully human monoclonal antibodies have significantly reduced immunogenicity and therefore generate low levels of human antihuman antibodies (HAHAs). The low titers of HAHAs usually do not impact significantly the pharmacokinetics of the human monoclonal antibodies and generate low levels of infusion-related reactions (<5% of patients) [7].

18.3 Monoclonal Antibodies, a Versatile Platform for Cancer Therapies

In 1982, Levy et al. demonstrated for the first time that tumor responses could be obtained in patients with B-cell lymphoma after administration of an antitumor monoclonal antibody designed to target specifically the BCR of their B-cell malignancy [2]. These first therapeutic monoclonal antibodies have inaugurated the concepts of tumor-targeted therapy and personalized medicine. The limitation of this approach was that it needed the generation of a personalized anti-BCR monoclonal antibody for each patient. In order to circumvent this limitation, a

monoclonal antibody was generated against CD20, an antigen commonly expressed by B cells and therefore by B-cell lymphomas. This monoclonal antibody, called rituximab, became the first monoclonal antibody to gain FDA approval for the treatment of cancer. Since then, many other tumor-targeting monoclonal antibodies have been approved in oncology and other indications (Fig. 18.3). The most prescribed tumor-targeted antibodies are directed against CD20 in B-cell malignancies (e.g., rituximab), against HER-2 (e.g., trastuzumab) in HER2-positive breast cancers, and EGFR (e.g., cetuximab) in head and neck squamous carcinomas and RAS wild-type colorectal cancers. All types of antigens could be in theory targeted by antibodies, including non-peptide ones such as gangliosides. Gangliosides are molecules composed of a glycosphingolipid (ceramide and oligosaccharide) with one or more sialic acids linked on the sugar chain. Recently, it has been demonstrated that a monoclonal antibody targeting the ganglioside GD2 could improve the survival of children with high-risk neuroblastoma [8].

18.3.1 Mechanisms of Action of Tumor-Targeting Antibodies

Monoclonal antibodies have multiple intrinsic properties which can be exploited against cancer cells. Antitumor monoclonal antibodies can have a direct cytotoxic effect on cancer cells by directly inducing tumor cell death upon ligation to the tumor-specific antigen expressed on their outer membrane. Monoclonal antibodies can also be selected for their antagonist properties on tumor growth factors, binding either the

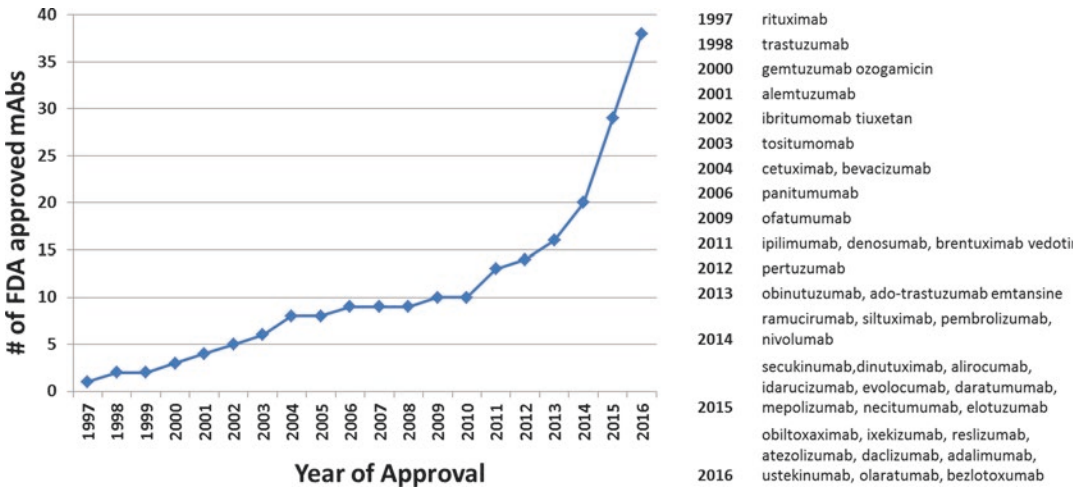

Fig. 18.3 FDA approvals for monoclonal antibodies. Monoclonal antibodies developed for autoimmune or inflammatory disorders could be of interest for treating immune-related adverse events of immune-targeted antibodies. Molecular targets: rituximab, CD20; trastuzumab, HER2; gemtuzumab ozogamicin, CD33; alemtuzumab, CD52; ibritumomab tiuxetan, CD20; tositumomab, CD20; cetuximab, EGFR; bevacizumab, VEGFR; panitumumab, EGFR; ofatumumab, CD20; denosumab, RANKL; brentuximab vedotin, CD30; ipilimumab, CTLA4; pertuzumab, HER2; obinutuzumab, CD20; HER2; ofatumumab, CD20; ramucirumab, VEGFR2; siltuximab, IL6; pembrolizumab and nivolumab, PD-1; secukinumab, IL-17A; dinutuximab, GD2; alirocumab and evolocumab, PCSK9 (proprotein convertase subtilisin kexin type 9) inhibitor; idarucizumab, dabigatran; daratumumab, CD38; mepolizumab, IL-5; necitumumab, EGFR; elotuzumab, SLAMF7; obiltoxaximab, bacillus anthracis; ixekizumab, IL17A; reslizumab, IL-5; atezolizumab, PD-L1; daclizumab, CD25; adalimumab, TNF; ustekinumab, IL-2 and IL-23; olaratumab, PDGFR-α; bezlotoxumab, clostridium difficile toxin B

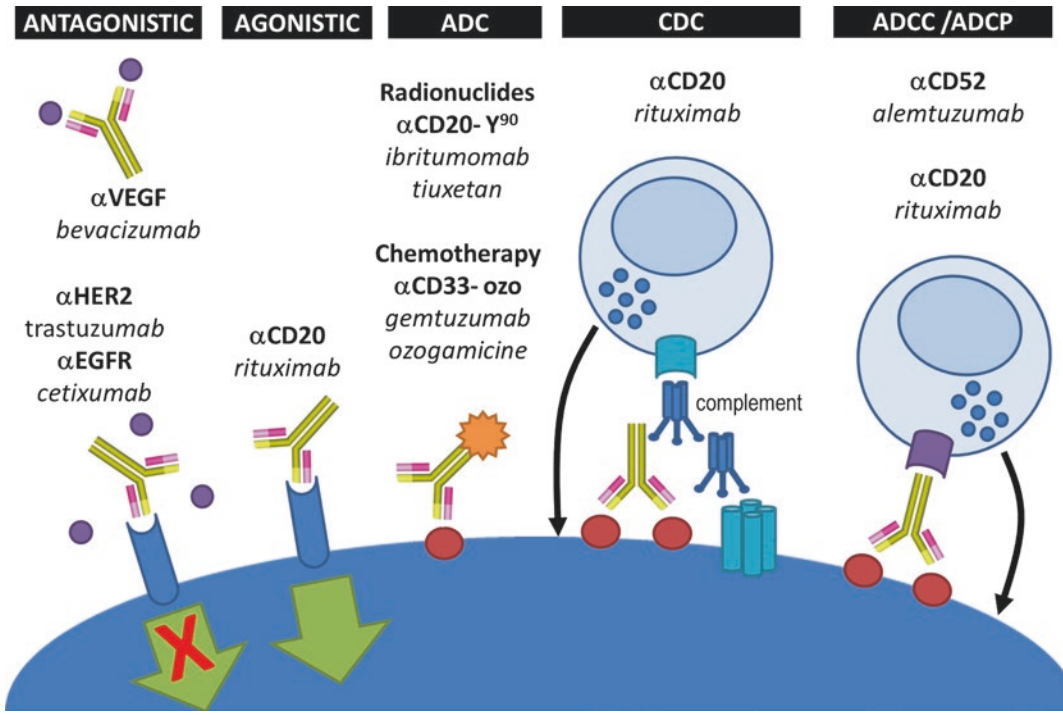

Fig. 18.4 Mechanisms of action of tumor-targeted monoclonal antibodies. Antibodies can be designed to either block a receptor or its ligand (antagonistic), or they can have intrinsic agonistic activities by directly stimulating receptors (e.g., pro-apoptotic signal). Tumor-targeted antibodies can be bound to either radioactive or chemotherapies to deliver the cytotoxic effect directly into the tumor bed (*ADC* antibody drug conjugate). The engineering of the Fc domains of antibodies can also enhance their ability to trigger complement-derived cytotoxicity (CDC), antibody-derived cell cytotoxicity (ADCC), or antibody-derived phagocytosis (ADCP)

ligands or blocking their cognate receptor (Fig. 18.4). Also, when monoclonal antibodies are opsonizing the surface of cancer cells, their distal part (the Fc domain) can trigger the destruction of the targeted cell upon engagement of the host immune effector mechanisms. First, they can activate the enzymes of the complement system which can directly attack the membrane of cancer cells or recruit immune effector cells via the complement receptor (CDC or complement-dependent cytotoxicity). Second, they may also activate cytotoxic innate immune effectors such as NK (natural killer) cells via the activation of their Fc gamma receptors (ADCC or antibody-dependent cellular cytotoxicity) (Fig. 18.4). Finally, upon opsonization of the targeted cells, the Fc domain of monoclonal antibodies can induce phagocytosis of cancer cells via the activation of Fc gamma receptors (FcγR) expressed on monocytes/macrophages (ADCP or antibody-dependent cellular phagocytosis) [9].

18.3.2 Engineering of Tumor-Targeting Monoclonal Antibodies

The role of Fc-mediated cancer cell death has been demonstrated in retrospective clinical studies showing the prognostic value of FcγR polymorphisms in B-cell lymphoma patients treated with anti-CD20 monoclonal antibodies [10] and in children with neuroblastoma treated with anti-GD2 monoclonal antibodies [11]. Therefore, most tumor-targeted antibodies have been

designed with an IgG1 isotype known to have a good affinity for Fc gamma receptors, notably CD16a (also known as FcγRIIIa), and an ability to activate natural killer cells (NK cells) to perform antibody-dependent cellular cytotoxicity (ADCC). Although the rationale for using IgG1 has been mainly built on blood NK cells, recent data suggest that IgG2 can also perform ADCC via FcγR expressing myeloid cells [12].

Within the CH2 region of antibodies Fc domain, amino acids (notably Asn297) can undergo glycosylation. The type of glycosylation of antibodies has an impact on the antibody FcγR affinity and its ability to trigger antibody-dependent cellular cytotoxicity [13]. The modification of monoclonal antibodies glycosylation status can now be engineered in order to enhance ADCC (Fig. 18.5). For example, obinutuzumab, a defucosylated anti-CD20 monoclonal antibody with enhanced ADCC potency in comparison to rituximab, has been approved by the FDA in 2013 for the treatment of chronic lymphocytic leukemia.

Thanks to their ability to target specifically cancer cells, monoclonal antibodies are now also used to deliver cytotoxic compounds directly to cancer cells and avoid off target toxicities. Indeed monoclonal antibodies can be linked to a cytotoxic molecule such as the anti-CD33 gemtuzumab ozogamicin (Mylotarg*) currently developed in acute myeloid leukemia or the new anti-CD30 brentuximab vedotin (Adcetris®) currently approved for Hodgkin lymphoma. Monoclonal antibodies can also be used to deliver radioactive molecules in situ, such as 90Y-ibritumomab (Zevalin®), a modified anti-CD20 antibody developed in non-Hodgkin lymphomas.

18.4 From Tumor-Targeting to Immune-Targeting Monoclonal Antibodies (and Back)

Although tumor-targeting monoclonal antibodies have shown clinical benefit and become established therapies in several cancers, this success has not been universal. Many antitumor monoclonal antibodies have failed their clinical development, either because they could not effectively trigger the host effector mechanisms (e.g., low level of target antigen expression, low penetrance in the tumor microenvironment, etc.) or because of cancer cell escape via the downregulation of the targeted antigen (e.g., B-cell lymphoma cells becoming CD20 negative). Furthermore, like other passive therapies, tumor-targeting monoclonal antibodies last only as long as the monoclonal antibody is in the patients, and relapses can occur upon treatment discontinuation. Stimulating the adaptive immune system by targeting co-stimulatory/co-inhibitory signals expressed on T cells could potentially generate active and long-lasting antitumor immunity. Also, since tumor-specific antigens are not targeted, such immunomodulatory monoclonal antibodies are not disease specific and can potentially achieve broad, polyclonal antitumor immunity, directed against multiple tumor antigens, therefore addressing better the

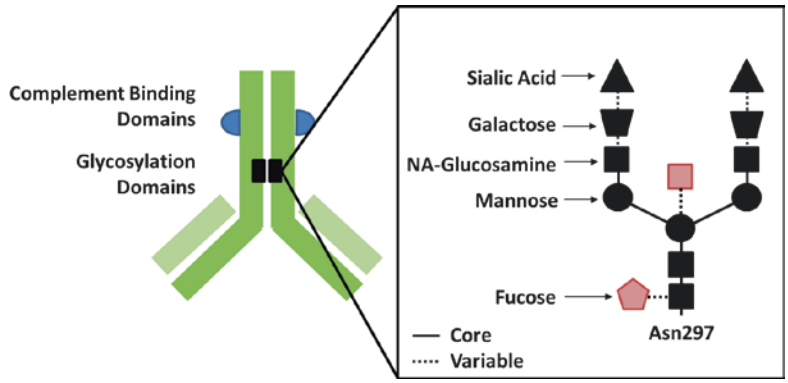

Fig. 18.5 Glycosylation status of monoclonal antibodies. Defucosylation or ablation of bisecting *N*-acetyl glucosamine (*in red*) enhances the affinity of monoclonal antibodies for Fc receptors and their ability to trigger antibody-derived cell cytotoxicity (ADCC)

heterogeneity of cancers and reducing the chances of immune escape. The first FDA-/EMA-approved immune-targeted monoclonal antibodies were targeting CTLA-4 (cytotoxic T-lymphocyte antigen 4) [14, 15], PD-1, and PD-L1 (programmed death-1 and its ligand) [16]. All (except one) anti-PD-1 and anti-PD-L1 antibodies currently in clinical development are purely antagonistic monoclonal antibodies because they have an IgG4 isotype (nivolumab from BMS, pembrolizumab from Merck/MSD) or bear a mutated IgG1 (atezolizumab from Roche and durvalumab from AstraZeneca) in order to prevent FcγR engagement. The only exception is avelumab (from Pfizer) which is an unmodified IgG1 anti-PD-L1. Avelumab could therefore also be used as a tumor-targeting monoclonal antibody against cancers with PD-L1-positive tumor cells [17]. With the same idea, ipilimumab, the first anti-CTLA-4 monoclonal antibody approved, is an IgG1 and could, in theory, also be used to treat CTLA-4-positive T-cell malignancies.

18.5 Bi-specific Antibodies: Tumor and Immune Targeted

The technical progresses in the design of monoclonal antibodies allow now to create antibodies with a dual specificity. This has led to the design of bi-specific antibodies with the aim of forcing the destruction of a target cell by a T cell. For instance, bi-specific T-cell engagers (BiTEs™) are fusion proteins made of two single-chain variable fragments (scFvs) of different antibodies. One of the scFvs binds to and activates T cells via the CD3 receptor. The other scFv binds to a tumor cell via a tumor-specific molecule. This is the case, for instance, of the anti-CD3/anti-CD19 blinatumomab antibody developed by Micromet/Amgen and currently approved for the treatment of relapsing B-cell leukemias [18, 19] and has also shown clinical activity in patients with B-cell lymphoma [20]. Many bi-specific antibodies are currently in clinical development, including in solid tumors. Some of them are full-length antibodies with two Fabs and an Fc domain [21, 22].

18.6 Challenges and Perspectives for Tumor-Targeted Antibodies

It is now well established that the immune system plays an important role in the tumor response to conventional chemotherapies [23]. However, the successive myeloablative chemotherapy cycles received by cancer patients may eventually hamper the ability of their immune system to fight against cancer cells. The impact of the immune system in the efficacy of radiotherapy has also been well described but, besides the very rare cases of abscopal responses, seems limited to the irradiated fields and do not allow the control of distant, unirradiated, metastatic tumor sites [24]. Tumor-targeted therapies such as tyrosine kinase inhibitors and tumor-targeted monoclonal antibodies can generate systemic tumor responses in patients bearing target-positive tumors with limited hematologic/immune toxicities. However, their antitumor efficacy is often limited in time due to the emergence of mechanisms of resistance. Clinically, this limited effect in time is translated by longer median progression-free survivals, but not by significantly improved long-term overall survivals. As opposed to tumor-targeted antibodies, immune-targeted antibodies seem to be able to generate long-lasting tumor remissions and to have a significant impact in the overall survival of patients in comparison to conventional therapies. The preclinical rationale built over the last 15 years in murine syngeneic tumor models strongly suggests that the combination of tumor-targeted antibodies might synergize with immune-targeted antibodies to improve both the objective response rates in cancer patients and their long-term survival. Anti-CD137 agonistic antibodies have been shown, for instance, to enhance NK cells' antibody-derived cell cytotoxicity and CD8 T cells' responses when used in combination with CD20, HER2, or EGFR tumor-targeted antibodies [25–27]. Anti-KIR antibodies have been shown to enhance tumor-targeted antibodies by mimicking missing-self signaling on NK cells [28]. Also, anti-CD47 monoclonal antibodies have been shown to enhance tumor-targeted

antibodies by triggering phagocytosis of macrophages following the disruption of this "don't eat me signal" [29]. All these immunomodulatory antibodies are currently tested in early phase clinical trials either alone or in combination, notably with tumor-targeted antibodies. This rapid clinical translation will allow to evaluate shortly the validity of these preclinical rationales.

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PD1 Checkpoint Blockade in Melanoma: From Monotherapy to Combination Therapies

19

Annette Paschen and Dirk Schadendorf

Contents

19.1 Malignant Melanoma

Malignant transformation of pigment-producing melanocytes leads to the development of melanoma in different tissues giving rise to distinct tumor subtypes (e.g., cutaneous, mucosal, uveal melanoma) each characterized by specific genetic alterations. UV light is a major risk factor for cutaneous melanoma which dominates in the Western World with an incidence of 15–25 per 100,000 individuals and a median age at diagnosis of 57 years, while mucosal melanoma is prevalent in Asia. Cutaneous melanoma is one of the most common cancers in young adults, and due to its early metastatic spread, melanoma is responsible for 75% of deaths related to skin cancer [1].

Tremendous progress has been made over the last decade in elucidating the genetic alterations involved in melanoma development and progression [2]. Notably, melanomas are characterized by the highest somatic mutation rate of all human malignancies (>10 mutations per megabase of DNA), showing a typical UV-induced mutation signature which is in line with UV radiation as a risk factor for melanoma [3, 4]. However, occurrence of melanomas in non-sun-exposed skin or in the mucosa argues against its absolute UV dependency. The large majority of mutations are so-called passenger mutations which are irrelevant for disease development and progression. In contrast, mutations in the mitogen-activated protein kinase (MAPK)

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cascade (NRAS-BRAF-MEK-ERK) are major drivers of the oncogenic process. In cutaneous melanomas, *NRAS* (*NRAS*^{Q61L} or *NRAS*^{Q61R}) mutations are present in about 15–20%, whereas 50% harbor mutations in *BRAF* (*BRAF*^{V600E}). These mutually exclusive *NRAS* and *BRAF* mutations mediate constitutive activation of the MAPK pathway, and its inhibition induces cell cycle arrest and apoptosis in melanoma cells. While constitutive MAPK signaling is considered a first step in the development of melanoma, additional mutations affecting other pathways, such as PTEN loss contributing to PI3K pathway activation, are required to achieve full malignant transformation. The genes driving this process are listed elsewhere [2].

Until recently, the median survival of patients with distant metastatic melanoma (stage IV) was only 6–12 months. However, progression-free survival (PFS) and overall survival (OS) were strikingly prolonged due to the clinical implementation of two different types of agents: (a) inhibitors targeting the MAPK pathway at the level of BRAF(V600E) (like vemurafenib, dabrafenib) and MEK (such as trametinib or cobimetinib) [5–7] and (b) antibodies (anti-PD1 and anti-CTLA-4) unleashing antitumor T cell responses. Among those antibody-based immunotherapies, usually termed immune checkpoint blockade, the highest durable tumor response rates have been achieved with anti-PD1 and anti-PD1-based combination therapies which will be addressed in this review. To gain insight into the underlying mechanisms, we will at first give some background information on the recognition of melanoma cells by adaptive cytotoxic CD8$^+$ T cells, which are reactivated in the course of the treatment and initiate clinical responses.

19.2 CD8$^+$ T Cell Responses Against Melanoma

Melanoma is a highly immunogenic tumor. Many patients develop spontaneous adaptive antitumor CD8$^+$ T cell responses prior to any treatment. Such T cells recognize melanoma cells with their T cell receptor (TCR) that binds to specific cell surface complexes consisting of peptides (epitopes) presented on HLA class I molecules. Those peptides originate from the degradation of endogenous proteins (antigens) in the tumor cells, followed by peptide loading onto HLA class I molecules for transportation and presentation at the cell surface. HLA class I-antigen peptide complexes are expressed on all nucleated cells of the body, but compared to normal cells, tumor cells show qualitative and quantitative differences in the antigen repertoire, allowing CD8$^+$ T cells to recognize them as "altered self" [8]. Upon binding to specific antigen peptide-HLA class I complexes, CD8$^+$ T cells release cytolytic granules onto their targets, thereby initiating apoptosis, and in addition secrete interferon-γ (IFNγ) that induces cell cycle arrest and/or death in surrounding cells [9].

Since 1991, many tumor antigens of distinct categories have been identified in melanoma [8], which are described in more detail in another chapter of this book. Among those, the category of neoantigens is of highest therapeutic interest: First, neoantigens are truly tumor-specific since they are derived from expressed somatic tumor mutations minimizing the risk of normal tissue destruction in the course of an antitumor CD8$^+$ T cell response. Second, neoantigen-specific CD8$^+$ T cells express high affinity TCRs for their cognate antigen-HLA class I complexes and have been demonstrated to be very potent antitumor effectors [10]. As indicated above, melanomas show a high mutational load that is mandatory for efficient neoantigen generation. In fact, not all somatic tumor mutations are expressed, and only a few of the expressed mutations pass the intracellular degradation machinery to end up as neoantigen peptide epitopes at the cell surface. Interestingly, recent studies suggest a correlation between melanoma mutational load and response to antibody-based immune checkpoint blockade [11].

CD8$^+$ T cells specific for distinct tumor antigens, including neoantigens, have been detected in the peripheral blood and in metastatic lesions of melanoma patients [12–14]. But in general,

disease progresses even in the presence of such tumor-reactive T cells. It is now well established that melanoma progression is due to a multitude of mechanisms that diminish T cell function. Among those the inhibition of T cell activity by direct tumor cell contacts seems to play a major role.

19.3 Checkpoint Control of T Cell Activation

Tumor antigen-specific $CD8^+$ T cells infiltrating melanoma lesions receive activating signals via their TCR. As a consequence, IFNγ is secreted that induces expression of PD-L1 (also known as B7-H1 and CD274) on surrounding melanoma cells as shown in Fig. 19.1 [15]. PD-L1 engages the inhibitory co-receptor PD1 that is induced on $CD8^+$ T cells upon TCR-dependent activation, thereby attenuating the T cell's effector function (cytolytic granule and cytokine release) as well as their proliferation [16]. PD1 also binds to PD-L2, not that broadly detectable on melanoma cells. Under normal conditions, PD1 signaling in T cells is of importance for the maintenance of self-tolerance as indicated by development of spontaneous autoimmunity in PD1 knockout mice [17]. Thus, PD1 is a checkpoint of $CD8^+$ T cell activation, and by acquisition of PD-L1 surface expression, melanoma cells acquire the capacity to directly diminish T cell activity (Fig. 19.2). PD1 expression has been demonstrated also on neoantigen-specific $CD8^+$ T cells isolated from tumor tissue as well as from peripheral blood of melanoma patients [13, 14], providing a rationale for the development of therapeutic strategies blocking the interaction between PD1 and PD-L1 (Fig. 19.2).

Another checkpoint of T cell activation is CTLA-4, the ligands of which are CD80 and CD86 that in contrast to PD-L1 are not expressed on melanoma cells. Instead, both ligands are expressed on professional antigen-presenting

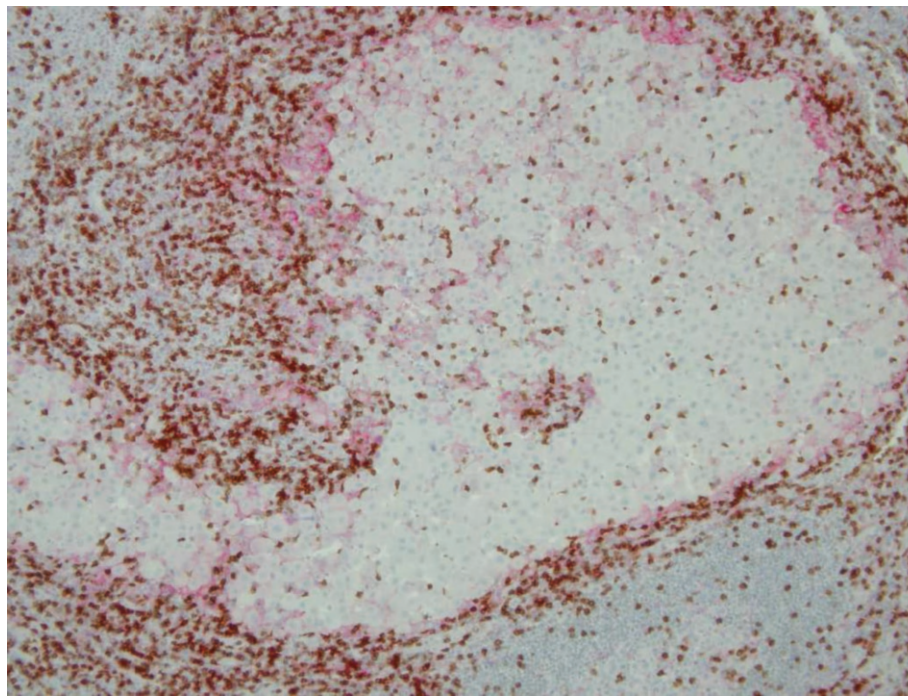

Fig. 19.1 Melanoma cells localized next to $CD8^+$ T cells express PD-L1. Immunohistochemistry staining of a melanoma lymph node lesion for CD8 (*brown color*), labeling T cells, and PD-L1 (*red color*), labeling melanoma cells

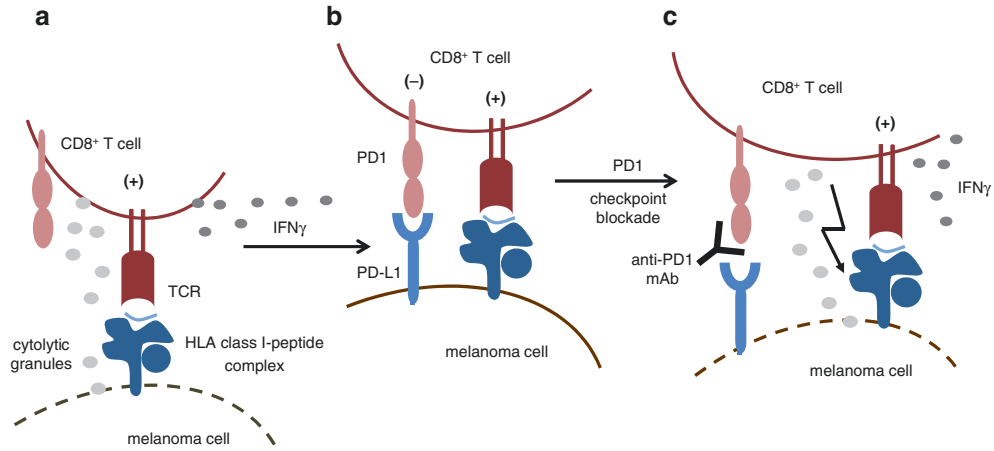

Fig. 19.2 PD1-dependent inhibition of T cell activity by melanoma cells. (**a**) T cells infiltrating tumor lesions recognize melanoma cells with their T cell receptor (TCR) that binds to specific HLA class I-antigen surface complexes. This leads to the activation of T cells associated with the release of cytolytic granules and the secretion of IFNγ. (**b**) IFNγ induces expression of PD-L1 on melanoma cells that binds to the inhibitory receptor PD1, thereby dampening T cell activation. (**c**) Blockade of the interaction between PD1 and its ligand PD-L1 by antibodies releases T cells from inhibitory signaling and restores their activity

cells (pAPC) such as dendritic cells (DC). DCs are equipped to sample tumor antigens in the periphery followed by their migration to draining lymph nodes, where primary T cell activation by tumor antigen-presenting DC takes place [18]. Upon TCR ligation, CTLA-4 is expressed on the surface of T cells that engages CD80/CD86 on pAPC thereby dampening T cell activation [19]. CTLA-4, like PD1, is of importance for the maintenance of self-tolerance. Mice deficient in CTLA-4 show massive lymphoproliferation and early lethality [20]. While expression of CTLA-4 is restricted to T cells, including regulatory T cells (Treg), PD1 is expressed also on natural killer (NK) cells and B cells, and the activity of these cells is most likely affected by antibodies targeting the immune checkpoints. Within this review, we cannot in detail address the complex biology of the receptors and their ligands, but one should keep in mind that PD1 and CTLA-4 can be considered as immune brakes (checkpoints) that limit the effector function of tumor-reactive CD8+ T cells. Release from these brakes (checkpoints) with therapeutic antibodies blocking the receptor-ligand interactions can induce striking and long-lasting clinical responses in melanoma patients as described in the following.

19.4 Checkpoint Blocking Therapy

Before 2011, melanoma patients with advanced metastatic disease received standard palliative treatments with chemotherapeutic agents (dacarbazine, temozolomide, fotemustine). However, large randomized trials demonstrating an impact on overall survival were lacking for these drugs. In 2011, the first checkpoint blocking antibody (ipilimumab, fully human IgG1 antibody) targeting CTLA-4 was approved for therapy of advanced disease patients by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA), based on two randomized trials. One trial compared ipilimumab to a peptide-based vaccine in patients with previously treated unresectable advanced melanoma [21]. In the second trial, patients were treated with ipilimumab plus dacarbazine or dacarbazine plus placebo [22]. Both studies demonstrated a significant improvement for patients being treated with ipilimumab, with response rates between 10 and 15% [21, 22]. Due to the role of CTLA-4 in the maintenance of self-tolerance, its systemic blockade is also associated

with toxicity. Severe in some cases, life-threatening adverse events (AE), defined as grade 3–4, occurred in 10–15% of patients receiving ipilimumab monotherapy. The most common grade 3–4 AE were colitis, skin rash, and endocrinopathies [21]. Overall immune checkpoint blockade with ipilimumab was the first therapy with a documented improved overall benefit in a subgroup of melanoma patients. This was a milestone in melanoma immunotherapy (details on CTLA-4-based therapies are given in another chapter of this book) that was quickly followed by success stories based on antibodies targeting the PD1 immune checkpoint.

19.5 Approved Anti-PD1 Antibodies

Subsequent to ipilimumab, two antibodies targeting the checkpoint PD1 were approved for the treatment of non-resectable and metastatic melanoma. Approval by the FDA and the EMA was given for nivolumab and pembrolizumab, a fully human and a humanized IgG4 antibody, respectively. Basically, nivolumab- and pembrolizumab-based therapies show much higher durable response rates, and AE occur at a much lower frequency compared to ipilimumab therapy. In the following, we will list the results of some clinical trials relevant to the approval of the antibodies (Table 19.1).

Table 19.1 Phase III trials related to the approval of PD1 checkpoint blocking therapies

Antibody		Trial	Patient cohort	Treatment arms	Outcome
Monotherapy	Nivolumab	CheckMate 066 (NCT01721772) Robert et al. NEJM 2015 [23]	Unresectable stage III or IV melanoma, no prior systemic treatment, no BRAF mutation (n = 418)	Nivolumab vs. dacarbazine	1-year OSR: 72.9% vs. 42.1% Median PFS (m): 5.1 vs. 2.2 ORR: 40% vs. 13.9% AE grade 3 and 4: 11.7% vs. 17.6%
		CheckMate 037 (NCT01721746) Weber et al. Lancet Oncol. 2015 [24]	Unresectable stage III or IV melanoma, after ipilimumab or after ipilimumab and BRAFi for BRAF mutants (n = 405)	Nivolumab vs. investigator's choice of chemotherapy (ICC): dacarbazine or paclitaxel + carboplatin	ORR: 31.7% vs. 10.6% AE grade 3 and 4: 5% vs. 9%
	Pembrolizumab	KEYNOTE 066 (NCT01866319) Robert et al. NEJM 2015 [28]	Unresectable stage III or IV melanoma, no more than one prior systemic treatment (n = 834)	Pembrolizumab (every 2 or every 3 weeks) vs. ipilimumab	6-month PFS: 47.3%, 46.4% vs. 26.5% Estimated 12-month SR: 74.1%, 68.4% vs. 58.2% RR: 33.7%, 32.9% vs. 11.9% AE grade 3–5: 13.3%, 10.1% vs. 19.9%
Combination therapy	Nivolumab + ipilimumab	CheckMate 067 (NCT01844505) Larkin et al. NEJM 2015 [29]	Unresectable stage III or IV melanoma, no prior systemic therapies (n = 945)	Nivolumab plus ipilimumab or nivolumab vs. ipilimumab	Median PFS (m): 11.5 vs. 6.9 vs. 2.9 AE grade 3 and 4: 55% vs. 16.3% vs. 27.3% OS not yet available

OSR overall survival rate, *OS* overall survival, *PFS* progression-free survival, *ORR* overall response rate, *SR* survival rate, *RR* response rate, *AE* adverse event

19.6 Nivolumab

The FDA approved nivolumab for treatment of advanced melanoma in December 2014, followed by an EMA approval in June 2015 based on the following two studies: CheckMate 066 [23] (randomized, double blind phase III study, 418 melanoma patients included) and CheckMate 037 [24] (open-label phase III study, 405 melanoma patients included) (Table 19.1). In both studies, nivolumab was administered at a dose of 3 mg/kg body weight every 2 weeks.

In the CheckMate 066 trial, patients without prior treatment received either nivolumab or dacarbazine. The 1-year overall survival (OSR) for patients treated with nivolumab was 72.9% compared to 42.1% for dacarbazine. The median progression-free survival (PFS) was 5.1 months for nivolumab and 2.2 months for dacarbazine. The overall response rate (ORR) was 40.0% with nivolumab and 13.9% with dacarbazine, and complete response was observed in 7.6% of the patients treated with nivolumab.

In the CheckMate 037 trial, patients were treated with either nivolumab or chemotherapy (dacarbazine or carboplatin and paclitaxel [investigators choice]). Patients enrolled in this study received pretreatment with nivolumab or BRAF inhibitor (in case of BRAF(V600E) mutant melanoma). The ORR was 31.7% for the nivolumab and 10.6% for the chemotherapy group. Notably, this trial demonstrated that patients pretreated with ipilimumab can still be nivolumab responders. This activity led to accelerated FDA approval of nivolumab in December 2014.

In summary, response rates to nivolumab ranged between 30 and 40%, strikingly higher compared to those reported for ipilimumab treatment. Nivolumab is also much better tolerated. Of course side effects occur, since PD1, like CTLA-4, is also involved in the maintenance of self-tolerance. However, these are less frequent and less severe. In both the CheckMate 066 and the CheckMate 037 studies, the most common side effects in the nivolumab treatment groups were fatigue, pruritus, and nausea. Drug-related AEs of grade 3 or 4 were 11.7% (CheckMate 066) and 5% (CheckMate 037).

19.7 Pembrolizumab

The FDA approved pembrolizumab (formerly known as MK3475 or lambrolizumab) for treatment of advanced melanoma in September 2014, followed by an EMA approval in July 2015 based on the following three trials: the phase I trial KEYNOTE-001 [25, 26], the phase II trial KEYNOTE-002 [27], and the phase III trial KEYNOTE-006 [28].

In the KEYNOTE-001 trial, melanoma patients (n = 135) with or without prior systemic treatment were treated with pembrolizumab at a dose of 10 mg/kg body weight every 2 or 3 weeks or 2 mg/kg body weight every 3 weeks. Across all dose, cohorts of the ORR was 38%, and the overall median PFS was >7 months [25]. In a randomized expansion cohort, melanoma patients (n = 173) showing treatment failure to ipilimumab and BRAF inhibitors (for BRAF(V600) mutant melanoma) were treated with pembrolizumab 2 mg/kg body weight or 10 mg/kg body weight every 3 weeks. There was no significant difference in ORR, median PFS, and 1-year survival rate between both dose cohorts [26]. Notably, this trial demonstrated that patients pretreated with ipilimumab could respond to nivolumab.

In the KEYNOTE-002 randomized trial, patients (n = 540) received pembrolizumab at a dose of 2 or 10 mg/kg body weight or were treated with chemotherapy (investigator's choice). Patients enrolled in this study were pretreated with ipilimumab and BRAF inhibitors (for BRAF(V600) mutant melanoma). Patients receiving either of the two pembrolizumab doses showed a significantly higher PFS rate after 9 months in comparison to patients treated with chemotherapy: 24 and 29% for pembrolizumab 2 and 10 mg/kg body weight, respectively, compared to 8% in the chemotherapy arm [27].

In the phase III randomized trial, KEYNOTE-006 patients (n = 834) with no more than one prior systemic therapy received either pembrolizumab or ipilimumab (Table 19.1). Pembrolizumab was administered at 10 mg/kg body weight either every 2 or every 3 weeks,

whereas ipilimumab was given in four doses of 3 mg/kg body weight every 3 weeks. The 6-month PFS was 47.3 and 46.4% for pembrolizumab administered every 2 and 3 weeks, respectively, compared to 26.5% for ipilimumab. The estimated 1-year survival rate was 74.1% and 68.4% for pembrolizumab given every 2 and 3 weeks, respectively, and 58.2% for ipilimumab. The response rates for pembrolizumab were around 33 and 12% for ipilimumab. Two interim analyses revealed an increase OS for patients treated with pembrolizumab. This led to an early stop of the study, and patients receiving ipilimumab were offered to cross over to treatment with pembrolizumab.

Regarding treatment toxicities, AE were less frequent and less severe in patients treated with pembrolizumab compared to ipilimumab (or chemotherapy). Most common AE were fatigue, diarrhea, endocrine disorders, rash, and pruritus. Drug-related AE of grade 3 or 4 developed in 10–13% of the pembrolizumab-treated patients (administered in different weekly intervals) and in 19.9% of patients receiving ipilimumab.

In summary, response rates to pembrolizumab ranged between 30 and 40%, again strikingly higher compared to those reported for ipilimumab treatment. The activity in pembrolizumab melanoma patients pretreated with ipilimumab led to an accelerated FDA approval. Since testing of different doses and schedules did not reveal significant difference, pembrolizumab received approval in a dose of 2 mg/kg body weight administered every 3 weeks.

19.8 Approved Combined Anti-PD1/Anti-CTLA-4 Combination Therapy

Since PD1 and CTLA-4 receptors bind to different ligands expressed also on distinct cell types, combination therapies were initiated to test the checkpoint blocking antibodies for their synergistic or additive effects. In the CheckMate-067 trial, melanoma patients (n = 945) without prior treatment received a combination of nivolumab and ipilimumab or monotherapy with either ipilimumab or nivolumab (randomization 1:1:1) [29]. The combination therapy mediated a significantly higher PFS (11.5 months) and ORR (57.6%) compared to monotherapy with nivolumab (PFS, 6.9 months; ORR, 43.7%) or ipilimumab (PFS, 2.9 months; ORR, 19.0%). Of the patients treated with the nivolumab/ipilimumab combination, 11.5% developed a complete response compared to 8.9 and 2.2% of the patients receiving nivolumab and ipilimumab monotherapy, respectively. Patients treated with the combination experienced a 58% reduced risk of death or tumor progression compared to ipilimumab monotherapy. The higher response in the combination therapy arm was associated with a higher toxicity: grade 3 and 4 AE occurred in 55% of patients treated with the combination and in 16.3 and 27.3% of patients receiving nivolumab and ipilimumab, respectively. Interestingly, it turned out that in patients with PD-L1-negative tumors (defined by immunohistochemistry), the response rate to the nivolumab/ipilimumab therapy was higher compared to the nivolumab monotherapy, while this difference was not observed for patients with PD-L1-positive tumors. Based on this observation, the FDA approved the nivolumab/ipilimumab combination therapy in 2016 for advanced-stage melanoma patients with PD-L1-negative tumors, while in Europe the combination therapy received approval independently of the tumor's PD-L1 status.

Overall anti-PD1 and anti-PD1/anti-CTLA-4 therapies induce striking clinical responses in a patient subgroup. But still, a majority of patients is primary resistant to checkpoint blockade, and also acquired resistance is a relevant topic.

19.9 Biomarkers Associated with Response to Anti-PD1 Therapy

To define the patient population that will most likely benefit from anti-PD1 treatment, pretreatment biopsies have been heavily screened for predictive biomarkers that, however, have not

been defined yet. Within several clinical trials, expression of PD-L1 in tumor samples has been determined by immunohistochemistry. However, different staining antibodies and different cutoff values were chosen to define PD-L1 positivity rendering results hardly comparable. It seems that response rates to PD1 checkpoint blockade are higher in patients with PD-L1-positive tumors, although patients with PD-L1-negative tumors can respond to anti-PD1 therapy [30, 31]. The presence of infiltrating lymphocytes within the tumor parenchyma but also at the margin of the lesion has been demonstrated to be associated with response to anti-PD1 therapy [31]. This is in line with the concept that $CD8^+$ T cells are reactivated in the course of the treatment and initiate clinical responses. T cells specific for neoantigens might be of highest relevance in this process. According to this, melanoma mutational load determining the amount of neoantigens has been associated with response to anti-CTLA-4 therapy, but so far, this association has not been seen for anti-PD1 treatment [11, 32]. Most likely, it will be necessary to combine multiple biomarkers, some of them to be defined in future studies, in order to predict response to PD1 checkpoint blockade [33].

19.10 Resistance to Therapy

Intensive cooperations between clinicians and scientists are ongoing in order to elucidate the mechanisms that mediate resistance to PD1 immune checkpoint blockade. The studies published so far suggest that the mechanisms are multifaceted as excellently summarized in several recent reviews [34, 35]. Basically, it can be distinguished between tumor-intrinsic and tumor-extrinsic factors that contribute to resistance. The latter include the activity of different immune-regulatory cells in the tumor microenvironment (regulatory T cells, myeloid-derived suppressor cells, neutrophils), which are addressed within different chapters of this book. Here we focus on melanoma-intrinsic factors that have been identified in the context of anti-PD1 therapy resistance, either primary or acquired resistance.

Genetic instability of melanoma, while on the one hand generating therapy-relevant neoantigens, allows resistant tumor clones to evolve under therapy. As such HLA class I-negative melanoma cells have been isolated from a progressing lesion of a patient initially responding to anti-PD1 antibody treatment [36]. These tumor cells genetically acquired a deficiency in *B2M*. Since B2M is an essential component of all HLA class I-antigen complexes, the cells developed an irreversible HLA class I-negative phenotype that could no longer be attacked by T cells, a phenomenon previously described for melanoma indicating that complete T cell resistance is a relevant topic [37, 38].

Recently, mutations abrogating IFNγ signaling in melanoma cells have been demonstrated to play a role in primary and acquired anti-PD1 therapy resistance [36, 39]. By acquisition of deficiency in JAK1 and JAK2, components of the IFNγ signaling pathway, tumor cells were no longer sensitive to the antiproliferative and pro-apoptotic activity of IFNγ that is released upon TCR-dependent T cell activation and essentially contributes to the efficacy of immunotherapies.

Interestingly, also non-genomic mechanisms seem to play a role in resistance to anti-PD1 therapy. It was demonstrated that a specific transcriptomic signature in melanoma cells defines a dedifferentiated tumor phenotype that is characterized by the downregulation of antigen presentation and low T cell infiltrates [32]. This suggests that melanomas can acquire a reversible state of resistance due to their phenotype plasticity. Ongoing studies will most likely identify additional mechanisms and phenotypes associated with resistance to anti-PD1 treatment.

19.11 Perspective

The approval of PD1 and PD1/CTLA-4 checkpoint blockade was a milestone that changed the landscape of melanoma therapy, inducing striking clinical responses in patients with advanced-stage disease. Follow-up data from the large clinical trials are now needed to determine the overall long-term clinical benefit achieved via these treatments.

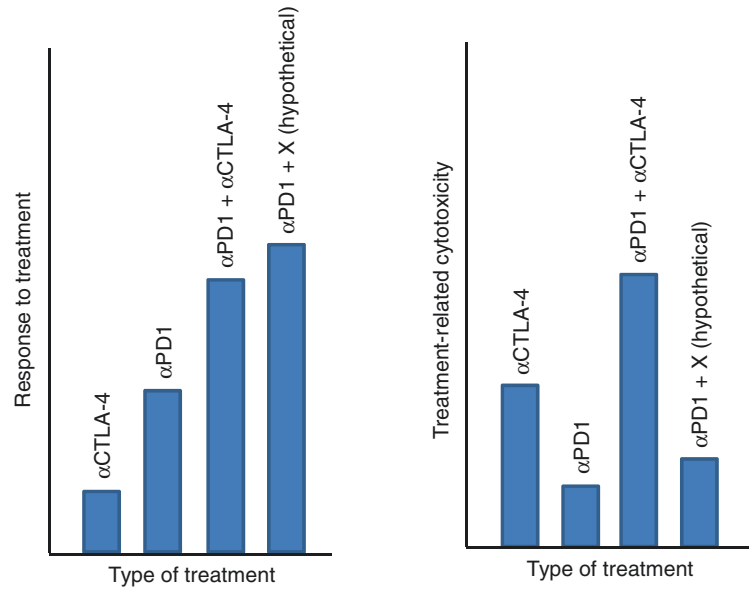

Fig. 19.3 Clinical responses and cytotoxicity related to immune checkpoint blockade. PD1-based therapies show highest clinical response rates. Anti-PD1/anti-CTLA4 combination therapy strongly increases treatment-related cytotoxicity. In the future, combination of PD1 checkpoint blockade with other agents (x) might lead to high response rates in the presence of low cytotoxicity

It is expected that a remarkable fraction of patients will be long-term responders [40]. But still, a majority of patients is primary resistant to PD1 and PD1/CTLA-4 checkpoint blockade. Furthermore, the high toxicity associated with anti-PD1/anti-CTLA-4 therapy demands for alternative combinations, which are heavily screened for in ongoing clinical trials (Fig. 19.3). In this regard, the combination of BRAF/MEK inhibitors with PD1 checkpoint blockade has attracted some interest. Patients with BRAF(V600) mutant melanoma (approximately 50%) receive combination therapies of BRAF and MEK inhibitors [5–7]. Response to these inhibitors is associated with an infiltration of different immune cells including $CD8^+$ T cells into the lesion, providing a rationale to combine inhibitor treatment with immune checkpoint blockade [41, 42]. First clinical trials have been initiated (https://ClinicaTrials.gov; NCT02130466). Besides PD1 and CTLA-4, activated tumor-reactive $CD8^+$ T cells can express additional checkpoints including LAG3, TIM3, and TIGIT [43]. Interestingly, IFNγ has recently been demonstrated to drive expression of the different checkpoint ligands on melanoma cells [44]. Since these receptors seem to have nonredundant functions in the control of T cell activity, their combination with anti-PD1 antibodies is currently tested in clinical trials (https://ClinicaTrials.gov; NCT01968109: combination of anti-PD1 and anti-LAG3 antibodies). Another approach combines anti-PD1 treatment with the intratumoral administration of an oncolytic virus (T-VEC), in order to induce antigen release and uptake by pAPC to further enhance T cell activation [45]. In addition to this, various other strategies are followed that can be screened for at https://ClinicaTrials.gov. First results of some trials might be available soon, but it may take much more time to optimize schedules and dosing for combination therapies. Overall, it can be expected that some of these trials will impact on melanoma therapy and provide improved treatment options for different patient's subgroups.

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Immune Checkpoint Inhibition in Lung Cancer

20

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Contents

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20.1 Introduction

Lung cancer is the leading cause of cancer deaths both in the United States and worldwide with estimates by the American Cancer Society for the year 2016 and GLOBOCAN for the year 2012 of 158,000 and 1.6 million deaths, respectively [1, 2]. Among patients with lung cancer, approximately 87% of patients have a broad group of histologies called non-small cell carcinoma (NSCLC), whereas approximately 13% have small cell lung cancer (SCLC) [3]. NSCLC may be further subdivided into adenocarcinoma (ADC), squamous cell carcinoma (SQCC), and large cell carcinoma (LC). Most patients with NSCLC present with advanced stage, which is essentially incurable and treated with palliative intent. With the exception of selected patients with activating epidermal growth factor receptor (*EGFR*) mutations or anaplastic lymphoma kinase (ALK) translocations, for which targeted therapy with small molecule tyrosine kinase inhibitors is associated with a prolonged clinical benefit [4–7], the standard of care for most patients with metastatic NSCLC is a platinum-based chemotherapy doublet [8, 9]. Monoclonal antibodies against either the vascular endothelium growth factor (VEGF) or EGFR have been approved based on a modest benefit over chemotherapy alone and are commonly used in the

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absence of contraindications [10, 11]. Virtually, all patients develop tumor progression after first-line therapy, and the standard second-line option has been docetaxel, which provides an overall response rate (ORR) of less than 10%, median progression-free survival (PFS) of less than 3 months, and median overall survival (OS) of 5.7–7.9 months [12–14]. Although ramucirumab, a monoclonal antibody against the extracellular domain for VEGF receptor 2, was approved in combination with docetaxel in previously treated patients with advanced stage NSCLC, the benefit over single agent docetaxel was statistically significant but clinically modest, with increased median PFS from 3 to 4.5 months and median OS from 9.1 to 10.5 months [15]. New treatment modalities were clearly needed to improve the outcomes for patients with advanced stage NSCLC.

20.2 Immune Checkpoint Blockade

The amplitude and quality of the antigen-specific T-cell responses are regulated by a balance between co-stimulatory and co-inhibitory signals [16, 17]. Under normal physiologic conditions, the co-inhibitory molecules, also known as immune checkpoint molecules, maintain self-tolerance preventing autoimmunity and limit the tissues from collateral damage during response to infections. The cancer cells, however, may co-opt these molecules to evade immune destruction. Among the key checkpoint molecules are the cytotoxic lymphocyte antigen 4 (CTLA-4) and the programmed death-1 (PD-1).

PD-1, also known as CD279, is a 288 amino acid type I transmembrane protein receptor composed of one immunoglobulin superfamily domain, a 20 amino acid stalk, and an intracellular domain containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) tyrosine motifs [18]. Although PD-1 is not expressed on resting T cells, it may be induced, appearing within 24 h from T-cell activation and declines once the antigen is cleared

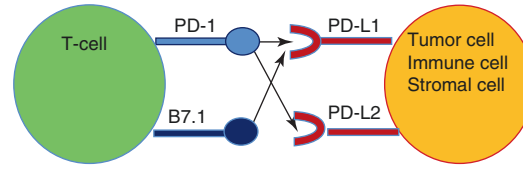

Fig. 20.1 The ligands for PD-1 are PD-L1 and PD-L2. PD-L1 and PD-L2 may also bind to B7.1 and RGMb

[19]. However, when the T cells are continually stimulated by the antigens during chronic infection or cancer, the PD-1 expression remains elevated, and the cells differentiate into a state of exhaustion. The two ligands for PD-1 are the programmed death ligand-1 (PD-L1) also known as CD274 or B7-H1 and the programmed death ligand-2 (PD-L2) also known as CD273 or B7-DC (Fig. 20.1). While PD-L1 is expressed in both hematopoietic and non-hematopoietic cells, PD-L2 has a more restricted expression, occurring mostly on dendritic cells, macrophages, and mast cells. Both ligands can bind another molecule in addition to PD-1, with PD-L1 binding CD80 (B7.1) on the T cells and PD-L2 binding the repulsive guidance molecule b (RGMb) [20, 21]. Binding to PD-L1 or PD-L2 leads to phosphorylation of ITIM and ITSM tyrosine motifs on PD-1 with recruitment of SHP-2 and reduced phosphorylation of the T-cell receptor (TCR) signaling molecules [22]. Signaling through PD-1 inhibits the PI3K and MAPK pathways, T-cell proliferation, production of cytokines, and cytotoxic effector molecules.

20.3 Initial Studies with Single Agent Immune Checkpoint Inhibitors

In the first-in-human phase I clinical trial with nivolumab, a fully human immunoglobulin G4 (IgG4) against human PD-1 previously known as MDX-1106 or BMS-936558, 39 patients with advanced metastatic NSCLC, melanoma, renal cell carcinoma, colorectal carcinoma, or castrate-resistant prostate cancer were treated in four escalating single dose cohorts of 0.3, 1, 3, and 10 mg/kg [23]. All patients had progressive

and refractory disease, with a median of four of prior therapies. The primary objectives were safety and tolerability of single dose therapy. Patients without tumor progression or adverse events grade 3 or higher received additional doses at weeks 12 and 16, with the option of repeated treatment at 3 months. The treatment was well tolerated, with no dose-limiting toxicities after the single dose. Twelve patients received multiple doses of nivolumab after achieving stable disease or tumor regression after the initial dose. This study was followed by a dose-expansion cohort trial in patients with the same malignancies using 1, 3, or 10 mg/Kg every 2 weeks for up to 2 years in the absence of unacceptable adverse events, complete response (CR), or progressive disease (PD) [24]. The updated report of the NSCLC cohort included 129 patients, of which 70 (54.3%) had been previously treated with three or more prior lines of systemic regimens [25]. The treatment was well tolerated, with grade 3 or 4 treatment-related adverse events (TAEs) occurring in 14% of patients. Nevertheless, there were three treatment-related deaths due to pneumonitis. The ORR was 17% across all doses, without differences between squamous and non-squamous histologies. The median PFS was 2.3 months, with 1-year and 2-year PFS of 22% and 9%, respectively. The median, 1-year, 2-year, and 3-year OS were 9.9 months, 42%, 24%, and 18%, respectively. The 0.3 mg/kg cohort was associated with the best outcomes, with ORR of 24.3%, median OS of 14.9 months, and 1-year, 2-year, and 3-year OS rates of 56%, 42%, and 27% respectively.

The Keynote-001 study assigned 495 NSCLC patients to different doses of pembrolizumab, previously known as MK-3475, a humanized IgG4 monoclonal antibody against PD-1 [26]. The treatment was well tolerated, with grade 3 or higher TAEs occurring in 47 patients (9.5%). The ORR was 19.4%, with median PFS and OS of 3.7 months and 12 months, respectively. The percentage of neoplastic cells showing membranous staining for PD-L1 by immunohistochemistry was used as a biomarker selection in the 182 patients assigned to the training group. PD-L1 expression in at least 50% of tumor cells was selected as the cutoff. The ORR, median PFS, and OS for patients PD-L1 expression of at least 50% in the validation cohort were 45.2%, 6.3 months, and not reached, respectively. There were no differences in efficacy among the regimens tested. Among the 824 patients who had samples evaluated for the study, the prevalence of PD-L1 scores of 50% or more, 1–49%, and less than 1% were 23.2%, 37.6%, and 39.1%, respectively.

In the initial study with atezolizumab, a human IgG1 monoclonal antibody against PD-L1 previously known as MPDL-3280A, 277 patients with solid tumors were treated with escalating doses by intravenous infusion every 3 weeks for up to 16 cycles of 1 year [27]. Among the 53 patients with NSCLC, the ORR and 24-week PFS were 23% and 45%, respectively. There was a correlation between responses and PD-L1 expression in the pretreatment specimens, which for NSCLC reached statistical significance in the tumor-infiltrating immune cells (ICs) but not in tumor cells (TCs). Most patients with tumor progression had no PD-L1 expression in TCs or ICs, and the growing tumors had a pattern or immune ignorance with little to no tumor-infiltrating immune cells, nonfunctional immune response with immune infiltrate expressing minimal to no expression of PD-L1, or excluded immune infiltrate which was located around the outer edge of the tumor.

20.4 Randomized Clinical Trials in Previously Treated Patients

The encouraging results from single agent monoclonal antibodies against PD-1 or PD-L1 in patients with advanced stage NSCLC led to randomized clinical trials using docetaxel 75 mg/m^2 every 3 weeks as the control arm. All studies had OS as the primary endpoint.

In the CheckMate-017 trial, 272 patients with advanced squamous cell lung cancer with one prior line of therapy were randomized to nivolumab 3 mg/kg every 2 weeks or docetaxel

[28]. Nivolumab was better tolerated than docetaxel, with no new safety concerns identified. Treatment with nivolumab was associated with increased ORR (20% vs 8%, $P = 0.008$), median PFS (3.5 vs 2.8 months, hazard ratio [HR] 0.62, $P < 0.001$), and median OS (9.2 vs 6.0 months, HR 0.59, $P < 0.001$) compared to docetaxel. The benefit from nivolumab on both PFS and OS was independent of PD-L1 expression in TCs. The CheckMate-057 had a similar design and randomized 582 patients with advanced NSCLC to standard dose of either nivolumab or docetaxel [29]. Patients were required to have one prior line of platinum-based chemotherapy doublet, and those with known *EGFR* mutation or *ALK* translocation were allowed to have received one additional line of tyrosine kinase inhibitor. Similarly to the CheckMate-017 trial, nivolumab was better tolerated than docetaxel and associated with improved ORR (19% vs 12%, $P = 0.02$). The median PFS, however, was numerically superior for the docetaxel arm (2.3 vs 4.2 months; HR 0.92, $P = 0.39$), whereas the 1-year PFS favored nivolumab (19% vs 8%). Both the median (12.2 vs 9.4 months; HR 0.73, $P = 0.002$) and 1-year OS (51% vs 39%) were better with nivolumab. The results of these two studies led to the approved nivolumab in previously treated patients with squamous cell and non-squamous cell histologies.

The Keynote-010 randomized 1034 patients with previously treated NSCLC- and PD-L1-positive tumors to docetaxel, pembrolizumab 2 mg/kg, or pembrolizumab 10 mg/kg every 3 weeks [30]. Pembrolizumab was better tolerated than docetaxel. There were no significant differences in OS between the two pembrolizumab arms, and the dose of 2 mg/kg was chosen for further studies. When compared to docetaxel, pembrolizumab 2 mg/kg was associated with increased ORR (18% vs 9%, $P = 0.0005$), similar median PFS (3.9 vs 4 months, HR 0.88, $P = 0.07$), and improved median OS (12.7 vs 8.5 months, HR 0.71, $P = 0.0008$). The benefit from pembrolizumab at the recommended dose was more pronounced in patients with PD-L1 proportion score of at least 50%, with increased ORR (30% vs 8%, $P < 0.0001$), median PFS (5 vs 4.1 months, HR 0.59, $P = 0.0001$), and median OS (14.9 vs 8.2 months, HR 0.59, $P = 0.0002$). On subgroup analysis, the OS favored pembrolizumab in all variables except for *EGFR*-mutant patients (HR 0.88, 95% confidence interval [CI] 0.45–1.70), where a trend toward worse PFS was also found (HR 1.79, 95% CI 0.94–3.42).

The POPLAR was a randomized phase 2 trial where 287 patients with advanced stage NSCLC previously treated with one line of platinum-based chemotherapy were randomized to atezolizumab 1200 mg or docetaxel at standard dose every 3 weeks [31]. PD-L1 was scored for both TCs and ICs. Atezolizumab was well tolerated, with no new safety signals. Although atezolizumab was not associated with differences in ORR (15% vs 15%) or median PFS (2.7 vs 3 months, HR 0.94; 95% CI 0.72–1.23), the primary endpoint was met with a significant improvement in OS in the experimental arm (12.6 vs 9.7 months, HR 0.73, $P = 0.04$). The OS benefit for atezolizumab was observed only in patients with at least TC1 or IC1. The OAK was a phase 3 study which randomized 1125 patients with advanced NSCLC previously treated with one or two lines of chemotherapy to atezolizumab 1200 mg or docetaxel [32]. Atezolizumab was better tolerated than docetaxel with fewer grade 3 or 4 adverse events (34% vs 54%). The study showed no improvement from atezolizumab in ORR or median PFS (2.8 vs 4 months, HR 0.95; 95% CI 0.82–0.10) in the intent to treat population. Nevertheless, the median OS was significantly longer with atezolizumab (13.8 vs 9.6 months, HR 0.73. $P = 0.0003$). The improved OS was observed regardless of PD-L1 expression levels, including patients with TC0 and IC0 (12.6 vs 8.9 months, HR 0.75, $P = 0.02$), TC1-3 or IC1-3 (15.7 vs 10.3 months, HR 0.74, $P = 0.01$), and TC3 or IC3 (20.5 vs 8.9 months, HR 0.41, $P < 0.0001$). The median OS favored atezolizumab in all prespecified groups except for patients with *EGFR* mutation (HR 1.24; 95% CI 0.71–2.18).

20.5 First-Line Therapy

With the established safety and efficacy of single agent immune checkpoint inhibitors in previously treated patients, with some patient achieving an unprecedented prolonged survival, the next step was to evaluate this strategy in the first-line setting.

The CheckMate-012 is a phase I multi-cohort study evaluating the use of nivolumab alone or in combination with ipilimumab, platinum-based chemotherapy, erlotinib, or bevacizumab maintenance in patients with advanced NSCLC. In the first-line nivolumab monotherapy arm, ORR was observed in 12 out of 52 patients (23%), with 14 additional patients achieving SD for a confirmed disease control rate (DCR) of 50% [33]. The median PFS and OS were 3.6 months and 19.4 months, respectively. Although patients with PD-L1 less than 1% had lower responses compared to those with PD-L1 positive (14% vs 50%), there were no significant differences in 1-year OS (79% vs 83%). In the combined immune checkpoint study, 78 chemotherapy naïve patients with advanced NSCLC were randomized to nivolumab 3 mg/kg every 2 weeks plus ipilimumab 1 mg/kg every 6 or 12 weeks [34]. Both arms had a tolerable safety profile. The ipilimumab every 12-week cohort was associated with numerically superior confirmed ORR (47% vs 38%) and median PFS (8.1 vs 3.9 months). Nevertheless, the ipilimumab every 6 weeks regimen was chosen for further development in NSCLC based on observation from studies in melanoma and small cell lung cancer where greater exposure to ipilimumab was associated with improved activity of the combination. Pooled results from both cohorts showed that there were 12 confirmed and 1 unconfirmed response among the 13 patients with PD-L1 ≥50%. In the third cohort of CheckMate-012 reported, 56 patients with previously untreated advanced NSCLC were treated with nivolumab 10 mg/kg plus a platinum-based doublet chemotherapy every 3 weeks for four cycles followed by maintenance nivolumab monotherapy until tumor progression or unacceptable toxicity [35]. The chemotherapy regimens used were gemcitabine plus cisplatin for squamous carcinoma, pemetrexed plus cisplatin for non-squamous carcinomas, and carboplatin plus paclitaxel for any histology. An additional arm included was nivolumab 5 mg/kg plus carboplatin and paclitaxel. There were 35 patients (95%) with TAE of any grade and 25 (45%) with grade 3 or 4 toxicity. Grade 3 or 4 pneumonitis was observed in four patients (7%). The confirmed ORR, median PFS, median OS, 1-year OS, and 2-year OS were 33–47%, 4.8–7.1 months, 11.6 to not reached, 50–87%, and 25–62%, respectively. The outcomes were considered particularly promising for the 14 patients receiving nivolumab 5 mg/kg plus carboplatin and paclitaxel, with confirmed ORR of 43%, estimated duration of response of 19.6 months, median OS not reached, 1-year OS of 86%, and 2-year OS of 62%.

In the randomized open-label phase 2 Keynote-021 trial, 123 patients with chemotherapy naïve advanced stage non-squamous NSCLC were randomized to carboplatin plus pemetrexed with or without pembrolizumab [36]. The pembrolizumab arm was associated with increased grade 3 or higher adverse effects (40% vs 26%). The primary endpoint of objective response was met with a significant increase in the ORR for the triplet therapy (55% vs 29%, $P = 0.001$). Although the median PFS was numerically higher for the pembrolizumab arm (13.0 vs 8.9 months), there was no difference in OS, with median not reached and estimated 6-month OS of more than 90% in both groups.

The Keynote-024 was an open-label randomized phase 3 trial comparing pembrolizumab 200 mg every 3 weeks to platinum-based chemotherapy in previously untreated patients with advanced stage NSCLC and PD-L1 expression of ≥50% in tumor cells (Fig. 20.2) [37]. Patients with *EGFR* mutation or *ALK* translocations were excluded from the study. The chemotherapy options were cisplatin plus pemetrexed, carboplatin plus pemetrexed, cisplatin plus gemcitabine, carboplatin plus gemcitabine, and carboplatin plus paclitaxel. Among the 1934 patients screened, 1653 had samples evaluated for PD-L1, of which 500 (30.2%) had PD-L1 proportion ≥50%. The primary endpoint was PFS. There were 305 patients eligible for the study, with 154 random-

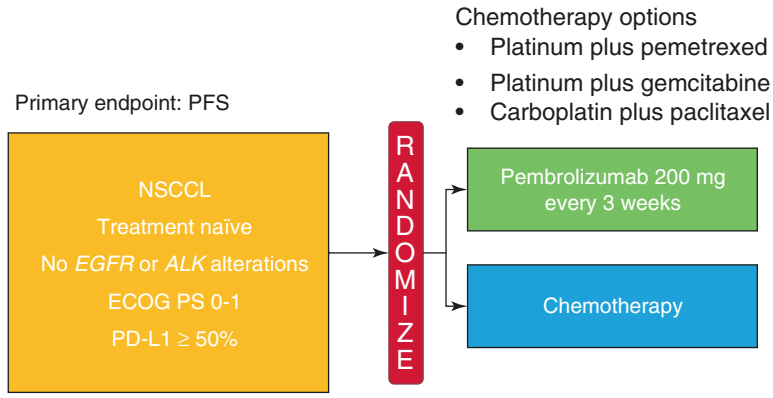

Fig. 20.2 Design for the Keynote-024. Patients with untreated stage IV NSCLC were randomized to pembrolizumab or chemotherapy. The chemotherapy choices included cisplatin plus pemetrexed, carboplatin plus pemetrexed, cisplatin plus gemcitabine, carboplatin plus gemcitabine, and carboplatin plus paclitaxel

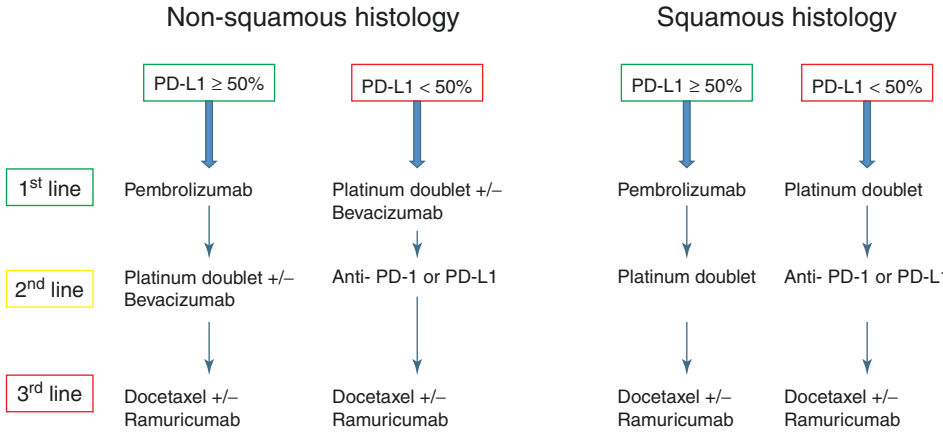

Fig. 20.3 New algorithm for the treatment of advanced stage NSCLC without oncogene-driven tumors, according to PD-L1 expression

ized to pembrolizumab and 151 to chemotherapy. The most common chemotherapy used was carboplatin plus pemetrexed. Pembrolizumab was associated with decreased incidence of adverse events of any grade (73.4% vs 90%) and grade 3 or higher (26.6% vs 53.3%). There were 45 (29.2%) immune-mediated adverse events in patients treated with pembrolizumab, most commonly hypothyroidism (9.2%), hyperthyroidism (7.8%), and pneumonitis (5.8%). Adverse events leading to death were found in one patient (0.6%) treated with pembrolizumab and three patients (2%) treated with chemotherapy. ORR was higher in patients treated with pembrolizumab (44.8% vs 27.8%). The median FPS was significantly longer in the pembrolizumab group (10.3 vs 6 months, HR 0.50, $P < 0.01$). Although the median OS was not reached in either group, the estimated percentage of patients alive at 6 months was also higher in patients treated with pembrolizumab (80.2% vs 72.4%). The results from the Keynote-024 established a new standard of care for patients with previously untreated advanced stage NSCLC (wild type for *EGFR*-activating mutations or *ALK* translocations) and tumor PD-L1 ≥50%, with better toxicity profile and improved ORR and PFS compared to standard chemotherapy (Fig. 20.3).

20.6 Predictors for Response to Immune Checkpoint Inhibitors

Since prolonged tumor control from immune checkpoint inhibitors is achieved in only a small percentage of patients, there has been a great interest in defining the predictors for benefit.

The most studied biomarker is PD-L1, which is detected by immunohistochemistry (IHC) and is commonly upregulated on the surface of tumor cells. Multiple studies have shown an association between tumor response and PD-L1 expression, although patients with no PD-L1 expression also achieve benefit. In a meta-analysis including 1567 NSCLC patients treated with immune checkpoint inhibitors, the ORR was 29% for 652 PD-L1-positive tumors and 13% for PD-L1-negative tumors (relative ratio [RR] 2.08, 95% CI 1.49–2.91, $P < 0.01$) [38]. The differences in ORR were also observed for both squamous (29% vs 14%, RR 2.12; 95% CI 1.37–3.29, $P < 0.01$) and non-squamous histologies (32% vs 11%, RR 3.14; 95% CI 2.15–4.54, $P < 0.01$). Nevertheless, although the 24-week PFS was significantly higher in patients with PD-L1-positive tumors (35% vs 26%, $P < 0.01$), there were no differences in 1-year OS (28% vs 27%, $P = 0.39$). Similar findings were observed in an analysis of 511 NSCLC patients treated on seven studies, where the ORR was higher in patients with PD-L1-positive tumors (23.2% vs 14.5%) [39].

Direct assessment of PD-L1 expression on tumor cells represents the most logic and straightforward biomarker for prediction of benefit from immune checkpoint inhibitors against PD-1 or PD-L1. However, there are several factors that should be taken into account for the proper interpretation of PD-L1 results, including the mechanisms of PD-L1 upregulation, presence of tumor-infiltrating cells (TILs), and methodology of IHC testing.

PD-L1 expression in tumor cells may be constitutive or adaptive (inducible). These mechanisms are not mutually exclusive and may coexist in the same tumor microenvironment (TME) [40]. Constitutive PD-L1 expression occurs through alterations in oncogenic pathways including amplification of 9q24 which contains the locus for PD-L1, PD-L2, and JAK-2 (PDJ amplicon), *PTEN* deletions, *PI3K* or *AKT* mutations, and *MYC* overexpression [41]. PD-L1 expression may also be induced by interferons (INFs), particularly INFγ, which is produced by activated Th1 helper CD4 cells, activated CD8 cells, and natural killer (NK) cells. INFγ results in PD-L1 expression in surrounding cells with interferon receptors including cancer cells and cells within the TME such as myeloid-lineage, stromal, and T cells (Fig. 20.4). Inducible expression occurs more commonly than constitutive and is characterized by patchy patterns of PD-L1 at the interface between tumor cells and TILs [42]. In contrast, constitutive PD-L1 expression is

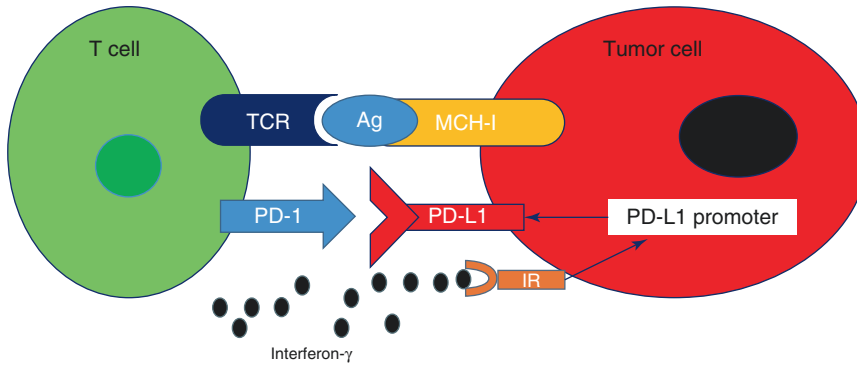

Fig. 20.4 Adaptive immune resistance. Binding of interferon-γ to the interferon receptor (IR) leads to increased PD-L1 expression

characterized by a more homogeneous PD-L1 staining in most cells.

Tumors may be classified into four distinct groups based on the PD-L1 status and presence of TILs (Fig. 20.5) [43]. Type I tumors have TILs and are PD-L1 positive, whereas type III tumors have PD-L1 expression in the absence of TILs. Types II and IV have PD-L1 negative with TILs absent in the former and present in the latter. Type I tumors represent the adaptive mechanism from the tumor cells expressing PD-L1 in response to INFγ secretion by the infiltrating immune cells, whereas type III tumors represent the constitutive PD-L1 expression, which is not related to the interaction with INFγ secreting cells. Type II tumors with neither TILs nor PD-L1 expression may represent immune ignorance, whereas in type IV tumors, the presence of TILs without induction PD-L1 in the tumor cells may indicate the presence of immune tolerance [44].

Since there are only two small hydrophilic regions that can be used as the binding site for antibody detection of PD-L1 by IHC, these antibodies bind to a unique site at PD-L1 compared to therapeutic anti-PD-L1 antibodies [45]. Furthermore, the use of different IHC cutoffs for the definition of positivity and availability of multiple assays without a standardized framework for comparison among the methods, makes the interpretation of the results and extrapolation of findings from individual studies difficult. The Blueprint Death Ligand 1 (PD-L1) IHC Assay Comparison Project was initiated to address the standardization of the antibody assays. In the phase 1 of the project, 38 NSCLC were stained for the four most commonly used antibodies including the Dako 28-8, Dako 22C3, Ventana SP142, and Ventana SP263, which have been used in studies with nivolumab, pembrolizumab, atezolizumab, and durvalumab, respectively [46]. Tumor sections were stained by Dako and Ventana for their antibodies with immunostained slides evaluated by three pathology experts who independently scored the slides for the percent-

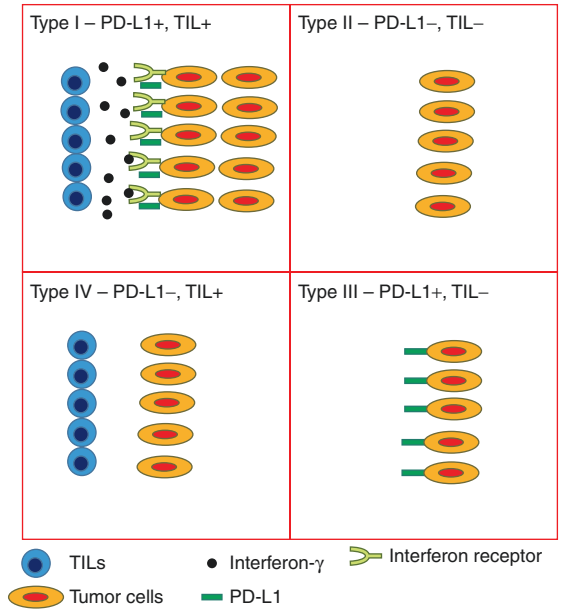

Fig. 20.5 Tumor classification based on PD-L1 expression and TILs. Type I is characterized by the presence of PD-L1-positive tumor cells at the interface with TILs, representing the adaptive expression in response to cytokines secreted by the infiltrating immune cells. This type of tumor is the most likely to respond to immune checkpoint blockade. Type III represents the constitutive PD-L1 expression by the tumor cells in the absence of TILs. It is unclear whether constitutive PD-L1 expression predicts for benefit from monoclonal antibodies against PD-1 or PD-L1. Types II and IV are characterized by PD-L1-negative tumors, with the former most likely caused by immune ignorance whereas the latter may be due to other immunosuppressive mechanisms

age of PD-L1 in the TCs and ICs. The percentage of PD-L1 stained tumor cells was comparable for the 22C3, 28-8, and SP263, with lower overall staining with the SP142 assay. Nineteen cases (50%) had PD-L1 staining above the cutoffs for all the four assays representing positivity concordance regardless of the method used, whereas five (13%) were below the cutoff according to all four methods. Nevertheless, 14 cases (37%) had discordant levels of PD-L1 expression, leading to different PD-L1 status classification for some patients. PD-L1 expression was demonstrated by all four assays, with greater variance among the assays compared to the TC expression.

Another concern with the PD-L1 test is the tumor heterogeneity. In a study using 49 NSCLC biopsy tissue specimens evaluated for PD-L1 expression in the tumors and stroma by chromogenic IHC and automated quantitative immunofluorescence using the antibodies E1L3N and SP142, some tumors had discordant results with PD-L1 according to the biopsy site, with positive and negative areas [47]. In a study involving 109 patients with pulmonary adenocarcinoma stained with the E1L3N antibody, the expression of PD-L1 was discrepant between the primary tumor and N1 lymph nodes in 15–18% of cases and between the primary tumor and N2 lymph nodes in 9.4–38% of cases depending on the cutoff for positivity [48].

Other potential biomarkers include mutation burden and presence of driver mutations. Since the antigenicity of neopeptides is not related to their function, passenger mutations with no functional role may have a role as tumor antigens, suggesting the possibility that higher mutation load would generate more neoantigens and increase the probability of benefit from immune checkpoint inhibitors [49]. In fact, tumors associated with the highest frequency of somatic mutations such as melanoma, NSCLC, and bladder cancer [50] are associated with increased probability of response to immune checkpoint inhibitors compared to those with lower mutation burden. In a study evaluating 34 patients with NSCLC, divided into discovery and validation cohorts, the mutation burden using whole exome sequencing was evaluated as a predictor for benefit from pembrolizumab [51]. In the median number of somatic nonsynonymous mutations, the 16 patients in the discovery and 18 patients in the validation cohorts were 209 and 200, respectively. In both cohorts, the probability of durable clinical benefit (DCB), defined as PR or SD lasting more than 6 months, and median PFS were significantly higher among patients with mutation burden above the median. Among patients with PD-L1-positive tumors and high nonsynonymous mutation burden, defined as above 200, the DCB was higher than in those with low mutation burden (91% vs 10%).

Patients with *EGFR* mutation or *ALK* translocations are usually never or light smokers [52, 53]. Since the average mutation frequency is ten times lower in never smokers compared to smokers [54], the former group of patients is expected to have a lower probability of benefit from immune checkpoint inhibitors. In a study involving 58 patients treated with anti-PD-1 or PD-L1 inhibitors, patients with wild-type *EGFR* and *ALK* had higher ORR (23.3% vs 3.6%, $P = 0.05$) and median PFS (2.58 months vs 2.07 months (HR 0.5, $P = 0.01$) compared to those with *EGFR* mutation or ALK translocation [55]. One possible explanation is the lower rates of concurrent PD-L1 expression and CD8-positive TILs within the TME in patients with *EGFR* or *ALK* alterations. In a meta-analysis including 1903 patients from three studies comparing nivolumab, pembrolizumab, or atezolizumab to docetaxel, the outcomes were evaluated according to *EGFR* status [56]. Among patients with wild-type *EGFR*, immune checkpoint blockade with anti-PD-1 or PD-L1 inhibitors was associated with a significant improvement in OS (HR 0.66; 95% CI 0.58–0.76). In contrast, there was no significant benefit from immunotherapy among patients with *EGFR* mutant tumors (HR 1.05; 95% CI 0.70–1.55).

20.7 Summary

Immune checkpoint inhibitors targeting PD-1 or PD-L1 represent a major step in the therapy of patients with advanced stage NSCLC, with clear benefit over docetaxel in previously

treated patients and, more recently, improved survival compared to a first-line platinum-based doublet in patients with previously untreated tumors and TC PD-L1 staining of at least 50%. In addition, randomized clinical trials showed better tolerability with anti-PD-1 or PD-L1 antibodies compared to standard chemotherapy, further increasing the enthusiasm for the use of these agents. Nevertheless, since this benefit is observed in only a small percentage of patients, there has been a great effort to identify predictors for outcomes. PD-L1 staining by IHC in TCs is an imperfect biomarker, with response rates observed in both positive and negative tumors. Several factors may account for the suboptimal role for PD-L1 staining, including the setting of PD-L1 expression in relation to TILs, methodology differences among the existing antibodies, cutoffs for positivity, and tumor heterogeneity. As an example, patients with PD-L1 positive and TILs (tumor type I) are expected to have adaptive PD-L1 expression and a higher probability of benefit from immune checkpoint blockade than those with PD-L1 positive but few or no TILs (tumor type III), for which it is unclear whether the constitute PD-L1 expression predicts for benefit from immunotherapy.

Although patients with NSCLC and negative IHC staining for PD-L1 in TCs are expected to have lower ORR compared to those with positive staining, the results are still similar to standard chemotherapy with docetaxel, and none of the randomized clinical trials showed survival detriment from immunotherapy administration prior to chemotherapy in the second-line setting. Furthermore, there was neither benefit nor detriment in the PFS from immune checkpoint inhibitors compared to docetaxel among patients with *EGFR* mutation, indicating that these patients should not be prevented from receiving anti-PD-1 or PD-L1 antibodies. Patients with tumors lacking PD-L1 and TILs (type II) are unlikely to respond to single agent immune checkpoint blockade and may be the best candidates for combination therapy including vaccines, anti-CTLA-4, or other checkpoint inhibitors.

Immunotherapy with single agent monoclonal antibodies against PD-1 or PD-L1 is now an established modality for the treatment of patients with advanced stage NSCLC, with selected patients achieving an unprecedented benefit. Nevertheless, there is still room for improvement, and future studies may help define the best candidates for such therapy and optimal rationally designed combinations.

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PD-1 Blockade in Renal Cell Carcinoma

21

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Contents

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21.1 Introduction

Renal cell carcinoma (RCC) is the most common form of kidney cancer and the eighth most common cancer overall in the United States, with a peak incidence between 60 and 70 years and male predominance [1]. Metastatic renal cell cancer (mRCC) is mainly resistant to cytotoxic chemotherapy and relatively also to radiotherapy. RCC was considered an immune-responsive tumor based on several interesting observations. First of all, spontaneous regression of metastatic lung lesions was noted in some patients [2, 3]. Second, immunotherapy with immunostimulatory cytokines such as interferon α (IFN-5 α) and interleukin 2 (IL-2) has demonstrated activity in RCC and has been the standard treatment for mRCC for decades [4]. Although IL-2 was applied in low- and high-dose regimens, the US Food and Drug Administration (FDA) approved only the high-dose regimen in 1992. A retrospective analysis published following 259 mRCC patients treated with high doses of IL-2 between 1986 and 2006 reported an overall 20% objective response rate (ORR) (9% complete responses (CR) and 11% partial responses (PR)) [5]. The high rate of CR by IL-2 therapy is one reason

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why immunotherapy in mRCC is so attractive. Unfortunately, due to its nonspecific antitumor activity, IL-2 administration was associated with a broad range of toxicities that includes systemic inflammatory response syndrome with hepatic and renal toxicities [6]. Due to these toxic side effects, today the therapeutic use of IL-2 is limited to a selected group of young and healthy patients with minimal comorbidities. Standard medical therapy for advanced RCC includes vascular endothelial growth factor (VEGF) receptor, tyrosine kinase inhibitors (TKI), and mammalian target of rapamycin (mTOR) inhibitors [7]. However, despite successes in improving clinical outcomes with the approval of these targeted therapies for mRCC, median overall survival (OS) for patients with newly diagnosed mRCC is 22–29 months, highlighting the need for additional therapies in this patient population [8].

21.2 Role of PD-1 and Its Ligand in RCC

RCC usually show a prominent immune cell infiltrate, consisting of a number of cell types: T-cells, natural killer (NK) cells, dendritic cells (DCs), and macrophages. Despite strong lymphocyte infiltration, immune dysfunction promotes RCC tumor growth and evasion. The tumor-induced changes in DC differentiation and the induction of anergy-associated genes in T-cells can partially explain the impaired antitumor response [9]. Cytokines failed to impact on immune system anergy, but increased evidence suggests that novel immune modulating therapies can significantly enhance antitumor immunity. Generally, activated T-cells, B-cells, NK cells, DCs, and monocyte cells express PD-1 in order to restrict autoimmunity during inflammatory states such as infections. However, many tumors express the PD-L1 to take advantage of this mechanism, downregulating the T-cell response. Several studies have investigated the biological and prognostic role of PD-1 and PD-L1 expression in RCC. The studies confirmed that PD-L1 is expressed in a significant proportion of both primary and metastatic RCC specimens but not in normal kidney tissues [10–12]. The immunohistochemical (IHC) expression of PD-1 and PD-L1 was evaluated in a large series of 196 nephrectomy specimens, by Thompson and colleagues [13]. PD-1 was expressed in 56% of patients whose tumor contained mononuclear cell infiltrates, while it was not expressed on RCC tumor cells. In addition, PD-L1 expression was correlated with aggressive tumor features such as higher tumor-node-metastasis (TNM) stage, the presence of necrosis or sarcomatoid differentiation, and increased risk of cancer-specific mortality. In addition, quantifying soluble PD-L1 by ELISA in a series of 172 RCC patients, Frigola et al. showed a significant association between higher levels of PD-L1, aggressive pathological features, and increased risk of death [14]. PD-1 and PD-L1 are both expressed on CD4 + CD25+ regulatory T-cells (Tregs), but if they influence function of this, regulatory T-cells are not completely clear. Tregs infiltration theoretically promotes the pro-angiogenic phenotype of RCC and is associated with stage and grade of RCC [15]. Interestingly, the percentage of tumor-infiltrating Tregs is reduced by both sunitinib and sorafenib treatment, while it is improved by everolimus, an immunosuppressor and inhibitor of the tyrosine kinase [16–18]. As observed in other tumor histologies, increased numbers of (FOXP3 + CD4 + CD25high) Tregs or tumor-infiltrating PD-1-positive lymphocytes are seen in peripheral blood and tumor-infiltrating lymphocyte populations in RCC patients and are associated with worse outcome [19, 20]. Furthermore, a high presence of NK cells among the lymphocytic infiltrate predicts a better prognosis in RCC [21]. Sorafenib and sunitinib have been shown to increase tumor cell sensitivity to NK cell killing on RCC cells [22]. Choueiri et al. in a sub-analysis from the COMPARZ study comparing sunitinib and pazopanib as first-line therapy for mRCC patients showed that increased PD-L1 tumor expression, as well as tumor CD8+ T-cell count, was associated with shorter OS in patients treated with pazopanib or sunitinib [23].

All these data support the need for prospective trials to investigate and validate PD-L1 as a potential biomarker before and after TKI therapy for RCC.

21.3 Anti-PD-1 Studies in RCC

21.3.1 Phase I

Safety and tolerability of nivolumab, a fully human monoclonal IgG4 antibody specific for PD-1, were first demonstrated in a phase I dose-escalation study of 39 patients with treatment-refractory mRCC, melanoma, colorectal cancer, castration-resistant prostate cancer (CRPC), or non-small cell lung cancer (NSCLC) [24]. Finally, only one patient in this study had RCC. After a dose-escalating six-patient cohort at 0.3, 1, 3, or 10 mg/kg, the study enrolled 15 patients in an expansion cohort at the maximum tolerated dose (MTD) or at 10 mg/kg. Patients received one dose of nivolumab and were monitored weekly for toxicity. Radiographic restaging occurred at weeks 8 and 12. Patients who did not have either progressive disease (PD) or adverse events (AEs) of grade 3 received additional doses (at the initial dose) at weeks 12 and 16. There were no dose-limiting toxicities (DLT) in this trial, and the MTD was thus not determined. The 15-patient expanded cohort was therefore enrolled at the 10 mg/kg dose. Overall, the drug was well tolerated with the most common grade 2 or greater toxicities being decreased CD4+ lymphocyte count (35.8%), lymphopenia (25.7%), fatigue (15.4%), and musculoskeletal events (15.4%). The only grade 3 immune-related AE was inflammatory colitis (one patient) and responded well to treatment with corticosteroids and infliximab. Overall, one patient (2.5%) had a CR, 2 (5%) had PR, and 2 (5%) had a mixed response defined as regression in some lesions with simultaneous progression in others. The unique RCC patient had a prolonged PR (more than 16 months) after receiving three doses at the 10 mg/kg. Further demonstration of safety and efficacy of nivolumab in RCC was in an expanded phase I trial which enrolled patients with advanced RCC, NSCLC, melanoma, colorectal cancer, or CRPC [25]. Sequential dosing cohorts of 1.0, 3.0, or 10.0 mg/kg every 2 weeks in 8 week cycles were planned. There was no MTD in the study which allowed for significant cohort expansion. Two hundred ninety-six patients were ultimately enrolled, of which 34 patients had advanced RCC previously treated with nephrectomy, immunotherapy, and/or TKI. In this trial, nivolumab appeared to have an excellent safety profile: only 15 of 296 patients (5%) discontinued therapy due to AEs. In total, 70% of patients reported treatment-related AEs and 14% reported grade 3 or 4 events. The only treatment-related AEs that occurred in at least 10% of patients were fatigue (24%), rash (12%), diarrhea (11%), and pruritus (10%). No more than 1% of grade 3/4 treatment-related toxicities occurred. Severe immune-related AEs were infrequent and were observed in less than 1% of treated patients. However, three drug-related deaths secondary to pneumonitis in the NSCLC (two patients) and colorectal cancer (one patient) groups occurred. Antitumor activity was observed across all tested doses. ORR for the entire RCC cohort was 27%. Five patients who started therapy more than 1 year before data analysis and achieved an objective response had a durable response of at least 1 year. PFS at 24 weeks was 56% for the RCC cohort. Three years later the last patient enrolled on this expanded phase I study in a subsequent report; additional efficacy, survival, and long-term safety were reported for the 34-patient mRCC cohort [26]. At the time of analysis, median duration of survival follow-up was 45.2 months (range, 25.9–57.9). In total, objective responses were noted in 10 of 34 patients (29%). Median time to response was 16 weeks (range, 8–48 weeks). Forty percent of the responders achieved a response at 8 weeks (first radiographic assessment), and the majority (70%) had achieved their response by 16 weeks. Median duration of response was 12.9 months. Five of the ten responders discontinued therapy for reasons other than PD. Four of these patients were

followed off therapy with durable responses ranging from 19 to 56+ weeks. In addition to the ten (29%) patients who had an objective response, nine patients (27%) had stable disease (SD) for at least 24 weeks. At the time of analysis, 19 of 34 (56%) patients had died. Median OS was 22.4 months (95% CI, 12.5–NR). The 3-year survival rate was 44%. Median PFS was 7.3 months (95% CI, 3.6–10.9) with a 2-year PFS rate of 12%. Overall, 85% (29/34) of patients reported treatment-related AEs of any grade. The most common were fatigue (41%), rash (27%), diarrhea (14%), and pruritus (14%). Grade 3 or 4 treatment-related AEs were reported in six (18%) patients but were reversible.

21.3.2 Phase II

A randomized phase II trial of nivolumab was conducted, based on the favorable toxicity data and promising efficacy from the phase I trials [27]. One hundred and sixty-eight patients were randomized to receive nivolumab every 3 weeks at three different doses: 0.3 mg/kg (60 patients), 2 mg/kg (54 patients), or 10 mg/kg (54 patients) once. Primary endpoint was a comparison of PFS across dose groups to determine whether there is a dose-response relationship. Secondary endpoints included ORR, OS, time to response, duration of response, and safety. There was no dose-response relationship in PFS with median PFS of 2.7 months (80% CI, 1.9–3.0), 4.0 months (80% CI, 2.8–4.2), and 4.2 months (80% CI, 2.8–5.5) in the 0.3, 2, and 10 mg/kg cohorts, respectively (p value 0.9). ORR was 20% in the 0.3 mg/kg, 22% in the 2 mg/kg, and 20% in the 10 mg/kg groups (p value 1.0). Time to response was similar in all groups with a median of 2.8–3.0 months and a range of 1.3–10 months. Duration of response was not reached (NR) in the 0.3 mg/kg and 2 mg/kg groups but was 22.3 months (80% CI, 4.8–NR) in the 10 mg/kg cohort. Median OS was 18.2 months (80% CI, 16.2–24.0) in the 0.3 mg/kg group, 25.5 months (80% CI, 19.8–28.8) in the 2 mg/kg group, and 24.7 (80% CI, 15.3–26.0) in the 10 mg/kg group. At the time of analysis, 40% of the 35 patients with objective response were still responding at least 24 months after treatment initiation. Seventy-three percent of patients reported at least one treatment-related AE, and 19 (11%) experienced a grade 3 or 4 treatment-related AE. Fatigue was the most commonly reported AE (81% of patients). In general, there was no significant difference between the incidences of treatment-related AEs across the cohorts. There were no grade 3 or 4 cases of pneumonitis. Treatment-related AEs led to drug discontinuation in 11 patients (7%). One (2%), six (11%), and four (7%) patients discontinued therapy due to treatment-related AEs in the 0.3, 2, and 10 mg/kg cohorts, respectively. The treatment-related AEs that led to discontinuation involved cardiac, endocrine, respiratory, thoracic, or nervous system disorders. In contrast to the phase I trials, there were no treatment-related deaths. In summary, the randomized phase II trial demonstrated that heavily pretreated mRCC patients could safely tolerate nivolumab with an ORR of 20–22% and with 40% of responders having a sustained response for over 2 years. There was no dose-response relationship between the 0.3, 2, and 10 mg/kg cohorts, and the safety profile was manageable across all groups. Based on the clinical efficacy and safety of various dosing combinations in the phase I and II trials, 3 mg/kg every 2 weeks was the selected dose for additional studies.

21.3.3 Phase III

The CheckMate 025 study was a phase III randomized trial of nivolumab versus everolimus in patients with advanced RCC [28]. Eight hundred twenty-one patients with advanced RCC previously treated with at least one (72% of patients) but not more than two total prior regimens of TKI were randomized in a 1:1 ratio to receive nivolumab at 3 mg/kg in a 60-min intravenous infusion every 2 weeks versus everolimus 10 mg orally until disease progression or unacceptable toxicities. The primary objective was OS. Key secondary objectives included ORR, investigator-determined PFS, and duration of response (DoR).

The interim OS analysis was conducted when 398 deaths occurred (70% of the planned number of events for final analysis) as of the trial cutoff date of June 18, 2015. There was a statistically significant improvement in OS for patients in the nivolumab arm compared with patients in the everolimus arm, with a 5.4-month difference in median OS (from 19.6 to 25.0 months) and a HR of 0.73 (95% CI, 0.60–0.89; *p* value 0.0018). Per investigator assessment, PFS confirmed ORR was 21.5 and 3.9% with median response durations of 23.0 months and 13.7 months in the nivolumab and the everolimus arm, respectively. ORR was higher in the nivolumab group than in the everolimus group (25% vs 5%; odds ratio 5.98; 95% CI, 3.68–9.72; *p* value < 0.001). Of the patients in the nivolumab arm who responded, 31% had a treatment response of more than 12 months. Finally, median time to response appeared similar between the two arms. At the time of the OS analysis, PFS per investigator assessment was also examined. No statistically significant difference in PFS between the two treatment arms was observed. The median PFS was 4.6 months in the nivolumab arm and 4.4 months in the everolimus arm. The stratified HR was 0.88 (95% CI, 0.75–1.03) with a two-sided log-rank *p* value of 0.11. Considering subgroup analyses, the benefit with nivolumab versus everolimus was noteworthy for patients with poor MSKCC risk (HR 0.48, 95% CI 0.32–0.70) [29].

The side effect profile in this study mirrored the prior experiences: 79% of patients treated with nivolumab had treatment-related AEs of any grade, and frequently they were fatigue, nausea, and pruritus. Only 19% of patients had grade 3 or 4 AEs (2% anemia and 1% fatigue, pneumonitis, and diarrhea). Study therapy was discontinued for adverse reactions in 16% of nivolumab-treated patients and 19% of everolimus-treated patients. Forty-four percent of patients receiving nivolumab had a drug delay for an adverse reaction. Rate of death on treatment or within 30 days of the last dose of study drug was 4.7% on the nivolumab arm versus 8.6% on the everolimus arm. Based on evidences shown in this study, in November 2015, nivolumab was approved by the FDA for the treatment of advanced kidney cancer after prior anti-angiogenic treatment and further approved by EMA in 2016.

21.4 PD-L1 as Predictive Biomarker

Because response rates to nivolumab are of approximately 25%, predictive biomarkers are needed to identify those patients most likely to benefit from therapy.

In metastatic melanoma and in squamous cell non-small cell lung cancer, PD-L1 expression has been associated with improved outcomes in patients treated with nivolumab or pembrolizumab [30–32]. Investigation of PD-L1 biomarker has been included into the prospective RCC clinical trials. In the phase I trial of 296 patients with a variety of tumors treated with nivolumab, PD-L1 cell-surface expression was analyzed in 42 tumors (5 RCC). Tumors were considered positive for PD-L1 expression if any cells stained positive by immunohistochemical (IHC) staining. Overall, 60% of these samples had positive PD-L1 expression, and 36% of these positive patients had an objective response. No objective response was noted in PD-L1-negative patients [25]. In RCC patients, in the randomized phase II trial of nivolumab at different doses, PD-L1 expression was determined by IHC using a rabbit antihuman monoclonal antibody. Cutoff for PD-L1 expression positivity was membranous staining of 5% of the tumor cells. Of the 107 (64%) patients whose tumors expressed any PD-L1, 29 (27%) had PD-L1 expression in 5% of the tumor cells, and 78 (73%) had membranous staining of PD-L1 in <5% of cells. There were no significant outcome differences between PD-L1-positive and PD-L1-negative patients when a cutoff for PD-L1 expression of 1% was used. Expression of PD-L1 alone may be associated with improved outcomes in patients treated with anti-PD-1 therapy [27]. In the phase III trial comparing nivolumab and everolimus, 370 (90%) of the 410 patients in the nivolumab group and 386 (94%) of the 411 everolimus patients had quantifiable PD-L1 expression. In patients with PD-L1

expression of 1%, mOS was 21.8 months in the nivolumab patients and 18.8 months in the everolimus patients. However, in patients with PD-L1 expression of less than 1%, the median OS was 27.4 and 21.2 months in the nivolumab and everolimus groups, respectively. Therefore, higher PD-L1 expression is prognostic for poorer outcome in patients with mRCC, but does not appear as a predictive biomarker for response [27].

Two recent meta-analyses investigated prognostic and predictive role of PD-1 expression in RCC. The first involved 1323 cases and indicated that a higher level of PD-L1 expression has a negative prognostic impact and increases the risk of death by 53% (HR 1.53; 95% CI 1.27–1.84; *p* value <0.001), while in metastatic patients, PD-L1 expression on primary tumors retained its prognostic role (HR 1.45; 95% CI 1.08–1.93; *p* value 0.01) [33]. The second study did not show any difference in response according to PD-L1 expressions [34]. In summary, there is no evidence for the routine use of PD-L1 expression as a predictive biomarker in patients with advanced RCC treated with nivolumab. Additional investigation is required using alternative antibodies to stain for PD-L1, as well as staining of additional cell populations (e.g., infiltrating immune cells) to determine the predictive value of PD-1/PD-L1 expression in RCC.

21.5 Future of PD-1 Blockade in RCC

There are an increasing number of trials investigating PD-1 blockade based on the encouraging results of single-agent nivolumab. Other checkpoint inhibitors are being actively investigated, including pembrolizumab (anti-PD-1), atezolizumab, and avelumab (two anti-PD-L1) as well as combination strategies of PD-1/PD-L1 inhibitors with other immune checkpoint inhibitors or with TKI.

Combination approaches have the potential to improve outcome when compared to the inhibition of PD-1/PD-L1 pathway alone [35]. There is a rationale to support combination of immunotherapy strategies to maximize clinical benefit, given the complexity of the regulation of multiple immune checkpoints.

In fact, PD-1 and CTLA-4 appear to play distinct and complementary roles when regulating adaptive immunity. CTLA-4 is upregulated early on, following T-cell activation to inhibit T-cell function, whereas PD-1 contributes to T-cell anergy and exhaustion [36]. Preclinical data indicate that the combination of PD-1 and CTLA-4 receptor blockades results in increased effector T-cell infiltration, decreased regulatory T-cell infiltration, and increased IFN-ϒ production, leading to more pronounced antitumor activity. The combination of nivolumab with ipilimumab, a fully humanized IgG1 antibody to CTLA-4, was evaluated in a phase I study at different doses in 44 patients with RCC (nivolumab 3 mg/kg + ipilimumab 1 mg/kg every 3 weeks × 4 doses versus nivolumab 1 mg/kg + ipilimumab 3 mg/kg every 3 weeks × 4 doses followed by continuous nivolumab 3 mg/kg every 2 weeks). Eighteen percent of patients discontinued therapy due to treatment-associated AEs, including pneumonitis, diarrhea, amylase/lipase, and alanine aminotransferase increase. The ORR for both treatment arms was 45%, and a high percentage of patients (80%) have ongoing responses. These encouraging results led to an ongoing phase III trial comparing this combination to sunitinib in first-line treatment for mRCC.

VEGF has been implicated in the development and regulation of myeloid-derived suppressor cells (MDSCs), and sunitinib has demonstrated ability to reverse tumor-induced immunosuppression via reduction of MDSCs. This reduction was associated with an improvement of effector T-cell function and T-cell interferon-gamma production and a decline in regulatory T-cell numbers [37]. Other VEGF inhibitors have shown the ability to augment the immune system by inducing autophagy and suppressing activation of tumor-associated macrophages, which correlates with tumor microvessel density and VEGF levels in renal cell carcinoma [38]. Additionally, VEGF has shown the ability to dramatically affect the functional maturation of dendritic cells, which are the most effective APCs in the induction of primary immune responses [39]. These results

highlight that VEGF inhibition may not only inhibit angiogenesis but may also improve the functional potency of antigen presentation and, consequently, assist in the development of immunity against cancer cells themselves.

Given the preclinical rationale for combination of VEGF inhibition and PD-1/PD-L1 blockade, a phase I trial investigated the combination of escalating doses of nivolumab (2 mg/kg dose escalated to 5 mg/kg every 3 weeks) with sunitinib (50 mg daily 4 weeks on, 2 weeks off; 33 patients) or pazopanib (800 mg daily, 22 patients) in patients with mRCC [40]. Although the pazopanib arm was closed because of dose-limiting liver toxicity, the sunitinib combination arm was dose escalated to a higher nivolumab dose and expanded to include patients who were treatment naive. Grade 3–4 treatment-related AEs were observed in 82% of patients receiving nivolumab and sunitinib and in 70% of patients receiving nivolumab and pazopanib. Overall, 36% of patients in the sunitinib arm and 25% of patients in the pazopanib arm discontinued treatment given adverse events. The ORR was 52% in the sunitinib arm and 45% in the pazopanib arm. Although toxicity was increased, the response rates observed in this study are significantly higher than those observed with the single agent, especially considering that a discrete number of patients received prior systemic therapies. However, due to toxicities, these combinations have not been pushed further.

Additionally, atezolizumab in combination with bevacizumab, a monoclonal antibody that directly inhibits VEGF, was investigated in a phase Ib study (atezolizumab 20 mg/kg and bevacizumab 15 mg/kg every 3 weeks) [41]. Ten patients with RCC were evaluable for response and the ORR was 40%. Forty percent of patients experienced SD for greater than or equal to 24 weeks. The combination was well tolerated, with 33% of patients (4 out of 12) experiencing grade 3–4 AEs. A phase II randomized trial of atezolizumab plus bevacizumab versus atezolizumab versus sunitinib (IMmotion 150 trial) was recently presented at the 2017 Genitourinary Cancers Symposium [42]. The trial enrolled 305 patients with previously untreated RCC and randomly assigned to receive intravenous atezolizumab plus bevacizumab, atezolizumab alone, or oral sunitinib alone until disease progression or unacceptable toxicity. Among patients whose tumors expressed PD-L1 (164 in total), those treated with dual immunotherapy had a 36% reduction in the risk of disease progression or death compared with sunitinib alone (HR 0.64; 95% CI, 0.38–1.08; p value 0.095); median PFS was 14.7 months vs 7.8 months, respectively. No PFS advantage with atezolizumab plus bevacizumab compared with sunitinib alone was observed in the overall population (HR, 1.00; 95% CI, 0.69–1.45).

The ongoing phase III IMmotion 151 study (ClinicalTrials.gov Identifier: NCT02420821) is recruiting participants to evaluate atezolizumab combined with bevacizumab vs sunitinib alone in the same population.

Other VEGF and PD-1/PD-L1 inhibitor combinations are currently under investigation in several ongoing phase III clinical trials (Table 21.1).

Table 21.1 Ongoing phase III clinical trials in first-line setting

Setting	Name	Study design	Primary endpoints	Estimated end of study
First line	CAG209-214 NCT02231749	Nivolumab + ipilimumab vs Sutent Phase III	PFS and OS	June 2019
First line	IMmotion 151 NCT02420821	Atezolizumab + bevacizumab vs Sutent Phase III	PFS and OS	June 2020
First line	Javelin Renal 101 NCT02684006	Avelumab + axitinib vs Sutent Phase III	PFS and OS	June 2018
First line	KEYNOTE-426 NCT02853331	Pembrolizumab + axitinib vs Sutent Phase III	PFS and OS	December 2019
First line	NCT02811861	Lenvatinib + pembrolizumab vs lenvatinib + everolimus vs Sutent Phase III	PFS	November 2020

Pembrolizumab, a humanized monoclonal antibody against PD-1, was also investigated in a phase I trial enrolled patients with advanced solid tumors including RCC [43] and is currently being investigated in combination with VEGF-targeted agents.

The combination of pembrolizumab with axitinib is being explored in a phase Ib study of patients with treatment-naive advanced RCC presented at 2016 ESMO Congress [44]. The results reported at ESMO were for 52 patients with advanced RCC who received frontline treatment with axitinib plus pembrolizumab. The ORR was 67.3% (35 patients), including 2 CR and 33 PR. This preliminary analysis indicates that this combination is well tolerated and exhibits antitumor activity in treatment-naïve patients with RCC. An ongoing phase III of this combination versus sunitinib will try to confirm these encouraging results.

Additionally, the combination of pembrolizumab and pazopanib has been investigated in an ongoing phase I/II study. Preliminary data from 20 patients enrolled on the phase I trial were presented. The combination caused significant hepatotoxicity, with 65% of patients (13 out of 20) experiencing grade 3 or higher liver dysfunction (all of whom recovered to grade 1 or lower). The confirmed ORR was 40% (8 out of 20) for the total cohort, 60% for the pazopanib 800 mg group, and 20% of the pazopanib 600 mg group. There was one CR in the pazopanib 800 mg group. New dosing schemas are currently being explored to reduce the risk of hepatotoxicity for the combination of pazopanib with pembrolizumab [45].

Axitinib was further investigated in combination with the anti-PD-L1 avelumab in a phase Ib trial in first-line advanced RCC. Confirmed PR was observed in six patients.

Both pembrolizumab and avelumab were considered encouraging and are being tested in phase III trials in untreated RCC (NCT02853331 and NCT02684006).

Cabozantinib, a dual VEGF/MET inhibitor, was recently demonstrated in a randomized phase III trial after progression on first-line VEGF-targeted therapy to improve ORR and PFS in addition to OS for patients with mRCC [46]. Preclinical evidence showed that MET can promote increased survival of renal cancer cells through the regulation of PD-L1 [47]. The combination of cabozantinib plus nivolumab alone or in combination with ipilimumab in patients with genitourinary tumors is currently accruing patients (NCT02496208).

Lenvatinib is a multitarget TKI of VEGF with activity against FGFR, PDGFR, RET, and KIT. A phase II randomized, open-label study investigated lenvatinib (24 mg/day), everolimus (10 mg/day), or the combination (lenvatinib 18 mg/day and everolimus 5 mg/day) as second-line treatment in mRCC (150 patients) [48].

Safety and efficacy of lenvatinib in combination with pembrolizumab, a humanized monoclonal antibody to PD-1, are currently being explored in a phase Ib/II study (NCT02501096).

Tivozanib is a potent and selective inhibitor of all three VEGF receptors. In a phase I/II trial in combination with nivolumab in RCC, known as the TiNivo trial, the safety of tivozanib in combination with nivolumab at escalating doses of tivozanib is being evaluated, and, assuming favorable results, the trial will be followed by an expansion phase II cohort at the established combination dose (EudraCT2016002310-44).

A summary of the activity of VEGF inhibition and PD-1/PD-L1 blockade in phase I trials is shown in Table 21.2.

Table 21.2 Activity of the combination of VEGF inhibition and PD-1/PD-L1 blockade in RCC phase I trials

Phase I trial	Patients, number	ORR, %	CR, %	PR, %
Nivolumab + sunitinib [40]	33	52	Not reported	Not reported
Nivolumab + pazopanib [40]	20	45	Not reported	Not reported
Avelumab + axitinib [52]	6	100	0	100
Pembrolizumab + axitinib [44]	52	71.2	5.8	65.4
Nivolumab + cabozantinib [53]	23	43	4	39
Pembrolizumab + lenvatinib [54]	13	69.2	0	69.2

Conclusion

PD-1 blockade has produced encouraging response rates in RCC. Especially nivolumab has met its primary endpoint of benefit in OS compared to everolimus in the CheckMate 025 trial becoming a new standard of care. Combination of anti-PD-1/PD-L1 and other agents against RCC has demonstrated higher response rates than single agent alone for the price of higher toxicities.

Development of predictive biomarkers to determine which patients will derive clinical benefit from these treatments remains an important need and is an area of active investigation. PD-L1 expression by IHC has been extensively evaluated as a predictive biomarker. Multiple unsolved issues have confounded its use: different antibodies, variable IHC thresholds to determine positivity, adequate tissue preparation and processing, divergent expression between primary and metastatic foci, and evaluation of staining of tumor versus immune cells [49].

In spite of efficacy and toxicity profile of immune checkpoint inhibitors, a series of open questions remains.

- First, what is the role of PD-1 blockade in RCC subpopulations, such as patients with non-clear cell RCC? Non-clear cell RCC patients have largely been excluded from the trials mentioned above. Recently, PD-L1 status of patients with non-clear cell RCC was assessed and correlated with OS, suggesting that blockade of PD-1/PD-L1 axis may show beneficial in this population [12].
- Second, are traditional measures of assessing response to therapy still predictive of the benefit of checkpoint inhibitors? In the phase III trial of nivolumab, a significant benefit in OS was observed in patients who received nivolumab compared to patients who received everolimus, without a PFS benefit [28]. This modest effect in PFS is probably the result of the pseudoprogression, which could occur due to infiltration of tumors by activated immune cells and could mimic progression. Immune-related Response Evaluation Criteria in Solid Tumors (iRECIST) have been proposed to address this phenomenon [50].
- Third, the optimal duration to continue immunotherapy is also unknown. Some trials have stopped therapy at time of progression or demonstrating intolerance. Others had proposed a predefined stopping point of 2 years of therapy. Whereas immunotherapy response patterns differ from traditional therapies, some patients may benefit from nivolumab also after RECIST progression [51].

Probably, an improved understanding of the host immune system and tumor microenvironment will better elucidate which patients take advantage from these promising agents. Complementary PD-1 pathway blockade and targeted therapy or other immunologic agents have the potential to reduce the tumor-induced immunosuppression and improve clinical outcomes for patients with mRCC. Given the encouraging clinical activity and safety profile of the current PD-1/PD-L1 inhibitors, it is likely that combination approaches will become key components.

Studies exploring novel agents, combinations, as well as biomarkers of response are currently ongoing and will likely inform the future treatment landscape for patients with mRCC.

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BCG and Anti-PDL-1 Ab in Bladder Cancers

22

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Contents

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22.1 Introduction

Immune checkpoint inhibitors has become an exciting field in cancer research demonstrating striking antitumor activity and survival benefits across multiple tumor types, including metastatic advanced urothelial carcinoma (UC). There is actually a strong rationale for the use of immune checkpoint inhibitors in UC, which has already benefited from the use of immune-based therapies with the intravesical bacillus Calmette-Guerin (BCG) for over four decades. Long before the recognition of immune checkpoints as cancer targets, UC was one of the original cancers to demonstrate a benefit from immunotherapy, and patients with non-muscle-invasive UC of the bladder (NMIBC) still benefit nowadays. Recent studies in metastatic UC showed that immune checkpoint blockade will represent a new backbone of advanced UC treatment with the potential to improve outcomes across all stages of the disease. While there are many other immune-based treatments such as cancer vaccines, chimeric antigen receptors are also investigated in UC; this review will focus on program cell death 1 (PD-1) and programmed death-ligand 1 (PD-L1) inhibitors in UC introduced by a brief history of development and use of BCG in bladder cancer. The biology of PD-1 and PD-L1 axis will not be covered in the present chapter as specific chapters are focusing on the field.

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22.2 BCG

22.2.1 Current Indication of BCG

Immune therapy has been established in early-stage bladder cancer for four decades with the use of intravesical BCG for NMIBC. BCG is a live attenuated vaccine derived from *Mycobacterium bovis*, which was initially developed as a potential vaccine to prevent tuberculosis. BCG immunotherapy administered after transurethral resection of bladder tumor (TURBT) is currently the most effective treatment of NMIBC and one of the most successful applications of immunotherapy to the treatment of cancer. The notion that BCG might have a role in cancer treatment dates back to 1929, when a study [1] reported a reduced incidence of cancer among patients with tuberculosis at autopsy. Subsequent studies [2] showed that the bladder responded to BCG with a delayed-type hypersensitivity reaction seen with the skin, and a striking clinical case reported [3] the observation of a response of melanoma metastatic to the bladder treated with intralesional BCG. This success in melanoma led to the evaluation of BCG in an animal model of bladder cancer [4]. The results of that study found six weekly intravesical plus percutaneous administrations to result in a 12-fold reduction in bladder tumor recurrence [5]. Then, the first BCG controlled trial showed statistically significant reduction in tumor recurrence in 54 evaluable patients [6]. Similar results were reported in much higher risk patients in another study [7]. Since then, two meta-analyses demonstrated that BCG therapy prevents, or at least delays, the risk of tumor progression in intermediate and high-risk non-muscle-invasive bladder cancers following TURBT [8]. BCG is currently recommended for patients with intermediate-risk tumors and patients with high-risk tumors (grade 3, carcinoma in situ [CIS], T1 stage). The current rationale for BCG use includes preventing or delaying recurrence, reducing progression, and avoiding radical cystectomy.

22.2.2 Mechanisms of Action

The mechanism by which BCG induces a favorable immune-mediated antitumor response is not fully understood. BCG attaches to bladder tumor cells as well as urothelial cells by means of specific fibronectin and integrin receptors [9] leading to the internalization of BCG. Antigens specific for BCG are expressed on the surface of tumor cells and major histocompatibility complex (MHC) class II antigen expression is upregulated [10, 11]. BCG efficacy needs a competent immune system and functional T-cell subtypes among other immune cell populations (NK cells, macrophage, granulocyte, and dendritic cells in addition to various cytokines and interleukins), which are recruited to the bladder and found in large amounts in the urine following therapy [12]. BCG induces an inflammatory response within the bladder wall, which is mediated by both local and systemic immune responses. The local inflammatory response is associated with the infiltration of granulocytes followed by macrophages and lymphocytes, particularly helper T cells. Neutrophils and monocytes are the key mediators of initial response to BCG by producing chemotactic factors. Neutrophils regulate migration to the bladder wall of effector cells, such as monocytes and the CD4+ T lymphocytes. The CD4+ T lymphocytes are activated through their T-cell receptor (TCR) and induce a T helper 1 initial immune response. BCG animal studies have confirmed the requirement of both CD4+ and CD8+ tumor-infiltrating lymphocytes (TIL), with depletion of either resulting in loss of BCG-mediated antitumor activity. A wide range of cytokines are induced, including interleukin (IL)-1, IL-2, IL-6, IL-8, IL-10, IL-12, TNF-a, interferon (IFN) g, granulocyte/macrophage colony-stimulating factor, and soluble intercellular adhesion molecule I [13]. For optimal efficacy, BCG must be given in maintenance schedule after the first induction instillations. In the EORTC-GU group meta-analysis, only patients who received maintenance BCG benefited from BCG therapy, the meta-analysis however was unable to determine

which BCG maintenance schedule was the most effective [8].

22.2.3 Mechanisms of Resistance to BCG Therapy

However, 40–50% of patients experience disease recurrence and/or progression (BCG failure). The question therefore arises as to what factors are responsible for BCG-refractory NMIBC. Some hypothesis have been envisioned: (1) the immune cell infiltrate is broad based, and the tumor-associated antigen-specific component is a minor component, or (2) there is an antigen-specific antitumor T-cell response, but it is impaired by PD-1/PD-L1 upregulation in response to the release of high concentrations of Th1 cytokines (i.e., IFNγ) by NK cells in the tumor microenvironment. Increasing tumor cell PD-L1 expression actually predicts localized UC stage progression, independent of tumor grade [14]. Also, PD-L1 levels are highest in CIS and within granulomata of bladder tissues of patients failing BCG therapy. PD-L1 is present in nearly 40% of CIS tumors before BCG treatment; the amount of expression by these tumor cells is relatively low (about 5% of tumor cells). By contrast, CIS cases that ultimately failed BCG therapy exhibited 15- to 20-fold higher levels of PD-L1 expression, predominantly within BCG granulomas [14]. PD-L1 expressed by bladder tumors (including CIS) may thus provide urothelial tumor cells with a molecular mechanism to impair host antitumoral immune cells and to progress toward aggressive cancer progression. Moreover, increased expression of T-cell-inhibitory PD-L1 by mononuclear cells that are recruited into bladder tissues in response to BCG therapy may contribute to a decline in the effectiveness of BCG therapy over time.

An aggressive, bioluminescent orthotopic bladder cancer model, MB49 tumor cells transfected with luciferase (MB49(luc)), has been used to study the antitumor effects of avelumab, an antibody to PD-L1 [15]. MB49(luc) bladder tumors are highly positive for the expression of PD-L1, and avelumab administration induced significant antitumor effects. These antitumor effects were more dependent on the presence of CD4 than CD8 T cells, as determined by in vivo immune cell depletions. The findings suggest that in this bladder tumor model, interruption of the immune-suppressive PD-1/PD-L1 complex releases a local adaptive immune response that, in turn, reduces tumor growth.

Taken together, these data indicate that the presence of PD-L1 could conceivably play a role in impairing host immune-related responses and result in bladder cancer progression, which stress the potential biologic role for the PD-1/PD-L1 interaction as a new immunotherapeutic target in NMIBC. Several phase 1 trials are currently investigating PD-1 or PD-L1 inhibitors in BCG-refractory NMIBC (NCT02625961) or in association with BCG for BCG-naive high-risk NMIBC (NCT02792192).

22.3 PD-1 and PD-L1 Inhibitors

22.3.1 Rationale for PD-1 and PD-L1 Inhibitors

As discussed above, immune therapy has been established in bladder cancer for several decades with the use of intravesical BCG for NMIBC. Beyond the clinical efficacy of BCG, there are strong biological features in urothelial cancer that suggest that PD-1 and PD-L1 inhibitors may be effective in bladder cancers. First, the association of neoantigens, immune regulation of cancer, and clinical efficacy of PD-1 axis blockade has been shown in various types of cancers including melanoma and lung cancer. In these reports, patients with high tumor mutational load and high neoantigens are more likely to respond to either CTLA-4 inhibition [16, 17] or PD-1 and PD-L1 inhibitors [18]. Overall, these studies demonstrated that overall mutational load, neoantigen load, and expression of cytolytic markers in the immune microenvironment were significantly associated with clinical benefit. Like in lung cancer, cigarette smoking in bladder cancer

patient induces multiple protein-level errors resulting in a high level of malignancy-associated neoantigen presentation within the tumor microenvironment. Recent genomic studies confirmed that urothelial cancer is highly mutated with a median number of eight mutations per megabase [19–21]. When listing the cancers from highest to lowest mutational load, UC came in third on the list, and the only cancers with a higher median mutation burden were melanoma and lung cancer [19].

Second, PD-L1 expression is frequent in UC at all stages and is associated with poorer outcome for both NMIBC and MIBC. Tumor cell (TC) PD-L1 status is associated with advanced disease and decreased survival in patients undergoing radical cystectomy for non-metastatic MIBC [22]. A larger study in 302 patients has reported a similar trend of association between PD-L1 status and increased risk of death for patients with organ-confined disease after cystectomy [23]. More recently, immune cells (IC) PD-L1 expression was associated with improved survival in patients with metastatic platinum-refractory UC [24]. Another study demonstrated the correlation of PD-L1 staining by immunohistochemistry (IHC) in cisplatin-based neoadjuvant chemotherapy responders and non-responders [25]. Non-responders to neoadjuvant frequently exhibit PD-L1 expression in the tumor (40%) and suggested that these could be candidates for anti-PD-L1 therapy. Taken together, these data suggest that PD-L1 expression either on TC or IC and high mutation loads are observed in UC paving the ground to investigate immune checkpoint inhibitors in UC.

22.3.2 Proof of Concept Trials with PD-1 and PD-L1 Inhibitors

Atezolizumab was granted the breakthrough status in UC based on the data from a phase 1 expansion trial [26]. Sixty-eight heavily pretreated metastatic UC patients were included in the trial. PD-L1 was scored as 0, 1, 2, and 3, corresponding to staining of <1%, 1–5%, 5–10%, and >10%. Both immune cells and tumor cells were assessed. The prevalence of positive PD-L1 expression (IHC score 2 or 3 (2/3)) in tumor-infiltrating IC in the prescreened population was 27%. Patients were initially selected by PD-L1 IHC on tumor-infiltrating IC to test the hypothesis that PD-L1-positive patients might specifically respond to atezolizumab. Then, the trial was subsequently expanded to treat patients regardless of PD-L1 status to determine whether PD-L1-negative patients could also respond. Overall, 205 patients were screened, of whom 23% were considered positive for PD-L1 on IC, and 4% were PD-L1 positive on the TC. Overall, 68 patients with UBC received treatment and were evaluable for safety. Sixty-seven patients were evaluable for efficacy. The majority of patients (93%) were pretreated with previous cisplatin- or carboplatin-based chemotherapy, and nearly 70% had received two or more previous systemic treatments. Objective response rates were 43% for those with IHC 2/3 tumors and 11% for those with IHC 0 or 1 (0/1) tumors. The IHC 2/3 response rate included a 7% complete response rate. Among patients with IHC 2/3 tumors, a response rate of 52% was achieved. The duration of response was pretty long in this setting (ranging from 0.1+ to 30.3+ weeks for patients with IHC 2/3 tumors and from 0.1+ to 6.0+ weeks for patients with IHC 0/1 tumors). Only 4% reported a grade 3 treatment-related AE, which included one occurrence each of asthenia, thrombocytopenia, and decreased blood phosphorus.

With extended follow-up, single-agent atezolizumab continues to be well tolerated in a heavily pretreated metastatic UC cohort of this phase 1 study. Excellent clinical benefit was observed in a heavily pretreated metastatic UC population: 2-year overall survival (OS) rate was 30% for all patients and 43% for patients with IHC 2/3 tumors which outweigh historical survival on chemotherapy [27].

These early results have generated intense research studies for testing atezolizumab in a larger international phase 2 trial (IMVIGOR 2010 study). In a single-arm, two-cohort, phase 2 trial, patients with inoperable locally advanced or metastatic UC whose disease had progressed

after previous platinum-based chemotherapy were treated with atezolizumab 1200 mg given every 3 weeks [28, 29]. The co-primary endpoints were the independent review facility-assessed objective response rate according to RECIST v1.1 and the investigator-assessed objective response rate according to immune-modified RECIST, analyzed by intention to treat. In this trial, 310 patients received atezolizumab treatment. The primary analysis demonstrated that compared with a historical control overall response rate of 10% with chemotherapy, treatment with atezolizumab resulted in a significantly improved RECIST v1.1 objective response rate for each prespecified immune cell group (IC2/3: 27%; IC1/2/3: 18%) and in all patients (15%). As noted in the phase 1 study, many patients with a response demonstrated durable responses. With a median follow-up of roughly 2 years, ongoing responses were recorded in 84% of responders. Grade 3–4 immune-mediated adverse events were rare, occurring in 5% of 310 treated patients, with pneumonitis, increased aspartate aminotransferase, increased alanine aminotransferase, rash, and dyspnea being the most common.

Meanwhile, atezolizumab was investigated in earlier stage of UC in chemo-naïve metastatic patients. Around 40% of patients are not fit for cisplatin-based chemotherapy and then are treated with carboplatin-based chemotherapy or even with only supportive care. Given its excellent safety profile, atezolizumab was assessed in metastatic unfit UC with the overriding hypothesis that an earlier administration of atezolizumab could provide significant benefit to these patients. A single-arm, multicenter, phase 2 study was thus conducted in previously untreated 123 patients with locally advanced or metastatic UC who were cisplatin ineligible [30]. The primary endpoint was independently confirmed objective response rate assessed in prespecified subgroups based on PD-L1 expression and in all patients. Overall, 119 received one or more doses of atezolizumab. The objective response rate was 23%, the complete response rate was 9%, and 19 of 27 responses were ongoing at the time of analysis. Median OS was 15.9 months which was much better than the expected OS in this population of patients treated with chemotherapy (~9 months) [31]. Once again, the treatment was well tolerated with only 8% of patients experiencing an adverse event leading to treatment discontinuation and 12% patients with immune-mediated events.

Overall, these data from phase 1 and 2 studies with atezolizumab showed that PD-L1 inhibition is well tolerated in advanced UC patients and had significant activity in a subset of patients. Based on these reports, atezolizumab obtained the approval by the Food and Drug Administration in May 2016 for the treatment of patients with locally advanced or metastatic UC whose disease has worsened during or following platinum-containing chemotherapy or within 12 months of receiving platinum-containing chemotherapy, either before (neoadjuvant) or after (adjuvant) surgical treatment.

Beyond atezolizumab, many immune checkpoint inhibitors are currently investigated including PD-1 inhibitors pembrolizumab [32] and nivolumab [33, 34] and PD-L1 inhibitors durvalumab [35] and avelumab. Overall, phase 1 and phase 2 trials are consistent in reporting an overall response rate of ~20–25%, early response (within the two first months) and durable response in chemo-treated metastatic UC patients (usually not reached at the time of analysis of the trials) (Table 22.1). PD-1 and PD-L1 inhibitors have a good safety profile with few treatment-related grade 3–4 events (<15%) and almost no treatment-related deaths. Taken together, these data outweigh the data usually observed with second-line chemotherapies which achieve a historical response rate of 10% and have a poorer safety profile.

22.3.3 PD-1 and PD-L1 Inhibitors Will Change the Standard of Care in Metastatic Setting

Based on these data, two large phase 3 trials have been conducted to compare either atezolizumab or pembrolizumab with chemotherapy in second-line (2L) or third-line (3L) metastatic setting. The

Table 22.1 Early phase 1/2 studies of PD1 or PD-L inhibitors in metastatic UC

	PCD4989g	IMVIGOR 210	Checkmate 032	Checkmate 275	Durvalumab phase 1	KEYNOTE-012
Drug	Atezolizumab	Atezolizumab	Nivolumab	Nivolumab	Durvalumab	Pembrolizumab
Target	PD-L1	PD-L1	PD-1	PD-1	PD-L1	PD-1
Phase trial	1	2	1	2	1	1b
Treated patients (n)	68	310	78	265	61	33
Median follow-up (months)		11.7	9	7.00	6.5	13
Setting		≥2 L	≥2 L	≥2 L	≥1 L	≥2 L
Biomarkers selection	On PD-L1 IC (5%) and then none	None	No selection	On PD-L1 TC	No selection	On PD-L1 TC or IC
					PD-L1+ TC or IC	1%
				≥5 vs ≥1 vs <1%	25%	
ORR	26.1	15	24.4	19.6	31	26
ORR PD-L1+	43.3	26	24	28.4	46.4	NA
ORR PD-L1-	11.4	NA	26.2	16.1	0	NA
Duration of response (months)	NR	NR	9.4	NR	NR	10
OS (months)	NA	9.0	9.7	8.7	NA	13
OS in PD-L1+	NA	NR	16.2	11.3	NA	NR
Biomarkers	IC PD-L1	PD-L1 IC	NR	25-gene interferon-ɣ	PD-L1+ TC or IC	PD-L1 on TC or IC
		Luminal 2		Basal 1 subtype	25%	
		T-effector gene expression				
		CD8 expression		12-chemokine signature		
		Mutational load		CD8 expression		
% treatment-related grade 3–4	4	16	22	18	4.9	15
n treatment-related deaths	None	None	None	3	None	None

IC immune cells, *NA* not available, *NR* not reached, *ORR* overall response rate, *OS* overall survival, *PD-1* programmed cell death 1, *PD-L1* programmed cell death-ligand 1, *TC* tumor cells

primary endpoint studies are both OS and progression-free survival (PFS) in total and PD-L1 positive patients. Preliminary data of KEYNOTE-045 comparing pembrolizumab to chemotherapy (vinflunine, paclitaxel, or docetaxel) in 542 patients have been reported late 2016 [36]. After a median follow-up of 14.1 months at the time of secondary interim analysis, median OS was 10.3 months (95% CI: 8–11.8) in the pembrolizumab arm versus 7.4 months (95% IC: 6.1–8.3) in the chemotherapy arm leading to a 27% reduction of risk of death (HR: 0.73, 95% CI: 0.59–0.91, $p = 0.0022$). The difference was better when the analysis focused on patients with a combined positive score (CPS) depicting the level of PD-L1 expression on the tumor and surrounding immune cells ≥10% (HR: 0.57, CI 95%: 0.37–0.88, $p = 0.0048$). No difference was shown regarding to the progression-free survival. Once again, the response rate of pembrolizumab was nearly 20% (21.1%), which was much better than that of chemotherapy (11.4%), and the responses were durable. CPS ≥10% was not associated with a better response rate. A grade 3–5 adverse event was observed in 15% of patients treated with pembrolizumab, and four deaths were related to pembrolizumab. KEYNOTE-045 is thus the first phase 3 trials demonstrating a benefit of PD-1/PD-L1 inhibitors in UC. Pembrolizumab should be approved quickly in 2017. The results of the phase 3 trial comparing atezolizumab with chemotherapy in 2L or 3L are awaited in 2017.

22.3.4 Can We Select the Patients Who Are More Likely to Respond to PD-1 and PD-L1 Inhibitors?

Even PD-1 and PD-L1 inhibitors are being approved in metastatic UC, only 20% of patients will respond to checkpoint blockade. The first strategy for managing the use of immunotherapies in UC is to identify patients who are more likely to respond to these agents. As with many other tumor types, there is an intense research aiming at predictive factors. Clinical factors are not consistent enough to predict the response. Tumor type (i.e., bladder cancer vs upper tract disease), sex (male vs female), and number of previous line of therapies are not associated with response. Smoking status was not either except in the KEYNOTE-045 trial which reported that current smokers are more likely to benefit from pembrolizumab as compared to never or former smokers. The sites of metastases were consistently associated with response. Patients with lymph node only experienced a higher response rate to PD-1 and PD-L1 inhibitions than those with visceral disease raising the question as to whether the specific tumor site microenvironment may play a role in predicting the benefit.

The list of potential biomarkers is still growing including microenvironment-based and tumor cell-based biomarkers (Table 22.2). The most advanced biomarkers of response to PD-1 and PD-L1 inhibitors include mutational load, tumor or immune cells expression of PD-L1, and intratumoral immune infiltration. PD-1 and PD-L1 status are not yet recognized as a robust and reproducible marker to predict response and/or resistance to PD-1 and PD-L1 inhibitors. In 2L setting, there is an association of PD-L1 expression (either assessed on TC or IC) with response, but this association is not discriminant enough to recommend its current use in clinical practice. A major drawback for the use of PD-1/PD-L1 staining has been the heterogeneity of current methods for assessing PD-L1 positivity on IHC. Different antibodies manufacturers have developed multiple different assays as a companion diagnostic. The antibodies used for assessing PD-L1 are different in their characteristics, which affects inter-study comparisons. There are a number of open questions regarding the use of these antibodies: first, the location of positive PD-L1 staining is generally classified as TC versus IC, and thus a standardized consensus has not been established yet. There is also no consensus on which cut-off is to be used for positivity. The majority of studies consider strong PD-L1 staining of >5% (IHC staining scored 2/3) on either TCs or IC as positive, while other studies consider 1% as a positive cut-off. Harmonizing Companion Diagnostics is now urgently needed.

Table 22.2 Key biomarkers currently assessed in UC to predict response to PD1 or PD-L1 blockade

Biomarker	Assay	Endpoint	Drug	Results
PD-L1 on IC	IHC dako kit (22C3 clone)	1% of cell stained	Pembrolizumab	Higher expression associated with response
	IHC Ventana (SP263)	25% of cell stained	Durvalumab	Higher expression associated with response
PD-L1 on TC	IHC dako kit (22C3 clone)	1% of cell stained	Pembrolizumab	Higher expression associated with response
	IHC dako kit (28-8)	1% vs 5% of cell stained	Nivolumab	Higher expression associated with response
	IHC Ventana (SP263)	25% of cell stained	Durvalumab	Higher expression associated with response
T CD8 infiltration	IHC		Atezolizumab	High T CD8 infiltration associated with response
CD8 expression	Gene expression	High vs low	Nivolumab	High CD8 expression associated with response
CXCL9/CXCL10 expression	Gene expression	High vs low	Nivolumab	High expression associated with response
Mutational load	Exome sequencing	Number of mutations/megabase	Atezolizumab	High CD8 expression associated with response
TCGA classification	Gene expression	Luminal vs basal	Atezolizumab	Luminal 2 associated with better response to atezolizumab
			Nivolumab	Basal 1 associated with better response to nivolumab
INF-ɣ signature	25 gene expression	High vs low	Nivolumab	Better response to nivolumab
TCR sequencing	DNA sequencing of the CDR3 region of the TCR beta chain	Clonal dominance, clonal expansion and T-cell fraction	Atezolizumab	High T-cell infiltration and clonality in the tumor plus peripheral expansion of dominant tumor-resident TCR clones associated with response

IHC immunohistochemistry, *TCGA* the cancer genome atlas, *TCR* T-cell receptor

The Blueprint initiative will help build an evidence base for PD-1/PD-L1 companion diagnostic characterization in the pre-approval stage, such that once the tests are approved, the information generated can lay the groundwork for post-approval studies that will help inform patients, physicians, and pathologists on how best to use the test results to determine treatment decisions.

The burning question in UC emerged from the association of response with transcriptomic signatures according to the Cancer Genome Atlas (TCGA) classification [20]. Recent data suggest that MIBC may be classified into four subtypes based on the expression of gene set with two luminal subtypes (luminal 1 and luminal 2) and two basal subtypes (basal 1 and basal 2) (Fig. 22.1). Exploratory analyses of IMVIGOR 210 [28, 29] showed that PD-L1 IC expression was higher in the basal subtype (60%) as compared to luminal subtype (23%). Increased PD-L1 TC expression was found only in the basal subtype (39% in basal subtype vs 4% in luminal subtype). CD8 T-effector gene expression was increased in luminal cluster II and basal subtypes but not in luminal cluster 1. Consistent with these data, a higher baseline IFN-ɣ response gene was observed in atezolizumab responders. These data are consistent with Th1 and CTL immune response. Response to atezolizumab occurred in all TCGA subtypes but was significantly higher in the luminal cluster II subtype than in other subtype [28, 29]. Taken together, these data suggest that these subtypes have distinct tumor-immune landscapes that reflect responsiveness to atezolizumab: luminal I tumors have a low T_{eff}

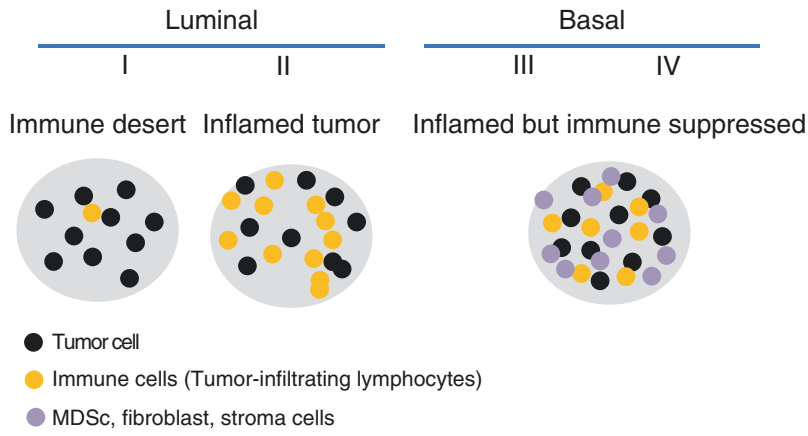

Fig. 22.1 Immune landscape across UC subtype

gene expression ("immune desert"), luminal II have a high T_{eff} and low stromal gene expression ("inflamed tumor"), and basal subtypes have a high T_{eff} as well as high stromal gene expression ("immune suppressed"). These preliminary data were not fully consistent with data reported in the CheckMate 275 study investigating the PD-1 inhibitor nivolumab [34]. IFN-ɣ signature was associated with higher response rate and higher PD-L1 expression. But the strongest IFN-ɣ signature was noted in patients with basal 1 subtype, which contained the highest proportion of responders and the highest CXCL9 or CXCL10 expression. These data need to be confirmed in the phase 3 trials investigating atezolizumab and pembrolizumab and may reflect the differential targets landscape between PD-1 and PD-L1 inhibitors.

22.3.5 How Can We Increase the Number of Responders?

The second burning question is how to increase the proportion of patients who benefit from PD-1 and PD-L1 inhibitions. Only 20% of patients with advanced platinum-based chemotherapy respond to PD-1 axis blockade. As in other tumors such as melanoma or lung cancer, combination immunotherapy may be more efficacious than monotherapy. Combining immunotherapy agents has been shown to result in improved response rates relative to monotherapy; for example, the concurrent administration of nivolumab and ipilimumab to patients with advanced melanoma induced higher response rate than those obtained with single-agent therapy. Importantly, responses appeared to be deep and durable [37, 38]. The rationale for evaluating the combination is that the mechanisms of CTLA-4 and PD-1 are nonredundant, suggesting that targeting both may have additive or synergistic activity [39] in both PD-L1-positive and PD-L1-negative populations. In addition, CTLA-4 antagonists have been shown to upregulate PD-1, and PD-1 has been shown to upregulate CTLA-4, further strengthening the rationale for combining tremelimumab and durvalumab [40]. Initial data from ongoing studies have demonstrated higher response rate and disease control rates in non-small-cell lung cancer patients with PD-L1-negative tumors receiving PD-1 inhibitor (nivolumab or durvalumab) + CTLA-4 inhibitor (ipilimumab or tremelimumab) combination therapy (30 and 70%, respectively) compared to durvalumab monotherapy (10 and 42%, respectively) [41, 42]. Such approaches are currently underway in UC in phase 1/2 trials [NCT02261220] [NCT01928394] [NCT02527434] [NCT02553642]. Phase 1 trials investigating the combinations of other inhibitor immune checkpoint [e.g., LAG-3, VISTA] or activator immune checkpoint [e.g., OX40, CD27] with a PD-1 inhibitor or a PD-L1 inhibitor as a backbone are currently conducted [NCT02528357] [NCT01968109] [NCT02608268]. Another strategy is to combine DNA damage agents like chemotherapy or radiotherapy with immune checkpoint inhibitors with the rationale that conventional agents may

Table 22.3 Main phase 3 trials in UC

Study code	ClinicalTrials.gov identifier	Indication	Treatment arms	Primary endpoints	Sample size
2L and 3L chemo-treated mUC					
Keynote-045	NCT02256436	Previously treated mUC	Pembrolizumab vs chemotherapy	OS	542
IMVIGOR 211	NCT02302807	Previously treated mUC	Atezolizumab vs chemotherapy	OS	932
1L chemo-naive mUC					
DANUBE	NCT02516241	Previously untreated mUC Cisplatinum eligible/ineligible	Durvalumab + tremelimumab vs durvalumab vs chemotherapy	OS and PFS	1005
CheckMate 901	NCT03036098	Previously untreated mUC Cisplatinum eligible/ineligible	Nivolumab + ipilimumab vs chemotherapy	OS and PFS in cis-ineligible patients	690
KEYNOTE-3475-361	NCT02853305	Previously untreated mUC Cisplatinum eligible/ineligible	Chemotherapy vs pembrolizumab vs chemotherapy + pembrolizumab	OS and PFS	990
CheckMate 901	NCT03036098	Previously untreated mUC Cisplatinum eligible/ineligible	Nivolumab + ipilimumab vs chemotherapy	OS and PFS in cis-ineligible patients	690
IMVIGOR 130	NCT02807636	Previously untreated mUC Cisplatinum eligible/ineligible	Chemotherapy + placebo vs atezolizumab vs chemotherapy + atezolizumab	OS and PFS	1200
JAVELIN	NCT02603432	Maintenance after response or stable disease on 1L chemotherapy	Avelumab vs best supportive care	OS	668
Adjuvant in non-metastatic MIBC					
IMVIGOR010	NCT02450331	High-risk MIBC after cystectomy	Atezolizumab vs observation	DFS	700
CheckMate 274	NCT02632409	High-risk MIBC after cystectomy	Nivolumab vs placebo	DFS	640

DFS disease-free survival, *MIBC* muscle-invasive bladder cancer, *mUC* metastatic urothelial carcinoma, *OS* overall survival, *PFS* progression-free survival

increase the mutational load in tumor cells resulting in higher response rate.

22.4 Perspective

Early-phase trials of checkpoint blockade in the heavily pretreated UC patients have demonstrated significant response rate, durable response, and good safety profile. Recent phase 3 trial confirmed that PD-1 blockade improved the outcome of lethal metastatic UC. Given these striking data, the potential application of PD-1/PD-L1 axis blockade to earlier disease states in chemo-naïve metastatic patients, in non-metastatic MIBC, and even in BCG-unresponsive high-grade NMIBC will be investigated in the coming months (Table 22.3). Thus, PD-1 and PD-L1 inhibitors will constitute the backbone of future immune-based therapies at least in advanced stage of UC. The development of rationale immune combinations is now the next steps of PD-1 and PD-L1 inhibitors. The emerging question is how to improve the number of patients who may benefit from immune checkpoint blockade. Multiple phase 1 trials investigating some combinations are ongoing. Ideally, these combinations should be based on potential mechanisms of resistance. Luminal I tumors are characterized by an immune desert and *FGFR2 and FGFR3* gene aberrations suggesting that educating quiescent T cells by inducing tumor cell death through treatment with chemotherapy, radiation therapy, or FGFR inhibitors may result in the release of tumor antigens for T-cell activation and in sensitivity to subsequent immune checkpoint inhibitor. An alternative approach would be to use vaccines carrying tumor-specific antigens for priming T cells before administration of immune checkpoint inhibitors, with potential to increase response rate. In basal tumors characterized by high myeloid cell infiltrations, combinations should aim at targeting immunosuppressive environment. Indoleamine 2,3-dioxygenase (IDO) and high intracellular concentrations of adenosine play a key role in pathways that induce a suppressive effect on cytotoxic T-cell function, so that targeting IDO1 and adenosine A2a receptor (A2aR) are also a promising target for therapy in UC [NCT02178722], [NCT02318277], and [NCT02655822].

Going forward, research efforts should focus on identification of predictive biomarkers of response to immune-oncology agents. Identification of innovative correlative biomarkers in future studies would hopefully help identify ways to predict on the individual patient level whether a patient would likely benefit from a specific therapy or combination. One can speculate that decision about single-agent therapy vs a particular combination may be decided by specific tumor or host markers (e.g., serum, microbiota) or both which stress the highly complex interaction of tumor and immune system for inducing antitumor response.

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PD-L1 and Other Immunological Diagnosis Tools

23

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Contents

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23.1 Introduction

As immunotherapy has joined the ranks of mainstay cancer treatment modalities, biomarkers of response and resistance have become highly sought after. Pretreatment biomarkers have the potential to facilitate rational patient selection, perhaps even for those patients who demonstrate unconventional response patterns such as a delayed response or tumor "progression" before evident regression. Immune-related adverse events, some severe, are also observed with this group of agents. Biomarkers of response could help practitioners avoid exposing patients who are unlikely to respond to these potential immune-related side effects. Additionally, the elevated cost of the therapies is another factor driving intensive study in this area.

The most well-studied factor influencing response to treatment is PD-L1 protein expression as measured by immunohistochemistry. It has been shown to enrich the objective response rate (ORR) to treatment with both anti-PD-1 and PD-L1 monotherapy across multiple solid tumor types, and most recently to combine anti-PD-1/CTLA-4 blockade in patients with non-small cell lung carcinoma (NSCLC). This chapter will review the utility of PD-L1 expression as a theranostic biomarker in multiple tumor types, as well as identify issues regarding its implementation in clinical practice, such as the different commercially available PD-L1 assays. Additional emerging tumor tissue-based biomarkers such as

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the mutational load of the tumor, densities of $CD8^+$ tumor-infiltrating lymphocytes (TIL), T-cell clonality studies, and gene expression signatures as well as peripheral blood biomarkers will also be introduced.

23.2 PD-1/PD-L1 Blockade and the Role of PD-L1 Expression as Biomarker

23.2.1 Early Experience in Solid Tumors

The first suggestion that PD-L1 expression measured by immunohistochemistry (IHC) might be associated with response to anti-PD-1 came from the phase I study of anti-PD-1 (MDX1106, nivolumab) reported in 2010 [1]. In this study, only nine patients had available tissue for study, and yet a correlation between membranous (cell surface) PD-L1 expression and response was noted. This finding was then further assessed on the expansion study where pretreatment tumor material was available: 42 patients with multiple tumor types, including melanoma, NSCLC, RCC, prostate cancer, and colorectal cancer. When tumor cell PD-L1 expression was assessed, none (0/17) patients who were PD-L1 negative responded, while 36% (9/25) patients whose tumors were positive by IHC for PD-L1 had an objective response ($p = 0.006$) [2]. Another landmark clinical trial that reported in 2014 evaluated the activity of an anti-PD-L1 antibody (atezolizumab) in different solid malignancies (NSCLC, melanoma, RCC, head and neck, breast, colorectal, among others) and also studied PD-L1 as a potential biomarker of response. However, they emphasized PD-L1 expression on immune cells rather than tumor cells. They reported a significant association between the expression of PD-L1 on immune cells and ORR (45% vs. 16% in PD-L1(+) and PD-L1(−) tumors, respectively) [3]. Subsequently, the majority of clinical trials assessing the response to PD-1/PD-L1 blockade have evaluated the protein expression of PD-L1 by the tumor and/or infiltrating immune cells in pretreatment tumor specimens and the relationship with clinical outcome. Some of these major clinical trials are summarized below, and composite response rates by PD-L1 status are shown in Fig. 23.1.

23.2.2 NSCLC

NSCLC is perhaps the most well-studied tumor type with regard to the activity of PD-1/PD-L1 blocking agents. Nine clinical trials, accounting for 1725 NSCLC-bearing patients treated with

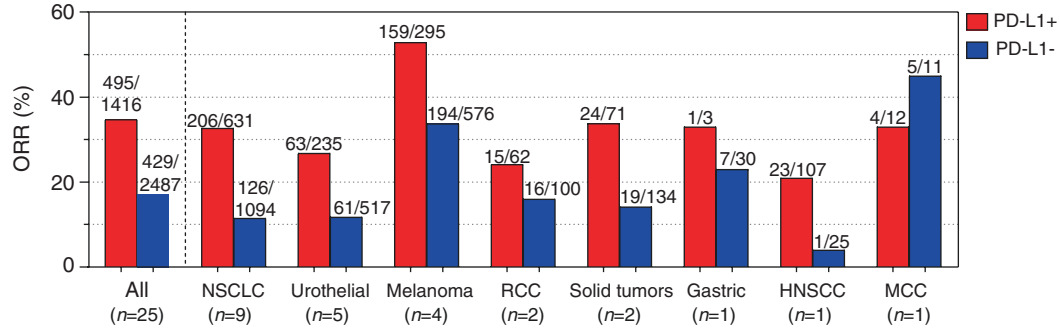

Fig. 23.1 PD-L1 expression measured by IHC in pretreatment tumor specimens enriches objective response rates (ORR) to PD-1/L1 blockade across multiple solid tumor types. The ORR to PD-1/PD-L1 blocking agents (nivolumab, pembrolizumab, atezolizumab, or durvalumab) for 25 clinical trials on eight cancer types is summarized for each tumor type and stratified by PD-L1 status. The aggregate number of responders over the total number of patients in each category is displayed at the top of each column, and the number of clinical trials (n) is displayed underneath the name of each cancer type. *NSCLC* non-small cell lung cancer, *RCC* renal cell carcinoma, *HNSCC* head and neck cancer, *MCC* Merkel cell carcinoma

one of three different anti-PD-1 or anti-PD-L1 therapies [nivolumab [4–8], pembrolizumab (human IgG4 anti-PD-1 monoclonal antibody) [9, 10], and atezolizumab [3, 11]], show a collective ORR of 17%. Data pooled from all patients—irrespective of whether they received the agents in the first-line or higher-order setting, or the assay used to determine PD-L1 positivity—demonstrates an ORR of 33% in patients with PD-L1(+) tumors and 11% for those with PD-L1(−) tumors. When each therapeutic was assessed individually, the relationship held constant: ORR 30% PD-L1(+) vs. 13% PD-L1(−) for nivolumab (five studies and 538 treated patients), ORR 33% PD-L1(+) vs. 11% PD-L1(−) for pembrolizumab (two studies and 1015 treated patients), and ORR 42% PD-L1(+) vs. 11% PD-L1(−) for atezolizumab (two studies and 190 treated patients).

A recent study comparing anti-PD-1 monotherapy to dual anti-PD-1/CTLA-4 blockade as first-line treatment for patients with NSCLC reported two interesting findings related to PD-L1 as a biomarker [12]. The first was the finding that increasing levels of PD-L1 in the pretreatment tumor microenvironment enriched for ORR to anti-PD-1. This finding is notable because it argues against dichotomous reporting of PD-L1 status. The second finding of note was that increasing levels of PD-L1 expression in the pretreatment tumor specimens were not only predictive of the improved response to anti-PD-1, but it was also associated with an increasing ORR in patients receiving the combinatorial regimen.

The histological subtype, underlying genetic alterations, and smoking status in patients with NSCLC have also been studied for their association with PD-L1 status and response to therapy. Subset analysis shows that the tumor histology may impact the association between PD-L1 expression and response to therapy, with PD-L1 enriching for response in non-squamous NSCLC but not in tumors displaying squamous histology [6, 9]. This may be due to the fact that PD-L1 expression in lung adenocarcinomas is associated with IFN-gamma, while PD-L1 expression in lung squamous cell carcinoma is not [13] (see below section on "*What is the meaning of PD-L1 expression in the tumor microenvironment?*"). Mutations in EGFR, TP53, and KRAS genes have all been associated with increased PD-L1 expression, often via activation of the PI3K-AKT-mTOR pathway [14]. Of these, TP53 and KRAS mutations have been correlated with higher ORR [15, 16], while EGFR mutations are thought to confer partial resistance to anti-PD-1 checkpoint blockade [4, 5, 9]. Lastly, several studies have reported higher ORR [4, 7, 10] or longer PFS [5] among patients who had history of smoking. The tumor expression of PD-L1 in this latter group is not clearly increased [17].

23.2.3 Urogenital Malignancies (Urothelial and Renal Cell Carcinoma)

Urothelial carcinoma (carcinoma of the bladder, ureters, and renal pelvis) is another tumor type that has displayed a high sensitivity to PD-1/PD-L1 blockade. Most clinical trials for this tumor type have evaluated the activity of anti-PD-L1 rather than anti-PD-1. An overall ORR of 16% has been observed in five different clinical trials, treating 752 patients [18–22]. When stratified by PD-L1 expression in the TME, patients with either PD-L1(+) tumor [18, 22] or immune cells [19–21] have an ORR of 27%, compared to an ORR of 11% in the patients with PD-L1(−) tumors. When subdivided by therapeutic agent, the association of PD-L1 expression with response is not dependent on the anti-PD-L1 agent received. For example, a 27% ORR in PD-L1(+) patients vs. 11% ORR in PD-L1(−) patients was observed in trials of atezolizumab (three studies and 695 treated patients) [19–21], and a 45% ORR in PD-L1(+) patients vs. 0% ORR for PD-L1(−) patients was noted in the one clinical trial on 42 patients [22]. Notably, the sole study focused on the efficacy of an anti-PD-1 agent (nivolumab, 67 treated patients) found similar ORR in patients with PD-L1(+) and PD-L1(−) tumors (24% vs. 26%, respectively) [18]. While differences in ORR were not observed, an improved median overall survival for patients with PD-L1(+) tumors when compared

to PD-L1(−) tumors was noted. The study focused on tumor cell PD-L1 display only and used a 1% threshold for assigning a tumor as PD-L1(+), which is lower than the studies using anti-PD-L1 agents. Future studies will undoubtedly focus on whether studying a component of PD-L1 expression on immune cells will better stratify patient response to nivolumab, as was seen for atezolizumab.

Two clinical trials focused on the treatment of metastatic renal cell carcinoma (RCC) have reported information regarding PD-L1 status and association with response. One study administered nivolumab (n = 107) and the other atezolizumab (n = 55), and the overall ORR for both studies was 19% [23, 24]. There was no clear benefit to PD-L1 expression in the TME in either of these studies. A separate small study with ten patients compared the activity of atezolizumab in combination with bevacizumab (humanized monoclonal anti-VEGF) between PD-L1(+) and PD-L1(−) tumors and also found no differences [25]. Like for lung SCC (discussed above), it is possible that this lack of enrichment may be due to the fact that PD-L1 expression in RCC is not associated with IFN-gamma expression, in contrast to many of the other solid tumor types [13].

23.2.4 Melanoma

Advanced melanoma has displayed one of the highest response rates to PD-1/PD-L1 blockade out of the solid tumor types studied. Four clinical trials including 871 patients with melanoma and annotated tumor PD-L1 expression demonstrated an overall ORR of 40% [26–29]. Patients with PD-L1(+) tumors (defined with a cutoff of 5% and focused on tumor cell expression in all clinical trials) showed an ORR of 53%, while those with PD-L1(−) tumors had an ORR of 34%. As some of the trials have matured, other endpoints such as progression-free survival (PFS) and overall survival (OS) can be assessed, rather than just the ORR. Patients with PD-L1(+) melanomas have now been shown to have longer PFS and OS than PD-L1(−) patients [30]. Further, melanoma patients demonstrating higher levels of PD-L1 expression are more likely to respond than those demonstrating lower level of expression [31], calling into question the use of a dichotomous scoring system for what may be considered a continuous variable. An association between the $BRAF^{V600E}$ mutation and PD-L1 expression or the patient's response to PD-1/PD-L1 blockade has not been identified [26, 32, 33]. This also has important biomarker implications, as it indicates that the $BRAF^{V600E}$ mutation and PD-L1 expression should be considered separate biomarkers when making treatment decisions for patients with advanced melanoma.

PD-L1 expression has also been evaluated as a potential biomarker for response to dual anti-PD-1/CTLA-4 checkpoint blockade in patients with melanoma. In contrast to patients receiving anti-PD-1 monotherapy, PD-L1 status does not impact the remarkably high response rates observed with this regimen, indicating that combinatorial blockade may be able to "overcome PD-L1(−) status" [26]. It has been suggested that PD-L1 status may be used on the future for sorting patients between regimens, with PD-L1(+) patients receiving anti-PD-1 monotherapy, which is associated with a lower side effect profile and PD-L1(−) patients receiving combined anti-PD-1/CTLA-4 checkpoint blockade.

23.2.5 Virus-Associated Cancers

Head and neck squamous cell carcinoma (HNSCC), gastric carcinoma, and Merkel cell carcinoma (MCC) are remarkable for their recognized association with viral infections—human papillomavirus, Epstein-Barr virus (EBV), and Merkel cell polyomavirus, respectively. In each of these tumor types, virus-positive tumors have been shown to be more likely to express PD-L1 than virus-negative tumors [32, 34–36]. The presence of viral antigens is thought to contribute to an antitumor immune response, and thus immune checkpoint blocking agents were anticipated to show activity in these tumor types. Initial studies suggest that like with the other solid tumor types discussed above, patients with

HSNCC show a greater likelihood of response to anti-PD-1/PD-L1 blockade if their tumors are PD-L1 positive vs. negative: ORR of 21% vs. 4% [37]. In a small study of patients with gastric cancer treated with pembrolizumab, a 22% ORR was observed. PD-L1 expression at a 1% cutoff was required for enrollment; thus, potential differential response between PD-L1(+) vs. (−) was not available, and EBV status was not assessed [38]. Patients with MCC on one study showed an impressive response to anti-PD-1, though notably, response to therapy was not significantly associated with either the presence of virus or PD-L1 expression [39]. It is possible that this is because virus-negative MCCs have very high mutational densities, a feature which is also recognized as a biomarker of response to anti-PD-1 (see *Mutational Load* section below). The relationship between viral status and response to immune checkpoint blockade is an area of ongoing, active investigation.

23.3 PD-L1 Assays

The initial reports of the correlating PD-L1 expression with response to anti-PD-1 were performed using a laboratory-derived IHC test [1, 2]. Four different proprietary, commercial PD-L1 assays were soon developed on two different immunostaining platforms and accompanied clinical trials of each respective company's anti-PD-1/PD-L1 agent, Table 23.1. Many of the assays used different scoring systems and different thresholds for considering a case to be PD-L1(+) vs. PD-L1(−). In addition to being confusing for pathologists and oncologists, this scenario is impractical for surgical pathology laboratories, as they are very unlikely to have the resources to support four different PD-L1 assays on two different immunostaining platforms. Most labs have a single immunostainer, and it may not necessarily be either the Dako Link 48 or Ventana BenchMark platform that is called for by the marketed assays.

The FDA recognized the potential hazard of such a "one assay/one drug" paradigm and helped support an effort to understand the comparative performance of the four different marketed PD-L1 assays. The four leading companies with anti-PD-1/PD-L1 therapies and the two companies that produced the PD-L1 IHC assays participated in this effort. In the resultant study, known as the "blueprint effort," 40 archival surgical pathology cases of NSCLC patients were gathered, and sequential FFPE tumor sections were stained with each of the assays. PD-L1 scoring of both tumor cell and immune cell PD-L1 expression was then assessed by three different pathologists, whose results were averaged. Importantly, it was not assessed using the companies' suggested scoring systems, but simply as a percentage of tumor cell or immune cell staining. They found that three of the assays performed very similarly (22C3, 28–8 and SP263 assays), while the SP142 assay clearly highlighted less tumor

Table 23.1 Four different commercially available PD-L1 immunohistochemistry assays

	Bristol-Myers squibb	Merck	Genentech/Roche	AstraZeneca/ Medimmune
mAb clone	28–8	22C3	SP142	SP263
Automated	Yes	Yes	Yes	Yes
Diagnostic partner	Dako	Dako	Ventana	Ventana
Machine	Link 48	Link 48	BenchMark ULTRA	BenchMark ULTRA
Scoring	Tumor cells (membrane)	Tumor cells (membrane)	Tumor and/or immune cells (membrane)	Tumor cells (membrane)
Positive cutoff	≥5% (also studied ≥1% and ≥10% thresholds)	≥1% for trial enrollment other analysis at ≥50%	• TC3 (≥50%) or IC3 (≥10%) • TC2/3 or IC2/3 (>5%) • TC 1/2/3 or IC 1/2/3 (>1%) • TC0 and IC0 (0%)	≥25%

cell PD-L1 expression than the other three assays [40].

There are two other notable studies focused on assay comparisons. One was led by the National Comprehensive Cancer Network, supported by Bristol-Myers Squibb, and involved 13 academic pathologists. This effort compared the 22C3 assay, the 28–8 assay, a laboratory-derived test (LDT) using the SP142 antibody clone designed to mimic the SP142 commercial assay, and an LDT using the E1L3N clone on 90 archival surgical pathology cases of NSCLC. In this larger and statistically powered study, the SP142 assay labeled not only less tumor cell staining of PD-L1 expression but also less immune cell expression [41]. Interestingly, in this comparison, when the scores for each case were averaged among the 13 pathologists, the 22C3 assay labeled slightly less tumor cells than the 28–8 and E1L3N assays. However, the variance of scoring between the individual pathologists was greater than the observed difference in PD-L1 labeling between the 22C3 assay vs. the 28–8 assay and E1L3N LDT; thus, in routine surgical pathology practice, this difference in assay performance would never be realized.

The third effort studied the largest number of cases—nearly 500 archival NSCLC cases—which were scored for PD-L1 expression by one primary pathologist. A proportion of the cases is also scored by a second pathologist. They showed a > 90% concordance between the SP263 assay, the 28–8 assay, and the 22C3 assay at multiple cut points for tumor cell staining [42]. The SP142 assay was not commercially available at the time this study was conducted and thus was not assessed for concordance with the other three assays.

The terminology with respect to commercially available PD-L1 assays is potentially confusing because the assays themselves include the clone name. For example, the "SP142 antibody clone" is different than the "SP142 assay," the latter of which includes the former as one of the many reagents which are used in a specific order and under precise technical conditions to make an assay system. Thus, observed performance differences in PD-L1 IHC assays could be due to primary antibody concentration, antibody affinity, the epitope that the antibody targets, antigen retrieval reagent, pH of specific reagents, or amplification steps, among other things. The assay systems for the commercially marketed assays are proprietary; so very little detail is available about the extended list of components. Two different studies have been performed which hold the other assay conditions essentially constant and focus on the concordance of the anti-PD-L1 antibody clones. For both NSCLC specimens and melanoma specimens, the SP142 antibody performed as well as the 28–8, 22C3, and the SP263 clones when all other assay conditions were held constant [43, 44]. When differences were observed between antibody clones, review of the slides showed that the differences were due to the focal and heterogeneous nature of PD-L1 expression that varied from one tumor slice to the next, rather than clear differences in assay sensitivity for tumor cell or immune cell labeling. Concordance was not significantly affected by whether the antibody labeled the intracellular or extracellular domain of the PD-L1 molecule, Fig. 23.2. These studies thus suggest that it is some other component of the assay system beyond the SP142 antibody clone itself that leads to the observed differences in SP142 assay performance, when compared to the other commercially available assays.

Taken together, these collective findings suggest that in the future, a surgical pathology labo-

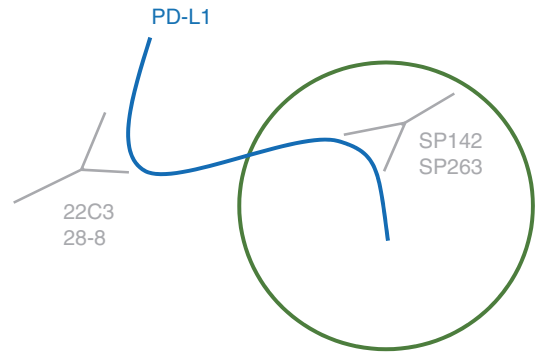

Fig. 23.2 Commercial PD-L1 assays contain different primary antibodies, some of which target the extracellular domain of the PD-L1 molecule (22C3 and 28-8), while others target the intracellular domain (SP142 and SP263)

ratory may be able to offer only one of the three commercial PD-L1 assays (28–8, 22C3, or SP263 assays) that show similar performance, or even an LDT with equivalent performance characteristics (including one that utilizes the SP142 clone), as long as the specific, *commercially designed, and clinically tested scoring system for PD-L1 expression is paired with the appropriate anti-PD-1/PD-L1 therapeutic agent.* Due to the focal nature of PD-L1 expression, as well as the closely related issue of temporal and spatial heterogeneity, additional studies are required to determine the ideal type of tissue (specimen size and number of lesions, etc.) to optimize the predictive value of PD-L1 expression in the pretreatment tumor microenvironment.

23.4 What is the Meaning of PD-L1 Expression in the Tumor Microenvironment (TME)?

The PD-1/PD-L axis, composed of programmed cell death protein 1 (PD-1) and its ligands (PD-L1 and PD-L2), is one of the most important physiologic mechanisms for the maintenance of peripheral tolerance [45, 46]. It is key in controlling the magnitude of cytotoxic T-cell responses and has been described in virtually all types of inflammatory conditions, unlike many other inhibitory receptor pathways [47]. PD-1 is expressed on activated T cells and B cells [48]. PD-1 expression by T cells is induced by TCR-dependent activation, though the levels of expression and the window of time where it is expressed are a function of the nature and continuity of the stimuli [49, 50]. PD-L1 and PD-L2 are typically expressed by a subset of macrophages but can be induced in a variety of both hematopoietic and non-hematopoietic cells by a diverse array of inflammatory cytokines [47]. PD-1/PD-L1 engagement inhibits the core functions of T cells, including cytokine production, proliferation, and cytotoxic granule formation [51–53].

Tumors may exploit the PD-1/PD-L1 axis to escape T-cell-mediated attack. Four different histologic patterns of PD-L1 expression in the TME were first described in melanoma and include: (1) PD-L1(+)/TIL+, (2) PD-L1(+)/TIL-, (3) PD-L1(−) and TIL+, and (4) PD-L1(−) /TIL- [54], Fig. 23.3A. Since this original description, these patterns have been recognized in solid tumor types as diverse as NSCLC, anal squamous cell carcinoma (SCC), and breast carcinoma [55–57]. Each of these patterns is thought to represent a distinct immune microenvironment, Fig. 23.3 [58].

The two PD-L1(+) patterns that are TIL- and TIL+ are thought to correlate with *innate* and *adaptive immune resistance* by tumor, respectively [59]. *Innate immune resistance* means constitutive, oncogene-driven expression of PD-L1. Histologically, this is represented by broad expression of PD-L1 on the tumor cell surface and is geographically unrelated to the presence of TIL. Genetic abnormalities that have been associated with such an increase in PD-L1 expression include PTEN deletions, PI3K-AKT mutations, and chromosome nine amplifications [14, 60, 61]. The *adaptive immune resistance* pattern of PD-L1 expression is characterized histologically by PD-L1 display on tumor cells or immune cells immediately adjacent to TIL [54]. This pattern is most often seen along the invasive margin of the tumor (where TIL are often the most dense). PD-L1 expression in this scenario is induced by interferon gamma (IFN-γ) and other inflammatory cytokines secreted by activated immune cells [13, 54, 62]. Importantly, tumors may also demonstrate a mixture of these two mechanisms, whereby there is an adaptive component superimposed on constitutive PD-L1 expression [55, 57, 61].

It is likely that the different biologic mechanisms underlying PD-L1 expression have contributed to the conflicting reports in the literature regarding the prognostic impact of PD-L1 expression. When the adaptive pattern of PD-L1 expression is studied, it is a positive prognostic feature in tumor types such as melanoma, NSCLC, breast carcinoma, and laryngeal SCC [62–65]. This is consistent with the notion that adaptive PD-L1 expression represents an ongoing host immune response against tumor. Notably, constitutive PD-L1 expression in both NSCLC and melanoma

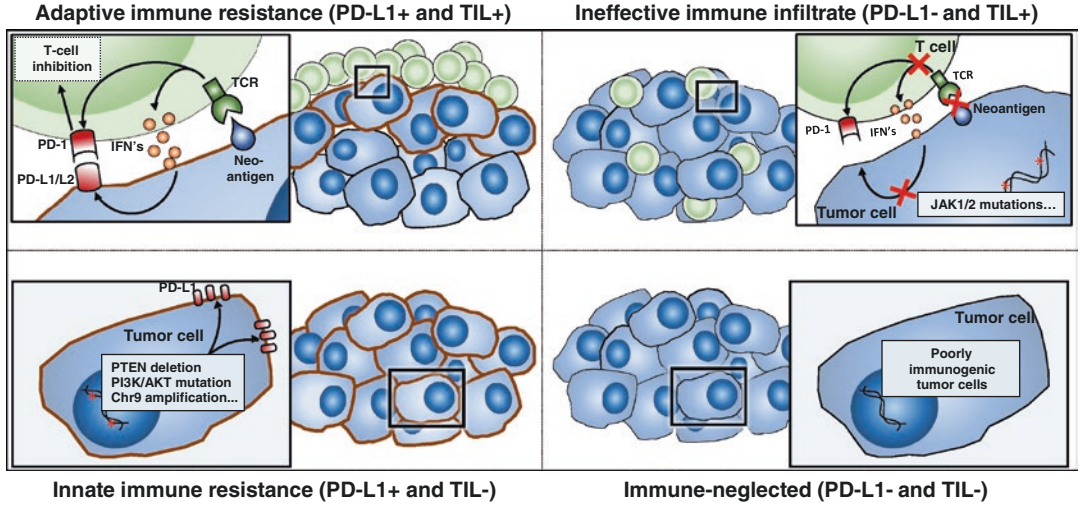

Fig. 23.3 Patterns of PD-L1 expression and TIL define distinct tumor microenvironments. In tumors demonstrating an *adaptive immune resistance* pattern of PD-L1 expression (*upper left*), activated, PD-1 expressing T cells at the host-tumor interface secrete IFN-ɣ and other inflammatory cytokines, which in turn, induces the expression of PD-L1 by tumor cells and associated immune cells. The subsequent engagement of PD-1 on T cells by PD-L1 induces T-cell inhibition, promoting immune tolerance. For tumors demonstrating an *innate* pattern of PD-L1 expression (*lower left*), mutations (e.g., PTEN) lead to constitutive PD-L1 expression by tumor cells that is independent of TIL. PD-L1(−)/TIL+ tumors are those with an *ineffective immune infiltrate* (*upper right*). Factors contributing to this phenotype include those that disrupt the adaptive immune resistance pathway—including the defective activation or function of TIL or mutations leading to the dysfunctional response to IFN-ɣ by tumors cells. PD-L1(−)/TIL− tumors are *immune-neglected* (*lower right*)

has been associated with a worse prognosis [56, 66]. These findings underscore the need for understanding the geographic context of PD-L1 expression as opposed to noting whether it is simply present or absent in the TME.

The two PD-L1(−) patterns that are TIL- and TIL+ also represent different underlying biologies. The mechanisms responsible for the PD-L1(−)/TIL+ type of microenvironment are more heterogeneous and still under investigation. Potential explanations include the different functional states of TIL, i.e., the TILs are not secreting IFN-γ [54, 62], as well as alterations in the IFN-γ receptor intracellular cascade in the tumor cells [67, 68]. It is also possible that the tumor is evading the immune system by another mechanism, including potentially an immune checkpoint other than PD-1/PD-L1. The tumors from TIL patients, on the other hand, are essentially *neglected* by the immune system.

Each of these PD-L1 expression patterns also likely has therapeutic implications [69]. For example, patients with an adaptive immune resistance pattern (PD-L1(+)/TIL+) are thought to be the most likely to respond to anti-PD-1/PD-L1 monotherapy [70, 71]. Individuals with immune-neglected tumors (PD-L1(−)/TIL-) or exhibiting a pure pattern of innate immune resistance (PD-L1(+)/TIL-) might require vaccination or other treatments that promote an antitumor T-cell response in addition to PD-1/PD-L1 checkpoint blockade [69, 72]. The sensitivity to anti-PD-1/PD-L1 of tumors demonstrating a combined adaptive and constitutive pattern is still unclear, but it is most likely related to the density and distribution of specific TIL subsets [73–75]. Finally, the approach to PD-L1(−)/TIL+ tumors will depend on additional characterization of the tumor and TIL. For example, tumors harboring mutations in JAK1 or JAK2 can interfere with the capacity of the tumor cells to upregulate PD-L1 in response to IFN-ɣ [67, 68] and thus can confer resistance to PD-1 blockade. Tumors evading the immune system via a

different checkpoint molecule or harboring TIL subsets other than cytotoxic T cells could also show a similar pattern. Depending on the underlying cause, these tumors could require blockade of another checkpoint, combination immunotherapy, or for the case of truly IFN-γ-resistant tumors, another form of therapy beyond immunotherapy [69].

23.5 Other Potential Biomarkers

23.5.1 Mutational Loads

One of the hallmarks of tumor cells is genomic instability [76]. In fact, a tumor cell exhibits in average ~120 non-synonymous mutations, a number that can increase up to ~2000 [77]. Higher mutational loads have been associated with an improved prognosis in certain cancer types [13, 78, 79]. This is thought to be because the mutations serve as potential sources of immunogenic neoantigens [80]. When the mutational landscapes for a broad array of cancer types were determined, many with the highest mutational loads (melanoma, NSCLC, and bladder carcinoma) were noted to be those that were responsive to PD-1/PD-L1 blockade therapies in early clinical trials [81, 82], further supporting this concept.

The relationship between mutational load and response to immune checkpoint blockade has now been formally tested in a number of clinical trials. In patients with NSCLC and treated with anti-PD-1, an increased number of tumoral non-synonymous mutations was associated with higher ORR and longest PFS [83, 84]. Similar findings have been reported for response to anti-CTLA-4 in patients with melanoma [85, 86] and to anti-PD-L1 in patients with urothelial carcinoma. [20] Perhaps the most striking example is provided by tumors that are microsatellite unstable (have defects in their DNA-repairing machinery) which exhibit much higher mutational burdens and correspondingly higher ORR to anti-PD-1 treatments than tumors from other etiologies [87]. It is currently unclear how much of this the improved survival seen after treatment is attributable to the fact that patients with an increased mutational load may have had an improved prognosis anyway [88].

Although mutational burden is often correlated with predicted neoantigen load, a given neoantigen may not always be immunogenic [80]. There is currently intense interest in identifying specific neoantigens that are the targets of individual host, antitumor T-cell reactivity. The identification of these would allow for the development of truly personalized medicine approaches.

23.5.2 Molecular Subgroups

The analysis of whole transcriptomic data from specific tumor types, for example, by The Cancer Genome Atlas project, has led to the description of cancer type subfamilies (or *molecular subgroups*) [89]. To date, the correlation between a tumor molecular subgroup and response to checkpoint blockade has only been assessed in urothelial carcinoma. Two independent studies have described that patients with the "luminal cluster II" subtype of urothelial cancer displayed higher chances of response to treatment [19, 20]. This is an emerging area of interest that will likely be extended in the future to other cancer types.

23.5.3 CD8$^+$ TIL

A large amount of evidence supports that the composition and distribution of the tumor immune infiltrate have an important impact on patient's clinical outcome [90]. A recent study by Becht et al. [89] reported more than 130 publications supporting a positive association between increased densities of CD8$^+$ and Th1-oriented TIL and good clinical outcome in more than 20 different cancer types. A similar association between CD8+ TIL densities and response to anti-PD-1 has been reported in patients with melanoma and colorectal cancer treated with pembrolizumab [70, 87] and patients with melanoma, initially treated with anti-CTLA-4 followed by nivolumab, at progression [91].

Interestingly, two independent studies have reported that the degree of $CD8^+$ TIL infiltration is not associated with response to anti-PD-1 in metastatic RCC [24, 62]. This could be related to the fact that in RCC—in contrast to the majority of cancer types—increasing cytotoxic $CD8^+$ TILs are associated with decreased survival [13]. This may be due to the co-expression of several inhibitory receptors on TIL and a polyclonal intratumor T-cell response [92, 93].

23.5.4 Gene Signatures

Several studies testing anti-PD-1/PD-L1 agents have evaluated the correlation between the abundance of a high number of immune- and nonimmune-related transcripts and clinical outcome in different cancer types. Consistent with previous evidence, the upregulation of IFN-γ signaling-related genes (e.g., IFNG, CD8A, IDO, and CX3CL1) has been associated with therapeutic response in several studies [3, 38, 94–96]. Other immune signatures correlated with clinical outcome include genes associated with TCR signaling and a cytotoxic immune response [94, 95]. As PD-L1 transcription can be induced by IFN-γ, it is reasonable to imagine that tumors exhibiting an IFN-γ-gene signature may overlap with those expressing PD-L1 protein [54], i.e., both methods could roughly identify the same group of tumors [62]. The validation of immune-related gene signatures is currently under intense investigation and commercial kits to estimate the patient's sensitivity to anti-PD-1/PD-L1 agents based on this method are anticipated in the near future.

Gene signatures associated with resistance to PD-1 blockade have also been reported. For instance, melanomas exhibiting resistance to pembrolizumab overexpress genes involved in mesenchymal transition (e.g., AXL and ROR2), immunosuppression (e.g., IL10 and VEGFA), and monocyte and macrophage chemoattraction (e.g., CCL2 and CCL13) when compared to melanomas that are sensitive to anti-PD-1 [88]. Nonimmune-related pathways may also contribute to the efficacy of checkpoint blockade. For example, PD-L1(+) renal cell carcinomas resistant to anti-PD-1 treatment overexpress genes involved in metabolic and solute transport functions, such as UGT1A [97].

23.5.5 T-Cell Repertoire

The mechanism of action of immune checkpoint inhibitors is most likely related to the enhancement of the in situ cytotoxic immune responses against neoantigens expressed on the tumor cells. In fact, many clinical trials have suggested that treatment with these agents facilitates an influx of $CD8^+$ TIL into the tumors [1, 70, 91, 98], accompanied by an intra-tumoral and peripheral change in the T-cell repertoires [83, 99, 100]. For this reason, the T-cell clonality index (which measures both the number and abundance of unique TCR CDR3 variable sequences) has been proposed as one potential biomarker of response to checkpoint inhibitors. For patients with melanoma treated with anti-CTLA-4, an oligoclonal response, i.e., a less diverse T-cell repertoire, in the pretreatment tumor specimens or in the peripheral blood following treatment initiation is associated with response [101, 102]. Patients with melanoma who responded to anti-PD-1 also exhibited more clonal T-cell populations when compared to nonresponders [70].

23.5.6 Peripheral Blood Markers

IFN-γ-driven PD-L1 expression in the local tumor microenvironment has been shown to associate with response to anti-PD-1, as discussed above. IFN-γ-responsive elements such as Bim expression on T cells in the peripheral blood have also been observed to be expressed at high levels in patients experiencing clinical benefit with anti-PD-1 [103]. Recently, a publication evaluating the activity of atezolizumab in metastatic RCC and advanced melanoma described that the serum concentration of some cytokines and acute-phase proteins could be associated

with response to treatment [24]. Another parameter that can be assessed in the patient's serum is the circulating tumor DNA (ctDNA) levels. One study reported that the levels of ctDNA correlated with clinical and radiologic outcomes in patients undergoing treatment with checkpoint blockade but its utility as biomarker of clinical response has yet to be formally tested [104]. Due to the minimally invasive nature of peripheral blood draws as well as the relatively easy potential implementation of a blood-based assay in routine clinical practice, this area is one of particularly intense scrutiny.

23.6 Conclusions and Future Directions

The majority of studies suggest a robust association between an increased in situ immune response against tumor and clinical outcome following therapeutic intervention with immune checkpoint blockade. Local PD-L1 protein expression, high mutational loads, increased infiltration with TIL, and overexpression of an IFN-γ-oriented gene signature are the biomarkers with the most experimental support. Most of the studies evaluating these parameters need further validation and extended testing in more cancer types. While most of the focus to date has been on cytotoxic T cells, other immune cell populations such as B cells and macrophages can facilitate tumor cell elimination. Additionally, populations such as regulatory T cells and myeloid-derived suppressor cells can dampen the antitumor immune response and promote malignant cell growth and tissue invasion [105]. New technologies such as multiplex immunofluorescence or immunohistochemistry will allow for the simultaneous identification, enumeration, and assessment of geographic relationships between numerous cellular populations, Fig. 23.4. Future studies will focus on integrating and prioritizing such spatial protein expression with genomic and transcriptomic signatures for improved patient selection, on treatment monitoring, and adaptive adjustment of therapeutic agent administration.

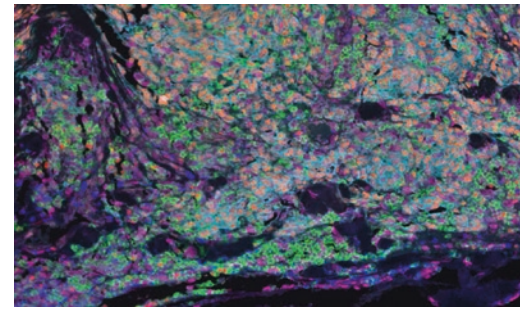

Fig. 23.4 Formalin-fixed paraffin-embedded melanoma specimen stained and imaged using multispectral immunofluorescence. This approach facilitates the simultaneous visualization of different cellular subsets and studies of co-expression on the same slide. Shown here are cytotoxic CD8+ T cells (*blue*), FoxP3+ regulatory T cells (*red*), CD68+ macrophages (*magenta*), and Sox10+ melanoma cells (*orange*). PD-L1 (*aqua*) expression is seen on tumor cells, macrophages, and lymphocytes in close proximity to PD-1 expression by both CD8+ and FoxP3+ T cells

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Oncolytic Viruses: T-VEC and Others

24

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Contents

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24.1 Background

Immunotherapy has become a standard for treatment of several advanced cancers such as melanoma, lung cancer, and head and neck cancer. Lymphocyte-rich tumor microenvironment is a predictor factor for response to immunotherapy [1]. Although theoretically, these tumor-infiltrating lymphocytes should be highly immunogenic, it has been shown that the tumor microenvironment by itself is immunosuppressive, and the tumor-associated antigens (TAAs) are weakly immunogenic, thus limiting spontaneous antitumor immunologic activity in most tumor types [2]. Therefore, strategies to shift the balance between tumor growth and immunosuppressive tumor microenvironment while at the same time enhancing the magnitude of antitumor immune response are highly desired. Much of the immunotherapies available currently such as ipilimumab, pembrolizumab, and nivolumab to name a few disrupt these immunoregulatory circuits such as CTLA-4 and PD-1 and enhance antitumor immunity. CTLA-4 blockade with ipilimumab and PD-1 blockade with pembrolizumab or nivolumab produces response rates of 11–15% and 30–40%, respectively. Therefore, studies with combination of ipilimumab and nivolumab were sought. Although response rates are far superior, frequency of serious adverse events has also risen as a result of dual checkpoint blockade therapy. Thus, therapies with good

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clinical activity at the same time less toxic are highly desired [3].

24.1.1 What Are Oncolytic Viruses?

Oncolytic viruses include both unmodified wild type and genetically engineered agents that selectively infect and replicate within tumor cells resulting in lyses of infected cells. In addition, the proinflammatory cytokines and tumor-derived antigens released as a result of tumor lysis trigger the immune system and activate antitumor immunity [4–6]. Oncolytic viruses are useful in cancer therapy because they not only have an innate tropism for cancer cells and result in tumor death but also the TAAs released serve as targets for mounting tumor-specific antitumor immunity [3].

24.1.2 History of Oncolytic Viruses

Even before oncolytic viruses were designed or used, some researchers made an observation as early in the mid-1950s that some viruses had the potency to infect and thereby result in peripheral leukemic blood cell death in vitro [3]. In addition, historically it was shown that viral syndromes due to chicken pox, measles, or hepatitis resulted in remission of advanced malignancies such as leukemia and lymphoma [7–9]. In 1949, 22 patients with Hodgkin's disease were treated with parenteral injection of human serum containing hepatitis B virus, causing infection in 13 of those treated. Seven of these had partial tumor response [10]. In 1952, 34 patients with advanced refractory tumors were treated with Egypt 101 virus, and evidence of oncotropism was noted with 4/34 showing temporary tumor regression [11]. In 1956, adenoidal-pharyngeal-conjunctival (APC) virus was used to treat 30 cervical cancer patients with tumor response seen in 26 of them [12]. Lindenmann and Klein did the sentinel studies on oncolytic viruses in 1967 and demonstrated that virotherapy using influenza virus enhances the immunogenicity of tumor cell antigens [2]. In the 1970s, Asada demonstrated antitumor response in more than 40% of patients with terminal cancers treated with nonattenuated mumps virus [7, 13]. Later in 1999, Martuza, Toda, and others showed that HSV G207, a genetically engineered oncolytic virus, induced antitumor immunity in CT26 colon cancer model [2]. Currently, multiple clinical trials with several different types of viruses are being conducted.

24.1.3 What Are the Characteristics of This Therapy?

Tumor selectivity and activation of antitumor immunity through release of TAAs in conjunction with other signals and cytokines via immunogenic cell death are characteristics of oncolytic viruses. The signals that work in conjunction with TAAs are damage-associated molecular pattern (DAMP) and oncolytic virus-derived pathogen-associated molecular pattern (PAMP) [2].

Since oncolytic viruses activate antitumor immune response, durable responses are possible with such therapies [4]. Given that oncolytic viruses target multiple oncogenic pathways resulting in cytotoxicity, generation of resistance is less likely [14]. Tumor selectivity results in minimal systemic toxicity unlike that seen with most immunotherapy such as checkpoint blockade inhibitors [14]. Most of these viruses are developed as intralesional therapies, although a few can be used systemically [15]. A particular challenge with oncolytic virotherapy is the inefficient delivery of these to tumor nodules and further spread to distant micrometastases, which limits its efficacy [2]. Anti-oncolytic virus antibodies that may be pre-existing as part of innate immunity in human serum or develop as a result of multiple administrations of this therapy can limit their use systemically. In addition, oncolytic viruses poorly extravasate in tumors and are sequestered by the liver [14].

Treatment efficacy of oncolytic viruses is monitored by factors such as viral shedding, intratumoral viral replication, viremia, viral genome, viral load, immune infiltrates, and circulating immune cells [16].

24.1.4 Mechanism of Action of Oncolytic Viruses

Tumor selectivity of oncolytic viruses is dependent of several factors. Firstly, there is receptor-mediated virus-specific cell entry naturally targeting tumor cells expressing receptors such as epidermal growth factor receptor (EGFR), folate receptor, Her2-neu, prostate-specific antigen, CD20, COX-2, and osteoclastin to name a few, or the viruses can be engineered to specifically target tumor cells with these receptors [7, 17, 18]. CD155 considered as a poliovirus receptor is present on cells of many tumor types and facilitated poliovirus entry [19]. Secondly, metabolically active cells such as tumor cells provide a good homing ground for oncolytic viruses to replicate. Tumor driver mutations can also specifically aid viral replication [16, 17, 20]. Adenovirus specifically replicates in the S phase. The wild-type virus encodes the E1A protein that facilitates S phase entry via retinoblastoma signaling. Retinoblastoma signaling mutations and enriched S phase populations are seen in many cancer types; thus, deletion of *E1A* gene in the oncolytic variant helps reduce its pathogenicity in normal cells [16]. Thirdly, a defective antiviral type I interferon signaling in tumor cells helps viral replication [16, 21]. Reovirus preferentially targets cells with activated Ras signaling [16, 22]. Some other altered signaling pathways that predispose cancer cells to the oncolytic effect of these viruses include RB/E2F/p16, p53, PKR, EGFR, Ras, Wnt, anti-apoptotic, hypoxia, defective IFN, and other innate immune signaling pathways [2]. This viral replication in the tumor microenvironment activates innate and tumor-specific immunity resulting in cytotoxicity of cancer and stromal cells [2] (Fig. 24.1). Other modes of cancer cell death are (1) direct oncolysis of cancer cells by the virus which could be a result of apoptosis, necrosis, pyroptosis, or autophagy mostly in combination and (2) the antiangiogenic properties of oncolytic viruses which can result in necrosis and apoptosis of uninfected cells [2].

On similar lines, the proposed resistance mechanisms to oncolytic virotherapy are the presence of neutralizing antibodies in the host and innate immune response against oncolytic viruses [16].

24.1.5 Types of Oncolytic Viruses

There are two types of oncolytic viruses: (1) viruses that preferentially replicate in cancer cells and leave nonpathogenic cells unaffected due to either dependence on oncogenic signaling pathways or heightened sensitivity to innate antiviral signaling and (2) viruses that have undergone genetic manipulation and/or been genetically engineered for insertion or deletion of genes necessary for replication in normal but not cancer cells. Autonomous parvovirus, myxoma virus (poxvirus), Newcastle disease virus (paramyxomavirus), reovirus, and Seneca Valley virus (picor-

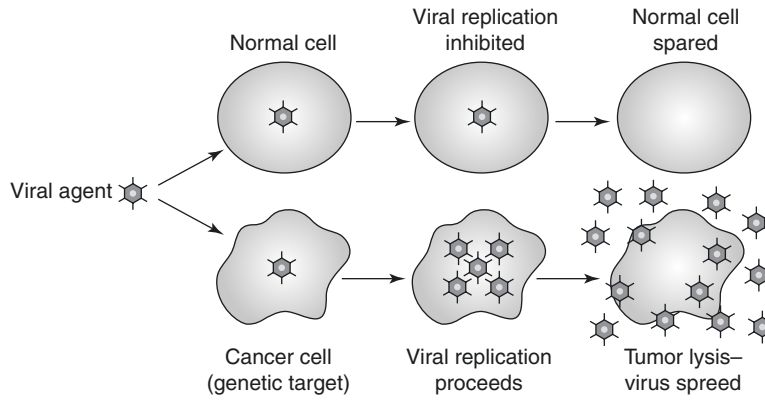

Fig. 24.1 Mechanism of action of oncolytic viruses: selective replication in cancer cells, tumor lysis, release of virus, and spread within cancer tissue (Reproduced from Kirn D et al. (2001) Nat Med 7: 781–787) [57]

navirus) are viruses that are specific to replication in cancer cells. On the other hand, viruses such as measles virus (paramyxomavirus), poliovirus (picornavirus), vaccinia virus (poxvirus), adenovirus, herpes simplex virus, and vesicular stomatitis virus are either genetically manipulated or engineered [14]. Several oncolytic viruses are currently being tested in clinical trials. The only oncolytic virotherapy to be USFDA approved is talimogene laherparepvec (T-VEC) derived from HSV-1 in the treatment of melanoma. Other therapies being developed are coxsackievirus (CVA21), adenovirus (K901, CG0070), vaccinia (JX-594, GM-CSF RV, Pexa-Vec), and reovirus (pelareorep, REOLYSIN®) [4, 15].

24.2 T-VEC

24.2.1 What Is T-VEC

Talimogene laherparepvec (T-VEC) previously also known as OncoVEX$^{GM\text{-}CSF}$ is a modified herpes simplex virus type 1-derived oncolytic immunotherapy that has been approved for use as intralesional therapy in melanoma [15, 23]. It is currently being developed for use in multiple solid tumors.

24.2.2 Structure and Proposed Mechanism of Action of T-VEC

This oncolytic virus is derived from HSV-1. Given that HSV-1 is well characterized and its biology is well known, it serves as a suitable vector. Its large non-integrating double stranded DNA genome can be easily manipulated to insert large genetic inserts. The structure of T-VEC (Fig. 24.2) shows deletion of two HSV genes: *ICP34.5* and *ICP47*. *ICP34.5* confers neurovirulence to HSV-1. Thus, deletion of *ICP34.5* provides selective cancer cell replication, and its replacement with two copies of the *GM-CSF* gene enhances local production of GM-CSF. GM-CSF is responsible for maturation and recruitment of dendritic cells and macrophages within the tumor, and these in turn allow for tumor antigen presentation leading to T-cell-mediated cytotoxic effect. Deletion of *ICP47* causes an early activation of the *US11* promoter which enhances viral replication and facilitates antigen presentation leading to better antitumor immune response [3, 15, 24].

Similar to other oncolytic viruses, T-VEC has been proposed to have both local and systemic effects (Fig. 24.3). Through mechanisms outlined above, T-VEC selectively replicates in tumor cells resulting in oncolysis. This in turn releases more progeny viruses and the cycle repeats itself. In conjunction with production of immunomodulatory cytokine GM-CSF, release of tumor-specific antigens induces recruitment and maturation of dendritic cells. Tumor cell antigen presentation to T cells occurs leading to activation and expansion of $CD8^+$ T cells eliciting a systemic immune response [15].

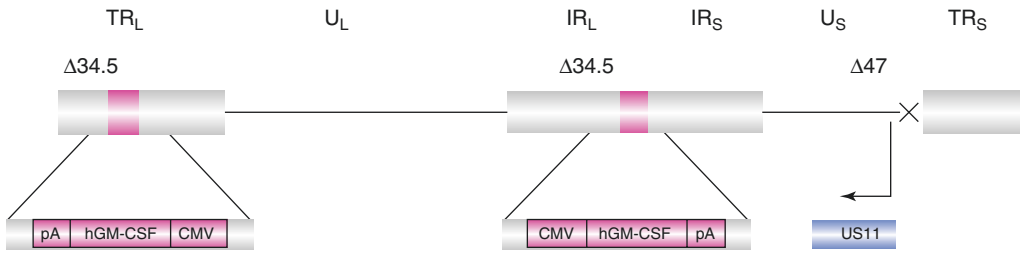

Fig. 24.2 Structure of T-VEC genome. Positions of deletions of ICP34.5 and ICP47 are marked as Δ34.5 and Δ47, respectively. The area marked in *pink* is the insertion site for the human *GM-CSF* gene (Reproduced from Hughes et al. (2014) Oncolytic Virotherapy 3: 11–20) [58]

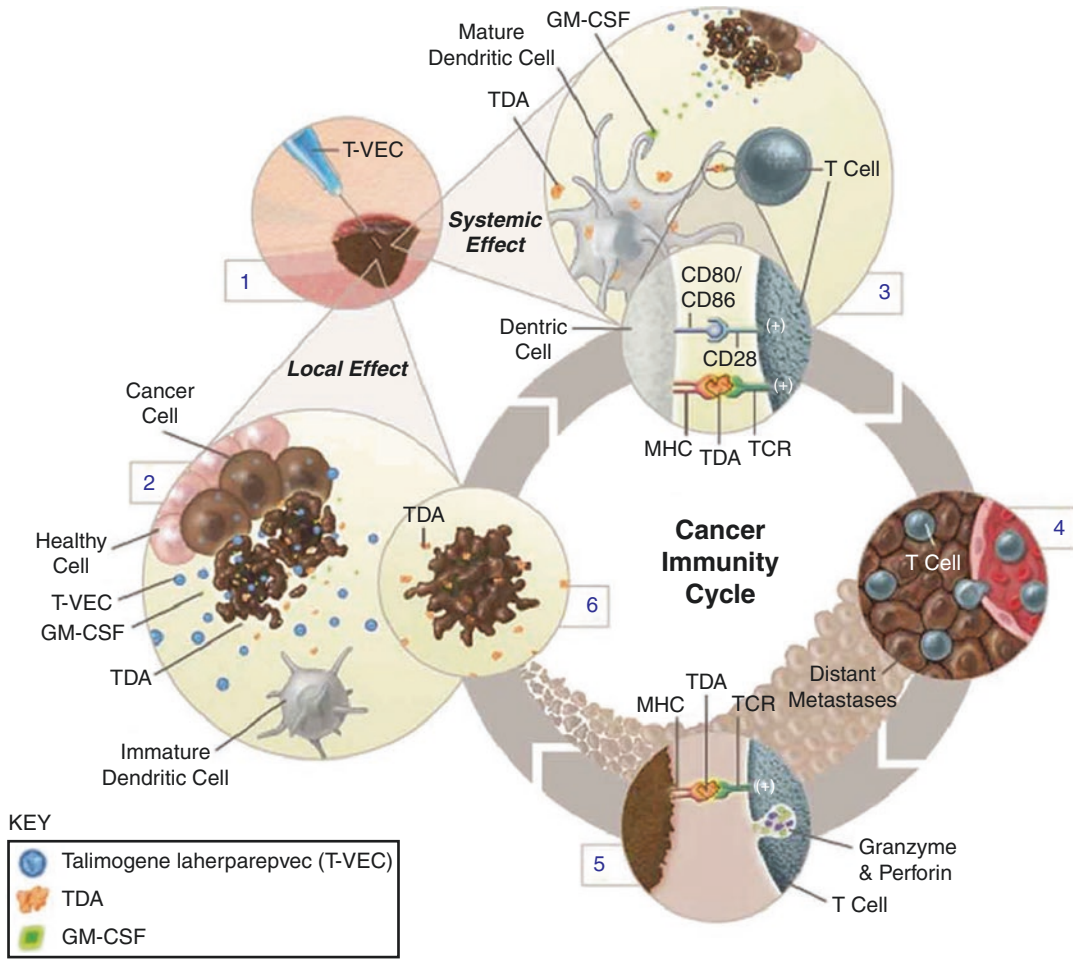

Fig. 24.3 Mechanism of action of T-VEC. After intratumoral administration of T-VEC (*1*), the virus replicates selectively in cancer cells (*2*). T-VEC also expresses GM-CSF, which promotes maturation and function of dendritic cells eliciting a systemic T-cell-mediated antitumor response (*3*). The virus can proliferate systemically to distant tumor sites (*4*). Tumor cell antigen presentation to T cells results in activation of innate immune system (*5*). Tumor cell lysis releases tumor-derived antigens (TDAs) (*6*) (Reproduced from Harrington et al. (2015) Expert Rev. Anticancer Ther 15: 1389–1403) [15]

24.2.3 Administration, Handling of T-VEC

A maximum injectable volume is determined based on the tumor dimension (Table). The volume of T-VEC administered varies by the lesion size: 0.1 ml for tumors up to 0.5 cm in longest dimension, 0.5 ml for tumors ranging from 0.5 to 1.5 cm, 1 ml for tumors ranging from 1.5 to 2.5 cm, 2 ml for tumors ranging from 2.5 to 5 cm, and 4 ml for tumors greater than 5 cm in longest dimension. The maximum volume per visit is 4 ml, and the volume to be administered is based on volume calculations for the tumor using its longest dimension. When multiple lesions are present, it is recommended that the largest lesions receive priority first followed by any lesions and then symptomatic lesions. Prior to injecting T-VEC, the area to be injected must be cleaned with alcohol swab and allowed to air-dry. If the lesion is not

superficially palpable, then ultrasound guidance should be used for deeper subcutaneous tumors. Usually a single insertion site for the needle with T-VEC is recommended, but multiple sites may be necessary for larger tumors. Premedications and local anesthetics are usually not necessary prior to administration unless there is prior history of pain at the injection site. After treatment, holding pressure for 30 s at the site of injection is recommended. This should be covered with gauze and occlusive dressing and should remain covered for 1 week after each treatment. All materials in contact with the treatment site should be disposed of using universal precautions.

T-VEC is handled as a biosafety level 1 agent, which implies that it does not consistently harm normal healthy humans. It is prepared in a sterile biosafety cabinet. It is available in two different types of vials: the yellow-green vial with a concentration of 10^6 PFU/ml and the blue vial with a concentration of 10^8 PFU/ml. These are stored at −70°C or colder and thawed at room temperature until it converts to liquid form for use. Once thawed, the vials can be refrigerated for 12 h (10^6 PFU/ml concentration) to 48 h (10^8 PFU/ml concentration). Once thawed, the vials cannot be refrozen. Protective personal equipment with universal precautions must be followed while handling T-VEC. In case of accidental exposure, area should be cleaned with water for 15 min. In the event of a spill, 10% bleach solution should be used with absorbent materials to clean the surface [25].

24.2.4 Preclinical Studies Using T-VEC

A murine A20 tumor model was studied. HSV-1 viral strains with and without GM-CSF gene expression were injected into single side tumors in a bilateral flank system. HSV-1 viral strains both with and without GM-CSF expression resulted in tumor regression; however, only GM-CSF expressing HSV-1 resulted in tumor regression on the contralateral side. Cytotoxic T cells primed to parental A20 cells were seen only with treatment with HSV-1 with GM-CSF expression suggesting long-term antitumor immune response generated by this strain. This laid the foundation of further genetic modification of HSV-1 and the generation of T-VEC [3, 26].

Therapies such as T-VEC are considered ideal for melanoma treatment because (1) the presence of in-transit metastases denotes spread of melanoma cells to dermal lymphatics at the same time making surgical resection difficult and (2) metastases from melanoma preferentially spread to the skin making it optimal for targeting through intralesional therapies [24]. Therefore, much of the advancements in T-VEC therapy have been in melanoma. In keeping in line of intralesional therapies, agents such as BCG, GM-CSF, IL-2, PV-10, and IFN-alpha have been used and demonstrated responses in injected and noninjected lesions but in less than half of patients treated. Cutaneous toxicity and lack of systemic benefit have limited their use for treatment [24].

Administration of T-VEC was shown to increase local and systemic MART-1-specific $CD8^+$ effector T cells, while at the same time there is decrease in both $CD4^+$ $Foxp3^+$ regulatory T cells and myeloid-derived suppressor cells (MDSC). Proportion of Tregs is lower in injected sites as compared to uninjected sites [3, 27].

24.2.5 Early Phase Clinical Trials

In a Phase I trial, T-VEC was evaluated for safety, optimal dose, and schedule in 30 patients including advanced breast cancer, head and neck cancer, colorectal cancer, and melanoma with cutaneous, subcutaneous, or superficial nodal sites of disease. Out of 19 evaluable histological specimens, inflammation and necrosis were noted in 73% of biopsies from the site of injection. The necrosis stained strongly for HSV protein. Tumor specificity of T-VEC was demonstrated by no evidence of necrosis in the non-tumor cells within the tumor microenvironment. The optimum dose and schedule derived from this study were initial dose of 10^6 PFU/ml followed by a dose of 10^8 PFU/ml after 3 weeks and repeated every 2 weeks until clinical response, toxicity, or disease progression. Clinical activity was similar between HSV-seronegative and HSV-seropositive patients [28].

After demonstration of good biologic activity of T-VEC in the Phase I study, a Phase II study was conducted in patients with stage III and IV melanoma. Twenty-six percent of patients achieved some form of response as assessed using RECIST criteria including eight patients achieving complete response. Durable responses were seen in 92% of patients ranging from 7 months to 31 months. The survival rate at 1 year was 58% in the intention to treat population, while it was 93% in those with initial objective response [3, 29].

In a Phase Ib study [1] (NCT01740297) conducted in the USA, 19 patients received T-VEC in combination with ipilimumab. T-VEC was administered at a dose of 10^6 PFU/ml on day 1 and second dose in 3 weeks at a dose of 10^8 PFU/ml repeated every 2 weeks. Ipilimumab was administered at a dose of 3 mg/kg every 3 weeks for four doses starting on day 1 of week 6. The regimen was overall well tolerated, except in five patients in which ≥Grade 3 adverse events (AE) were reported. Nausea was a common Grade 3 adverse event. Other AEs reported were fever, fatigue, flu-like symptoms, dehydration, diarrhea, and vomiting. Overall response rate assessed using the immune-related response criteria (irRC) [30] was 56%, with four (33%) complete responses and five partial responses. This overall response rate is comparable to 56% that was seen with ipilimumab and nivolumab, except that 55% of participants had Grade 3/4 AEs [31]. Durable response rate (responses seen for ≥6 months) was 44%. Twelve-month progression-free survival (PFS) was 50% and overall survival (OS) 72%. Activated $CD8^+$ T cells ($CD3^+$, $CD4^-$, $HLA\text{-}DR^+$) increased 1.5-fold from baseline after treatment and correlated with response.

On similar lines a Phase Ib/III study (NCT2263508) studying the combination of T-VEC with pembrolizumab in previously untreated, unresectable stage III/IV melanoma patients is being conducted. Based on results published so far from the Phase Ib portion of the study, the confirmed overall response rate as assessed using irRC was 57.1% with CR rate of 23.8%. Participants received T-VEC at a dose of 106 PFU/ml on day 1, 108 PFU/ml on day 22 and every 2 weeks thereafter, while pembrolizumab was administered at a dose of 200 mg IV on day 36 and every 2 weeks thereafter. Grade 3/4 AEs were noted in 33% of patients, and no Grade 5 AEs were noted [32].

In another Phase I trial studying the safety of T-VEC in advanced pancreatic cancer patients, it was demonstrated that T-VEC can be administered directly to visceral lesions via endoscopic, ultrasound-guided, fine needle injection (EUS-FNI) safely. The trial was designed such that patients would receive their first dose ranging between 10^4 PFU/ml and 10^6 PFU/ml followed by every 2 weeks of additional two doses of T-VEC ranging 10^7–10^8 PFU/ml all administered endoscopically. Four cohorts were planned; however, the study was stopped prior to enrollment of cohort 4 for reasons other than safety. Two of four in cohort 3 showed tumor reductions greater than 30%. Most common AEs observed were ascites, dehydration, anemia, abdominal pain, constipation, nausea, and vomiting. Although two Grade 5 AEs were noted, these were not attributed to the study drug. Interestingly, 59% of participants discontinued the study before receiving the planned treatment [33].

T-VEC has also been studied in combination with radiotherapy and cisplatin in a Phase I/II study in patients with untreated stage III/IV squamous cell cancer of the head and neck. Participants received chemoradiotherapy 70 Gy in 35 fractions with cisplatin dosed at 100 mg/m^2 on days 1, 22, and 43. T-VEC was administered as an initial dose of 10^6 PFU/ml followed by two additional doses of 10^6 PFU/ml, 10^7 PFU/ml, or 10^8 PFU/ml in cohorts 1, 2, and 3, respectively. The regimen was overall well tolerated with no reported DLTs. All patients underwent neck dissection after 6–10 weeks. Response rate as assessed by RECIST was 82% (four complete responses, ten partial responses), and pathological complete response was noted in 93% of patients at the time of neck dissection. At 29 months, the disease-specific survival was 82% and relapse-free survival of 76% [34].

Other selected ongoing trials are listed in Table 24.1.

Table 24.1 Summary of selected ongoing trials with oncolytic viruses[a]

Trial name/ID	Phase, sponsor	Tumor type	Study population	Treatment	Primary end points	Secondary end points	Estimated number of participants
Herpesvirus							
(A) T-VEC							
NCT02211131	Phase II, Amgen	Melanoma	Resectable stage IIIB/C and IVA melanoma	Immediate surgical resection versus six doses of neoadjuvant T-VEC followed by surgical resection	Efficacy	PCR, OS, RFS, rate of R0 resection, overall response, safety	150
NCT02453191	Phase Ib/II, Amgen	Sarcoma	Locally advanced soft tissue sarcoma	T-VEC with concurrent preoperative external beam radiotherapy	Phase I—safety and tolerability Phase II—efficacy as assessed by PCR rates	ORR, TTP, OS	32
NCT02509507	Phase I, Amgen	HCC/liver metastases	Progressive HCC or liver metastases for solid tumors	T-VEC injected into liver tumors	Determine MTD	Safety	100
MASTERKEY232/ KEYNOTE-137/ NCT026260000	Phase Ib/III, Amgen	Squamous cell carcinoma of head and neck	Recurrent metastatic squamous cell carcinoma of head and neck	T-VEC in combination with pembrolizumab	Safety	Incidence of AEs, objective response rate, best overall response, duration of response, disease control rate, PFS, OS	40
(B) HSV 1716 (SEPREHVIR)							
NCT00931931	Phase I, Nationwide Children's Hospital	Non-CNS solid tumors	Refractory localized or advanced non-CNS tumors from ages 7 to 30	Intratumoral for localized disease or intravenous for metastatic disease of HSV 1716	Safety	Measure antiviral immune response	30
(C) HF10							
NCT02272855	Phase II, Takara BioVex Inc.	Melanoma	Unresectable or metastatic melanoma	Intratumoral HF10 plus intravenous ipilimumab	Best overall response rate	Safety, tolerability, objective response rate, PFS, durable response rate, 1-year OS, tumor-infiltrating lymphocytes, peripheral blood cytokines	46

(D) G207							
NCT02457845	Phase I, UAB	Brain tumors	Children with progressive or recurrent supratentorial brain tumors	Catheter-infused G207 with or without single dose of radiation	Safety and tolerability	Immunologic response, viral shedding, PFS, OS, change in PS, QoL	18
Reovirus							
(A) REOLYSIN®							
NCT02620423	Phase I, Oncolytics Biotech	Pancreatic adenocarcinoma	Patients with advanced pancreatic adenocarcinoma who has progressed after (or did not tolerate) first-line treatment	REOLYSIN® and chemotherapy (gemcitabine *or* irinotecan *or* 5FU) in combination with pembrolizumab	Safety and DLTs	ORR, analysis of pre- and posttreatment biopsies and blood-based immune markers to determine effects of REOLYSIN® and pembrolizumab administered together	9
NCT01274624	Phase I, Oncolytics Biotech	Colorectal cancer	FOLFIRI naïve KRAS mutant metastatic colorectal cancer	Intravenous REOLYSIN® in combination with FOLFIRI and bevacizumab	DLTs, pharmacokinetic parameters for irinotecan and 5FU when combined with REOLYSIN®	CEA and objective response, clinical benefit rate, PFS, OS, safety, tolerability, correlative studies to identify biomarkers of response and efficacy	32
Adenovirus							
(A) DNX-2401							
CAPTIVE/ KEYNOTE-192/ NCT02798406	Phase II, DNAtrix, Inc.	CNS tumors	Recurrent glioblastoma or gliosarcoma	Intratumoral DNA-2401 followed by pembrolizumab	Objective response rate	OS, time to response, duration of response	48
(B) VCN-01							
NCT02045602	Phase I, VCN Biosciences, SL	Solid tumors	Advanced or metastatic solid tumors or pancreatic adenocarcinoma	Intravenous VCN-01 alone or in combination with nab-paclitaxel/ gemcitabine	Safety, tolerability, RP2D	Viral genome analysis in tumor, viral pharmacokinetics, viral shedding, neutralizing antibodies anti-VCN-01, ORR, PFS	36

(continued)

Table 24.1 (continued)

Trial name/ID	Phase, sponsor	Tumor type	Study population	Treatment	Primary end points	Secondary end points	Estimated number of participants
(C) ONCOS-102							
NCT02879669	Phase I/II, Targovax Oy	Mesothelioma	Unresectable malignant pleural mesothelioma	Intravenous ONCOS-102 plus cyclophosphamide in combination with pemetrexed/cisplatin versus pemetrexed/ cisplatin alone	Safety and tolerability	Determine and compare tumor-specific immunological activation. ORR, PFS, OS	30
NCT03003676	Phase I, Targovax Oy	Melanoma	Advanced or unresectable melanoma progressed after PD-1 blockade	Intravenous cyclophosphamide priming with intratumoral ONCOS-102 followed by pembrolizumab	Safety	Objective response rate, correlation of TILs and ORR, changes in immune subsets in tumor tissue and peripheral blood before and after ONCOS-102 and pembrolizumab, PFS, clinical benefit rate	12
Vaccinia virus							
(A) Pexa-VEC (JX-594)							
PHOCUS/ NCT02562755	Phase III, SillaJen, Inc.	HCC	Advanced HCC not received prior systemic therapy	Pexa-Vec followed by sorafenib versus sorafenib alone	OS	TTP, PFS, ORR, DCR, AEs, time to symptomatic progression	600
(B) PROSTVAC							
NCT02649855	Phase II, NCI	Prostate cancer	Metastatic castrate-sensitive prostate cancer	Standard ADT followed by simultaneous docetaxel + PROSTVAC or sequential docetaxel and PROSTVAC	Determine if immune response induced by PROSTVAC in combination with docetaxel is greater than docetaxel alone	Immune subsets of immunologic response, antigen-specific immune response, TTP, pharmacogenomics, OS	38

PCR pathological complete response, *ORR* overall response rate, *OS* overall survival, *RFS* recurrence-free survival, *PFS* progression-free survival, *TTP* time to progression, *DCR* disease control rate, *AE* adverse event, *NCI* National Cancer Institute, *HCC* hepatocellular carcinoma, *TILs* tumor-infiltrating lymphocytes, *RP2D* recommended Phase II dose, *PS* performance status, *QoL* quality of life, *DLT* dose-limiting toxicity, *5FU* 5-fluorouracil, *FOLFIRI* 5FU + irinotecan + leucovorin

[a]These trials are listed as currently "recruiting" as of February 26, 2017, on clinicaltrials.gov. Studies that are "active but not recruiting" or "not yet recruiting" or "completed" have not been listed here

24.2.6 Phase III Clinical Trials

In the randomized Phase III OPTiM trial [35] of 436 patients with unresectable stage IIIB–IV melanoma, responses were compared between intralesional T-VEC and GM-CSF administration. Durable response rate (DRR) defined as a response assessed by WHO criteria [36] and lasting for ≥6 months within the first 12 months of treatment initiation was the primary end point. DRR was significantly superior in the T-VEC arm compared to the GM-CSF arm (16.3% versus 2.1%; $p < 0.001$). Overall response rate (ORR) was also higher in the T-VEC arm compared to the GM-CSF arm (26.4% versus 5.7%), with 10.8% CR in the T-VEC arm. There was a trend for better overall survival (OS) in the T-VEC arm with median OS being 23.3 months as compared to 18.9 months in the GM-CSF arm. Exploratory analyses showed that effect of T-VEC was more pronounced on DRR and OS in patients with early-stage disease (stage IIIB, IIIC, or IVM1a) and those that were treatment naïve (Fig. 24.4).

In a separate study [37] reporting the patterns of response with T-VEC administration in the OPTiM study, Andtbacka et al. noted fair response rates even in the uninjected non-visceral and visceral lesions. ORRs were 32%, 18%, and 14% in injected lesions, uninjected non-visceral lesions, and visceral lesions, respectively, with corresponding CR rates being 15%, 6%, and 3%. Injected lesions took shorter time to response about 9.3 weeks, while uninjected sites took 12.3–12.9 weeks to respond. Sixteen percent of patients treated with T-VEC had durable response. Progression prior to response (PPR) was not a predictor of duration of DR or OS. However, almost 50% of T-VEC durable responders were shown to have PPR mostly due to development of new lesions.

24.2.7 Adverse Events

Only mild adverse events are usually encountered with T-VEC administration. In the Phase I trial by Hu et al., major side effects noted were Grade 1/2 pyrexia, which was more pronounced in the HSV-seronegative patients. Some other common side effects were fatigue, nausea, vomiting, and anorexia [3, 28]. In the Phase II study, 85% of patients with advanced melanoma experienced adverse events that were most commonly limited to Grade 1/2 flu-like symptoms. Three patients with response developed vitiligo, a known occurrence with other immune therapies such as IL-2 [3, 24, 29]. In the Phase III OPTiM study as well, T-VEC was overall well tolerated in the study population with frequent adverse events (AEs) reported as fatigue, chills, and fever. Grade 3/4 AEs were rare with the only AE reported in >2% of subjects in the T-VEC arm being cellulitis [35]. Acetaminophen or indomethacin can be used to prevent or treat pain, fever, or chills resulting after T-VEC treatment. Meperidine can be used to control rigors. Patients with adrenal insufficiency or those with hypophysitis on prednisone 10 mg daily or less can

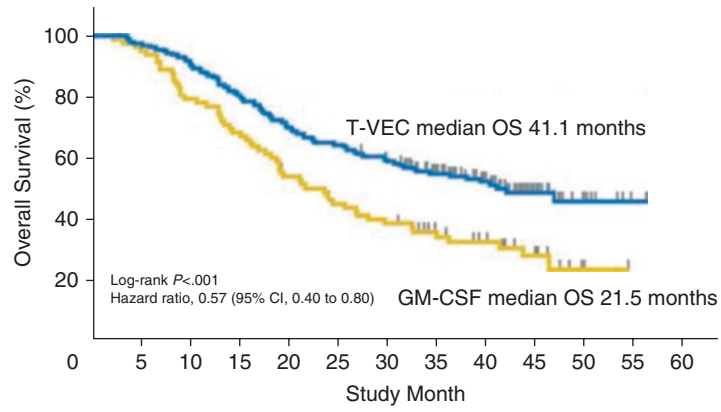

Fig. 24.4 Kaplan–Meier curve depicting overall survival in patients with stage IIIB, IIIC, and IVA disease in the Phase III OPTiM trial (Reproduced from Andtbacka et al. (2015) J Clin Oncol 33: 2780–2788) [35]

receive T-VEC. Dose of corticosteroids can be increased if adverse events such as fever or flu-like symptoms develop after the injection. Cellulitis is a known adverse event with T-VEC administration. Herpes cellulitis is usually self-limiting and clears within a maximum of 48 h. If there is fever and leukocytosis, superimposed bacterial infection must be considered. In case encephalitis or viremia is suspected, PCR testing for HSV DNA from blood and CSF is reliable. Acyclovir or similar antivirals can be considered in case of accidental exposure to T-VEC [3]. Although a theoretical concern remains that the virus may mutate and regain pathogenicity, this has not been clinically observed.

Based on some studies, T-VEC should not be offered as therapy to individuals with active herpetic infection or those taking daily antivirals such as acyclovir. Given that T-VEC is a modified live, attenuated virus, its use is not recommended in individuals with severely compromised immune status such as those with human immunodeficiency virus infection, leukemia, or lymphoma or on high-dose immunosuppressive therapy. There is not enough evidence to define the safety of T-VEC in pregnant women or children, and therefore use in this population is not recommended [3, 25].

24.2.8 Approved Use

T-VEC is the first approved oncolytic virus by the FDA in the USA for melanoma patients with cutaneous, subcutaneous, and nodal lesions that were recurrent after initial surgery. This approval was announced in October 2015. Shortly thereafter in December 2015, the European Medical Agency (EMA) approved its use in Europe. T-VEC received Australia's Therapeutic Goods Administration (TGA) regulatory approval for use in melanoma in May 2016 [3]. Approval in Europe and Australia has been limited to melanoma patients with unresectable stage III or stage IVa disease based on superior response rates in this subset of population as seen in the OPTiM study [35].

BioVex, Inc. initially developed T-VEC under the name OncoVEX$^{GM\text{-}CSF}$. After its acquisition by Amgen in 2011, it is now manufactured and marketed as Imlygic. Two major limitations of its use are (1) failure to improve overall survival and (2) no effect on visceral metastases. At this time, there is no defined biomarker that will help clinicians select patients suitable for T-VEC therapy. Some suggested criteria for patient selection for T-VEC therapy are patients with accessible disease, those with low burden visceral disease that can initially receive T-VEC therapy for local control of disease followed by systemic therapy such as checkpoint blockade agents, elderly or those with significant autoimmune conditions or other comorbid illnesses not considered suitable for other forms of therapy, and those that have progressed through other lines of therapy.

24.2.9 Future of T-VEC

While T-VEC shows significant effect on local disease control, systemic effects are weak which provides the rationale for combination therapy. Similar to other immunotherapies, differentiating true progression from pseudoprogression is challenging using conventional methods of assessing tumor response such as RECIST and WHO. It has now been widely discussed in the immuno-oncology community that immune-based response criteria be specifically used when assessing response with immunotherapies [37].

Further studies are necessary to determine the appropriate combination of T-VEC with other immunotherapy agents. Also, from ongoing studies we will have more information in the future about sequencing of therapy involving T-VEC. Most of the success with T-VEC has been intralesional. Further studies using alternative methods of administration are necessary to explore its therapeutic benefit for non-cutaneous malignancies.

24.3 Other Oncolytic Viruses

24.3.1 Reovirus

Pelareorep (REOLYSIN®) is a live type 3 Dearing (T3D) strain of reovirus that is capable of repli-

cating in tumor cells with activated Ras pathway. Protein kinase R (PKR) is a serine/threonine kinase that is involved in defense against viral infection and is not activated/phosphorylated in Ras transformed cells. Since many tumors harbor an activated Ras pathway with inactivated or non-phosphorylated status of PKR, it has been proposed that reovirus oncolytic therapy can be used for treatment in solid tumors [38].

In a Phase I study (REO 003) of intratumoral injection of reovirus in patients with recurrent malignant gliomas, no dose-limiting toxicities were noted. Of the 11 patients with evaluable disease, 10 had disease progression, and 1 had stable disease. Median survival was 21 weeks with median time to progression 4.3 weeks [39]. In a Phase IIb trial (GOG 186H) comparing REOLYSIN® plus paclitaxel to paclitaxel alone in women with recurrent or persistent ovarian, tubal, or peritoneal cancer, overall survival did not differ between the two arms with comparable toxicity profile in both arms [40]. In a randomized Phase II trial of NSCLC (both squamous and adenocarcinoma histologies) treated with docetaxel or pemetrexed with or without REOLYSIN®, PFS was significantly better in the females with adenocarcinoma treated with REOLYSIN® plus pemetrexed as compared to pemetrexed alone [41].

REOLYSIN® has received USFDA's orphan drug designation for malignant gliomas, pancreatic cancer, peritoneal cancer, ovarian cancer, and fallopian tube cancer.

24.3.2 Coxsackievirus

Coxsackie A21 also known as CAVATAK is a naturally occurring picornavirus that has shown to result in mild respiratory illness in humans. It has been shown to preferentially infect ICAM-1-expressing cells commonly seen in melanoma in addition to other tumor types such as prostate cancer, breast cancer, and multiple myeloma. It is a genetically unmodified virus, and preliminary results from the Phase II CALM study in unresectable stage III/IV melanoma showed promising response rates [42]. The best overall response rate as assessed by irRC was 28% (eight complete responses, eight partial responses). The irPFS rate at 6 months was 39% and durable response rate was 21%. Twelve-month OS rate was 73%. Activity was noted both in injected sites and noninjected sites including lymph nodes, lungs, and other distant sites [43]. Another Phase I trial of CVA21 in combination with pembrolizumab is planned in advanced non-small cell lung cancer.

Another coxsackievirus that is still being tested preclinically has shown promise for treatment of advanced non-small cell lung cancer (NSCLC). Coxsackievirus B3 (CVB3) has shown selective tropism for NSCLC cell lines and shown to be potent and efficacious. This correlates with the expression of coxsackie and adenovirus receptors (CARs) in NSCLC. Antitumor effect has been seen in A549 adenocarcinoma xenografts that are radioresistant as well as resistant to EGFR blockade with gefitinib [7, 44].

24.3.3 Adenovirus

ONYX-015 is engineered by deleting the *E1B* gene in adenovirus to selectively replicate and destroy tumor cells that are p53 deficient. This has shown efficacy in the treatment of relapsed/refractory squamous cell carcinoma of the head and neck, alone or in combination with chemotherapy. It is approved for treatment of head and neck cancers in China under the name H101 [16, 45, 46].

Delta-24-RGD (DNX-2401) is an adenovirus engineered to selectively target integrin on tumor cells. Preclinically, it has shown great promise in glioblastoma models. Currently, there is a clinical trial underway testing the combination of DNX-2401 and pembrolizumab in recurrent glioblastoma [16, 47]. A chimeric adenovirus with expression of human GM-CSF (Ad5/3-D24-GMCSF) has been tested in a Phase I trial in 21 patients with advanced solid tumors. Sixty-seven percent of patients experienced objective clinical benefit as assessed using RECIST criteria. Overall, the drug was well tolerated with mostly Grade 1/2 AEs [48]. Currently a Phase I/II study

of Ad5/3-D24-GMCSF with low-dose metronomic cyclophosphamide is ongoing. Another Phase I trial in early-stage NSCLC with recombinant DNA and adenovirus expressing L523S protein is ongoing [7].

24.3.4 Vaccinia Virus

Pexastimogene devacirepvec (Pexa-Vec or JX-594) is a genetically engineered Wyeth strain of vaccinia virus that expresses GM-CSF, but also has deletion of thymidine kinase gene, which aids the selective replication of this virus in cells with high levels of thymidine kinase as that seen in tumor cells with altered *ras* or *p53* genes. In a Phase II study of advanced HCC with Pexa-Vec followed by sorafenib, a disease control rate of 62% was achieved with Pexa-Vec alone and 59% after initiation of sorafenib. Transient Grade 3/4 lymphopenia was observed. Otherwise, the regimen was well tolerated with flu-like symptoms, nausea, and abdominal pain [49].

In another Phase Ib study in treatment-refractory colorectal cancer, intravenous administration of Pexa-Vec achieves stable disease rate of 67%, and most common AEs were Grade 1/2 pyrexia and chills [50].

PROSTVAC® is a prime-boost regimen for subcutaneous use that has been studied in minimally symptomatic castrate-resistant prostate cancer patients. A vaccinia virus serves as the primary immunotherapy agent followed by a series of six boosters of fowl pox virus, both engineered to express the prostate-specific antigen (PSA) and three costimulatory molecules: ICAM-1/CD54, LFA-3/CD58, and B7.1/CD80 (termed as TRIad of COstimulatory Molecules or TRICOM). In a Phase II study, participants were randomly assigned to receive PROSTVAC plus GM-CSF or control with empty vectors and saline injections. Although the two arms did not differ significantly for PFS, at 3 years, the study drug arm had 8.5 months of longer survival than the control arm with a hazard ratio of 0.56 ($p = 0.0061$) [51]. This has led to the conduct of the Phase III PROSPECT trial, which has completed enrollment and results are expected soon.

24.3.5 Measles Virus

MV-NIS is an engineered form of measles virus of Edmonston lineage expressing the human thyroidal NIS (sodium iron symporter). The NIS is a membrane ion channel expressed in thyroidal tissue that aids in iodine trapping a feature that has been advantageous for thyroid scanning (^{123}I or Technitium 99^{m}) or ablation (with ^{131}I). This induces selective cancer cell death through syncytia formation [7]. In a Phase I study of recurrent, progressive epithelial ovarian cancer or primary peritoneal carcinoma, 16 patients treated with MV-NIS demonstrated a median OS of 26.5 months with most common AEs being Grade 1/2 abdominal pain, fever, fatigue, and neutropenia [52].

24.3.6 Poliovirus

PVSRIPO is a genetically engineered poliovirus Sabin type 1 in which the internal ribosomal entry site (IRES) is replaced by the IRES from human rhinovirus type 2. This modification abolishes the neurovirulence of poliovirus and selectively replicates in cells expressing CD155/nectin-like molecule 5 (Necl5), which is seen across solid cancers. Preliminary results from a Phase I study of a single intratumoral injection of PVSRIPO in recurrent glioblastoma demonstrated a 1-year OS of 56% with most common Grade 3 AEs being hyperglycemia, lymphopenia, and hemiparesis [53]. In 2016, USFDA granted PVSRIPO breakthrough designation status for potential treatment of glioblastoma to promote accelerated development of this drug.

24.3.7 Retrovirus

Vocimagene amiretrorepvec or Toca 511 is a non-lytic retrovirus based on murine leukemia virus engineered to encode the cytosine deaminase transgene. Given along with a novel oral extended-release prodrug 5-fluorocytosine (Toca FC), it catalytically converts this prodrug to 5-fluorouracil. Seventeen patients with recurrent

high-grade gliomas treated with Toca 511 and Toca FC showed good tolerability with only dose-limiting toxicity of Grade 3 being vasogenic edema in one participant. Viral DNA was observed in 76% of resected tumors after treatment. The regimen also had a favorable OS of 13.6 months compared to historical control of 7.1 months with external lomustine [54]. In 2015, Toca 511 and Toca FC received orphan drug designation for glioblastoma by USFDA. Now in February 2017, USFDA provided break through designation to these drugs for the glioblastoma. A Phase II/III trial (Toca 5) comparing Toca 511 and Toca FC with standard of care in recurrent gliomas undergoing surgery has completed enrollment, and results are expected in the first half of 2018.

24.3.8 Parvovirus

Parvovirus H-1 (H-1PV) is a rodent single-stranded DNA virus that is nonpathogenic in humans and known to activate caspin-mediated death pathway in cancer cells [55]. In a single-center Phase I/IIa trial with intratumoral or intravenous H-1PV in patients with progressive primary or recurrent glioblastoma, no dose-limiting toxicities were noted. PFS 6 months or greater was noted in 33% of the participants, while 80% had an OS of 6 months or greater [56]. This trial has completed enrollment and final results are awaited. In the meantime, a Phase I/II trial is currently open with H-1PV for patients with inoperable metastatic pancreatic cancer.

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Part IV

Developing Fields

Innate Immune Receptors in the Regulation of Tumor Immunity

25

Sho Hangai, Yoshitaka Kimura, Tadatsugu Taniguchi, and Hideyuki Yanai

Contents

25.1 Introduction

Cancer immunosurveillance, or the process by which the immune system searches and recognizes cancerous and cancer cells in the body, has a history going back to the early twentieth century when Paul Ehrlich predicted that the immune system represses the growth of carcinomas. The concept of cancer immunosurveillance has since been elaborated and contentiously debated by immunologists over many decades. After much aspiration and frustration, experimental evidence now unequivocally shows that such immune responses are indeed critical for the host defense against cancers [1–3]. Related to this are the interesting observations that the innate and adaptive immune systems contribute to the antitumor effects of conventional chemotherapy- and radiotherapy-based cancer treatments with such effects thought to be mediated at least in part through activation of innate immunity by the exposure of tumor-derived antigens during these treatments [4]. Thus, these classical cancer therapies are intimately tied to anticancer immune responses of the host.

The development of new and effective methods that harness the power of the immune system to treat cancers has become a very attractive and intense field of research. Recent years have seen remarkable progress in cancer immunotherapies including tumor-targeting monoclonal antibody, immune cell checkpoint therapy, tumor vaccine, and chimeric antigen receptor (CAR) T cell

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therapies [5–7]. While these therapies have brought about remarkable and in some cases durable response rates across many types of malignancies, the numbers of patients that benefit are still limited. As such, new therapies and combinations of existing ones that can potentiate the host's antitumor immune responses with minimal or manageable adverse effects remain the focus in the field [8, 9].

The innate immune system employs many of the same mechanisms for the host defense against invading pathogens as it does in the eradication of cancer or cancerous cells [10, 11]. Perhaps the most well-studied cell type in these processes is the natural killer (NK) cell [12, 13]. In the context of anticancer innate responses, NK cells can directly kill virus-infected and cancerous cells. Indeed, animal models with impaired or deficient NK cells display increased incidences of various types of cancer [14]. Additional innate cell types including monocytic- and granulocytic-derived cells are known also to regulate, either positively or negatively, the antitumor adaptive immunity within and without the tumor microenvironment (TME) [15, 16]. In parallel to cellular immunity, the importance that the signal-transducing innate immune receptors play to the regulation of adaptive immune responses [17] has increased the attention on these receptors for their role in the regulation of oncogenesis [11]. Relative to the T cell receptor and B cell receptor, PRRs are a limited number of germline-encoded receptors that recognize pathogen-associated molecular patterns (PAMPs) or endogenous stress signals termed damage-associated molecular patterns (DAMPs) [11, 18]. As such, PRRs are not as discriminative as those for T cells and B cells [18–20].

Pattern recognition receptors (PRRs) have been subdivided into (1) membrane-associated PRRs, which include toll-like receptors (TLRs) and C-type lectin receptors (CLRs); (2) cytoplasmic PRRs, which include the RNA-sensing retinoic acid-inducible gene-I (RIG-I)-like receptor (RLR) family and DNA-sensing receptors; and (3) soluble PRRs, which include complement receptors, collectins, pentraxin proteins, and others [18–21]. The first two PRRs classes, but not the soluble PRRs, can transmit signals to the nucleus upon engagement with their cognate ligand(s) to alter gene expression profile of the cell. Most typically, this involves the induction of type I interferon (IFN) genes and inflammatory cytokine genes for mediating antimicrobial responses [18, 20, 22]. The innate signaling also directly drives dendritic cell (DC) maturation, antigen presentation, as well as cytotoxic $CD8^+$ T cell functions [18–20].

There is accumulating evidence that antitumor immunity is regulated by the activation of PRRs [23]. Stimulation of PRRs by exogenously administered ligands induces robust antitumor immune responses in animal models [24–26], while excessive tumor growth has been observed in some PRR-deficient mice models [27, 28]. On the other hand, that the activation of PRRs evokes inflammatory responses raises the question of whether PRRs may actually hinder cancer immunity as the progression of some types of tumors is often associated with inflammation [29, 30]. The chronic activation of innate PRRs by endogenous ligands released from tumor cells may promote tumor progression through pro-inflammatory responses, which augments the proliferative, anti-apoptotic, and pro-fibrogenic signals within the TME [23, 29]. These seemingly contradictory reports indicate that innate immune activation by PRRs confers a dual role, i.e., an immune-enhancing role that potentiates antitumor immune responses and a tumor-promoting role through the induction of chronic inflammation.

In this review, we summarize our current understanding of innate PRRs which play a role in tumor immunosurveillance and regulation of oncogenesis. We also discuss the potential therapeutic implications of targeting tumors using modulators of the signal-transducing innate PRRs. For those interested in the broader biology of innate immune cells and PRRs, we recommend excellent reviews written by others [4, 11, 31, 32].

25.1.1 Role of TLRs in the Regulation of Tumor Development

The TLR family of receptors are by far the best understood classes of innate signaling PRRs

which function as sentinels of pathogen infection [19, 20, 33]. TLRs can recognize microbial PAMPs and signal through recruitment of cognate adaptor proteins [34]. In general, TLRs commonly utilize for their signaling pathway the adaptor called myeloid differentiation primary response gene 88 (MyD88); however, TLR3 instead utilizes the TIR domain-containing adaptor-inducing IFN-β (TRIF, also called TICAM) signaling pathway, although there is evidence that it also utilizes the MyD88 pathway [34, 35]. TLR4 requires both MyD88 and TRIF pathway for full-blown activation of the downstream signaling cascades [34]. Upon binding to TLRs, the adaptor proteins engage additional downstream proteins that mediate the activation of transcription factors and protein kinases, such as nuclear factor-kappa B (NF-κB), IFN regulatory factors (IRFs), and mitogen-activated protein kinase (MAPK) to modify transcription of target genes such as those for type I IFNs and inflammatory cytokines [20, 34].

That TLRs are highly expressed in antigen-presenting cells and the activation of some TLRs induces antitumor mediators such as type I IFNs led to efforts to harness TLR agonists for cancer therapies. In fact, TLRs are prominent therapeutic targets for the activation of anticancer immune responses, originally highlighted by Coley toxin and bacillus Calmette-Guérin (BCG) in association with anticancer responses [36]. On the other hand, TLR signaling also induces inflammatory responses, thereby potentially favoring tumor growth as well [37]. An emerging notion is that, in addition to PAMPs, TLRs also recognize a wide range of self-derived molecules called as DAMPs released upon cellular stress that can alter TME [37, 38]. Thus, as described below, TLRs apparently show both pro-tumor and antitumor functions [37, 39]. This may be attributed to different tumor-inducing protocols or mice facilities, which can affect developmental process of tumor and commensal microbiota.

TLR1/2/6 These TLRs are expressed on the cell surface [34]. TLR2 forms heterodimers with TLR1 or TLR6 and recognizes a variety of PAMPs including lipopeptides, peptidoglycan, lipoteichoic acid, lipoarabinomannan, zymosan, glycosylphosphatidylinositol-anchored mucin-like glycoproteins from *Trypanosoma cruzi*, and hemagglutinin protein from bacteria or yeast [34]. TLR2 has also been shown to recognize endogenous ligands such as biglycan, hyaluronic acid, versican, and surfactant protein A [34]. The role of TLR2 in the regulation of oncogenesis has also been studied in animal models, with evidence that TLR2 signaling may play antitumor or pro-tumor function depending on model studied.

TLR2-deficient mice show exacerbated tumor growth in a model of hepatocellular carcinoma (HCC) in which a single injection of N-nitrosodiethylamine (DEN) is followed by repeated administration of carbon tetrachloride (CCl_4) (hereafter called DEN/CCl_4 model) [40]. It is suggested that an attenuated antitumor immune response including impaired IFN-γ expression, loss of cellular senescence, and autophagy are responsible for the expansion of tumor growth [40]. Similarly, a different study shows that TLR2-deficient mice develop more and larger intestinal tumors in the DEN/CCl_4 model [41]. Tumor development is characterized by an increase in levels of IL-6, IL-17A, and signal transducer and activator of transcription 3 (STAT3) phosphorylation in the intestinal TME [41].

On the other hand, in a mouse gastric cancer model in which cancer cells display hyperactivation of STAT3, TLR2 deficiency results in reduced tumor burden, indicating a pro-tumor role of TLR2 [42]. This effect is independent of inflammation and is characterized by impaired proliferation and increased apoptosis of the gastric epithelial cells of the host [42]. These phenomena are explained by the suppression of phosphatidylinositol-3 kinase (PI3K), serine-threonine kinase 1 (AKT), extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK), and NF-κB pathways in these cells due to the TLR2 deficiency [42]. In addition, TLR2 signaling may also play a role in maintenance of "stemness" in normal stem cells as well as gastric tumor cells [42]. TLR2-deficient mice also show slower metastatic growth of Lewis lung carcinoma (LLC) cells as

compared to wild-type (WT) mice in a model of lung cancer [43]. Enhanced tumor growth in WT mice may be due to the activation of TLR2:TLR6 complex by LLC-derived versican, resulting in secretion of tumor necrosis factor-α (TNF-α) by myeloid cells which is known to promote tumor growth [43].

TLR3 TLR3 is localized within endosomes where it recognizes endocytosed double-stranded RNA (dsRNA), typically derived from viruses [34] as well as self-derived messenger RNA released from dead cells [34]. There is accumulating evidence to suggest that TLR3 functions to promote antitumor immunity. In an implanted transgenic adenocarcinoma model of prostate cancer, TLR3-deficient mice exhibit increased tumor growth as compared to controls [44]. In this model, TLR3-type I IFN signaling pathway enhances the activation of NK cells for antitumor responses [44]. Consistent with this, several studies have examined potential therapeutic effects of polyinosinic-polycytidylic acid (poly I:C), a synthetic TLR3 ligand, in the treatment of cancers. The proposed mechanisms of the poly I:C-TLR3 axis are (a) induction of IRF3-dependent NK-activating molecule (INAM) on DCs [45], (b) skewing of tumor-infiltrating macrophages toward a M1 phenotype [46], and (c) activation of DCs including the production of type I IFNs for enhancing effective cytotoxic T cell immune responses [47].

On the other hand, TLR3 has also been implicated in enhancing the growth of tumors in a spontaneous lung metastasis model in which lung metastasis is suppressed in TLR3-deficient mice [48]. It is further suggested that an RNA(s) derived from tumor exosomes activates TLR3 expressed in lung epithelial cells, leading to neutrophil recruitment and development of pre-metastatic niche, which results in the promotion of tumor progression [48].

As mentioned above, the TLR3 agonist poly I:C has been considered a promising adjuvant for cancer immunotherapy for several decades. Although effective, this therapy has been shown to cause life-threatening side effects, such as cytokinemia [36, 49, 50]. In this context, a recent study reported a new type of synthetic RNA that was designed to selectively activate the TRIF pathway, thereby effectively activating NK cells and cytotoxic T cells in tumor-loaded mice without inducing a severe cytokine storm induced by other types of dsRNA [51].

TLR4 TLR4 is expressed on the cell surface and recognizes lipopolysaccharide (LPS), a component of gram-negative bacteria [34]. It has also been reported that TLR4 recognizes various endogenous ligands, such as high-mobility group box protein 1 (HMGB1), heat shock proteins (HSPs), biglycan, hyaluronic acid fragments, and oxidized low-density lipoprotein [34].

In a mouse colon cancer model in which a single dose of azoxymethane (AOM) is combined with exposure to dextran sodium sulfate (DSS) (termed AOM/DSS model hereafter), TLR4-deficient mice show decreased tumor burden, suggesting its pro-tumor role [52, 53]. Mechanistically, it is proposed that TLR4 signaling (presumably activated by commensal bacteria) in colonic epithelial cells induces (a) immunosuppressive cyclooxygenase-2 (COX-2), (b) amphiregulin that activates epidermal growth factor receptor (EGFR) signaling [52], and (c) TME formation by the recruitment of COX-2-expressing macrophages [53]. In accordance with above results, mice carrying a transgene for a constitutively active TLR4 protein under the villin promoter in intestinal epithelial cells are more susceptible to tumor development in the same mouse model [54]. In another colon cancer model, however, TLR4 exhibits an antitumor effect. Mice harboring constitutively active TLR4 in the intestinal epithelial cells show decreased tumor burden in the $APC^{min/+}$ mouse model of spontaneous intestinal tumorigenesis (APC^{min} model) [55]. Tumor cells isolated from the intestine of these mice show elevated expression of IFN-β and caspase-3 activation, which correlate with increased apoptosis in vivo [55].

In a HCC model, i.e., the DEN/CCl_4 model, diminished tumor development is observed in TLR4-deficient mice as compared to WT mice, implicating the pro-tumor function of TLR4 signaling [56]. Microbiota of the intestine and TLR4

on liver-resident cells are thought to mediate increased proliferation, preventing apoptosis in tumor cells and expression of epiregulin, a hepato-mitogen [56]. Similarly, a reduction in the development of HCC is observed in TLR4-deficient mice in DEN-induced HCC model [57]. One study, however, shows that TLR4-deficient mice exhibit higher tumor burden in DEN-induced HCC model [58]. This exacerbation of carcinogenesis is explained by impaired DNA repair and subsequent oxidative stress [58].

In a skin cancer model, which is induced by the combination of 7,12-dimethylbenz[a]anthracene (DMBA) and croton oil, TLR4-deficient mice also show resistance to carcinogenesis, indicating a tumor-promoting role of TLR4 signaling [59]. Mechanistically, it is argued that HMGB1, released from dying keratinocytes, activates TLR4 and enhances inflammation, thereby promoting tumor development [59]. In addition, in a genetically engineered mouse model of melanoma, i.e., the HGF-CDK4(R24C) mice, which harbor deregulated receptor tyrosine kinase signaling of hepatocyte growth factor (HGF) and impaired cell cycle control by an oncogenic CDK4(R24C) mutation, TLR4 deficiency abrogates the UV-induced enhancement of lung metastasis [60]. In this experimental setting, TLR4 is activated by extracellular HMGB1 released from UV-damaged keratinocytes causing the recruitment of neutrophils that induce angiogenesis and migration of melanoma cells toward endothelial cells [60]. On the other hand, another study indicates that TLR4-deficient mice exhibit enhanced tumor burden in the DMBA skin cancer model, and this is accompanied by elevated levels of serum IL-17 and decreased level of IFN-γ, suggesting impaired Th1-mediated antitumor responses [61].

Reports describing roles of TLR4 in other organs are rather limited. In the pancreas, mice lacking TLR4 in hematopoietic cell compartment show reduced lesions of pancreatic intraepithelial neoplasia in the $p48^{Cre}$;$Kras^{G12D}$ pancreatic cancer model [62]. Contrary to this, TLR4 deficiency results in exacerbated tumor in the lung after injection of 3-methylcholanthrene and butylated hydroxytoluene (BHT) [63]. TLR4 has also been shown to be protective for both DMBA-induced mammary cancer model [64] and 4 T1-inoculated metastasis model [65]. The pro-tumor or antitumor outcome of TLR4 signaling may depend on the activation status of the MyD88 and TRIF signaling pathways which have the tendency to play pro-tumor and antitumor growth roles, respectively [50, 66].

TLR5 TLR5 is expressed on the cell surface [34] where it recognizes flagellin, a component of bacterial flagella [34]. One study reported that the ectopic expression of flagellin by tumor cells induced detectable antitumor immune response against EL4 murine lymphoma, thus utilizing flagellin as a tumor vaccine [67]. This effect is mediated by TLR5 signaling and inflammasome induced by the activation of nucleotide-binding oligomerization domain (NOD) containing-like receptor (NLR) [67]. However, a recent report demonstrated that TLR5 could enhance tumor growth. Here, TLR5-deficient mice show abrogated tumor growth in a genetically engineered mouse sarcoma model [68]. TLR5 deficiency is associated with decreased expression of IL-6, which leads to reduced recruitment of myeloid-derived suppressor cells (MDSCs), which cause γδ-T cells to release galectin-1, which suppresses antitumor adaptive immune responses and accelerates tumor progression. Interestingly, TLR5-dependent acceleration of the tumor growth is mediated through interactions with commensal microbiota [68].

TLR7/8 TLR7 recognizes single-stranded RNA (ssRNA), typically derived from RNA viruses, within endosomes [34]. It is highly expressed on plasmacytoid DCs (pDCs) and is crucial for the massive release of type I IFNs against RNA viruses that define this cell type [34]. Human TLR8 also recognizes viral ssRNA [34]. However, TLR8-deficient mouse cells show no defects in cytokine production against viral ssRNA [34]. TLR7 can also recognize self-derived ssRNA bound to autoantibodies [34].

Small molecule agonists of TLR7/8 have been proposed as antitumor immunotherapy drugs. Imiquimod has been known to activate TLR7-MyD88

signaling to exert antitumor effect [69, 70]. Several studies have elucidated detailed mechanisms of antitumor function of this compound. For example, imiquimod makes DCs produce type I IFNs and transform themselves into cells which can directly eliminate tumor cells via TNF-related apoptosis-inducing ligand (TRAIL) and granzyme B activities [71, 72]. In addition, immunostimulatory RNA oligonucleotides with specific sequence also induce antitumor immune response through NK cells in a TLR7-dependent manner [73].

Genetic studies, however, show an opposite role of TLR7 on tumor development. One study reveals that TLR7 deficiency in hematopoietic cells abrogates tumor development in a mouse pancreatic cancer model in which a mutated K-Ras gene is expressed [74]. Another study shows that TLR7-deficient mice are associated with less tumor burden and prolonged survival in LLC lung cancer metastasis model [75]. There is no clear explanation for the seemingly discrepant results between studies employing synthetic ligands and genetic studies in terms of effects of TLR7 signaling on tumor progression; it may depend on the magnitude and duration of TLR7 activation between synthetic and endogenous ligands, and this may also be the case for other PRRs (see below).

TLR9 TLR9 within endosomes recognizes unmethylated CpG DNA motifs, which are frequently found in viral and bacterial genome [34]. TLR9 can also recognize self DNA bound to autoantibodies in a manner similar to TLR7 [34].

The role of TLR9 signaling in antitumor immunity has been underscored by numerous reports. Most notably, a therapeutic effect of CpG-oligodeoxynucleotide (CpG-ODN), a TLR9 ligand, on tumor growth has been extensively studied [76–78]. CpG-ODN treatment induces a significant antitumor effect in C3 murine model of cervical cancer and C26 murine colon cancer model, wherein the tumor regressions and extended survival resulting from this therapy requires the participation of $CD8^+$ T cells [76, 77]. CpG-ODN also has a suppressive effect on a murine neuroblastoma cell line neuro2a, which seems to be mediated by NK cells [78]. Furthermore, combination of another TLR9-stimulating ODN with trastuzumab, a monoclonal antibody against human epidermal growth factor receptor 2 (HER2), is efficient for the control of trastuzumab-resistant human breast cancer cells in a mouse xenograft model [79]. Interestingly, this ODN modulates the interaction of TLR9 with HER receptors at the membrane level, thereby inhibiting the HER-dependent growth signal [79].

On the other hand, TLR9 may promote tumor growth. Orthotopically implanted pancreatic cancer cells carrying mutated genes for K-Ras and p53 show delayed growth in TLR9-deficient mice as compared with WT mice [80]. This effect is specific to the pancreatic TME, as pancreatic cells implanted subcutaneously do not show reduced growth in TLR9-deficient mice [80]. Mechanistically, TLR9 activation of pancreatic stellate cells (PSCs) results in chemokine (C-C motif) ligand 11 (CCL11) production, leading to tumor cell proliferation via its receptor chemokine (C-C motif) receptor 3 (CCR3). Moreover, it is shown that PSCs can recruit regulatory T cells (Tregs) into the peritumoral site in a TLR9-dependent manner, possibly through the CCL3-CCR5 axis. Additionally, TLR9 in immune cells is also responsible for MDSC recruitment to the TME, further exacerbating tumor progression [80].

25.1.2 Role of Cytosolic Nucleic Acid Sensors in Tumor Development

Cytosolic nucleic acid-sensing PRRs are expressed in almost all cell types and detect RNA and DNA or their mimetics to evoke innate immune responses [81]. The induction of type I IFNs is the hallmark of the activation of these cytosolic PRRs, and this induction underlies effective antiviral responses through the PRR's recognition of virus-derived nucleic acids [81]. Since the antitumor function of type I IFNs has been well appreciated, the role of these PRRs in antitumor immunity has also been the focus of attention [82].

RLRs RIG-I (also known as DDX58) and melanoma differentiation-associated 5 (MDA5) sense dsRNA, a replication intermediate for RNA viruses, leading to the robust production of type I IFNs in infected cells [83]. Another member of this family, laboratory of genetics and physiology 2 (LGP2) acts as a negative feedback regulator of RIG-I and MDA-5 [84], but it may promote the MDA5 signaling pathway in some cases [85, 86]. Despite the overall structural similarity between these two activatory PRRs, they detect distinct viral species, because of different structural features of the virus-derived RNAs [83]. RIG-I binds specifically to ssRNA containing 5′-triphosphate such as viral RNA and in vitro-transcribed dsRNA [87, 88]. It has also been shown that RIG-I binds preferentially to short dsRNA, while MDA5 preferentially recognizes long dsRNA [89]. Both RLRs share in common signaling features. Upon recognition of dsRNA, they are recruited by the adaptor MAVS (also known as IPS-1, CARDIF, or VISA) to the outer membrane of the mitochondria leading to the activation of several transcription factors including IRF3, IRF7, and NF-κB [81]. While IRF3 and IRF7 primarily mediate the induction of type I IFNs, NF-κB regulates the gene induction for inflammatory cytokines (for further details, see [90–92]).

That the activation of these RLRs results in the robust induction of type I IFNs prompted many investigators to assess their role in antitumor responses, as type I IFNs modulate a plethora of important cellular functions other than antiviral responses including effects on cell growth, differentiation, and antitumor immunity [82]. In humans, low RIG-I expression in HCC tissue predicts a poorer prognosis and a higher resistance to IFN-α therapy [93]. The tumor-suppressive role of RIG-I has been validated in RIG-I-deficient mice in the model of HCC [93]. It has also been reported that RIG-I activation induces the secretion of extracellular vesicles (EVs) from melanoma cells, which exhibit expression of the NKp30-ligands on their surface, thus triggering NK cell-mediated elimination of melanoma cells [94]. Since MDA5 triggers similar if not identical signaling pathways as RIG-I, there is the direct implication that MDA5 is also involved in tumor development [83]. Since LGP2 enhances the survival and activation of $CD8^+$ T cells [95], it is also possible that LGP2 plays a direct regulatory role in antitumor immune responses.

The fact that the RIG-I activation also induces intrinsic apoptosis through BH3 family, which occurs independently of type I IFN signaling, led to the study of exploiting RIG-I-mediated apoptosis to selectively eliminate malignant cells [96]. As such, much effort has been devoted to the development of targeting drugs of the RLR signaling pathway for cancer therapy with the working hypothesis that the induction of type I IFN, apoptotic, or both pathways may play a crucial role(s) [97–102].

DNA Sensors Among DNA-sensing PRRs reported thus far, the cyclic GMP-AMP (cGAMP) synthase (cGAS) is one of the best characterized molecules for its role in antiviral immunity. Viral DNA released into the cytosol is catalyzed by cGAS and converted to cGAMP which in turn binds to stimulator of IFN genes (STING) to activate its downstream signaling pathways including type I IFNs [103]. Perhaps expectedly, some studies have implicated the involvement of cGAS in the host immune responses against tumors [104–106]. cGAS-expressing macrophages are essential for IFN-β production in response to tumor-derived DNA [104]. Consistent with this, DCs are stimulated by irradiated tumor cells to release IFN-β, and this promotes antigen-specific $CD8^+$ T cell activation in cGAS-dependent manner [105]. In addition, cGAS is also required for IFN-β production triggered by the treatment with anticancer drugs, cisplatin and camptothecin [106].

As well as cGAS-mediated pathway, other DNA-sensing mechanisms are also important for the response to cytosolic nucleic acids. DNA-dependent activator of IRFs (DAI) associates with TBK1 and IRF3 and responds to cytosolic DNA for type I IFN induction [107], and its antitumor function has been reported [108]. Another DNA-sensing PRR, human IFN-γ-inducible protein 16 (IFI16), and its mouse ortholog p206 induce IRF3 activation and IFN-β production

upon cytosolic DNA stimulation [109], wherein recruitment of STING to IFI16 may be a critical step in the signaling pathway [109]. A similar association between STING is also observed with another PRR, DDX41 [110]. DDX41 recognizes various DNA and functions as a direct sensor for cyclic-di-GMP and cyclic-di-AMP, which are second messengers for STING activation as cGAMP [111]. The tumor-suppressive role of DDX41 has been reported in hematopoietic neoplasia caused by mutations in *DDX41* gene [112]. Furthermore, it is implicated that some DNA damage-responding molecules, such as DNA-dependent protein kinase (DNA-PK) and meiotic recombination 11 (MRE11), are involved in cytosolic DNA sensing [113, 114]; however, whether these DNA sensors regulate tumor development is largely unknown. Overall, as tumor-derived DNA is taken up by antigen-presenting cells in TME and activates DNA-sensing PRRs, their antitumor functions have been the focus of much attention in basic and clinical cancer immunology [104].

The role of STING in antitumor immune response has been the particular focus of attention [103]. In the mice inoculated with tumor cells expressing an immunogenic peptide, STING appears to contribute to antitumor response to these cells, wherein tumor-derived DNAs, which are taken up by antigen-presenting cells in TME, stimulate STING and induce IRF3-mediated IFN-β production for the expansion of antigen-specific $CD8^+$ T cells [104]. Similarly, STING-dependent IFN-β production and $CD8^+$ T cell activation are triggered in irradiation-treated tumor [105]. It is suggested that such IFN-β upregulation is induced by dead cell-derived DNA via IRF3 activation [115]. Furthermore, STING promotes IL-18 and IL-22BP expression in tumor tissue and suppresses AOM/DSS colon carcinogenesis [116]. STING signaling, on the other hand, has been reported to downregulate the expression of pro-inflammatory IL-6 that activates the pro-tumor transcription factor STAT3 [117]. A study using a glioma model shows that STING ameliorates the associated cancer burden with enhanced $CD8^+$ T cell activation and reduced infiltration of immunosuppressive cells such as MDSCs and Tregs in the brain [118].

In general, the targeting of STING for its activation has been beneficial for the treatment of cancer in many mouse models. Administration of cGAMP decreases tumor growth of colon 26 cells in association with DC maturation [119]. Moreover, cGAMP also retards B16 melanoma cell growth in vivo with the activation of $CD8^+$ T cells in TME through type I IFN signaling [120].

The activation of STING by a chemical compound ML RR-S2 can exert antitumor activity in several mouse models [121]. ML RR-S2 is also experimentally useful as an adjuvant for anticancer vaccine with GM-CSF-expressing dead tumor cells [103]. This therapeutic strategy, called as STINGVAX, has been shown to be effective against B16 mouse melanoma tumor that are resistant to a checkpoint therapy [103]. Related to this, it is also noteworthy in this context that some anticancer chemical drugs are also DNA adduct-forming agents that trigger cell death and release STING agonists [122]. It has been discovered that 5,6-dimethylxanthenone-4-acetic acid (DMXAA), a vascular-disrupting agent, directly binds to STING and it is now used as a STING agonist for cancer therapy [123]. DMXAA induces IFN-β production from antigen-presenting cells and shows potent therapeutic activity against in vivo growth of tumor cells through enhancement of adaptive immunity [121]. In addition, macrophages are targeted by DMXAA and polarized from an M2-type to M1-type [124, 125]. Furthermore, DMXAA treatment promotes immunologic memory against tumor [121], and mice that rejected tumors by DMXAA administration are resistant to secondary-challenged tumor. Notably, intratumoral administration of this drug inhibits the tumor growth developed at other distant site [121]. As such, the STING-activating molecules, such as cytosolic cyclic dinucleotides and chemical compounds, may pave the way(s) for the establishment of effective cancer immunotherapy.

On the other hand, there are reports showing a pro-tumor role of STING [106, 126]. DNA released into cytosol in carcinogen-damaged cells stimulates STING to induce inflammatory cyto-

kine expression and exacerbates DMBA-induced skin carcinogenesis [106]. It has also been reported that intradermal growth and lung metastasis of LLC tumor cells are enhanced by STING in immunosuppressive host [126]. This study also shows that antitumor responses are induced against gp100-transduced LLC cells in STING-dependent manner, implicating that STING-mediated antitumor or pro-tumor response is dependent on the immunogenicity of tumor cells.

25.1.3 Role of CLRs in Tumor Development

CLR family members are primarily characterized by their detection of carbohydrates on bacteria, fungi, and viruses [127], while some CLRs can also recognize oxidized lipids and other DAMP molecules exposed by damaged cells [128, 129]. CLR activation leads to immunoreceptor tyrosine-based activation motif (ITAM)/immunoreceptor tyrosine-based inhibition motif (ITIM)-dependent or ITIM-independent signal transduction to induce host immune responses [129]. Some ITAM-based CLRs such as Dectin-1 and C-type lectin domain family 2 (CLEC-2) possess hemITAM motif and recruit spleen tyrosine kinase (Syk) to activate NF-κB via caspase activation and recruitment domain 9 (CARD9) [129]. Syk further transduces MAPK and nuclear factor of activated T cell (NFAT) pathways and induces reactive oxygen species (ROS) production which contributes to NACHT, LRR, and PYD domain-containing protein 3 (NALP3) activation [129]. Other ITAM-based CLRs, represented by Dectin-2, Mincle, and macrophage C-type lectin (MCL), associate with ITAM-containing adaptor protein such as Fc receptor γ (FcRγ) chain, leading to Syk-dependent signal transduction [129]. ITIM-containing CLRs inhibit the activation of NF-κB and STAT5 as well as ITAM-based signaling pathway through Src-homology 2 domain-containing phosphatase-1 (SHP-1) and SHP-2 [129]. In addition to signal transduction, CLRs also drive the phagocytotic system of myeloid cells to promote the uptake of invading pathogens and abnormal self-derived molecules [130]. Through these mechanisms, CLRs play critical roles in regulating innate and adaptive immune systems.

CLR involvement in tumor development has not been intensely analyzed. Some recent studies have revealed that CLRs control tumor growth and metastasis, functioning as key innate receptors to trigger both anti- and pro-tumor host responses.

Dectin-1 Dectin-1 mainly recognizes β-glucan structures and contributes to the defense against bacterial and fungal infection [127]. Activation of Dectin-1 with such carbohydrates leads to Syk-dependent signal transduction through hemITAM motif on itself [129]. In the control of subcutaneous tumor growth and lung metastasis, Dectin-1 induces antitumor responses through the enhancement of NK cell cytotoxicity against tumor cells [27]. This antitumor mechanism is triggered by Dectin-1 recognition to N-glycan structures on tumor cells [27]. Although Dectin-1 is a β-glucan receptor, a variety of glycan structures on cancer cells likely also bind to Dectin-1 [27]. Cancer cells activate Dectin-1 signaling in myeloid cells and promote antitumor killing of NK cells in cell-to-cell contact-dependent manner [27]. Supporting this, the expression of INAM, a membrane protein to drive NK cell activation, is upregulated by Dectin-1 in the presence of cancer cells [27]. NK cell-mediated tumor killing can also be induced against liver-metastasizing cancer cells. Dectin-1 enhances cytotoxic activity against SL4 colon cancer cells by NK cells to suppress liver metastasis [28].

Dectin-1-dependent antitumor immune responses can be useful for cancer therapy. The administration of lentinan, a purified β-glucan isolated from shiitake mushroom, suppresses gastric cancer development in human study [131]. In addition, sizofiran, *Schizophyllum commune*-derived β-glucan, improves the prognosis of ovarian cancer patients when combined with cisplatin, adriamycin, and cyclophosphamide [132]. The therapeutic effect of β-glucans has been also reported using murine in vivo models of tumor growth and for breast and liver metastasis [133]. The mechanisms of β-glucan-induced antitumor

response have been intensely studied. Dectin-1 agonist curdlan activates DCs to elicit potent $CD8^+$ T cell responses and markedly reduces lung metastasis of B16 melanoma cells [134]. Moreover, β-glucan-containing extracts from a mushroom, which promotes the expansion of NK cells and upregulates the expressions of antitumor cytokines such as IFN-γ and IL-12, decrease tumor burden of colon cancer 26 cell-inoculated mice [135].

In addition to instigating antitumor immunity, β-glucans can inhibit pro-tumor host responses. Whole β-glucan particles (WGP) from *Saccharomyces cerevisiae* downregulate the immunosuppressive activity of MDSCs and dampen the expansion of MDSCs and Tregs in the TME [136]. WGP also converts immunosuppressive M2-like macrophages to M1-like cells in a Dectin-1 and CARD9-dependent manner [137]. CARD9 is a signaling molecule activated by ITAM-associated CLRs like Dectin-1 that can induce antitumor responses [129]. Mice treated with mushroom-derived β-glucans also show loss of immunosuppressive TME [138].

Dectin-2 Dectin-2 binds to mannose-rich carbohydrates to transduce Syk-dependent signaling [127]. Unlike Dectin-1, Dectin-2 does not possess an ITAM motif [129]. Instead, it associates with FcRγ chain which itself possesses ITAM motif to activate downstream pathways [129]. The role of Dectin-2 in tumor immunity is relatively unique as compared to other CLRs. Although Dectin-1 inhibits subcutaneous tumor growth and lung metastasis [27], Dectin-2 is not involved in these responses [28]. Notably, however, Dectin-2 contributes to the suppression of liver metastasis [28]. The underlying mechanism is unique in that Dectin-2 promotes phagocytosis of Kupffer cells, liver-residing macrophage, against cancer cells in vitro, although such Dectin-2-dependent uptake of cancer cells is not observed in bone marrow-derived macrophages and alveolar macrophages [28]. Consistent with this, tumor metastasis to liver is notably enhanced in Dectin-2-deficient mice [28]. Interestingly, the expression of IL-6 and CXCL1, which is induced when Dectin-2 is activated by fungi, remained unaffected when Dectin-2-deficient Kupffer cells interact with cancer cells [28]. This observation suggests that antitumor Dectin-2 signaling pathway(s) in Kupffer cells is distinct from the conventional one for antimicrobial responses.

Mincle Macrophage-inducible C-type lectin (Mincle) binds mannose and trehalose-6,6′-dimycolate (TDM), a mycobacterial glycolipid [129]. Mincle also recognizes endogenous ribonucleoprotein spliceosome-associated protein 130 (SAP-130), which is released from dying cells [139]. Stimulation of Mincle with its ligand induces Syk-dependent signaling pathway through ITAM-possessing FcRγ chain [129]. In pancreatic oncogenesis of $p48^{Cre}$; $Kras^{G12D}$ mice, Mincle establishes an immunosuppressive TME and promotes tumor development [140]. Mincle signaling enhances the production of IL-10 from T cells and the infiltration of MDSCs and M2-like macrophages in tumor [140]. This oncogenic process is associated with necroptosis and the induction of SAP-130 expression in pancreas [140]. Since SAP-130 administration into pancreas aggravates tumor growth, the ligation of Mincle with dead cell-released SAP-130 promotes oncogenic process with immunosuppression [140]. Interestingly, in spite of its relative importance in pancreatic tumor progression, Mincle doesn't control liver metastasis of SL4 colon carcinoma cells [28].

CLEC-2 CLEC-2 is a receptor for a snake venom toxin, rhodocytin [127], and stimulation with rhodocytin induces similar signaling pathways to ITAM-dependent ones [129]. CLEC-2 also recognizes podoplanin, a mucin expressed on some cancer cells [141]. Although the study of CLEC-2 in vivo is challenged by difficulties of embryonic lethality of CLEC-2-deficient mice [142], the CLEC-2-podoplanin interaction is thought to be a key event in promoting cancer metastasis [141]. Platelets expressing CLEC-2 are activated by podoplanin on cancer cells triggering their aggregation [143]. The platelet aggregation supports the evasion of cancer cells from immune

system essentially by masking their detection while also promoting metastasis by facilitating adhesion to the endothelial wall [144]. Furthermore, CLEC-2 activation induces the secretion of growth factors, chemokines, matrix proteins, and angiogenic factors, which can promote metastasis [141]. In addition, CLEC-2 also activates podoplanin signaling in cancer cells, which leads to the downregulation of E-cadherin and promotes metastasis [145, 146]. Furthermore, the interaction of podoplanin with platelet accelerates subcutaneous tumor growth of podoplanin-expressing PC-10 lung cancer cells [147]. As such, it is strongly suggested that CLEC-2 plays an important role in multiple steps of tumor development.

DC-SIGN DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN) shows high affinity for mannose, fucose, N-acetylglucosamine, and Lewis antigens [127, 148]. DC-SIGN activation drives ITAM/ITIM-independent signaling through lymphocyte-specific protein 1 (LSP1) [129] and promotes cross-presentation of antigens to $CD8^+$ T cells [149]. Consistent with this, DCs treated with Lewis X oligosaccharides-heparanase complex enhance $CD8^+$ T cell response and possess the ability to reduce tumor growth of G422 glioblastoma cells in vivo [150].

On the other hand, DC-SIGN plays pro-tumor roles in some cases. DC-SIGN recognizes Lewis carbohydrates on carcinoembryonic antigen (CEA) and Mac-2-binding protein (Mac-2BP) which are mainly expressed by colon cancer cells [151–153]. Since ligation of DC-SIGN on macrophages to cancer cells induces IL-10 production [154], DC-SIGN likely helps to establish immunosuppressive TME. This notion is further supported by a report showing that DC-SIGN-deficient mice exhibits slower subcutaneous tumor growth of LLC cells compared to WT mice [155].

MGL Macrophage galactose C-type lectin (MGL) has specificities to galactose and N-acetylgalactosamine for its ligand recognition [127, 129]. MGL binding to such carbohydrate moieties on microbes promotes their uptake and leads to antigen presentation [129]. Further, MGL recognizes carbohydrate antigens, Tn and sialyl Tn, on mucin 1 (MUC1) which are expressed on a variety of cancer cells [156, 157]. While MGL activation triggered by sialyl Tn-associated MUC1 enhances DC maturation and migration [158], anti-MGL blocking antibody inhibits the dissemination of foot pad-inoculated cancer cells to lymph nodes [159]. Therefore, MGL may have a pro-tumor role in lymph node metastasis.

MR Mannose receptor (MR) recognizes mannose and promotes its endocytosis [127]. It has also been known that ovalbumin (OVA) conjugated with synthetic MR ligands, 3-sulfo-Lewis A and tri-N-acetylglucosamine, primes DCs to induce antigen-specific $CD8^+$ T cell proliferation and Th1 polarization of $CD4^+$ T cells [160]. Therefore, MR ligation with its ligand likely induces antitumor immune responses. Indeed, treatment by OVA conjugated with anti-MR antibody instigates $CD8^+$ T cell responses in vivo and, together with CpG treatment, markedly reduces subcutaneous tumor growth of OVA-expressing B16 cells in MR-transgenic mice [161].

Nevertheless, MR also possesses pro-tumor activity. MR is involved in the recognition of tumor-specific mucin CA-125 and TAG-72 [162]. Stimulation of MR on tumor-associated macrophages (TAMs) with these molecules induces IL-10 production and inhibits IL-12 expression [162]. Moreover, MR expressed on tumor-activated liver sinusoidal endothelial cells (LSECs) inhibits antitumor cytotoxicity of liver sinusoidal lymphocytes (LSLs) [163]. MR further decreases and increases IFN-γ and IL-10 production, respectively, from LSLs of tumor-bearing mice [163]. These reports evoke the notion that MR promotes cancer development.

MCL MCL is an FcRγ-associated receptor for TDM and a heterodimeric counterpart of Dectin-2 [164, 165]. Consistent with this and like Dectin-2, MCL enhances the phagocytotic activity of Kupffer cells and suppresses liver metastasis of SL4 colon carcinoma cells [28]. Interestingly,

although MCL induces Mincle expression in bone marrow-derived macrophage [166], Mincle is not involved in the control of liver metastasis, as described above [28].

Other CLRs The roles of other CLRs in cancer have been studied, albeit not extensively. Some studies indicate that DAMPs released in TME can regulate immune responses to cancer [167, 168]. Additionally, some DAMPs act as a ligand for CLRs. DC NK lectin group receptor-1 (DNGR-1) recognizes F-actin and actin complex with spectrin β and α-actinin, both of which are exposed by damaged cells [169, 170]. Furthermore, F-actin can drive Syk-dependent signaling in DNGR-1-expressing cells [169], implicating its involvement of DNGR-1 in host immune responses to tumor. In addition, CLEC12A binds to uric acid crystals from dead cells and inhibits neutrophil-associated inflammation in vivo after the challenges with necrotic cells or irradiation [171]. Uric acid also contributes to DC maturation, $CD8^+$ T cell priming, and Th17 differentiation [172–174]. Therefore, CLEC12A may regulate tumor development in dead cell-enriched microenvironment.

CLRs might have roles beyond sensing DAMPs in the regulation of tumor immunity. Mannose-binding lectin (MBL) recognizes Lewis antigens on SW1116 human colorectal carcinoma cells [175, 176]. Moreover, blood dendritic cell antigen 2 (BDCA-2), which is exclusively expressed on pDC and activated by asialo-oligosaccharides with terminal galactose to suppress type I IFN production, binds to several kinds of human cancer cells such as ovarian and colon carcinoma cells [177]. ITAM-based CLRs other than those described above, for example, SIGN-related gene 3 (SIGNR3) and myeloid DAP12-associating lectin (MDL-1), can also be involved in tumor growth, since CARD9 promotes colon tumorigenesis in males of APC^{min} mice [178]. Further analysis for the functions of these receptors in immune response to cancer promotes a better understanding of CLR-mediated regulation of tumor development. In line with this, how ITIM-associated CLRs are involved in the regulation of tumor growth and metastasis, which is obscure so far, is an intriguing question to be clarified.

25.1.4 Role of NLRs in Tumor Development

The inflammasome is a multiprotein complex comprised of a PRR, the adaptor protein apoptosis-associated speck-like protein containing CARD (ASC), and caspase-1 [179]. These PRRs include members of NLR and absent in melanoma 2 (AIM2)–like receptor families [179] and recognize various PAMPs and DAMPs upon infection or cellular damage, respectively, to recruit ASC and trigger caspase-1 activation [179]. Activated caspase-1 subsequently cleaves pro-IL-1β and pro-IL-18 into their mature forms [179]. Inflammasomes are involved in host defense against pathogens as well as in the pathogenesis of auto-inflammatory, neurodegenerative, and metabolic diseases [180–182]. Not surprisingly, there is evidence to suggest the involvement of inflammasomes in the development of cancer [183].

NLRP3 NLRP3 recognizes a variety of ligands including bacterial DNA:RNA hybrids, bee venom, ATP, uric acid crystals, aluminum hydroxide, and asbestos [179]. The NLRP3 inflammasome requires two signals for its full activation. The first signal is mediated by several receptors, of which TLRs are well known to activate NF-κB-mediated upregulation of NLRP3 [179]. The second signal is mediated by its ligands which subsequently promote inflammasome assembly, caspase-1 activation, and release of IL-1β and IL-18 [179].

The role of NLRP3 in tumor development seems to be context dependent and organ specific. Many studies have focused on the role of NLRP3 in colon cancer development, and these studies generally describe a role that is antitumor. In AOM/DSS colon cancer model, for example, NLRP3-deficient mice show increased tumor burden [184]. This effect is explained by attenuated levels of IL-1β and IL-18 at the tumor site. It

is also shown that NLRP3 in hematopoietic cells is responsible for that effect [184]. Another study reports that reduced level of IL-18 caused by NLRP3 deficiency leads to impaired production of IFN-γ and insufficient antitumor immunity [185]. Similarly, in the liver metastasis model of colon carcinoma cell line MC38, NLRP3-deficient mice are more susceptible to metastatic tumor growth [186]. This event is attributed to impaired NK cell's tumoricidal activity due to insufficient induction of IL-18 [186]. Another report also shows that NLRP3-deficient mice develop more severe tumor in DMBA/TPA skin papilloma model than WT mice [187].

On the other hand, a report shows a role in promoting tumor growth via NLRP3. NLRP3-deficient mice show reduced tumor burden in an MCA-induced fibrosarcoma model [188]. The decreased tumor development is also found in lung metastasis models of E0771 murine mammary adenocarcinoma cells, B16F10 murine melanoma cells, and RM-1 murine prostate cancer cells. Reduction of tumor burden is associated with an increased frequency of NK cells; it is suggested that NLRP3 on $CD11b^{+}Gr\text{-}1^{int}$ myeloid cells are responsible for the recruitment of NK cells [188].

NLRP3 has also been the focus as a key molecule determining the efficacy of anticancer chemotherapy, although the results are controversial. One report shows that the anticancer chemotherapy with oxaliplatin is inefficient against EL4 murine lymphoma established in NLRP3-deficient mice [189]. Mechanistically, dying tumor cells release ATP, activate NLRP3 via purinergic receptors on DCs, and successfully prime $CD8^{+}$ T cells [189]. Conversely, another report reveals that activation of NLRP3 on MDSCs leads to the release of IL-1β, dampening anticancer effect of chemotherapy with 5-fluorouracil (5-FU) on EL4 lymphoma [190]. As a result, NLRP3-deficient mice show enhanced response to anticancer chemotherapy [190].

NLRC4 The flagellin and inner rod proteins of the type III secretion system of several bacterial species are the proposed ligands of NLRC4 [179]. These ligands do not interact with NLRC4 directly. Rather, they bind to several NAIP proteins, which can activate the NLRC4 inflammasome and lead to caspase-1 activation [179].

In AOM/DSS colon cancer model, NLRC4-deficient mice develop more tumors, possibly due to increased proliferation and reduced apoptosis in the colonic epithelial cell [191], while no contribution of NLRC4 in colon cancer development is reported [184]. Another report demonstrates that NLRC4-deficient mice exhibit enhanced tumor growth in a B16F10 melanoma model [192]. The exacerbated tumor growth is inflammasome independent, and NLRC4 on macrophages is required for the induction of antitumor adaptive immunity [192].

NLRP6 and NLRP12 Ligands of NLRP6 and NLRP12 are still enigmatic and they can function as both activator and inhibitor of inflammation [179]. NLRP6 is protective against experimental colitis via its induction of IL-18. On the other hand, NLRP6 suppresses MAPK and NF-κB signaling in macrophages infected by *Listeria monocytogenes*, *Salmonella typhimurium*, and *Escherichia coli.* NLRP12 can activate caspase-1 in *Yersinia pestis* and *Plasmodium chabaudi* infection. However, it can also suppress NF-κB pathway in *Salmonella typhimurium* infection [179].

NLRP6-deficient mice are susceptible to tumorigenesis in AOM/DSS colon cancer model, suggesting its antitumor role [193]. Interestingly, this susceptibility is transmittable by cohousing of mice. These results are attributed to microbiota-induced CCL5, leading to the promotion of epithelial cell proliferation through activation of the IL-6-mediated signaling [193]. NLRP12-deficient mice also suffer from more severe tumor development due to elevated noncanonical NF-κB activation in AOM/DSS model [194]. It is suggested that NLRP12 inhibits noncanonical NF-κB pathway via regulation of NF-κB-inducing kinase (NIK) and TNF receptor-associated factor 3 (TRAF3) function [194]. Another study also reports the tumor-suppressive role of NLRP12 in the same model and the negative regulation of canonical NF-κB and ERK pathway [195].

AIM2 AIM2 recognizes double-stranded DNA (dsDNA) by its positively charged HIN-200 domain and recruits ASC for caspase-1 activation via its PYD domain [179]. AIM2-deficient mice are more susceptible to tumor development in the AOM/DSS colon cancer model, implying an antitumor role [196]. Mechanistically, AIM2 deficiency causes proliferation of tumor-initiating stem cells via aberrant Wnt signaling. Furthermore, dysbiosis of gut microbiota in AIM2-deficient mice also contributes to the enhanced tumorigenesis [196]. Another report also reveals a protective role of AIM2 in the AOM/DSS and APC^{min} colon cancer models [197]. Interestingly, AIM2 can interact with DNA-PKcs and interrupt activation of Akt pathway for tumor progression [197]. Of note, both studies argue that tumor-suppressive activity of AIM2 is independent of its role in inflammasome activation.

ASC ASC is a bipartite adaptor protein that forms a speck upon activation of AIM2 and most NLRs and interacts with caspase-1 via its CARD domain, resulting in the activation of caspase-1 [179]. ASC-deficient mice show increased tumorigenesis in AOM/DSS colon cancer model and develop more severe tumors than NLRP3-decifient mice [184, 185], although the precise mechanism and compartment of ASC activity remain elusive. Another report reveals that susceptibility of ASC-deficient mice in the AOM/DSS-induced colon cancer model is transmittable by cohousing, indicating the involvement of intestinal microbiota in the same manner as NLRP6-deficient mice [193].

In the DMBA/TPA skin carcinogenic model, ASC functions in cell type-specific manner [198]. ASC deficiency in myeloid cells results in decreased tumor burden, suggesting its pro-tumor role [198]. This impaired tumor growth is accompanied by decreased production of inflammatory cytokines, such as IL-1α, IL-1β, TNF-α, and IL-6 [198]. Interestingly, epidermis-specific ASC-deficient mice develop more severe tumors, and ASC regulates the proliferation of keratinocytes possibly through the activation of p53, which is independent of inflammasome activity [198].

25.2 Concluding Remarks

In this review, we focused on the role of innate immune receptors in the regulation of tumor immunity. The immune system is intrinsically a double-edged sword in that, while essential to the host's homeostasis by eliminating undesirable entities (molecules, pathogens, and cancerous cells), it also contributes to a variety of harmful events when it is dysregulated. Since the activation of innate immune receptors generally evokes inflammatory responses, which may cause either antitumor or pro-tumor response, the role of these receptors in the regulation of tumor development is variable, as described above.

Notwithstanding, the deepening of our understanding on how these innate receptors function toward antitumor direction warrants further investigation to seek improving more effective ways to treat cancers by harnessing these receptors. It may be of particular interest that type I IFN system, whose antitumor activities have been known for many decades, is being "revisited" nowadays [82]. There is evidence that type I IFNs are involved in the context of rapidly emerging cancer checkpoint therapy field [104, 199]. In addition, the IRF7-IFN-β pathway appears to be critical for optimal antitumor activity [200]. Thus, one possibility may be the development of agonists, which selectively activate the innate receptors to induce type I IFNs.

Clearly, we can expect that further work will bring about the establishment of improved way(s) to harness the power of innate and adaptive immune systems for the treatment of cancer.

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Co-stimulation Agonists via CD137, OX40, GITR, and CD27 for Immunotherapy of Cancer

26

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26.1 Immune Synapses and the Co-stimulation of Anticancer T-Cell Immune Responses

T-cell- and NK-cell-mediated immunity are under tight control since their functions involve the elimination of cells showing signs of infection, genetic stress, and/or the promotion of tissue inflammation. Thymic selection processes ensure that the repertoire of antigen T-cell receptors (TCRs) is purged of self-reactivity and is biased to recognize foreign peptides bound to self MHC molecules [1].

Once in the periphery, T cells patrol in search of their antigen on antigen-presenting cells. If the cognate antigen is met under noninflammatory conditions, the usual outcome is apoptosis or anergy induction of the antigen-recognizing lymphocytes with the reactive lymphocytes left dysfunctional [2]. However, if the surrounding environment denotes intense tissue damage and destruction [3], infection [4], or inflammation, the professional antigen-presenting cells display, together with processed antigen epitopes, a set of activating signals which in conjunction with the TCR determine rapid cell division (clonal expansion) and acquisition of pro-inflammatory and cytotoxic functions. These accessory signals are mediated by soluble factors (cytokines or interleukins) and membrane attached receptor-ligand pairs (co-stimulatory molecules) [5]. The prevailing working conceptual scheme is based

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on the distinction of three types of signals [6] in T-cell activation: signal 1 emanating from the TCR-CD3 complex, signal 2 that procures co-mitogenic effects and protection from apoptosis, and signal 3 that results in the enhancement of pro-inflammatory and cytotoxic functions [7]. The conceptual dichotomy between signal 2 and 3 is usually very difficult to dissect, since many of the co-stimulatory pathways share effects on these two functional properties. To make things more complicated, the effect of surface receptors very often controls cytokine secretions which can act in an autocrine or paracrine fashion, therefore confounding the attribution of the functional effects. In conclusion, while the TCR detects the antigenicity of a scanned cell [8], the co-stimulatory molecules determine immunogenicity, depending on the context of recognition. Interestingly enough, another category of surface receptors and their ligands counteract the action of co-stimulatory molecules and are conceptually grouped as co-inhibitory molecules [9].

There are co-stimulatory molecules that are constitutively expressed on resting antigen-naïve T cells such as CD28 and CD27. Other surface co-stimulatory receptors are only present on the membrane once the lymphocyte is antigen-primed or raise their intensities of expression from very low baseline levels. Such is the case of ICOS, CD137, OX40, and GITR, among others. This is an important distinction for immunotherapy, since some co-stimulatory receptors are conceivably able to operate during priming, while other co-stimulatory functions can only take place on recently primed T cells.

It is important to keep in mind that these receptor-ligand interactions usually take place in the context of cell-to-cell contacts termed immune synapses that are well organized by the action of adhesion molecules (integrins and their ligands) [10, 11]. The floating cholesterol-rich domains of the plasma membranes play a role in these structures, as well as the tubulin and actin cytoskeletons [12]. Vesicles and secretion granuli are directionally reorganized toward the contacting region by the centrosome and the tubulin cytoskeleton [13]. It is in this subcellular context that both the TCR and the co-stimulatory molecules engage and signal. Other activating systems at the synapse level are mediated by cytokines that are directionally secreted. Five of these soluble proteins are best known for their functions: IL-2, IL-12, IFNα/β, IFNγ, and IL-15. In the case of IL-15, it acts more like a membrane-bound co-stimulatory molecule, since it is anchored to the plasma membrane by IL-15Rα to be transpresented to the contacting lymphocytes [14].

Co-inhibitory systems are also receptor-ligand pairs that operate at the level of the immune synapse but whose function is to mitigate or suppress the activation signals [5]. The action of such receptors is important to keep autoimmunity at bay and to prevent collateral damage of healthy cells in a tissue infected by an intracellular pathogen [15]. Often these co-inhibitory ligands and receptors are termed checkpoints, and these are extensively described in accompanying chapters in this book. A critical functional aspect to be kept in mind to understand the balance between co-stimulatory and co-inhibitory receptors is that such receptors control the stability and duration of the integrin-dependent cell-to-cell contact [16] and thus of the antigen presentation processes taking place in the immune synapse.

Co-stimulatory molecules lack any intrinsic enzymatic activity, and their functions are therefore dependent on dynamic recruitment of signaling adaptors. Dissection of intracellular signaling by co-stimulatory receptors is a difficult task because these receptors signal in the context of potent TCR-CD3 stimulation. In many instances it is found that the overall effect is mainly amplification or potentiation of TCR-dependent signals [17] with little evidence for exclusively elicited biochemical second messenger effects. The main biochemical events occurring immediately downstream of this type of co-stimulatory receptors are either tyrosine and serine/threonine phosphorylations [18] or polyubiquitination of proteins [19, 20]. Both mechanisms result in the formation of transient protein docking sites and multiprotein complexes that are usually followed by the activation of transcription factors.

Signaling is usually triggered by a natural ligand but can be also promoted by cross-linking of the receptors with bivalent antibodies or

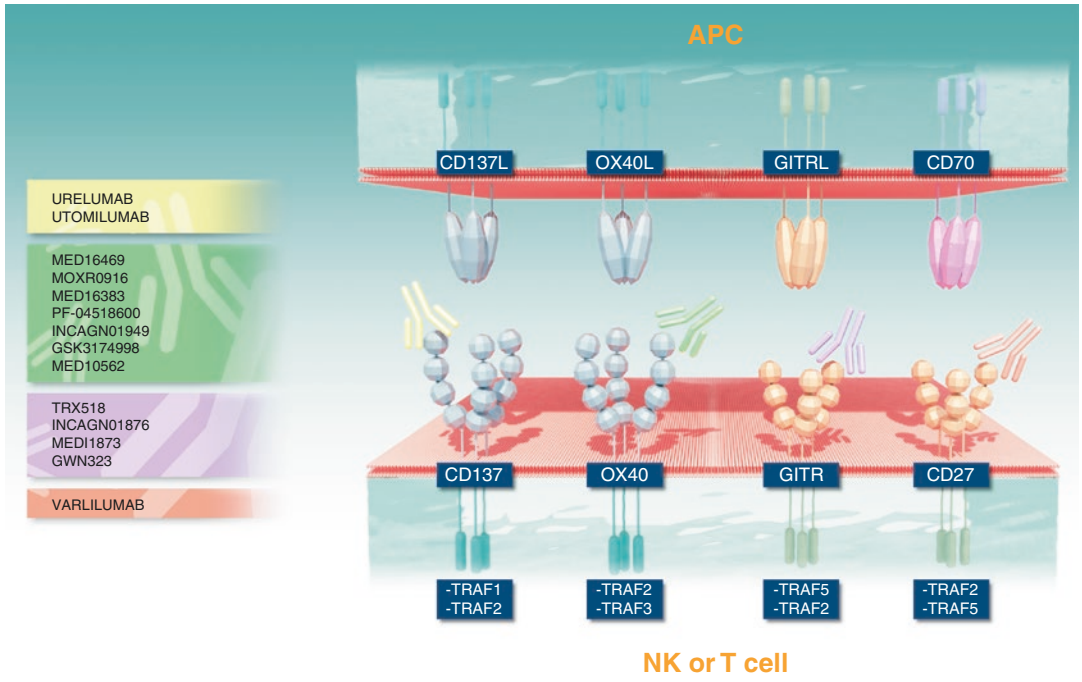

Fig. 26.1 Constitutive and activation-induced co-stimulatory members of the TNF and TNFR families expressed on T cells, NK cells, and antigen-presenting cells (APCs). Schematic representation of an immune synapse between effector T cells (Teff), regulatory T cells (Treg), and NK cells with an APC. TRAF adaptor usage and reference to the agonist monoclonal antibodies under clinical development are provided for each TNFR co-stimulatory molecule

recombinant forms of multimerized natural ligands, monoclonal antibodies (mAbs) [21], or other moieties such as nucleotide aptamers [22]. Signaling normally occurs in the immune synapses in a compartmental fashion and with the receptors frequently being internalized upon ligation.

The TNFR family encompasses two types of molecules: those that have an intracytoplasmic death domain, involved in eliciting apoptosis, and those that do not contain it [23]. In the immune system, this family of receptors and ligands regulates many functions such as apoptosis induction, inflammation, and lymphocyte activation [24]. In tumor immunotherapy the most important targeted receptors do not have death domains, which would recruit the caspase-activating machinery. TNFRs lack any intrinsic enzymatic activity and rely for signal transduction on adaptor proteins of the TRAF family and others [25, 26]. Figure 26.1 shows a schematic representation of the main TNFR members and their ligands as they would act in an immune synapse. The agonist antibodies under clinical development are also presented (Fig. 26.1).

26.2 Preclinical and Clinical Experience with Antibody Agonists of Co-stimulatory Receptors of the TNFR Family

To invigorate or reinvigorate at will an immune response in an antigen-specific fashion has been a long-term elusive goal in pharmacology. The objectives behind vaccination have been only successful in a prophylactic setting when able to elicit neutralizing antibodies to the infectious agents or their toxins prior to exposure but largely unsuccessful when the goal is to induce a protective cellular immune response. In cancer we often find the situation in which there is already an

ongoing baseline immune response [27, 28]. Such immune response is often too weak as to cause the eradication of the malignancy, but multiple lines of evidence in mouse and human indicate that it is actively controlling its progression [29]. In a way there is an ongoing equilibrium [30] of immunity and cancer that mainly manifests itself in the tumor microenvironment and potentially in the tumor-draining lymph nodes. In light of these concepts, it is conceivable to pharmacologically act on receptors of immune cells present in this microenvironment scenario to decisively potentiate the ongoing baseline immune responses, empowering them to impact the malignancy. Immunology research during the late 1990s found in a serendipitous way that co-stimulation via TNFR members could lead to tumor eradication in mouse models [31, 32]. We will review knowledge on the targeting of the main receptor of this family whose effects are under clinical investigation.

26.3 CD137 (4-1BB)

CD137 (4-1BB or TNFSR9) was originally discovered on activated T lymphocytes and found to favor proliferation and IL-2 production by T cells [33, 34]. Indeed, its presence is readily detected on CD8- and CD4-activated T cells (including Tregs) following antigen-mediated activation [35]. Its expression has also been reported on activated NK cells [36], B cells, and myeloid cells [37]. Experiments in knockout mice indicate that this molecule is important for optimal antiviral and alloreactive CTL responses [38, 39] and for the induction of long-lived memory T cells as demonstrated following virus infections [40, 41].

Only one transmembrane ligand (CD137L) has been identified, and this belongs to the TNF family [42]. The ligand is termed 4-1BBL, CD137L, or TNFSF9. The system is quite conserved in mouse and human. Ligand binding induces CD137 to productively signal using TRAF2 and TRAF1 as its main adaptors [43, 44]. The extracellular domain has been found to be kept to some extent pre-cross-linked by galectin-9 bound to its carbohydrates [45]. The resulting conformational features of such lectin-CD137 complex are likely important for signaling [46].

Early signaling by CD137 involves K-63 poly-ubiquitination reactions dependent on TRAF2 functions that ultimately result in activation of the NF-kB and MAP kinase pathways [47]. Preliminary evidence suggests that these pathways are actively regulated by specific de-ubiquitinases. The biochemical effects of TRAF1 in these complexes are far less clear [48], but a recent involvement of TRAF1 that has been discovered in the regulation of linear ubiquitin has been proposed in the contexts of other TNF family receptors [49]. It has been reported that CD8 T cells primed in the presence of CD137 agonists become favorably poised for future encounters with antigen. This is probably explained by epigenetic changes in miRNA expression and chromatin configuration (Angela Aznar, Submitted).

Agonist anti-CD137 mAb and the trimerized natural ligand have been reported to exert antitumor effects in mouse models [31]. The therapeutic effects elicited by agonist mAbs are mediated by a strong CTL response that more efficiently destroys the malignant tissue. A requirement for NK cells in some models has been reported [36]. The functional effects of CD137 ligation on Treg cells are controversial [50].

It is remarkable that multiple mouse tumor models are amenable to treatment with agonist anti-CD137 mAb [51]. In the resistant tumors, it is easy to find combination strategies with other therapies that ultimately result in synergistic, often curative, effects. These include combinations with vaccines [52], cytokines [53], and other immune-stimulatory mAbs [54, 55]. In addition, radiotherapy and chemotherapy have also been reported to be synergistic with anti-CD137 mAb [56, 57]. Agonist anti-CD137 single-chain mAb expressed on the plasma membrane of tumor cells results in powerful localized CTL- and NK-mediated antitumor immunity [58, 59]. Recent evidence suggests that the transcription factor eomesodermin partially explains this robust CTL phenotype [60].

In the case of other TNFR family members such as CD40, cross-linking of the receptor by

the agonist antibodies is dependent on CD32 Fc receptors on neighboring cells [61, 62]. However, in our hands this is not the case with CD137 as the antitumor effects are intact in *Cd32 (FcRγII)−/−* mice possibly because of the baseline pre-cross-linking by galectin-9.

Bivalent 4-1BB agonistic aptamers also induce antitumor effects in tumor-bearing mice [63]. This strategy has also been used in combination with a novel approach that was described to enhance tumor antigenicity, in which tumor cell nonsense mRNA-mediated decay (NMD) was inhibited with aptamer-siRNA chimeras targeted to tumor cells. The combination of 4-1BB agonistic aptamer and targeted inhibition of NMD showed a synergistic antitumor effect [64].

The cytoplasmic tail of CD137 has been used to construct chimeric antigen receptors (CARs) encompassing a single-chain antibody CD3ζ and the CD137 cytoplasmatic tail. In these constructs, the CD137 part is critical to support the persistence and expansion of the transfected T lymphocytes [65, 66]. In fact, the anti-CD19 CARs encompassing CD137 have produced the best clinical results against leukemias with long-term engraftment of the transduced T cells [67, 68].

Another intriguing aspect of CD137 co-stimulation is its largely unexplained interplay with the TCR signaling machinery on T cells and how it behaves in immune synapses [69]. Recent evidence indicates that it can rapidly internalize following ligation but that it seems to keep signaling from endosomal compartments [47].

It is a yet unsatisfactorily explained paradox that the very same anti-CD137 antibodies, which successfully treat cancer in mouse models, also ameliorate mouse autoimmune disease mediated by CD4 T cells such as EAE, lupus, and collagen-induced arthritis [70, 71]. This effect seems to depend on an overactivation of CD4 lymphocytes that ultimately die off [50]. In contrast, treatment of mice with these antibodies results in mild liver inflammation composed of periportal polyclonal infiltrates dominated by CD8 T lymphocytes [72]. Liver inflammation is dependent on CD137 (absent in CD137 KO mice), IFNγ, and TNFα. Interestingly, anti-CD137 monoclonal antibodies inhibit humoral (antibody-mediated) immunity in mice [73]. This activity is due to actions on follicular T helper cells that in human are the main CD137 baseline expressing lymphocytes in healthy volunteers [74]. Abrogation or attenuation of neutralizing antibody responses has been exploited in mouse models to permit repeated administration of tumor virotherapy together with anti-CD137 mAb [53].

On human and mouse NK cells, CD137 becomes expressed following exposure to IgG-coated target cells subjected to antibody-dependent cellular cytotoxicity [75]. This function is mediated by the CD16 Fcγ receptor. Once expressing CD137, the agonist anti-CD137 mAb strongly upregulates ADCC function capabilities for subsequent encounters. Indeed, this powerful effect produces synergistic antitumor cytotoxicity with antibodies such as anti-CD20 [75], trastuzumab [76], or cetuximab [77]. Upregulation of CD137 on NK cells infiltrating human head and neck cancer has been seen in patients treated with cetuximab [78].

Two anti-CD137 mAb antibodies are currently undergoing clinical trials. Urelumab (IgG4), developed by BMS, showed an excellent preclinical safety profile including results in cynomolgus macaques. Phase I studies of urelumab demonstrated a tolerable safety profile for doses escalated to 15 mg/kg. However, in subsequent phase II studies, several patients developed liver inflammation that resulted in a temporary halt in development and led to two fatalities [79, 80]. New phase I trials were started that identified safe doses at 0.1 mg/kg (a flat dose of 8 mg/kg every 2 or 4 weeks), and the antibody is currently undergoing further clinical evaluation in combination regimens with rituximab (NCT01775631), cetuximab (NCT02110082), elotuzumab (NCT02252263), and nivolumab (SITC meeting 2016). Urelumab monotherapy produced signs of clinical activity including objective responses in melanoma (ASCO 2014), renal cell carcinoma, and lymphoma (SITC meeting 2016), but its value is mainly foreseen to be in combination regimens.

PFZ-05082566 (utomilumab) is an IgG2 anti-CD137 and also under development after showing bioactivity in vitro assays and safety in

nonhuman primates [81]. A phase I clinical trial and its expansion are ongoing with escalating doses in combination with rituximab (NCT01307267) and pembrolizumab (NCT02179918). In the phase I trial, a number of patients with virally induced Merkel carcinomas are being treated with evidence of objective clinical activity, and no signs of hepatotoxicity have been observed in the dose levels reported thus far [82, 83]. Clinical responses suggest some degrees of at least additive clinical efficacy with pembrolizumab (ASCO 2016) that warrant expansions to explore combined immunotherapy strategies.

26.4 OX40 Co-stimulation

OX40 (also known as CD134 or TNFRSF4) is a type 1 transmembrane protein that belongs to the TNFR family and was first discovered in 1987 on the surface of activated $CD4^+$ T cells in rats [84]. The original publication showed that an anti-OX40 antibody (MRC OX40) could increase T-cell proliferation [84], and subsequent studies demonstrated that OX40 is expressed on $CD4^+$ and also on $CD8^+$ T cells (albeit at lower levels) 24–72 h following TCR engagement [85, 86]. Treg cells constitutively express OX40 in mice [87], but in human Treg cells, OX40 expression is upregulated upon activation [86]. Other cells belonging to the innate immune system such as NK, NKT cells, or neutrophils can express OX40 as well [88]. Stimulation in these cells via OX40 has shown a pro-inflammatory and pro-survival effect, suggesting that OX40 modulation contributes not only to adaptive but also to innate immune responses [88, 89].

OX40 ligand (OX40L, CD252) is predominantly expressed on activated APCs, but other hematopoietic (NK, mast, and activated T cells) and non-hematopoietic cells (smooth muscle, vascular endothelial cells) can express OX40L as well [90, 91]. OX40 ligation is known to recruit TRAF2 and TRAF3 to the intracellular domain of OX40, leading to activation of both the canonical and noncanonical NF-κβ pathways which ultimately induce the expression of pro-survival molecules and increase cytokine production associated with enhanced T-cell expansion, differentiation, and the generation of long-lived memory cells [92, 93]. The importance of noncanonical pathway needs further studies, comparing OX40 co-stimulation properties with other TNFR members.

The co-stimulatory nature of OX40 has been confirmed in autoimmune mouse models where blocking OX40/OX40L interactions diminishes the clinical signs of autoimmunity [94]. By contrast, overexpression of the OX40L in transgenic mice leads to increased signs of autoimmunity [95]. Additionally, in patients, OX40L expression is upregulated in hosts with autoimmune diseases, and the majority of OX40L expression appears to be confined to the lesions, suggesting that OX40L expression is the limiting factor in regard to OX40 signaling in T cells [96]. Based on these findings, and preclinical evidence that OX40 engagement can expand T-cell populations and increase effector functions, OX40 agonists (mAbs and soluble forms of OX40L) have been investigated for cancer immunotherapy.

In preclinical tumor models, OX40 monotherapy with agonist mAbs was shown to be effective in eradicating primarily immunogenic tumors including MC303 sarcoma, CT26 colon carcinoma, SM1 breast cancer, and small B16 melanoma, among others [97–99]. However, OX40 failed to provide adequate antitumor immunity in poorly immunogenic tumors [96]. Therefore, different combinatorial strategies have been explored to increase OX40 agonist antitumor efficacy. In line with this, combinations with cytokines such as interleukin 12 (IL-12) or IL-2 have been successfully tested with or without vaccination [100, 101]. Furthermore, based on the experience that stimuli signaling through Toll-like receptors (TLR) induce OX40L expression on APCs, combination of TLR agonists with OX40 co-stimulation-based strategies has demonstrated synergistic effects [102, 103]. In addition, anti-OX40 antibodies have been combined with other immunostimulatory monoclonal antibodies to treat lymphomas, sarcomas, hepatic colon metastasis, and spontaneous hepatocellular carcinoma [55, 104–106]. Combining OX40 agonist with cancer vaccines, chemotherapy or

radiation has also demonstrated an improvement in control of established tumors [107, 108] and enhanced expansion and prolonged survival of tumor-specific T cells [109].

It is believed that one of the main advantages of targeting OX40 is the abrogation of Treg-mediated immunosuppression by inhibition of the activity of Treg cells [110] or by direct depletion of these immunosuppressor cells [87, 111, 112]. Thus, combination of anti-OX40 mAbs with cyclophosphamide (chemotherapy that is capable of partially depleting Treg cells) showed a synergistic effect in a poorly immunogenic B16 murine melanoma tumor and was associated with a reduction of Treg at the tumor site [107]. Another mechanism reported to be associated with OX40 stimulation is the ability of OX40 engagement to trigger cytolytic activity in $CD4^+$ T cells. Data in preclinical models show that anti-OX40 mAb is synergistic in combination with anti-CD137 [113] or in combination with cyclophosphamide and adoptive T-cell therapy [114], resulting in an increase in cytotoxic activity and in a preferential Th1 polarization.

Clinical development of OX40 agonists started in 2006 with a murine antihuman OX40 (anti-hOX40) mAb. This murine antibody was first tested in nonhuman primates [115], and subsequently 30 patients with solid tumors received three doses (days 1, 3, and 5) from 0.1 to 2 mg/kg in the first-in-human clinical trial [116]. The treatment was very well tolerated, and no maximum tolerated dose was identified. Even though slight tumor size reductions were observed in some patients, none of the patients showed an objective response by RECIST criteria. Elevated levels of neutralizing human anti-mouse immunoglobulin antibodies were considered the most important limitation in this clinical trial. Nonetheless, combinations of the murine anti-hOX40 with chemotherapy and/or radiotherapy in clinical trials are currently ongoing in different solid tumors (Table 26.1). The obvious next step was the development of fully human OX40 agonists to allow repeated dosing in cancer patients. Human Fc:OX40L has demonstrated promising results in preclinical development [117], and clinical testing may begin in the near future. Two humanized anti-OX40 mAbs, MEDI6383 (AgonOx, AstraZeneca) and MOXR0916 (Genentech, Roche), are now undergoing clinical development (Table 26.1) as single agents and in combination with PD-1 and PD-L1 blocking agents.

26.5 GITR

Glucocorticoid-induced TNFR-related protein (GITR, TNFSFR18, CD357) belongs to the TNFR family. It was originally discovered in murine T-cell hybridomas treated with dexamethasone [118] thus explaining its name. Further studies concluded that glucocorticoid treatment has no effect on GITR expression in humans (and is not necessary in mice). In contrast, its expression is induced after TCR engagement in $CD4^+$ and $CD8^+$ T cells [119, 120]. GITR is expressed at low levels on resting $CD4^+$ and $CD8^+$ T cells, and it is upregulated 24–72 h after TCR engagement and remains expressed on the lymphocyte surface for several days. In contrast, Treg cells constitutively and brightly express GITR [121], where it is thought that GITR exerts an inhibitory activity on Treg suppressive functions [122]. Additionally, GITR expression has been found on NK cells, eosinophils, basophils, macrophages, and B cells, particularly upon activation [123].

GITR ligand (GITRL), as was the case with OX40L, is highly expressed on activated APC and endothelial cells. Upon ligation, GITR downstream signaling is exerted through a complex consisting of a single TRAF5 and two TRAF2 proteins, leading to NF-κβ and MAPK pathway activation [124]. GITR-mediated co-stimulation ultimately enhances T-cell proliferation and effector functions in part because of upregulation of IL-2Rα, IL-2, and IFNɣ [120, 125]. GITR ligation protects T cells from activation-induced cell death (AICD), leading to an increase in memory T cells. Initial studies demonstrated that GITR ligation could potentially overcome tolerance to self- and tumor-antigens, making it an attractive target for the development of cancer immunotherapies [126]. Subsequently, an antitumor effect of GITR stimulation has been demonstrated in different tumor models with an agonist anti-mGITR antibody

Table 26.1

Target	Name/alternative names (company)	Clinical trials.gov identifier	Phase	Disease	Single agent/combination
CD137	Urelumab/BMS-663513 (Bristol-Myers)	NCT00309023	I–II	Metastatic or locally advanced solid malignancies	Monotherapy
		NCT00351325	I	Advanced solid malignancies	With chemotherapy
		NCT00612664	II	Stage III–IV melanoma (2° line)	Monotherapy
		NCT00461110	I	Non-small cell lung cancer	With chemoradiation
		NCT00803374	I	Unresectable stage III–IV melanoma	With ipilimumab
		NCT02652455	Pilot clinical	Metastatic melanoma	With nivolumab and ACT[a]
		NCT02658981	I	Recurrent glioblastoma	Monotherapy or combination with anti-PD-1
		NCT01775631	I	B-cell malignancies	With rituximab
		NCT02420938	II	Relapsed, refractory, or high-risk untreated patients with chronic lymphocytic leukemia. Small lymphocytic lymphoma	With rituximab
		NCT02845323	II	Patients with cisplatin-ineligible muscle-invasive urothelial carcinoma of the bladder	With nivolumab
		NCT02110082	I	Colorectal cancer and head and neck cancer	With cetuximab
		NCT02252263	I	Multiple myeloma	With elotuzumab
	Utomilumab/PF-05082566 (Pfizer)	NCT02951156	Ib/III	Diffuse large B-cell lymphoma	With avelumab/rituximab/azacitidine
		NCTO1307267	I	Advanced solid tumor/ lymphoma	Monotherapy (solid tumors) and with rituximab (lymphomas)
		NCT02179918	I	Advanced solid tumor	With pembrolizumab

Target	Name/alternative names (company)	Clinical trials.gov identifier	Phase	Disease	Single agent/combination
OX-40	MEDI6469 (MedImmune)	NCT01644968	I	Advanced cancer	Combination with KLH[b] or tetanus vaccine
		NCT01303705	Ib	Progressive or metastatic prostate cancer after systemic therapy	With cyclophosphamide and radiation
		NCT01689870	I/II	Unresectable or metastatic melanoma	With ipilimumab
		NCT01416844	II	Metastatic melanoma	Monotherapy
		NCT02274155	Ib	Locoregionally advanced, oral head and neck squamous cell carcinoma	Monotherapy
		NCT02559024	I/IB	Metastatic colorectal cancer	With surgery/RFA[c] of metastases
		NCT01862900	I/II	Progressive metastatic breast cancer	With stereotactic body radiation therapy
		NCT02205333	Ib/II	Selected advanced solid tumors or aggressive B-cell lymphomas	Monotherapy or combined with durvalumab, tremelimumab, or rituximab
	MEDI6383 (AstraZeneca)	NCT02221960	I	Selected locally advanced or metastatic carcinomas	Alone and in combination with MEDI4736
	MOXR0916 (Roche)	NCT02410512	Ib	Metastatic or locally advanced solid malignancies	Atezolizumab with or without bevacizumab
	PF-04518600 (Pfizer)	NCT02315066	I	Selected locally advanced or metastatic carcinomas	Alone and in combination with utomilumab
	INCAGN01949 (Agenus)	NCT02923349	I/II	Advanced or metastatic solid tumors	Monotherapy
	GSK3174998 (GlaxoSmithKline)	NCT02528357	I	Selected advanced solid tumors	Monotherapy or with pembrolizumab
	MEDI0562 (MedImmune LLC)	NCT02705482	I	Selected advanced solid tumors	Combination with tremelimumab or durvalumab

(continued)

Table 26.1 (continued)

Target	Name/alternative names (company)	Clinical trials.gov identifier	Phase	Disease	Single agent/combination
GITR	TRX518/Anti-TNFRSF18 (Tolerx)	NCT01239134	I	Unresectable stage III or stage IV malignant melanoma or other solid tumor malignancies	Monotherapy
	MEDI1873 (MedImmune LLC)	NCT02583165	I	Selected advanced solid tumors	Monotherapy
	GWN323 (Novartis)	NCT02740270	I/Ib	Advanced solid tumors and lymphomas	Monotherapy or combination with anti-PD-1
	INCAGN01876 (Agenus)	NCT02697591	I/II	Advanced or metastatic solid tumors	Monotherapy
CD27	Varlilumab/CDX-1127 (Celldex Ther.)	NCT02335918	I/II	Squamous cell carcinoma of the head and neck, ovarian carcinoma, colorectal cancer, renal cell carcinoma, glioblastoma	With nivolumab
		NCT01460134	I	Selected refractory or relapsed hematologic malignancies or solid tumors	Monotherapy
		NCT02270372	Ib	Advanced ovarian cancer or breast cancer	With ONT-10
		NCT03038672	II	Relapsed or refractory aggressive B-cell lymphomas	With nivolumab
		NCT02413827	I/II	Patients with unresectable stage III or stage IV melanoma	Combination with ipilimumab and CDX-1401
		NCT02386111	I/II	Metastatic clear cell renal carcinoma	Combination with sunitinib
		NCT02924038	Pilot study	WHO grade II low-grade glioma	IMA950 vaccine plus poly-ICLC

[a]*ACT* adoptive cell therapy
[b]*KLH* keyhole-limpet hemocyanin
[c]*RFA* radiofrequency ablation
[d]*ONT-10* a liposomal synthetic glycopolypeptide MUC1 targeted antigen formulated with PET lipid A adjuvant

(rat monoclonal DTA-1) or GITRL manipulation. DTA-1 has been shown to be effective in treating 8-day established Meth-A sarcomas [127], CT26 [128], and small-established B16 tumors [129]. Additionally, GITR agonists have demonstrated a synergistic antitumor effect when combined with vaccines [129, 130], TLR agonists [131], and other immunostimulatory monoclonal antibodies such as anti-CTLA-4 [132]. Recombinant forms of GITRL have also been successfully employed by means of DCs engineered to secrete a soluble GITRL fusion protein [133].

Different mechanisms might contribute to the antitumor effects of GITR modulation. Perhaps the best defined is the action of GITR agonists in the abrogation of Teff cell suppression by Treg cells. Some reports have shown that GITR agonists increase Teff function in the tumor microenvironment by directly targeting Treg cells impairing Treg expression of FoxP3 and consequently abrogating Treg suppressive function [129] or by targeting antigen-specific $CD8^{+}$T, augmenting its resistance to Treg suppression [134, 135]. More recently it has also been reported that anti-GITR mAb (DTA-1) might directly deplete intratumor Tregs by activating myeloid cells through FcγRs as part of its mechanism [136]. In support of this hypothesis, most of the studies find increases in Teff/Treg ratios at the tumor site after treatment with DTA-1 that correlate with therapeutic benefit [129, 134, 135].

Several humanized agonist antihuman GITR mAbs are in clinical development (Table 26.1). TRX518 has been developed by Tolerx Inc. (now GITR Inc.), and a dose-escalation phase I clinical trial is ongoing this mAb at Memorial Sloan Kettering Cancer Center and other centers. More antibodies on the same specificity have also started clinical trials INCAGN01876 (Incyte) and MEDI1873 (Medimmune), and results are eagerly awaited (Table 26.1).

26.6 CD27

This molecule belongs to the TNFR family. A unique feature of CD27 among TNFR family members is its constitutive expression at significant levels on the majority of T cells (chiefly including naive T cells) [137, 138]. CD27 is also expressed on plasma cells, for which it is broadly accepted as a marker [139]. Due to the constitutive expression pattern of CD27, the expression of its only known ligand, CD70, has to be tightly regulated. In fact, CD70 is only transiently expressed on activated APC, T cells, and NK cells under physiological conditions [40, 140]. However, constitutive expression of CD70 has been documented during chronic inflammation [141].

The use of CD27 as a target in cancer immunotherapy is compromised by the inhibitory and co-stimulatory mechanisms related to the CD27-CD70 pathway in the different immune contexts. CD27 ligation by CD70 recruits TRAF2 and TRAF5 to the intracellular domain of CD27, activating the NF-κβ and c-Jun pathways, and, as a consequence, promotes cell survival, enhances T and B cell expansion, and increases effector functions [142]. By contrast, CD27 signaling has also been reported to lead to T-cell dysfunction in a context of a CD70 continuous expression, which can happen in the context of a chronic infection such as in the LCMV chronic infection model, in which blocking of the CD70/CD27 interaction has been shown to help to eradicate the infection [141].

Constitutive expression of CD70 has been documented in cancer [143]. It has been observed that constitutive CD70 expression on tumors or APCs improves antitumor immunity in murine lymphoma models, enhancing NK-mediated rejection [144]. In line with this observation, the administration of an agonistic anti-CD27 antibody has been shown to protect against intravenous injection of two different lymphoma cell lines [145] and to delay the growth of B16 subcutaneous melanoma [146]. However, intact CD27/CD70 signaling has been associated with decreased antitumor immune response and an increase in intratumoral Treg cells [147].

All in all, it seems that the effects of CD27 may depend on both the tissue context in which CD70 is expressed and also the duration of CD27-CD70 ligation. Clinical development of CD27-related agents will require a precise trig-

gering of certain molecules in selected environments to avoid exaggerating tumor-induced immunosuppression. A fully human agonist anti-CD27 monoclonal antibody, CDX-1127 (Celldex Therapeutics, Inc., Needham, MA, USA), is being evaluated in clinical trials in selected hematological and solid tumors (Table 26.1).

26.7 Future Perspectives

The clinical success of ipilimumab [148], anti-PD1 [149, 150], and anti-PD-L1 [150–152] mAbs has validated the principle that modulation of the immune response can overcome immune evasion mechanisms of tumors and produce objective antitumor responses. However, different mechanisms to evade immune attack can be developed by each patient's tumor, and now the challenge is to determine which mechanism is dominant and which is the most suitable individualized immunotherapy. Clinical experience has revealed that not all patients are sensitive to the CTLA-4 blockade or PD-1/PD-L1 pathway blockade. Elucidating the mechanisms that explain this primary resistance [153] and achieving a better understanding of the heterogeneity among patients' tumor immune infiltration are essential if we are to implement personalized medicine and establish logical rationales for combination in the cancer immunotherapy arena.

Combination of blocking co-inhibitory molecules (so called checkpoint blockade) and co-stimulatory mAbs seems to be the next most promising approach following CTLA-4 and anti-PD-1 combinations [154]. In line with this, clinical trials combining anti-PD-1 and anti-CD137 have been conducted (NCT02253992 and NCT02179918). Because CD137 is expressed on activated NK cells through FcγRs-Fc binding, the combination of anti-CD137 mAbs with anti-EGFR, anti-CD20, or anti-HER-2 mAbs has been successfully tested in mouse tumor models, and phase I clinical trials in lymphoma, head and neck, and colorectal cancer patients are ongoing (NCT01307267, NCT01775631). Combinations of different TNFRSF members have been successfully tested in preclinical models [55, 113], but concerns exist regarding translation of this approach to the clinical setting due to the toxicity profile of agonist immunostimulatory monoclonal antibodies (ISMAB) [155]. Thus, different strategies focusing on more specific delivery of the antibodies to the tumor site or intratumoral administration in accessible lesions [156–159] are being proposed to overcome this limitation. Agonist monoclonal antibodies targeting co-stimulatory receptors of T and NK cells are definitively part of the new wave of immunotherapies, which are currently changing treatment of cancer paradigms.

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The Impact of the Intestinal Microbiota in Therapeutic Responses Against Cancer

27

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27.1 Introduction

Only recently, the scientific community gained insights on the importance of the intestinal resident microflora for the host's health and pathophysiology [1–3].

The gut microflora exerts a variety of different fundamental functions, such as the degradation of nutrients to provide energy source, the elimination of xenobiotics, the education of the immune system, the growth and differentiation of epithelial cells of the barrier, the intestinal peristaltism, and the production of antimicrobial peptides to eradicate pathogens and ensure colonization resistance [4]. The intestine represents the largest compartment of the immune system. It is exposed to food and commensal antigens that our body needs to tolerate and is the portal of entry for many pathogens. The numbers of bacteria increase going down the gastrointestinal tract, with up to 10^{12} per ml in the colon. Aerobic species are prevalent in the upper small intestine, whereas anaerobic bacteria dominate in the colon. *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* are the most prevalent, together with members of the *Archaea* kingdom [4].

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Gut microbiota plays a crucial role in modulating innate and acquired immune responses and thus interferes with the delicate balance between inflammation and tolerance. Recent advances in sequencing technologies allowed the deep characterization of the human microbiota, thus greatly improving our knowledge on the role of the microbiome in human health and disease. Human microbiome project consortium studies (MetaHIT and HMP) demonstrated that healthy individuals have not only a high degree of bacterial diversity, dependent on their habitat (intestine, oral cavity, skin, or vagina), but that there is also a remarkable interindividual variability at the level of species [5, 6]. Despite the species diversity found at various locations, there is a certain constancy that preserves both the function and the bacterial gene profiling associated to specific tissue sites. Hence, the anaerobic *Firmicutes/Bacteroidetes* spp. dominate the intestine, whereas *Actinobacteria* and *Proteobacteria* spp. are highly abundant in the skin [7]. Defining the "normal microbiome" is a difficult task, considering that the same microbe may behave as commensal or as pathogen depending on the dietary components, the nutritional milieu, the genetic background of its host, and a potential coinfection. Nonetheless, "commensal" bacteria are expected to contribute to immune homeostasis, whereas "pathobionts" are associated with immune reactivities against themselves [8]. Perhaps paradoxically, it is possible to find in serum of healthy subjects antibodies specific to commensals as well as circulating T cells able to react against nonpathogenic bacteria [9–11].

Gut colonization at birth is indispensable to trigger the maturation of the mucosal immune system. At this phase, the intestinal microbiota plays a crucial role in setting up innate and acquired immune responses "imprinted" for life, interfering in the balance between inflammation, infection, and tolerance. Germ-free mice have hypoplastic Peyer's patches, lack isolated lymphoid follicles in the intestine, and have low levels of IgM natural antibodies in the serum, this phenotype being reverted by gut colonization [12]. Undoubtedly, colonization of the distal small intestine by segmented filamentous bacteria (SFB) is crucial for the development of resident lamina propria dendritic cells secreting IL-6 and IL-22 and the concomitant differentiation of Th17-T regulatory (Treg) cells in the newborn gut [13]. The discovery of a link between defined members of the microbiota (such as *Bacteroides fragilis* and TLR2) and the induction of Treg cells by *Clostridiaceae* generated a huge interest. However, the homeostasis of the colonic Treg compartment relies more on the synergistic effect of different bacterial strains and an interkingdom ecosystem creating a balanced microenvironment able to sustain the generation and maintenance of the anti-inflammatory milieu [14–16].

27.2 Evaluating Gut Dysbiosis in Cancer Bearers

Our knowledge of the genetic and functional diversity in gut microbes is far from being complete. Old methods consisting in qPCR or FISH analyses were neither comprehensive, specific, nor sensitive enough. The burst of recent knowledge emerged from the targeted 16S rRNA gene pyrosequencing followed by metagenomic shotgun sequencing, a costly method with heavy data mining. The catalog of reference genes in the human gut microbiome was reported, gathering data from MetaHIT, Human Microbiome Project (HMP), and a large Chinese diabetes study, as well as >500 sequenced genomes of gut-related bacteria and archaea. This nonredundant reference catalog of 9,879,896 genes is freely accessible through the website (http://meta.genomics.cn), and the data are deposited in the GigaScience Database. We may have reached saturated coverage of core gene content and functions. Of note, the number of genes present in more than 50% of the subjects remained below 300,000, pointing out the dominance of individual-specific genes. The individual-specific genes are enriched in the categories cell wall/membrane/envelope biogenesis and DNA replication, recombination, and repair. The common genes are enriched in functions such as signal transduction mechanism,

energy production, carbohydrate transport and metabolism, and amino acid transport and metabolism [17].

Several groups investigated the gut microbiota in healthy individuals and in cancer bearers to establish microbe-cancer relationships. For example, Goedert et al. analyzed gut microbiota using 16S rRNA sequencing in postmenopausal women. The authors observed a lower alpha diversity (number of different taxa) and altered beta diversity (between-subject community composition) in subjects suffering from a breast cancer, when compared to healthy individuals.

Dejea et al. analyzed colorectal mucosa from subjects bearing or not colorectal cancer (CRC), using FISH and 16S rRNA sequencing. They demonstrated that bacterial biofilm presence, but no consistent bacterial genus, was associated with colorectal cancers [18]. Other studies, using 16S rRNA or metagenomic sequencing, revealed case-control discrepancies in the gut microbiota composition, showing that feces from CRC subjects display higher prevalence and levels of *Fusobacterium* and *Porphyromonas* compared to controls [19, 20]. However, across studies many differences were observed in taxa that diverged between cases and controls. This could be related to characteristics of the population studied (diet, medication) as well as to setting parameters (experimental methods or case-control matching). Furthermore, in complex ecosystems, current metagenomic studies are unable to detect bacteria at concentrations below 10^5 bacteria/gram. Moreover, only 15% of identified species were concomitantly detected by the culturomics and the metagenomics, highlighting the complementarity between culture-dependent and culture-independent methods. The diversification of culture conditions coupled with the identification of the bacterial repertoire by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry [MALDI-TOF MS] enabled significant progresses in taxonogenomics. These efforts will help defining "cancer-associated gut fingerprints" in the near future in different cohorts of cancer types to establish the impact of cancer topography on gut dysbiosis.

27.3 Microbiome and Cancer Incidence

Commensal microbial communities inhabiting the intestine, as well as other places in the body, appear to play an unappreciated role in intestinal and extraintestinal carcinogenesis by providing yet to be characterized environmental signals [21]. Pioneering studies performed in germ-free, gnotobiotic, or antibiotic-treated rodents revealed an unsuspected role for commensals in tumorigenesis—irrespective of the role of inflammation. In the genesis of colon cancer or hepatocarcinoma, microbes can be direct transforming agents [22, 23] by providing a toxic metabolite, an oncogenic product, or by inducing an inflammatory milieu which will culminate in genomic instability and/or DNA damage response and/or immune escape [24–26]. Commensals can also form cooperative biofilms that facilitate cross-feeding or cross-metabolism, redefining the cancer landscape [18, 27]. Recently, the development of extraintestinal (breast and ovarian) neoplasia was linked to TLR5-mediated, IL-6- or IL-17-driven systemic inflammation provoked by intestinal microbes [28]. In contrast, other observations support a beneficial role for bacteria in combatting cancer. Prolonged antibiotic treatment with a combination of metronidazole and ciprofloxacine subsequently tripled breast cancer (BC) incidence in proto-oncogene HER2/neu-driven transgenic mice [29]. In humans, epidemiological studies suggested a dose-dependent association between antibiotic use and risk of BC [30]. Antibiotic use disrupted the intestinal microfloral metabolism of estrogens [31, 32]. Knekt et al. first reported an epidemiologic study on the association of antibiotic uptake and BC incidence. Women <50 who self-reported previous and/or present antibiotic use for urinary tract infections but did not harbor a bacteriuria had an elevated risk of breast cancer, compared with women without this usage [33].

By extension, associations between antibiotic exposure and cancer risk were reported in various cancers [34–36]. In contrast, the use of antivirals or antifungals did not affect the cancer risk [37]. Multiple studies reported an association between

an altered composition of local microbiomes at portals of entree and cancer occurrence (such as oral in head and neck cancers [38], bronchial in NSCLC [39], intestinal and colorectal malignancies [40] and vaginal in cancer of the cervix [41]).

These studies are often flawed or biased by (a) the lack of rigorous methodologies (old methods of PCR versus 16S pyrosequencing analyses versus deep sequencing (metagenomics) versus culturomics), (b) overlooking lifestyle factors (such as social milieu, sedentary way of life, malnutrition, alcoholic or smoking behavior, etc.) that may modify the microbiome and increase cancer risk independently from each other, (c) the lack of a validation cohort (according to the REMARK criteria), and (d) the lack of cause-effect relationship between microbial dominance and the underlying pathophysiology. The current momentum for the impact of the gut microbiome in cancer will reinvigorate clinicians to launch prospective studies evaluating the impact of ATB uptake, as well as nutrition, genetic, and environmental factors in driving or influencing the course of oncogenesis processes.

27.4 Radiotherapy and Microbiota

The effects of radiotherapy on the microbiome and cancer immunotherapy have not been studied extensively. Most studies to date address radiation-induced bowel injury and its modulation by the host microbiome.

Crawford et al. reported first that the intestinal microbiome is an important modifier of radiation-induced intestinal injury, showing that germ-free animals are more resistant to radiation enteritis [42]. Microbial composition in mice was significantly altered by radiation. Modulation of the intestinal microbiome by fecal microbial transplantation (FMT) after whole-body irradiation increased survival and partly restored intestinal epithelial integrity through stimulation of angiogenesis [43, 44]. TLR signaling may play an important role in intestinal radioprotection. Ciorba et al. showed that *Lactobacillus* administration in a preclinical model of radiation-induced intestinal injury exerted protective effects through TLR2 and migration of Cox2-expressing mesenchymal stem cells [45]. Several clinical studies reported the modification of the intestinal microbiota post-radiation-induced intestinal injury [46–48]. Wang et al. showed that pelvic irradiation induced the release of inflammatory markers in the intestine of patients, associated with asthenia. Low microbial intestinal diversity also predicted radiation-induced colitis development [49]. Demers et al. showed that the administration of *Bifidobacterium/Lactobacillus* significantly lowered diarrhea [50]. However, no single bacterial strain was consistently shown to be beneficial in radiation-induced intestinal injury.

The beneficial role of the microbiome in the efficacy of radiotherapy remains to be demonstrated. There is good rationale to believe that disruption of the mucosal barrier facilitating the translocation of mucosal microbiota may activate the innate arm of immunity [51]. This was first reported by Paulos et al. showing that total body irradiation promoted a LPS-TLR4-dependent activation of antigen-presenting cells facilitating the efficacy of adoptive T cell transfer [52]. Radiation-induced intestinal injuries promoted the translocation of commensals and the release of LPS resulting in the priming of dendritic cells and activation of adoptively transferred T cells. This was correlated with the treatment efficacy and with the long-term cure of mice harboring melanoma B16F10 tumors. Thereby, modulating the homeostatic balance between the gut microbiota and the immune system could enhance cell-based tumor immunotherapy. Conversely, a subsequent study by Espinosa-Carrasco showed that LPS translocation only partly accounts for the effect seen by Paulos et al. [53]. To date, there is no data supporting the role of microbiome components as response modifiers in radio-immunotherapy.

27.5 Cyclophosphamide and Gut Microbiota

It is well known that chemotherapy can induce mucositis as well as neutropenia, two major side effects that could lead to the use of antibiotics

and result in dysbiosis [54, 55]. The question of the impact of the gut microbiota on the efficacy on chemotherapeutic agents, and especially those capable of affecting the intestinal homeostasis, was addressed [56, 57]. The efficacy of cyclophosphamide (CTX), a DNA-alkylating agent belonging to the family of nitrogen with immunomodulatory and anti-angiogenesis properties currently used in clinics for the treatment of breast cancer, sarcoma, and pediatric malignancies [58], depends on its ability to induce the translocation of selective Gram-positive bacteria niching in the small intestine such as *Enterococcus hirae* or *Lactobacillus johnsonii* into secondary lymphoid organs. Indeed, CTX is responsible for disrupting the gut barrier integrity as well as intestinal homeostasis (both epithelial and immune compartments). Patients treated with CTX have a weaker intestinal barrier which breaks the tolerance toward the intestinal microbiota and leads to its immunization against some bacterial strains. This immunization is composed of $CD4^+$ effector lymphocytes called "pathogenic Th17" producing IFNγ and IL-17, which helps tumor-infiltrating Th1 lymphocytes to control the tumor growth in mice. Interestingly, broad-spectrum antibiotics as well as vancomycin (which mainly kills Gram-positive bacteria) and colistin (which mainly eliminates Gram-negative bacteria) all compromised the polarization of pTh17 in the spleen and the full-blown anticancer activity of CTX in vivo in P815 mastocytoma- and MCA205 sarcoma-bearing mice, supporting the notion that the efficacy of cyclophosphamide was microbiota related [59]. Further study showed that among the translocating Gram-positive bacteria, *E. hirae* induces Th17 and Th1 $CD4^+$ T lymphocytes and stimulates tumor-specific $CD8^+$ T cells, as it reduces immunosuppressive intratumoral Tregs and IL-17-producing γδ T cells (Fig. 27.1). Mono-association of antibiotic-treated mice with *E. hirae* greatly

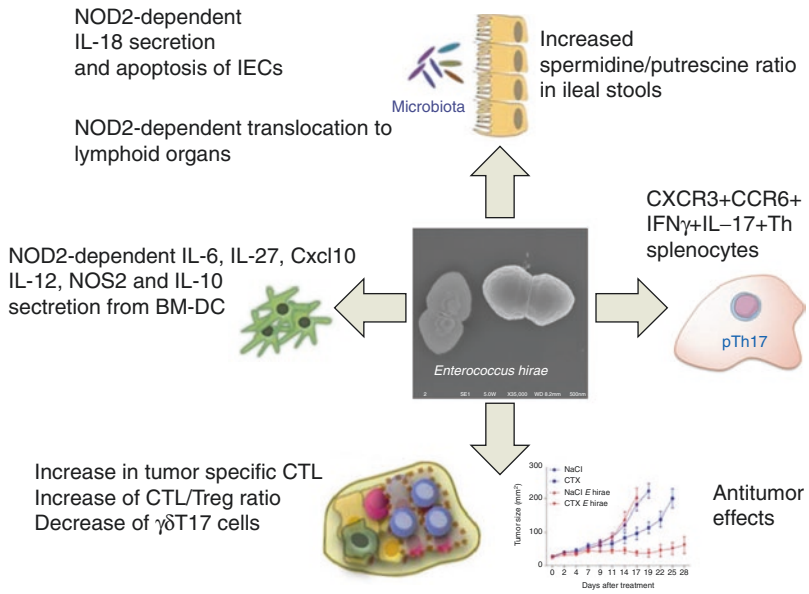

Fig. 27.1 The immunogenicity of *E. hirae*. *E. hirae* resides in the jejunum and colon in about 25% individuals. It is very dominant in mouse guts at Gustave Roussy Villejuif. This *Enterococcus* is not pathogenic and benefits from cyclophosphamide-mediated increased gut permeability to translocate in secondary lymphoid organs, prime specific pathogenic CXCR3 + CCR3+ T cells that traffic to tumor lesions, and reprogram the tumor microenvironment, i.e., decreasing regulatory T cells and γδT17 cells and enhancing cancer-specific CTL responses. *E. hirae* also acts on the intestinal epithelial cells, modulating apoptosis and IL-18 secretion in a NOD2-dependent manner. *E. hirae* is recognized by bone marrow dendritic cells to induce IL-12 and IL-27 production in a NOD2-dependent fashion. All these properties participate to its adjuvant effects to boost cyclophosphamide-mediated immunostimulatory capacities and anticancer efficacy

improved tumor growth reduction by CTX, and this effect was blocked by antibodies depleting $CD8^+$ T cells or neutralizing IFNγ, indicating that it requires an anticancer immune response. In parallel, we have demonstrated that the antitumor efficacy of CTX was greatly ameliorated in mice presenting a defect in NOD2 expression in intestinal epithelial cells, demonstrating the role of NOD2 as a gatekeeper and a gut immune checkpoint restricting the immunogenicity of *E. hirae*. The characterization of the gut microbiota in NOD2 animals highlighted that Gram-negative bacteria, from the *Porphyromonadaceae* family, dominated the colon, genus *Barnesiella.* Mice that were mono-associated with *Barnesiella intestinihominis* developed high numbers of polyfunctional $CD4^+$, $CD8^+$, or γδT cells, in the spleen and the tumor bed. *B. intestinihominis*-fed mice contained more abundant IFNγ-producing γδ T cells at the expense of immunosuppressive IL-17-producing γδ T cells compared with non-fed (or *E. hirae*-fed) animals. Moreover, CTX combined with *B. intestinihominis* reduced the growth of transplantable cancers (RET melanoma, MCA205 sarcoma, MC38 colon cancers) in mice through a pathway requiring $CD8^+$ T cells and IFNγ, but not IL-17. Finally, *E. hirae* and *B. intestinihominis*-specific memory Th1 cell immune responses selectively predicted longer progression-free survival in advanced lung and ovarian cancer patients treated with chemo-immunotherapy. At present, it is not known whether *E. hirae* can synergize with *B. intestinihominis* with respect to its tumor growth-blocking activity [60].

Therefore, part of the immunomodulatory effects of the most well-known immunomodulator (CTX) require a functional microbiome, at least a Gram-positive small intestine residing *Enterococcus* and a colon residing *Barnesiella*.

27.6 Platinum Salts and Gut Microbiota

The demonstration of the importance of the gut microbiota on the efficacy of anticancer agents was also reported by the Dr. Giorgio Trinchieri's group. Iida et al. demonstrated that bacteria accounted for the ROS-mediated antitumor effects of tumor-infiltrating myeloid cells during platinum-based anticancer therapies [61]. Indeed, the authors showed that the efficacy of platinum salts (oxaliplatin, cisplatin) relied on the production of reactive oxygen species (ROS) by intratumoral myeloid cells (myeloid suppressor cells, monocytic and immature dendritic cells) through a mechanism involving MyD88. ROS induce DNA damage culminating in DNA adduct formation and apoptosis of tumor cells and account for part of the tumoricidal activity of oxaliplatin against MC38 or EL4 tumor-bearing mice. Interestingly, the efficacy of chemotherapy was abrogated in germ-free or ATB-treated mice compared to SPF animals. Next, the authors investigated the impact of ATB treatment on oxaliplatin-mediated ROS production. The expression of Cybb encoding reactive oxygen species (ROS)-generating nicotinamide adenine dinucleotide phosphate oxidase (NOX2) was attenuated by the ATBs [61]. Furthermore, microbial compensation of ATB-treated mice with *Lactobacillus acidophilus* restored efficacy of cisplatin-based chemotherapy and reestablished inflammatory gene expression related to cisplatin functions [62]. Altogether, these results support the notion that the gut microbiota impacts on the redox equilibrium of the tumor microenvironment, affecting the therapeutic effects of platinum salts currently used in clinic.

27.7 Gut Bacteria and the Efficacy of Immuno-oncological Compounds

Oncologists no longer consider to treat cancer by only targeting tumor cell clones but also by mobilizing the immune system and eliciting long-term memory T cell responses protecting against the minimal residual disease. Immune checkpoint blockade (ICB) became the backbone of cancer treatment modalities, but primary resistance to ICB concerns about 70% of all comers, suggesting that T cell responses culminating in T cell

infiltration of tumor lesions required for tumoricidal effects are quite a rare event. The immunity of a person is influenced by a complex set of factors, i.e., host, tumor, and environmental cues as well as prior histories of distinct therapies, that govern the threshold and timing of anticancer immune responses. Several lines of evidence point out to the critical role of the microbiome in dictating the "cancer immune set point" of a person, i.e., the threshold beyond which an immune response will ensure. Several groups indeed highlighted the role of distinct commensals in determining the antitumoral efficacy of various types of immunotherapeutics.

After administration of anti-interleukin-10 receptor (IL-10R) plus cytosine-phosphate-guanosine oligodeoxynucleotides (CpG-ODN) TLR9 agonists, *Alistipes shahii* was found overrepresented in the feces of colon cancer-bearing mice. Upon mono-association of previously sterilized mice with *A. shahii*, the immunotherapeutic response of subcutaneous colon cancers was improved, as compared to sterile mice. In this model, *A. shahii* led to an increase of TNFα production by intratumoral myeloid cells, and neutralization of TNFα abolished the therapeutic effect. Thus, *A. shahii* impact innate immune effectors of the myeloid lineage, reshaping the tumor microenvironment to improve the outcome of immunotherapy [61].

Other studies showed that the efficacy of immune checkpoint blockers (ICB), aimed at reactivating tumor-infiltrating T cells, is also dependent on the gut microbiota. Ipilimumab, a monoclonal antibody against cytotoxic-T-lymphocyte-associated protein 4 (CTL4-4), induced considerable improvement in the overall survival of patients with metastatic melanoma with up to 18% long-term control of the disease [63, 64]. The antitumoral efficacy of ipilimumab was abrogated in ATB-treated SPF mice or in germ-free animals bearing many types of transplantable tumors. Vétizou et al. showed that non-enterotoxin-producing strains of *B. fragilis* have anticancer properties in the context of immunotherapy with CTLA4 blockade. After neutralization of this immune checkpoint with specific monoclonal antibodies, *Bacteroidales* representation in the ileums and feces was affected, and *B. fragilis* spp. could take over, associated with DC migration and activation in the mesenteric lymph nodes and class II-restricted T cell memory responses against *B. fragilis* antigens. *B. fragilis*-mediated immune responses post-CTLA4 blockade were IL-12 dependent. The defect in preclinical response of GF tumor-bearing mice to anti-CTLA4 Ab was overcome by mono-association with *B. fragilis* as well as by adoptive transfer of $CD4^+$ T cells that were previously primed with *B. fragilis*-pulsed dendritic cells (DCs) that contributed to the antitumor immune rejection. Interestingly, mice that were mono-associated with *B. fragilis* exhibited a more mature DC phenotype in tumor beds than controls with respect to the expression of MHC class II as well as the co-stimulatory CD80 and CD86 molecules. It has been documented that *B. fragilis* cell walls contain the immunostimulatory polysaccharide A (PSA) that can act on DCs. However, it remains to be determined whether PSA alone would be as efficient as live *B. fragilis* with respect to its antineoplastic activity. Finally, we were able to confirm that these findings were of clinical relevance by analyzing the gut microbiota of metastatic melanoma patients before and after ipilimumab. The 16S pyrosequencing analyses of feces contents in 25 stage IV melanoma patients revealed three major enterotypes based on the abundance or relative representativity of distinct spp. of *Bacteroides* and *Prevotella* genera. By performing fecal microbial transplantation (FMT) of feces representative of each enterotype into tumor-bearing GF mice subsequently treated with anti-CTLA4 Ab, we demonstrated that the microbial composition of enterotype C, enriched in immunogenic *Bacteroides* species, was able to allow the niching or colonization of *B. fragilis (but not Bacteroides uniformis*, *vulgatus*, *or distasonis)* and to restore the efficacy of anti-CTLA4 Ab, otherwise lost in GF mice, while clusters A and B failed to do both [65]. Of note, melanoma patients tended to exhibit an enterotype C while being treated with ipilimumab.

In parallel, Gajewski's group showed that the antitumor efficacy of anti-PD-L1

Ab was influenced by the colon content in *Bifidobacterium* species (*Bifidobacterium breve* and *Bifidobacterium longum*). In this study, Sivan and colleagues compared relative antitumor CTL responses against a candidate tumor antigen in genetically similar C57BL/6 tumor bearers derived from two different mouse facilities (bought from two distinct vendors) differing in terms of microbiome composition. Contrasting mice from the Jackson Laboratory and from Taconic Farms, they revealed significant differences in the growth kinetics of subcutaneously implanted melanomas, with more aggressive tumors in Taconic Farms derived- mice attributable to lower dendritic cell maturation and IFN signatures associated with poor intratumoral cancer antigen-specific TIL accumulation. Interestingly, the tumor growth in Taconic Farms mice was reduced following FMT from mice originating from the Jackson Laboratory or cohoused with these littermates. Pyrosequencing of gene amplicons of Jackson and Taconic mice feces revealed a high content in *Bifidobacteriales* spp. in the colony that exhibited reduced growth of transplantable melanomas and improved CTL-mediated immunosurveillance [4]. Selective transfer of *B. breve* or *B. longum* into mice that are normally devoid of these strains was sufficient to reduce melanoma growth and restored anti-melanoma CTL responses. *B. breve* and *B. longum* stimulated the maturation of DC both in vitro and in vivo. As a consequence, the frequency of tumor-specific CTL accumulating in melanomas increased in mice carrying *B. breve* or *B. longum*, and such CTL-infiltrated tumors responded more vigorously to immunotherapy with an antibody targeting PD-L1 than did melanomas evolving on sterile mice or mice bearing a gut microbiome devoid of immunostimulatory *Bifidobacteriales* [66].

Hence, various strains of commensals were associated with the immunostimulatory effects of distinct I-O strategies. The future will tell us whether most of these commensals would mediate their bioactivity on innate or cognate immune responses regardless of the therapeutic compound or whether they would act within the mode of action and scope of the defined compound.

27.8 Role of Intestinal Microbes in Graft-Versus-Host Disease (GVHD)

Graft-versus-host disease occurs after allogeneic hematopoietic stem cell transplantation (allo-HSCT) and often limits the success of the therapy. GVHD results from the attack of the host cells by the transplanted donor immune cells. Numerous studies demonstrated that GVHD depends on several factors such as age, conditioning regimen, graft source, etc. Moreover, increasing evidences indicate that the gut microbiota plays a significant role in the pathogenesis of GVHD and could be associated to the relapse of hematologic malignancies after allo-HSCT. Previous studies performed in mice showed that the severity of GVHD is attenuated in GF mice or mice treated with antibiotics [67, 68] or in humans [69]. Characterization of the gut microbiota of GVHD patients revealed a significant decrease of diversity which was associated with an increase of *Lactobacillales* and decrease of *Clostridiales*, which in turn can modulate intestinal inflammation [70]. In a later study, Jenq et al. anticorrelated the abundance of *Blautia*, belonging to the *Clostridia* class, with GVDH-related mortality [71]. While bacterial species known for their health-promoting properties such as *Faecalibacterium* or *Ruminococcus* were deeply decreased, the abundance of *Enterococci* was markedly enhanced in GVHD patients, compared with patients without GVHD [72]. Indeed, an increase of *Enterococci* after transplantation in adult patients with severe GVHD was noticed. This was also observed in ten pediatric patients undergoing allo-HSCT. Interestingly, patients undergoing GVHD displayed lower amounts of *Bacteroides* and *Parabacteroides*, whose abundance positively correlated with the levels of SCFAs, especially propionate, before allo-HSCT [73]. Lately, Simms-Waldrip et al. demonstrated that the use of antibiotics targeting anaerobic bacteria in the course of allo-HSCT correlated with a significant decline of anti-inflammatory *Clostridia* and the development of GVHD [74]. As a result, manipulating the gut microbiota through the use

of probiotics or prebiotics could be considered as a therapeutic approach aiming at decreasing the risk of GVHD. Preliminary data indicated that administration of *Lactobacillus rhamnosus* before and after allo-HSCT promoted less severe GVHD and improved survival of mice [75]. Other studies observed that administration of *Lactobacillus johnsonii* reduced GVHD severity through prevention of *Enterococci* accumulation post allo-HSCT [76]. Moreover, FMT was used in a clinical pilot study to treat GVHD of the gut occurring after allo-HSCT [77]. The abundance of *Lactobacilli* increased post-FMT in most of the patients, and the gut microbiota shifted toward an anti-inflammatory state. Other studies showed that FMT was efficient in treating *Clostridium difficile* infection post allo-HSCT [78, 79]. Butyrate-producing *Clostridia* strains reduced GVHD of the gut that is induced by allogeneic BMT. This effect was correlated with improved junctional integrity and reduced apoptosis of intestinal epithelial cells [80].

In a recent study, Peled et al. reported that intestinal abundance of *Eubacterium limosum* was associated with a reduced risk of relapse/progression of hematologic malignancies after allo-HSCT. Interestingly, the strongest association between *E. limosum* and a lower risk of disease relapse was observed in patients receiving T cell replete grafts. Donor cell-mediated graft-versus-tumor (GVT) effect was virtually higher in these patients, when compared to T cell-depleted graft recipients, suggesting that gut microbiota might play a role in GVT activity [81].

Altogether, these studies highlighted the dominant role of gut microbiota in dictating the efficacy and toxicity of allo-HSCT and open up new avenues of interventions on GVHD/GVT by harnessing the gut microbiome.

27.9 Bacteria for Therapy of Cancer: Probiotics and Others

The development of anticancer agents based on live microbial agents traditionally focused on local or systemic parenteral routes. William Coley was the first who partially succeeded in obtaining anticancer effect using microbes, by injecting intratumorally a mixture of *Streptococcus pyogenes* and *Serratia marcescens* [82]. Many efforts were then made to develop microbial-based anticancer treatment. *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) was approved by FDA and EMA in 1990 for superficial bladder cancer treatment. BCG efficacy relies on the induction of a local immune response against residual cancer cells, reducing the probability of relapse [83, 84]. Since then, no other bacteria obtained marketing authorization despite Phase I clinical trials utilizing various strategies outlined in Table 27.1.

Investigators recently raised an alternative approach that consists in administering live bacteria (probiotics) per os to colonize the gut and consequently obtain anticancer activities.

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer health benefits on the host" [85]. They are known to reinforce natural defenses, protect against gastrointestinal disorders and pathogens, and modulate innate and/or adaptive immunity.

Lactobacilli are substantial probiotics, which belong to the group of lactic acid bacteria. Many studies demonstrated that various isolates of *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus GG*, and/or *L. acidophilus* mediate anticancer effects through different mechanisms, including activation of NK cells, maturation of DCs, or release of probiotic-derived ferrichrome [86–95].

Prohep, a probiotic mixture of *Lactobacillus rhamnosus* GG (LGG), *Escherichia coli* Nissle 1917, and heat-inactivated VSL#3, delivered orally to mice bearing subcutaneous hepatocellular carcinoma, prevented tumor progression, and shifted the gut microbial community toward *Prevotella* and *Oscillibacter*. These later are known to produce anti-inflammatory metabolites, which in turn decrease the Th17 polarization and enhance the differentiation of anti-inflammatory Treg/Tr1 cells in the gut [96].

Whereas these probiotics taken individually may elicit antitumor activities, it is not

Table 27.1 Clinical trials utilizing live bacteria

Bacterial species	Cancer	Clinical benefit	Refs
Streptococcus pyogenes and *Serratia marcescens*	Osteosarcoma	Coley's toxins: Injection of *Streptococcus pyogenes* and *Serratia marcescens* in sarcoma patients leading to tumor regression	[82]
Mycobacterium bovis BCG	Superficial bladder cancers	Intravesical administration of a live attenuated form of *Mycobacterium bovis* leading to decreased risk of local recurrence	[97]
L. Casei Shirota (found in the fermented milk Yakult)	Superficial bladder cancer	NK and macrophage stimulation leading to decreased tumor recurrence	[98–100]
IMM-101 (heat-killed Mycobacterium obuense; NCTC 13365) with gemcitabine	Melanoma Advanced pancreatic adenocarcinoma	Increased survival in metastatic disease in a randomized Phase II study	[101, 102]
Live-attenuated *Listeria monocytogenes expressing mesothelin (CRS-207)* with GVAX-cyclophosphamide	Advanced pancreatic ductal adenocarcinoma	Increased overall survival associated with mesothelin-specific CD8 Tc1 responses	[103]
IL-13-PE: Recombinant cytotoxin consisting of human interleukin-13 (IL-13) and a truncated form of *Pseudomonas* exotoxin A (PE)	Adrenocortical carcinoma Phase I	Feasibility and neutralizing antibody responses	[104]
IL4-PE: Chimeric fusion protein composed of IL-4 and *Pseudomonas* exotoxin	Astrocytoma Phase I	No toxicity, median survival of 8.2 months and evidence of necrosis on MRI in several patients	[105]
Attenuated strain of *Salmonella typhimurium*, *VNP20009*	Metastatic melanoma Refractory solid tumors Phase I	Evidence of bacterial tumor colonization but objective response	[106, 107]
TAPET-CD: An attenuated *Salmonella bacterium* expressing the *E. coli* cytosine deaminase gene	Three patients (one head and neck squamous cell and two adenocarcinoma of the esophagus). Intratumoral injection	Evidence of bacterial colonization and conversion of 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU) in 2/3 tumors	[108]
Tf-CRM107 is a conjugate of transferrin and a point mutation in the diphtheria toxin	15 patients with malignant brain tumor	MRI regression of tumor volume in 9/15 patients with no evidence of severe local and systemic complications at low dose	[109]

BCG Bacille Calmette-Guérin, *NK* natural killer cells, *MRI* magnetic resonance imaging

established if when optimally combined they can create an ecosystem with extensive antitumor properties.

As mentioned previously, some intestinal commensals modulate the antitumor immune responses generated by anticancer compounds as well as the efficacy of the treatment (CTX and *E. hirae*/*B. intestinihominis* [60], CpG-ODN + anti-IL10 and *Alistipes shahii* [61], anti-PDL1 and *Bifidobacterium longum* and *breve* [66], anti-CTLA4 and *B. fragilis*/*Burkholderia cepacia* [65]).

The mechanisms of action of the microorganisms described above are not fully deciphered at the molecular level. The identification of bacterial structures directly involved in anticancer

activity or immunosurveillance promotion will raise favorable circumstances to (a) improve antitumor effect via probiotic engineering or (b) to develop compounds that mimic their pharmacological activity.

27.10 Genetically Modified Bacteria

Din et al. demonstrated that synthetic engineering of bacteria improved the antitumor effects of the antimetabolite 5-FU in a mice model of liver metastases of colorectal cancers. Pulsatile delivery cycles of 5-FU by the bacteria were allowed via a synchronized lysis cycle of *E. coli* based on quorum sensing feedback loops [110]. In necrotic tumor characterized by low vascularization, chemotherapy has limited efficacy due to restricted accessibility. This can be partially restored using anaerobic bacteria as tumor-targeting vectors. Bacteria engineering increased 5-FU efficacy in liver metastasis, but this strategy was fully successful when combined with I-O or other anticancer drugs [110]. This unique way to deliver compounds in avascular tumors needs to be further assessed in patients resistant to traditional therapies.

27.11 Microbial Products with Cancer-Modulating Properties

Microbial agents can synthetize a wide range of molecules that affect either antitumor immunosurveillance or growth/survival of cancer cells. One distinguishes (a) toxins, (b) ligands of pattern recognition receptors (PRRs), and (c) metabolites (short-chain fatty acids, polyamines, vitamins, secondary bile products, AhR ligands). While toxins display direct cytotoxic properties, activation of PRRs stimulates immune response, and metabolites affect the host metabolism. However, certain metabolites can also behave as PRR ligands, as it was shown for N-acetylglucosamine (a sugar subunit of bacterial peptidoglycan) acting on hexokinase to activate the NLRP3 inflammasome [111] or for tryptophan derivatives acting on the aryl hydrocarbon receptor (AhR) [112]. These approaches have been detailed in a previous report [113]. We will only recapitulate the most prominent ones that can be harnessed for oncological purposes.

Bacterial toxins are usually peptides with amphipathic alpha-helices containing cationic charges that cause the lysis of non-protected bacterial membranes. Structural analogs of toxins were developed in order to kill cancer cells. LTX-315, a synthetic peptide developed for intratumoral therapy, targets mitochondria. It also triggers necrotic cell death with immunogenic properties. This means its direct tumoricidal effects are prolonged as a consequence of danger signal emission that activates specific antineoplastic immune responses [114].

Several PRR ligands are FDA and EMA approved. Monophosphoryl lipid A (MPL), a derivative of *Salmonella minnesota* LPS, is used as an adjuvant in a peptide-based vaccine for the prevention of cervical carcinoma-associated strains of human papillomavirus [115]. Imiquimod, a synthetic agonist of TLR7, is topically delivered for the treatment of actinic keratosis.

Short-chain fatty acids (SCFAs) such as acetate, butyrate, and propionate are synthetized from dietary fibers and polysaccharides by the *clostridial clusters IV* and *XIVa* of *Firmicutes*. While acetate supports the expansion of various human cancer types [116–118], propionate and butyrate may have anticancer properties through distinct mechanisms. Propionate and butyrate could repair gut dysfunction, by promoting regulatory T cell (Treg) differentiation and/or accumulation and mediating anti-inflammatory activities [119–121] or inhibiting histone deacetylases (HDACs), conferring to these metabolites anticancer properties. Butyrate also induced apoptosis in colorectal cancer and lymphoma cells, suggesting direct tumoricidal activities [122, 123]. As a result, a way to prevent or treat cancer would consist in developing dietary regimen that increases intestinal butyrate over acetate production.

27.12 Future Prospects

Over the past decade, microbiome raised a major interest, since it appears that these commensal communities influence the development and outcome of a wide range of disease, including cancer. Microbiota might affect tumor formation and progression. It may also determine anticancer treatment responses. Either indirect effects such as immunosurveillance or direct effects of microbial compounds (i.e., carcinogens, cytotoxic agents, and metabolites) may affect tumor cells via diverse processes (mutagenesis, epigenetic modulation, stimulation of receptors on host cells, effect on anabolic and catabolic pathways).

Given that the cancer immune set point may be largely controlled by the health of our gut microbiome, diagnosis tools to evaluate a patient's gut dysbiosis are urgently needed to be able to guide the indications of specific therapies and anticancer probiotics. Technologies based on culturomics, metagenomics, or PCR or mass spectrometry will lead to diagnosis tools for cancer-associated dysbiosis and predictors of primary resistance to ICB.

Increasing knowledge of the functional exploration of patient-derived microbiomes, coupled with relevant preclinical models, will allow the development of four alternative anticancer interventions: (a) orally administrable microorganisms (probiotics), (b) specific dietary or drug-based interventions that favor the expansion of beneficial microorganisms, (c) drugs that specifically target microbial enzymes that generate harmful toxins and metabolites, and (d) microbial products with anticancer properties. Each of these therapies could be used alone or in combination with conventional anticancer treatment. However, live microbes, especially when genetically modified, raise safety concerns with regard to their escape into the environment, their potential pathogenicity, and the acquisition of antibiotic or enzymatic chemoresistance. Hence, live microbial agents are subjected to regulatory and intellectual property-related unsolved issues. An alternative to eventually overcome such limitations would be the development of small molecules or chemically defined macromolecules that influence the natural gut microbiome in a favorable fashion or mediate beneficial effects on their own.

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Local Immunotherapies of Cancer

28

Thomas U. Marron, Linda Hammerich, and Joshua Brody

Contents

28.1 Introduction

The use of immune stimuli to eradicate cancer is not a novel concept; in the mid-nineteenth century, the German physician W. Busch noted a dramatic regression of a patient's cancer following a severe case of erysipelas in a patient infected with *Streptococcus pyogenes* [1]. This published observation was the impetus behind William Coley's injecting *Streptococcus pyogenes* extracts into cancer patients, later called "Coley's toxin," with which Dr. Coley and others had varying success [2]. Though this treatment fell out of favor with the introduction of radiation, more recent experiments based on these findings used endotoxin to induce a significant immune response; in early phase trials, some clinical responses were seen; however, the toxicity was predictably high and the approach was deferred [3–5]. Regardless, the responses demonstrated the possibility to activate an antitumor immune response with strong pro-inflammatory stimuli.

Cancer immunotherapy has progressed at a rapid pace in the past decade, with approval for multiple monoclonal antibodies (mAbs) targeting the CTLA-4 and PD-1/PD-L1 immune checkpoints, with small molecules and mAbs targeting numerous other immune checkpoints advancing rapidly through clinical trials. These approaches rely on reactivation of pre-existing antitumor immunity that has been silenced by immunosuppressive mechanisms within the tumor microenvironment. It is therefore unsurprising that most

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impressive clinical responses have been seen in either highly immunogenic tumors with the largest mutation burden and a number of possible neoantigens—e.g., melanoma and non-small cell lung cancer (NSCLC) in smokers—or in tumors which have an inherent immune infiltrate that is checked by high expression of PD-L1 on tumor cells, as is the case for Hodgkin's lymphoma and some subsets of diffuse large B-cell lymphoma (DLBCL) [6–9]. Still, for the majority of patients with solid cancers for which checkpoint inhibitors have been approved for clinical use (lung, bladder, and renal cell carcinoma and melanoma), there is either too strong immunosuppressive microenvironment or too weak immune response to result in a systemic immune response and significant tumor regression. In support of this theory, the degree to which many solid tumors have an "inflamed" phenotype with significant immune cell infiltration predicts response to immunotherapy [10, 11]. These findings suggest that combinatorial approaches in which inhibitory checkpoints are abrogated while inflammation is induced within the tumor microenvironment would potentiate the immune response to tumor antigens and improve clinical response rates.

The efficacy of CTLA-4 and PD-1 blockade, particularly when used in combination, clearly demonstrates the potential for success; however, these doublet therapies carry significant toxicity and still only achieve response rates approaching 50% in highly immunogenic tumors [12–14]. A large number of alternative combinatorial approaches are now under investigation to increase the efficacy: toxicity ratio. One category that offers promise is vaccination, which, similar to classical vaccines, introduces immune adjuvants alongside tumor-associated antigens (TAA) to focus the immune response on cancer-specific targets. Many ex vivo approaches have shown efficacy relying either on in vitro differentiation of antigen-presenting cells (APCs) with specific TAAs or whole tumor lysate or in vitro expansion of tumor-infiltrating lymphocytes which are responsive to TAAs. Though small studies of these in vitro methods have demonstrated potent clinical responses, these are resource-intensive and thus difficult to optimize and scale up.

One alternative is in situ vaccination (ISV), in which a "vaccine" is created within the tumor to achieve the same goals as have been achieved with in vitro techniques; specifically these therapies aim to induce damage to cancer cells, followed by uptake and processing of released tumor antigen and stimulatory "damage"-associated molecules, and finally antigen presentation—and cross-presentation—to CD4 and CD8 T cells (Fig. 28.1). There have been multiple approaches to ISV, but the general principles are the same, specifically (1) inducing immunogenic cell death; (2) recruitment of leukocyte infiltration, inducing the "inflamed" phenotype responsive to immunotherapy; (3) activation of APCs resulting in activation of innate lymphocytes and presentation of TAA to antigen-specific effector cells; and finally (4) modulation/abrogation of the immunosuppressive tumor microenvironment. Here we review some approaches to achieve these four aims.

28.2 Immunogenic Cell Death

Immunogenic cell death results in release of tumor antigens from dying cells into the microenvironment along with cell-stress signals and damage-associated molecular patterns (DAMPs) capable of activating receptors on and in APCs. As opposed to apoptosis, a comparatively tolerogenic form of cell death, immunogenic cell death, not only provides the antigen but also provides inflammatory stimuli, and as such, agents inducing this form of toxicity are occasionally able to elicit a vaccinal response on their own [15].

While immunotherapy is generally described as mechanistically distinct, in reality there may have been an immunostimulatory component to many of the cytotoxic therapies that have been employed over the last century. An early observation of the role of the immune system in cancer was the abscopal effect, in which localized radiation given to a patient resulted in distant regression of disease outside of the radiation field [16]. While rare, this abscopal effect offers a proof of principle that can be further optimized. Radiation has been used in a variety of

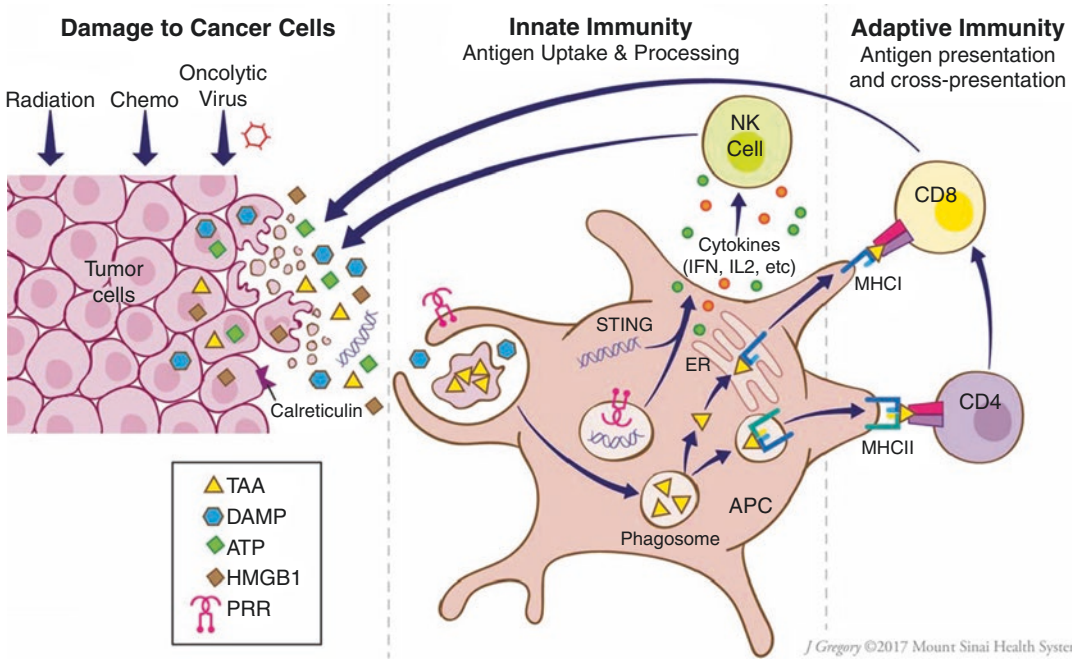

Fig. 28.1 The central tenants of induction of an in situ immune response to tumor antigen. First is a stimulus causing immunogenic cell death, which leads for resident or recruited APCs to take up antigen and become activated due to pro-inflammatory signals, which are both an innate immune response and antigen processing and presentation/cross-presentation to achieve an adaptive immune response

vaccine/immunotherapy regimens given its ability to induce immunogenic cell death. Radiation stimulates translocation of calreticulin to the cell surface and release of a multitude of DAMPs, including free DNA, high-motility group box 1 (HMBG1), and ATP; these signals stimulate pro-inflammatory receptors on dendritic cells (DCs), activating the inflammasome and Toll-like receptor (TLR) pathways among other pattern recognition receptors (PRRs) [17–21]. Upon uptake of dead or dying tumor cells by phagocytes, cell-free DNA is also capable of activating the stimulator of interferon (IFN) gene (STING) pathway, inducing IFN production and potentiating activation of a strong effector T-cell priming [22]. In parallel, radiation further induces expression of key chemokines that are critical for recruitment of effector $CD8^+$ T cells to the irradiated tumor, including CXCL9, CXCL10, and CXCL16 [23, 24]. At the same time, radiation has also been shown to suppress the immune response through recruitment and differentiation of regulatory T cells and myeloid-derived suppressor cells (MDSCs) to the tumor microenvironment, leading to secretion of cytokines such as TGF-β and conversion of immunogenic ATP into adenosine by CD39 and CD73, which suppresses T effector cell activation and survival via the A2a adenosine receptor [25–27].

Our group and others have demonstrated a significant upregulation of PD-1 and PD-L1 on intratumoral T cells and tumor cells, respectively, in response to ionizing radiation [28, 29]. Though these and other inhibitory ligands are also markers of T-cell activation, T cells upregulate these ligands and receptors upon activation as a physiologic check on the immune system to avoid autoimmunity—possibly explaining the rarity of clinically relevant abscopal effects and may offer rational targets for combinatorial approaches using radiation therapy. Numerous preclinical studies demonstrate that RT is synergistic with checkpoint-blocking therapies [30], though clinical success has been limited [31]. So far, most published studies have targeted CTLA-4, but there are encouraging preclinical studies combining radiation with blockade

of PD-1 and other key checkpoints and immunosuppressive pathways (reviewed extensively by Kang et al. [32]).

Finally, radiation is highly lymphotoxic, hence its continued use in some stem cell transplant-conditioning regimens for lymphoid leukemias. Thus, depending on dose and the scope of the treatment field, RT risks eliminating the very T cells the treatment aims to activate. This supports the use of limited field and hypo-fractionated dosing in order to achieve the optimal vaccinal effect; preclinical models have supported the benefit of hypo-fractionated dosing in optimizing antitumor immune responses [33]. A comprehensive review of radiation as an adjuvant to emerging immunotherapies is provided in this series by Professors Formenti and Demaria, leaders in this field.

Though radiation and chemotherapy are both typically considered lymphotoxic, chemotherapy can also serve multiple roles as an adjuvant to immune therapy. Like radiation, chemotherapy causes immunogenic tumor cell death resulting in the release of both immunogenic ligands and tumor antigen that can be taken up by intratumoral APCs as well as those in the surrounding tissue and draining lymphatics, extensively reviewed by Kepp et al. [34]. Aside from tumor cytotoxicity, lymphotoxic chemotherapies such as cyclophosphamide have been found to preferentially deplete regulatory T cells (Tregs), with a milder effect on T effector cells [35].

The role of chemotherapy in inciting the initial step of priming an immune response, as well as the role of old and new cytotoxic chemotherapies in combination with emerging immunotherapies, is reviewed elsewhere in this series by Professor Kroemer, an authority on immunogenic cell death.

28.3 Recruitment of Immune Infiltrate

Though immunogenic cell death is likely necessary to initiate and potentiate a tumor-specific immune response, the rarity with which the abscopal effect is observed, and the paucity of studies demonstrating that radiation or chemotherapy increases tumor-specific T cells, suggests that additional signals are needed to potentiate the anti-TAA immune response. To promote antigen uptake, one extensively used approach has been the addition of growth factors inducing APC differentiation and recruitment into tumor tissue. In contrast to systemic cytokine administration, direct intratumoral injection of cytokines and growth factors has also been tried and showed promise in augmenting the response to immune-based therapies, including granulocyte-macrophage colony-stimulation factor (GM-CSF) and FMS-like tyrosine kinase 3 ligand (Flt3L).

Preclinical studies have assessed the immunostimulatory capabilities of multiple cytokines and growth factors by transducing tumors with the corresponding genes in a murine melanoma model. These studies looked at a panel of genes including IL-2, IL-4, IL-5, IL-6, IFNγ, IL1-RA, ICAM, CD2, TNF-α, and GM-CSF; GM-CSF achieved the most profound tumor rejection and induction of tumor-specific $CD4^+$ and $CD8^+$ T cells which protected mice from rechallenge with non-transduced melanoma cells [37]. GM-CSF is a hematopoietic growth factor, and outside the marrow compartment, GM-CSF can be produced by lymphocytes and myeloid lineage cells, as well as non-hematopoietic cell types within the tumor microenvironment including fibroblasts, endothelial cells, and epithelial cells; GM-CSF is capable of activating both innate and adaptive immune response, and expression is typically promoted by inflammatory cytokines such as IL-6 and TNF-α, while immunosuppressive cytokines such as IL-10 decrease its expression [38–42]. Clinically, autologous tumor cells from 21 melanoma patients were transduced with GM-CSF, and subsequent tumor biopsies revealed greater T-cell infiltration, as well as tumor necrosis, fibrosis, and edema, in the majority of patients [43]. Comparable immune responses were seen in a subset of patients treated in similar trials of autologous GM-CSF-transduced tumor cells from patients with melanoma, as well as lung and prostate cancer [44–46]. Combinations of

GM-CSF with treatments that promote immunogenic cell death have also demonstrated induction of antitumor immunity. One ISV trial in follicular lymphoma combined local radiotherapy and intratumoral rituximab with intratumoral injection of immature autologous dendritic cells and GM-CSF; this permutation resulted in an overall response rate (ORR) of 36%, and those in whom there was a measurable tumor-directed $CD8^+$ response had the most profound and durable clinical responses [47].

Though attraction and maturation of DCs lead to a pro-inflammatory immunogenic signal, intratumoral GM-CSF has also been found to be a chemoattractant to neutrophils and other myeloid lineage cells capable of promoting angiogenesis and suppressing $CD8^+$ T-cell priming. GM-CSF furthermore directly promotes the growth of some tumors by enhancing expression of matrix metalloproteinases (MMPs) instrumental to growth and migration and metastases [48]. Some human and murine cancers have been shown to constitutively express GM-CSF, or in some cases G-CSF, as well as the respective receptor, leading to autocrine promotion of proliferation [49–55]. Some propose that the role of GM-CSF may differ based on the tissue involved, such that in some cancers, its use may be an immune adjuvant, and in others it may promote tumor growth and suppress the immune response. A large cohort of gene arrays of human colorectal cancers demonstrate that over a third of cancers produce GM-CSF, and colorectal cancer patients with an increased level of GM-CSF in serum have been shown to have a worse prognosis [55, 56]. In head and neck cancer, GM-CSF and other growth factors in the serum were associated with worse invasion and prognosis overall [57, 58]; similarly, invasive bladder cancers occasionally secrete GM-CSF, and some patients' cancer cells also express the GM-CSF receptor, and both these findings correlate with worse prognosis [53, 59]. Leukocytosis and elevated GM-CSF levels in blood or in tumor samples are seen in a variety of other cancers as well, including lung and glioblastoma multiform, some of which are the targets in immunotherapy trials incorporating GM-CSF [54, 60, 61]. These data prompt consideration of alternate growth factors capable of differentiating or recruiting APCs to the tumor site.

Flt3L is a hematopoietic growth factor crucial to the mobilization and differentiation of stem cells and progenitors [62]. Flt3L has been shown to facilitate the differentiation of both DCs and NK cells from hematopoietic stem cell precursors [63–66]. Specifically, Flt3L directly induces the differentiation of a proportion of $CD34^+$ CD45RA-early progenitor cells into a DC precursor and results in increased numbers of plasmacytoid DC (pDC) and $CD141^+$ and $CD1c^+$ (cDCs) [63, 67]. These DC subsets, particularly the $CD141^+$ subset, are especially capable of cross-presentation of tumor antigen to $CD8^+$ T cells, and their intratumoral presence correlates strongly with clinical outcomes [68, 69].

Numerous preclinical studies have demonstrated the ability of Flt3L to mobilize immune cell subsets, DCs predominant among these [67, 69–73], including models showing antitumor effect of Flt3L administration in melanoma, lymphoma, leukemia, and breast, colon, prostate, lung, and hepatocellular carcinoma [74–82]. Other models have demonstrated that single-agent Flt3L is ineffective, rather the optimal antitumor immune response requires combinatorial approaches which induce antigen release and stimulation of APCs; for instance, in a murine model of breast adenocarcinoma, Flt3L priming potentiated the abscopal effect elicited by low-dose radiation and eliminated systemic tumor burden in a T-cell-dependent fashion, while Flt3L alone had no effect without radiotherapy [83].

Clinically, a phase I study evaluated the use of autologous DCs that first were expanded in vivo with Flt3L, loaded ex vivo with carcinoembryonic antigen peptide, and reinfused; treatment was well tolerated and demonstrated that Flt3L expanded DCs 20-fold; 2 of 12 patients experienced dramatic tumor regression [84]. Clinical response correlated with the expansion of $CD8^+$ CEA $tetramer^+$ T cells, confirming the role of $CD8^+$ T cells in this treatment strategy. Similar robust increases in intratumoral and systemic DCs were seen in patients in additional early phase trials with peritoneal carcinomatosis, mesothelioma, and prostate cancer, all with

limited toxicity [85, 86]. These early phase trials suggested that Flt3L is able to significantly increase the presence and activity of intratumoral APCs and that—similar to GM-CSF—it appears that combinatorial approaches are necessary to prime clinical remissions. Indeed, while Flt3L administration promotes DC production systemically and intratumoral infiltration, these DCs are immature DCs, which lack costimulatory molecules needed to activate effector cells. This both limits their ability to promote an effector T-cell response and actually promotes tolerogenic pathways keeping tumor-specific T cells quiescent [73]. As such, though Flt3L appears to augment the number of intratumoral DCs, additional pro-inflammatory stimuli are necessary to activate these APCs upon arrival.

28.4 Activation of Dendritic Cells

Single-agent studies of GM-CSF and Flt3L have demonstrated low toxicity but also low efficacy, confirming what we know about the physiological role of DCs in priming and homeostasis; it is not enough to promote the ingress of APCs into a tumor; an activation signal is needed. While this can come from DAMPs released by immunogenic cell death, arguably the strongest innate immune responses can also be elicited by introduction of pathogen-associated molecular patterns (PAMPs). TLRs are evolutionarily conserved innate immune receptors capable of recognizing PAMPs, as well as some DAMPs, and likewise TLR ligands, such as lipopolysaccharide (LPS), free single- or double-stranded RNA (ssRNA or dsRNA), and hypomethylated DNA motifs found in pathogens, are able not only to bind and activate canonical TLR pathways but also other cytoplasmic inflammatory pathways [87]. The sum stimulus of these pathways results in a maturation signal potentiating DC antigen presentation and cross-presentation and subsequent T-cell activation.

TLRs are resident either on the cell surface (as is the case for TLRs 1, 2, 4, 5, and 6) or in the endosome (TLRs 3, 7, 8, and 9), and their distribution is key to the PAMP and DAMP ligands they recognize. TLR7 classically recognizes free ssRNA, and agonists for this TLR were the first PAMP analog to be FDA approved for clinic use in the form of imiquimod. TLR7 is highly expressed on multiple myeloid lineages, with highest expression on plasmacytoid DCs that play a key antiviral role through induction of type I IFNs (IFNα/β). Imiquimod is an imidazoquinoline derivative capable of activating TLR7 and induces a strong antiviral immune response; it was first approved for the treatment of genital warts caused by a cutaneous viral infection and subsequently for the treatment of basal cell carcinoma and actinic keratosis, with off-label indications for cutaneous viral infections such as molluscum contagiosum [88–90].

Preclinical studies have also investigated the use of imiquimod in other cancers that are not virally derived. In a murine model of cutaneous breast cancer, topical application of imiquimod combined with local radiotherapy resulted in complete regression of locally treated tumors with abscopal responses at distal sites suggesting induction of T-cell-mediated antitumor immunity [91]. In this model, response is associated with increase in T-cell infiltration into tumor lesions, and the abscopal effect was dependent on $CD8^+$ T cells. Similar trials have been performed in small series or case reports in patients with cutaneous involvement of metastatic melanoma or breast cancer; topical application of imiquimod induces a pro-inflammatory tumor microenvironment and some tumor regressions [92–94]. There are also many series or case reports of antitumor effects of imiquimod in cutaneous T-cell lymphomas [95–103] and B-cell lymphomas [104–107], now a common off-label use of this adjuvant.

In a combinatorial approach using imiquimod and a human cancer-testis antigen NY-ESO-1 peptide vaccine in melanoma, patients were pretreated with Flt3L, and imiquimod was applied to the vaccine sites [108]. Unfortunately, clinical response rates to this vaccine triplet regimen were suboptimal, and only 12 of 27 patients were evaluable, a minority of whom were treated with imiquimod, and only one patient experienced a partial response.

Systemic treatment with imiquimod has been assessed in preclinical models, with oral treatment leading to significant IFN induction and tumor growth inhibition in colorectal, sarcoma, and lung cancer models [109]. In a model of lymphoma, intravenous delivery of R848, a guanosine derivative which acts as an agonist for both TLR7 and TLR8 (another endosomal TLR which recognizes ssRNA), combined with local tumor irradiation induced durable $CD8^+$ T-cell antitumor immune responses, clearance of systemic disease, and protection from tumor rechallenge [110]. A novel imidazoquinoline, 3 M-052 or MEDI9197, which is also a TLR7 and TLR8 ligand formulated with a lipid tail to allow tissue retention, was shown in preclinical models to suppress tumor growth following intratumoral injection both at target and distal lesions [111]. This antitumor effect was enhanced by PD-L1 and CTLA-4 blockade, even in models in which checkpoint blockade alone was ineffective. A phase I basket trial of intratumoral MEDI9197 injections in metastatic disease is underway, with recent cohorts combining MEDI9197 with anti-PDL1 therapy, hoping to reproduce the preclinical synergistic activation of antitumor immune responses [112].

There has been extensive study of the role of type I IFNs in the control of tumor growth, and these IFNs appear integral to mount an immune response to tumors [113, 114]. Preclinical studies suggest that this is due not to the role of IFNs in stimulating the cytotoxic T cells but also the role IFNs has in inducing maturation of DCs, particularly $BATF3^+$ $CD8\alpha^+$ DCs in mice (the corollary of $CD141^+$ DCs in humans which also express the BATF3 and IRF8 characteristic transcription factors) which are the most capable of cross-presenting tumor antigen to $CD8^+$ T cells [115, 116].

TLRs are potent stimuli of IFNs; however, there are also cytoplasmic PRRs capable of inducing immune responses. One of these keys to the induction of the IFNs is the STING receptor, a receptor for cGAMP, a degradation product from cytoplasmic DNA [117]; STING activation has been shown to strongly activate type I IFN transcription [118]. Preclinical studies of intratumoral injection of a STING agonist DMXAA (also known as ASA404) into murine B16 melanoma were sufficient to achieve priming of tumor-specific $CD8^+$ cells and achieve immune rejection of the tumor [119, 120]. DMXAA was tried in conjunction with chemotherapy in a large phase III trial of NSCLC in humans with no appreciable activity, though that was later determined to be due to poor binding to human STING [121]. ADU-S100 is a novel cyclic dinucleotide that binds and activates both human and mouse STING [122], and this is currently being used intratumorally in early phase trials.

It has also been noted that cancer cells may downregulate the STING pathway in order to avoid induction of IFN in response to DNA damage commonly present in rapidly dividing cancer cells [123]. This has been exploited by the field of oncolytic viruses using either pathogenic viruses that have been engineered to decrease virulence in normal tissue (such as herpes simplex virus, HSV, and vaccinia) [123, 124] or viruses that are typically not virulent in human cells but are able to infect and propagate in cancer cells in which the IFN pathway has been silenced (such as Newcastle disease virus, NDV) [125]. Oncolytic viruses are not only capable of inducing tumor lysis and antigen release; the viral components can activate innate PRRs. Initial limited clinical efficacy of these viruses alone made it evident that an additional immune stimulus such as a growth factor or cytokine could potentiate the immunity induced by viral infection. JX-594, a vaccinia virus, was engineered to express GM-CSF in infected cells, has been shown to selectively replicate in tumor cells, and demonstrated antitumor immunity in preclinical models and early phase clinical studies after either intratumoral or systemic inoculation with virus [124]. These studies revealed increased infiltration of T cells into the tumor and disruption of tumor-associated vasculature in mice and humans, leading to reduced blood flow and rapid necrosis of tumor cells [126–128]. This effect is tumor-specific due to loss of normal IFN response, as endothelial cells of normal blood vessels are not affected. Clinical trials in liver cancer and melanoma have found JX-594 is well tolerated and response rates have

been encouraging [129–133]. Similarly, HSV, engineered to not express genes that block antigen presentation on MHC molecules and other genes key to viral replication in normal cells, demonstrated significantly enhanced immunogenicity by inserting GM-CSF into the viral genome [134]. Clinical trials of this construct referred to as talimogene laherparepvec or T-VEC (previously OncoVEX GM-CSF) have shown potent induction of necrosis in treated tumors and inflammation of distal tumors and significant response rates including complete responses to single-agent therapy in patients with melanoma, head and neck cancer, and breast and other metastatic malignancies [135–138]. A phase III study of T-VEC in patients with advanced melanoma demonstrated superior response rates compared to GM-CSF alone and a trend toward improvement in overall survival ($p = 0.051$), which led to approval of T-VEC for clinical use [139]. Combinations of oncolytic virus with immunomodulators such as checkpoint-blocking antibodies offer great promise; these trials (e.g., NCT03069378, NCT02965716) and the field of oncolytic viruses in general are reviewed elsewhere in this series by Professor Puzanov.

While STING is a cytoplasmic PRR recognizing free DNA, endosomal TLR9 is another PRR capable of sensing free DNA, specifically DNA containing hypomethylated CG-enriched oligonucleotide (CpG) islands commonly seen in the prokaryotic genome. TLR9 is expressed by pDCs and other DC subtypes to varying degrees and is also expressed on B cells, which can also be effective APCs. Interestingly, B-cell lymphomas are derived from mature B cells expressing high levels of TLR9, and CpG containing ligands can activate lymphoma B cells, just as they can with nonmalignant B cells, to increase expression of costimulatory molecules [140–142]. In a lymphoma model, CpG ligands inhibited proliferation of malignant B cells, and when combined with systemic chemotherapy capable of inducing immunogenic cell death, intratumoral CpG induced antitumor response and disease regression at site of injection as well as distal lesions in a $CD8^+$ T-cell-dependent fashion, suggesting CpG was potentiating priming of tumor-reactive T cells [142]. While tumor-resident DCs may also respond to TLR9 ligands, in this model, this effect was seen in mice with somatic TLR9 deficiency, carrying TLR9-expressing tumors, suggesting that antigen presentation by tumor cells, not host APC, may be sufficient to induce potent immune responses. The TLR9 agonist agatolimod (PF-3512676) has been used in clinical trials in humans, including three trials using a combination of local irradiation and intratumoral agatolimod administration which achieved clinical responses, including some complete responses, among the 45 patients with indolent B-cell lymphoma and 15 patients with mycosis fungoides that were treated [143–145].

CpG has been shown to also elicit tumor regression in non-B-cell malignancies; in a murine glioma model, intratumoral CpG increased T-cell infiltration, eliminated tumors, and protected from rechallenge [146], and similar antitumor T-cell-mediated responses are primed with CpG injection in murine models of mesothelioma, breast cancer, and melanoma [147–149]. In humans, another TLR9 agonist, litenimod (CpG-28), was given as an intratumoral injection into 34 patients with recurrent glioblastoma multiforme, achieving some partial responses [150]. The above CpG molecules are classified as CpG type B (CpG-B) in that they optimally activate B cells, whereas a CpG-C molecule SD-101—designed to comparably activate B cells and pDC—has shown promising preclinical and clinical results upon intratumoral administration and is currently being studied in lymphomas and solid tumors including combination studies with anti-PD1 antibodies (NCT02521870, NCT02731742, NCT02927964, NCT03007732).

As noted above, the importance of $CD141^+$ DCs in the tumor relates to their potent ability to cross-present antigen and prime a potent $CD8^+$ T-cell response to tumor antigen. It is noteworthy that these cells express very low levels of TLR7, TLR8, and TLR9; however, they express high levels of TLR3, another PRR located in the endosome which responds to dsRNA [151]. Polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethyl cellulose (poly-ICLC, also known as hiltonol) is a stabilized double-stranded RNA (dsRNA) “host-targeted” therapeutic viral mimic

that exhibits broad innate and adaptive immune-enhancing effects and was shown to be a promising vaccine adjuvant due to its strong induction of type I IFNs [152, 153]. As such, it was first used as an IFN inducer at high doses in cancer trials, with predictably high toxicity [154], while at lower doses, it achieved a broader host defense stimulation, potent adjuvant effect, and antiproliferative and antiviral effects [155–157].

The possible antitumor and antiviral activity of poly-ICLC is thought to be dependent on its stimulation of both TLRs and MDA5 (another cytoplasmic PRR [158]) and its concomitant induction of IFNs and other cytokines and its induction of MHC molecules. Likewise, dsRNA induction of various costimulatory factors such as B7-H2, CD40, and OX40 may help overcome evasions in the tumor itself [159].

Administration of poly-ICLC has been demonstrated to prolong survival in multiple murine tumor models. In a murine lymphoma model, IP administration of poly-ICLC significantly prolonged survival and correlated with peritoneal macrophage tumoricidal activity [160]. Poly-ICLC also prolonged survival in rodent models of gliomas, melanoma, and fibrosarcoma [161, 162]. Poly-ICLC incorporated into peptide-based vaccine for gliomas showed ability to induce tumor lymphocyte infiltration and induction of an IFN signature [163–165], and poly-ICLC used as an adjuvant with the E7 HPV tumor antigen in a murine model of cervical cancer demonstrated expansion of antigen-specific $CD8^+$ T cells as well as tumor regression [166]. While single-agent poly-ICLC can induce tumor responses, our group has developed a combined approach ISV in the murine lymphoma A20 model in which poly-ICLC is used to activate intratumoral DCs after treatment with low-dose radiation and intratumoral Flt3L (Fig. 28.2 is the

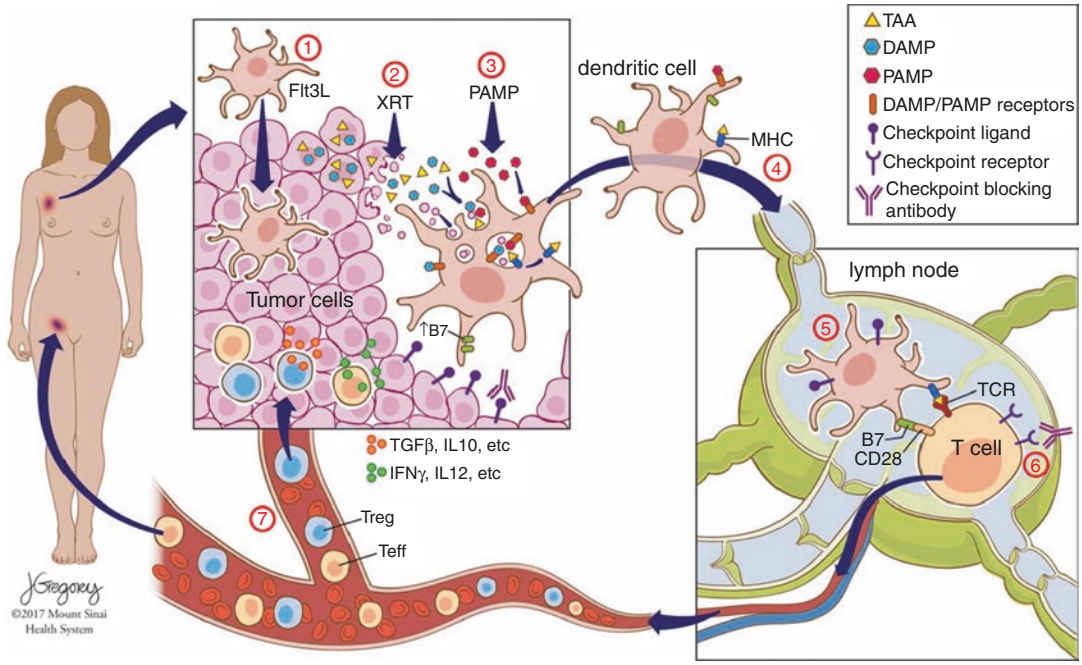

Fig. 28.2 Novel ISV protocol. Intratumoral Flt3L (*1*) induces recruitment of DCs to the tumor, which subsequently take up antigen after low-dose radiation (*2*), and DCs are activated by intratumoral injection of PAMPs such as poly-ICLC (*3*), inducing activation of antigen-loaded DCs, which then migrate to the draining lymph nodes (*4*) where they interface with tumor antigen-specific T cells (*5*). Activation threshold of senescent or exhausted tumor-specific T cells can be overcome through addition of checkpoint blockade (*6*), and activated T effector cells then travel to tumor sites throughout the body (*7*) to exert systemic antitumor effect and promote tumor-specific memory

human correlate). While this approach does induce tumor regression and cure in approximately one-third of animals, we have shown that addition of checkpoint blockade with an anti-PD-1 mAb results in regression of nearly all tumors, further supporting the importance of both inducing intratumoral inflammation *and* modulating the immunosuppressive tumor microenvironment to achieve the optimal antitumor immune response [167].

Poly-ICLC has been used in dozens of clinical trials in multiple solid malignancies and lymphoma which have been reported, and many more ongoing clinical trials in combination with chemotherapies, radiation, and/or other immune adjuvants, with varying clinical success, but with promising data, demonstrated the ability to elicit immune response to tumor antigen [165, 168–178]. Based on these and other studies in humans, poly-ICLC is thus emerging as a particularly potent immunogenic core for multiple cancer and HIV vaccines, given its adjuvant role in potentiating antigen uptake, cross-presentation, and skewing of immune response to tumor antigens toward Th1 phenotype [176]. A trial of 22 patients with recurrent malignant gliomas used autologous DCs loaded ex vivo with synthetic TAA peptides, administered with concurrent intramuscular poly-ICLC, resulting in potent antigen-specific T-cell responses, and a suggestion of prolonged survival [179]. A similar peptide-based vaccine trial in patients with advanced ovarian cancer used a vaccine that combined overlapping long peptides from NY-ESO-1 in conjunction with poly-ICLC; while the peptide alone induced no immune response, the investigators saw significantly higher induction of NY-ESO-1-specific antibodies as well as antigen-specific T-cell responses in 10 of 11 patients that received the poly-ICLC [174]. Another vaccine in multiple tumor types expressing NY-ESO-1 attached full-length NY-ESO-1 protein to DCs by conjugating the tumor antigen to an antibody for DEC-205 expressed on DCs; this antibody-peptide conjugate was administered in conjunction with poly-ICLC, again this adjuvant was found to be well tolerated, and a small minority had robust clinical regression of metastatic disease [180]. Another study of the cancer testes antigen MAGE-A3 similarly treated patients with whole peptide and poly-ICLC, followed by autologous stem cell transplant and adoptive transfer of ex vivo expanded T cells, and found that over three quarters of the 25 patients treated developed cellular and humoral antigen-specific responses [173].

Intratumoral injection of poly-ICLC to achieve an in situ vaccination is currently being studied as well, alone and in combination. In an ongoing trial of single-agent intratumoral poly-ICLC used in patients with metastatic malignancies, a patient with advanced facial rhabdomyosarcoma had significant intratumoral inflammation and necrosis followed by marked tumor regression [175]. In an ongoing study aimed at potentiating the abscopal response, intratumoral injection of recombinant Flt3L and poly-ICLC is administered in combination with low-dose radiotherapy; early reports demonstrate increased intratumoral DCs and regression of both treated tumors and systemic disease in some patients with advanced-stage follicular lymphoma [181, 182]. Given recent preclinical results demonstrating the potency of this approach in combination with checkpoint blockade, the upcoming iteration of this (intratumoral) ISV will study the combination along with (systemic) anti-PD1 mAb (Fig. 28.2).

28.5 Modulating Immunosuppressive Cellular Compartment

While most of the monotherapy and combinatorial approaches we have covered thus far are able to induce tumor-specific T cells and responses in a subset of patients, many patients remain refractory to these therapies. This is likely due to the T-cell response working in opposition to other driving immune force within the tumor microenvironment, e.g., subsets of myeloid and lymphoid cells exerting a strong immunosuppressive effect. Through secretion of immunosuppressive chemokines and cytokines as well as direct cell contact, malignant cells promote the development and recruitment of immunosuppressive Tregs, tumor-associated macrophages (TAMs),

and MDSCs [183–186]. Along with immunosuppressive costimulatory ligands and cytokines expressed by tumor cells, these leukocytes act in concert to limit immune reactivity to tumor cells; to effectively induce an immune response against tumor antigen, this immunosuppressive milieu will likely need to be disrupted (Fig. 28.3).

A critical cell responsible for modulating effector T-cell responsiveness is the Treg, and there is extensive literature demonstrating that malignant cells secrete chemokines recruiting intratumoral Tregs, which act to impair proliferation of T effector cells and impair their degranulation and cytotoxic activity [187, 188]. As mentioned previously, in addition to their cytotoxic effect on cancer cells, certain chemotherapies such as cyclophosphamide, anthracyclines, and platinum agents have been shown to exert preferential cytotoxicity to Tregs over effector T cells [15, 189]. In patients treated with chemoimmunotherapy, one study demonstrated a rapid reconstitution of the $CD8^+$ and NK cell number and function, while the $CD4^+$ compartment—particularly Tregs—remained depleted for up to a year [190]. While chemotherapy could play a role in conditioning before vaccination, most of the agents may also impede optimum lymphocyte responses, so to deplete Tregs in a more targeted manner, some vaccine trials have incorporated daclizumab, a humanized anti-CD25 antibody which depletes Tregs [191–193]. While the ultimate clinical effect in these early phase trials was negligible, the treatment effectively depleted Tregs in the circulation and could be considered for use in conjunction with other combinatorial immunotherapy-vaccine approaches.

Similar to tumor cells, TAMs and MDSCs express high levels of suppressive surface antigens including PD-L1 and through cytokines and direct modulation promote senescence of intratumoral T cells or skew the phenotype away from optimal cytotoxic effector functions; their presence and prevalence are unsurprisingly associated with a poor prognosis [194, 195]. While there is a paucity of antigen-specific surface markers on MDSCs and TAMs that would allow for targeting, Bruton's tyrosine kinase (BTK) is expressed in MDSCs, and treatment of mouse and human MDSCs with the Tec-kinase inhibitor ibrutinib significantly impaired cell migration

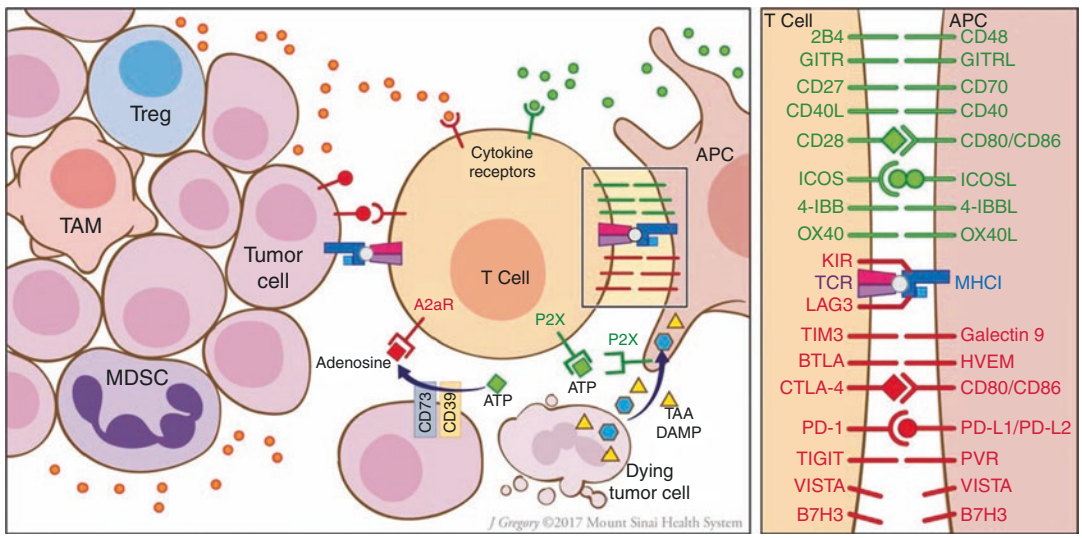

Fig. 28.3 Modulating the stimulatory/inhibitory components of the tumor microenvironment. The interface between the T cell and APC (*right panel*), which in many ways mirrors the ligand-receptor interactions seen between the T cell and the tumor cell, offers a multitude of targetable costimulatory and inhibitory ligands. Furthermore, tumor cells and recruited immunosuppressive cells such as Tregs, TAMs, and MDSCs promote immune evasion through release of cytokines skewing the effector response and through conversion of activating DAMPs such as ATP into inhibitory adenosine

and production of immunosuppressive cytokines [196, 197]. Though developed to target BTK, ibrutinib acts on other Tec-family kinases such as the IL-2-inducible T-cell kinase (ITK), which can also skew T-cell maturation toward an effector phenotype, further countering the immunosuppressive microenvironment [198]. This blockade of immunosuppressive pathways was further shown in a recent preclinical study of CpG-based ISV with adjuvant ibrutinib that demonstrated enhanced antitumor response [199]. Ibrutinib is now being used in combination with a variety of immunomodulatory compounds including checkpoint-blocking antibodies, with promising clinical responses.

There are also promising clinical and preclinical studies of other immunomodulatory compounds such as lenalidomide that suggest a possible adjuvant role in vaccine protocols. One preclinical trial looking at direct immunosuppressive activity of cancer cells found that tumor-infiltrating lymphocytes (TILs) developed impaired immune synapse formation with lymphoma cells upon incubation with malignant cells, blocking cytotoxic activity of the T cells, and that this defect in synapse formation was reversed after treatment with lenalidomide [200]. Recent studies of lenalidomide combined with rituximab in the treatment of lymphoma found increased ADCC-mediated killing by NK cells [201], and trials in multiple myeloma demonstrated improved efficacy of PD-1 blockade when given concomitantly with lenalidomide [202]. Clinical trials using lenalidomide with a variety of immunotherapies within and outside the hematological compartment are ongoing.

Conclusion

The field of cancer immunotherapy is rapidly evolving, with many new checkpoint-blocking and agonistic antibodies in the clinical trial pipeline. While these offer great promise, we know from studies of CTLA-4 and PD-1 blockade that inflamed tumors are most responsive to immune-mediated therapies. As such, local immunotherapies, such as ISV approaches that focus on homing APCs to a tumor, and stimulating immunogenic cell death and inflammatory pathways alongside checkpoint agonist/antagonist mAbs, tyrosine kinase inhibitors, or other immunomodulatory drugs will likely yield the rational combination therapies that allow us to achieve better clinical response rates and more durable immune memory.

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Strategies to Reduce Intratumoral Regulatory T Cells

29

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Abbreviations

A2aR	Adenosine 2a receptor
A2bR	Adenosine 2b receptor
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis

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APC	Antigen-presenting cell
ATLL	Adult T-cell leukemia–lymphoma
BCG	Bacille Calmette–Guérin
Bevacizumab	Anti-VEGF mAb
CCR4	C-C motif chemokine receptor 4
CCL22	C-C motif chemokine ligand 22
CEA	Carcinoembryonic antigen
CTCL	Cutaneous T-cell lymphoma
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
CTX	Cyclophosphamide
CXCL12	CXC chemokine ligand 12
CTL	Cytotoxic $CD8^+$ T cell
CDC	Complement-dependent cytotoxicity
DC	Dendritic cell
DD	Denileukin diftitox
DgA	Deglycosylated ricin A
EGFR	Epidermal growth factor receptor
eTreg	Effector Tregs
FDA	Food and Drug Administration
FDB	Fludarabine
FcγR	Fcγ receptor
FoxP3	Forkhead box P3
GITR	Glucocorticoid-induced TNF-related protein
GITRL	GITR ligand
HNSCC	Head and neck squamous cell carcinoma
ICOS	Inducible costimulator
IDO	Indoleamine 2,3-dioxygenase
IL-2	Interleukin-2
IL-2R	IL-2 receptor
LAG-3	Lymphocyte activation gene-3
mAb	Monoclonal antibody
MCP-1	Monocyte chemoattractant protein-1
mCRPC	Metastatic castration-resistant prostate cancer
MDSC	Myeloid-derived suppressor cell
MEDI4736/ atezolizumab/ durvalumab	Anti-PD-L1 mAbs
mRCC	Metastatic renal cell cancer
NIR	Near-infrared
NK	Natural killer cell
OS	Overall survival
OX40	CD134
OX40L	OX40 ligand
PD-1	Programmed death-1
PF-05082566	4-1BB agonist mAb
Pembrolizumab	Anti- PD-1 mAb
PTCL	Peripheral T-cell lymphoma
PTX	Paclitaxel
RCC	Renal cell carcinoma
Rituximab	Anti-CD20 mAb
SDF-1	Stromal cell-derived factor-1
Tconv	Conventional T cells
Teff	Effector T cell
TI	Tumor-infiltrating
TIL	Tumor-infiltrating lymphocyte
TME	Tumor microenvironment
TNF	Tumor necrosis factor
Treg	Regulatory T cell
Tremelimumab	Anti-CTLA-4 mAb
VEGF-A	Vascular endothelial growth factor-A

29.1 Introduction

29.1.1 Treg Characterization, in Mouse and Human

Regulatory T cells (Tregs) include diverse subsets of immunosuppressive cells that play a critical role in self-tolerance and immune homeostasis. Due to their immunosuppressive capacities, Tregs are able to suppress antitumoral responses through several mechanisms and therefore enhance tumor escape and progression [1].

Tregs are characterized by the expression of forkhead box P3 (**FoxP3**), which is essential for their development and function [2, 3]. While mouse Tregs express constitutively FoxP3 [2], human Tregs do not necessarily do. Moreover, activated human conventional T cells (Tconv) transiently express intermediate levels of FoxP3 [4]. That is why the characterization and identification of human Tregs is more complex and involves more combined markers [5]. Currently, in human studies, Tregs are identified by flow

cytometry as $CD3^+$ $CD4^+$ $CD25^{high}$ $CD127^{low}$ cells [5, 6].

Furthermore, in both mouse and human, Tregs are described to express high levels of inhibitory receptors (e.g., the cytotoxic T lymphocyte antigen-4 (**CTLA-4**) [7, 8], the programmed death-1 (**PD-1**) [9], and the lymphocyte activation gene-3 (**LAG-3**) [10]), costimulatory receptors (e.g., the glucocorticoid-induced TNF-related protein (**GITR**) [11, 12] and **OX40**, also known as CD134 [13]), and other important surface markers such as the inducible costimulator **(ICOS)** [14] and the ectonucleotidases **CD39** [15] and **CD79** [16].

29.1.2 Basic Treg Immunosuppressive Activities

Treg immunosuppressive functions can be summarized as four well-known mechanisms:

1. Release of cytokines known as immunosuppressive mediators such as **IL-10** [17], **TGFβ** [18], and **IL-35** [19]
2. Direct cytolytic activity through **granzyme A**, **granzyme B,** and **perforin** secretion [20]
3. Disruption of T-cell metabolism through their **CD25** receptor, **CD39** [21], and **CD73** ectonucleotidases [16]
4. Inhibition and modulation of dendritic cells (DC) through co-inhibitory receptors such as **LAG-3** [10] or **CTLA-4** [22, 23]

29.1.3 Prognostic Value of Treg Presence in Peripheral Blood and Tumor Microenvironment in Human Cancers

Tregs play a crucial role in the maintenance of self-tolerance, preventing autoimmunity and chronic inflammatory diseases in healthy individuals [24]. However, in cancer patients, the effect exerted by Tregs on tumor progression seems to vary according to the tumor type.

The presence of Tregs in peripheral blood and tumor sites has been extensively studied, and Tregs have been associated with a bad prognosis in many human cancer types including pancreatic [25], liver [26], lung [27], breast [25], and ovarian cancer patients [27, 28]. On the opposite, Tregs may exert a beneficial role in follicular lymphoma [29] and in head and neck carcinoma [30]. Finally, they seem to have no effect on survival in anal squamous cell carcinoma [31].

29.1.4 Treg Recruitment into Tumor Microenvironment

Tregs are recruited into the tumor in response to chemokines secreted by malignant cells and innate immune cells. Key chemokine–chemokine receptor pathways include **CCL17/CCL22–CCR4** [14, 32] and **CXCL12–CXCR4** [33]. Moreover, tumor-infiltrating (TI) Tregs exhibit enhanced suppressive capacity compared to Tregs from peripheral blood or healthy tissues, probably due to an activation by the tumor microenvironment (TME) [34–38].

29.1.5 Treg-Targeting Approaches in Cancer Therapy

As they mostly promote tumor progression and immune escape, Tregs offer promising targets for novel therapeutic strategies for cancer. A number of approaches have already been approved by the medicine agencies and many others exploring Treg depletion, or impairment of their suppressive functionality is currently under development [35, 39, 40].

The first strategies developed for Treg-targeting used CD25-specific antibody and certain chemotherapeutic drugs at metronomic doses. The disadvantage of these treatments is that they also target activated $CD4^+$ and $CD8^+$ T cells, as these cells share many phenotypic markers with Tregs. Today, several approaches have been developed with the aim to inhibit Tregs by either suppressing their function through targeting their immunosuppressive activities listed above or limiting their migration to the TME through targeting the chemokine–chemokine receptor pathways involved in Treg trafficking to tumor sites. Moreover, other drugs originally developed for other therapeutic

indications such as anti-angiogenic molecules and tyrosine kinase inhibitors, have recently been assessed for Treg inhibition.

In this chapter, all these approaches used in mouse tumor models and in human clinical trials will be developed in more details.

29.2 Treg Depletion by Low Dose of Chemotherapy

Anticancer chemotherapies used at their conventional and clinically effective dose have been historically thought to exert their antitumoral activity only through direct killing of tumor cells and to be generally immunosuppressive. However, recent data showed that chemotherapies could induce immune-mediated anticancer responses.

In the early 2000s, a metronomic regimen (a low and minimally toxic dose administration, over a long period of time) of certain conventional chemotherapeutic drugs, including **cyclophosphamide** (CTX), **paclitaxel** (PTX), and **fludarabine** (FDB), has first been used in patients with advanced chemotherapy-resistant cancers, to reduce tumor angiogenesis with minimal toxicity [41, 42].

In rodent and human studies, this approach was reported to have an additional immunomodulatory action through Treg depletion within the TME and peripheral blood, as well as by leading to functional impairment of these immunosuppressive cells [43, 44] (Fig. 29.1a). Therefore,

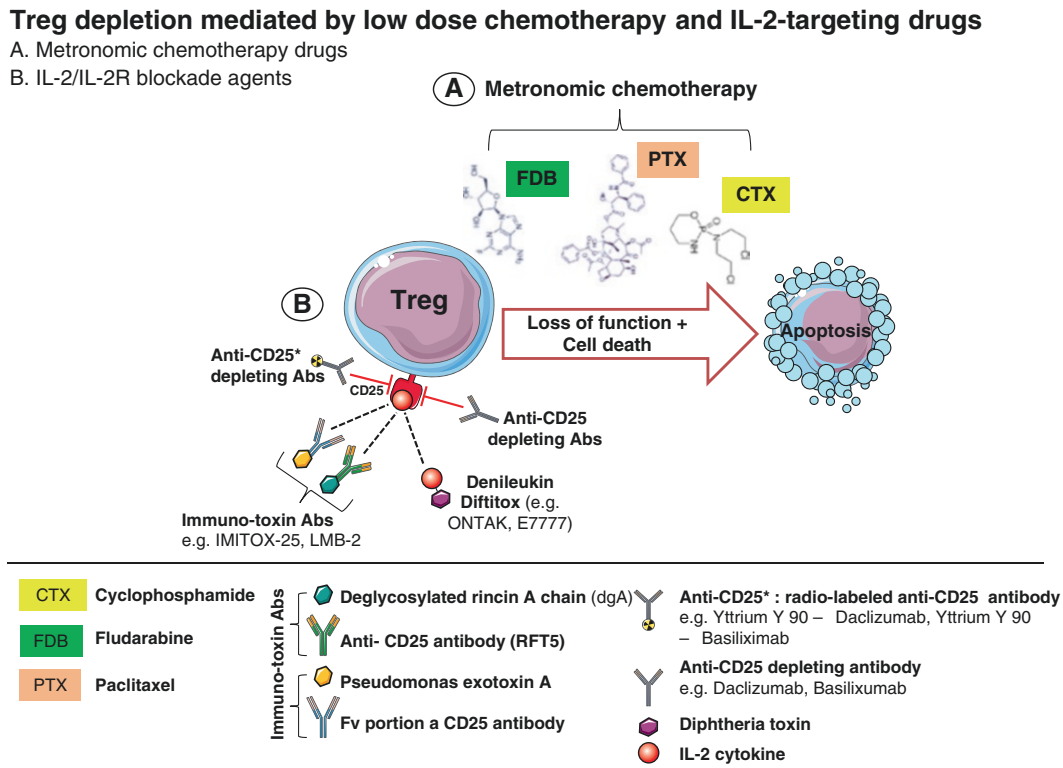

Fig. 29.1 Treg depletion mediated by low-dose chemotherapy and IL-2-/IL-2R-targeting drugs. (**a**) Low-dose oral metronomic chemotherapeutic agents including cyclophosphamide (CTX), paclitaxel (PTX), and fludarabine (FDB) induce a profound and selective decrease on Treg number. (**b**) IL-2/IL-2 receptor blockade agents: daclizumab and basiliximab, two anti-CD25 antibodies (radiolabeled or not), can invoke cell death (apoptosis) by mediating ADCC or CDC. Two CD25-directed immunotoxin antibodies are being tested in human cancers: IMITOX-25 (RFT5-dgA), a CD25 antibody RFT5 linked to deglycosylated ricin A, and LMB-2, a fusion protein composed of the Fv portion of CD25 antibody attached to a fragment of *Pseudomonas* exotoxin A. Due to its IL-2 component, denileukin diftitox/ONTAK is capable of binding to CD25 (high-affinity IL-2 receptor) causing its rapid endocytosis and resulting in Treg cell apoptosis

metronomic chemotherapies resulted in a restoration of tumor-infiltrating lymphocyte (TIL) antitumoral functions [45].

Ghiringhelli and colleagues were the first to demonstrate that a single dose of CTX (30 mg/kg, i.p.) can deplete Tregs from tumor-bearing rats compared with nontreated animals [46]. These preclinical results were further confirmed by the same group in patients with advanced solid tumors [43]. They showed a strong and selective decrease of peripheral immunosuppressive Tregs, improving the control of tumor progression. Moreover, these authors described metronomic CTX as a potent treatment for reducing tumor-induced immune tolerance before anticancer immunotherapy.

Furthermore, Yingzi Ge et al. monitored in treatment-refractory and metastatic-advanced breast cancer patients the immunological effect of metronomic CTX (50 mg/day per OS during 3 months) on circulating Treg number and function, as well as on the induction of tumor-specific T-cell responses. The authors measured a transient Treg depletion with a stable breast tumor-reactive T-cell response, which was correlated with good clinical outcomes in these patients [47].

Treg depletion by low-dose chemotherapy prior to vaccination showed a significant enhanced overall survival (OS) in patients and elicited an effective antitumor immune response, as reported in many murine tumor models and in different human cancers. For example, in a randomized phase II study using a peptide-based vaccine for renal cancer treatment, it has been shown that a single dose of CTX reduced the number of Tregs and induced specific immune responses, associated with longer OS [48].

In a recent phase I/II clinical trial, metastatic castration-resistant prostate cancer (mCRPC) patients received a long-term administration of a DC-based vaccine combined with chemotherapy drugs (metronomic CTX, docetaxel, and prednisone). A significant decrease in Tregs in the peripheral blood was observed. The long-term administration of the vaccine led to the induction and maintenance of tumor-specific T cells, but no immunological parameter was significantly correlated with better OS [49].

In contrast to these clinical outcomes, in a phase I clinical trial, 49 metastatic cancer patients (breast, lung, kidney, stomach, bladder, and prostate cancers) received a bacille Calmette–Guérin (BCG) vaccine plus CTX, to induce Treg depletion. Surprisingly, the number of peripheral Tregs was significantly increased upon treatment [50]. One possible explanation for the opposite effect of CTX in this study could be the difference between the routes of administration. In fact, in the clinical studies mentioned above, CTX was orally administrated, whereas in the latter example, patients received a single intravenous infusion of CTX.

However, other reports supported that metronomic drugs, even administrated orally, induced an increase of Treg number and suppressive activity. Koumarianou and colleagues showed not only an increase of Treg number in the peripheral blood of cancer patients but also an increase of Treg/effector T-cell (Teff) ratio and of the immunosuppressive activity of Tregs. They also found that these effects were more profound in metronomic than in standard chemotherapeutic approaches [51].

In a phase II trial, Ellebaek et al. also showed that despite using metronomic CTX in combination with a COX-2 inhibitor and a DC-based vaccine, Tregs did not decrease after the treatment in melanoma patients [52].

To date, the reasons for the contradictory effects of metronomic chemotherapy on Tregs are not clear [53]. The doses and the schedule of drug administration, the type and stage of cancer, the mode of action of the chemotherapeutic drugs, the basal status of the immune system of patients before treatment, and the choice of combinations should be taken into particular account by clinicians in the design of experimental approaches including metronomic chemotherapy drugs.

29.3 IL-2 Receptor (IL-2R)-Induced Treg Depletion

Tregs are characterized by the expression of IL-2R α chain or CD25, which is critical to their expansion [54]. IL-2R on Tregs also acts as a

regulatory mechanism. High-affinity IL-2R on Tregs limits the amount of IL-2 available for Tconv, hindering their expansion and activation [1]. This is the reason why some Treg-depleting strategies use the IL-2/IL-2R axis to deplete Tregs in cancer and, therefore, restore antitumoral immunity.

29.3.1 Anti-CD25 Antibodies (e.g., Daclizumab and Basiliximab)

Daclizumab is a humanized IgG1-κ monoclonal antibody (mAb) targeting human CD25. It is well tolerated when administered and causes no severe secondary effects [55]. Initially approved by the Food and Drug Administration (FDA) in 1997 for the prevention of renal allograft rejection, it is now been tested for the depletion of Tregs (Fig. 29.1b) in several cancer clinical trials [56] (Table 29.1).

The first group to investigate the effect of daclizumab on Treg depletion in cancer used a single low dose of 0.5 mg/kg, prior to a DC vaccination on metastatic melanoma patients [57]. They reported that daclizumab depleted $CD4^+$ $CD25^{high}$ $FoxP3^+$ T cells from patients' peripheral blood but measured a clearance of daclizumab associated with the reappearance of Tregs approximately 30 days after. However, the functional T-cell-specific response to the vaccine was less effective on patients pretreated with daclizumab, compared to the group who received only the vaccine.

Table 29.1 CD25-targeting agents in cancer clinical trials

Treatment	Cohort	Indication	Trial phase	Start date—status	NCT ID
LMB-2 an anti-Tac(Fv)-PE38 recombinant immunotoxin + peptide-based vaccine	26	Metastatic melanoma	Phase II	12/2005 (completed)	NCT00295958
IMTOX-25 (RFT5-dgA) anti-CD25 immunotoxin	41	Metastatic melanoma	Phase II	04/2006 (completed)	NCT00314093
Basiliximab (Simulect®) antihuman CD25 chimeric antibody + DC-based vaccine	18	Glioblastoma multiforme	Phase I	03/2007 (active, not recruiting)	NCT00626483
Daclizumab + peptide-based vaccine + **Prevnar**	11	Metastatic breast cancer	Phase I	11/2007 (completed)	NCT00573495
IMTOX-25	29	Advanced cutaneous T-cell non-Hodgkin lymphoma	Phase II	07/2008 (completed)	NCT00667017
LMB-2 + fludarabine + CTX	18	Adult T-cell leukemia	Phase II	10/2008 (active, not recruiting)	NCT00924170
Daclizumab + tumor lysate-loaded DC-based vaccine or + bevacizumab	67	Recurrent ovarian, primary peritoneal, or fallopian tube cancer	Phase I	05/2010 (active, not recruiting)	NCT01132014
Yttrium-90-labeled daclizumab + chemotherapy + auto-stem cell transplant	6	Advanced Hodgkin lymphoma	Phase I/II	10/2011 (active, not recruiting)	NCT01468311
Yttrium Y 90 basiliximab + chemotherapy (before autologous hematopoietic cell transplantation)	24	Mature T-cell non-Hodgkin lymphoma	Phase I	06/2015 (recruiting)	NCT02342782

In view of the significant number of cancer clinical trials involving CD25-targeting agents, we propose here a non-exhaustive list of recent clinical trials (since 2005) monitoring anti-CD25 mAbs and immunotoxin Abs. The choice of the samples was made based on the cancer indication and the combinatory therapies, providing an overall view of the current clinical applications of CD25-targeting agents. All clinical trials can be found in https://clinicaltrials.gov/

In another trial, Rech et al. investigated the effect of daclizumab on metastatic breast cancer patients in association with a peptide-based vaccine [58]. The authors observed that daclizumab permitted both a marked and durable depletion of Tregs in peripheral blood and an effective boosting of vaccine-induced specific T-cell responses.

Those studies demonstrated that antihuman CD25 daclizumab is capable of inducing Treg depletion in cancer patients. Treg depletion can lead to $CD25^-$ T-cell activation, and the remaining antibody in the system would likely be deleterious to newly activated T cells expressing CD25 [59]. In fact, daclizumab in vivo half-life is over 4 weeks, which could explain the lower rate of T-cell-specific response after vaccination observed in Jacobs et al. study.

Basiliximab, a chimeric antibody, is another antihuman CD25 mAb, which is assessed in cancer clinical trial for Treg depletion (Fig. 29.1b). It is currently being tested in different phase I clinical trials in glioblastoma, acute myeloid leukemia, and Hodgkin and non-Hodgkin lymphoma cancer patients (Table 29.1).

29.3.2 Denileukin Diftitox (ONTAK®)

Denileukin diftitox (DD)/ONTAK is a genetically engineered recombinant fusion protein composed of diphtheria toxin catalytic domain and the full-length human interleukin-2 (IL-2). Due to its IL-2 component, DD is capable of binding to high-affinity IL-2R causing its rapid endocytosis [60] and resulting in cell apoptosis within 40–72 h [61] (Fig. 29.1b). ONTAK has been approved by the FDA since 1999 for the treatment of patient with persistent or recurrent cutaneous T-cell lymphoma (CTCL), whose malignant cells express IL-2R α chain CD25 [62]. Since then, ONTAK has also been investigated in $CD25^-$ cancers, to deplete $CD25^+$ Treg populations. So far, ONTAK has been used in numerous clinical trials for Treg depletion in different cancers, dosages, and regimen, all with various outcomes (Table 29.2).

Several studies have been using ONTAK at a single dose of 18 μg/kg, in combination with DC or peptide vaccination. These trials include the treatment of carcinoembryonic antigen (CEA)-positive malignancies [63], metastatic renal cell carcinoma (RCC) [64], and metastatic melanoma [65]. Among those three studies, two reported no changes in the number of $CD4^+$ $CD25^+$ $FoxP3^+$ at this dose [63, 65]. On the other hand, the RCC trial observed a transient depletion of $CD4^+$ $CD25^{high}$ Tregs, with no apparent impact on other cell populations, including $CD25^{low}$ T cells [64].

Among the different trials about Treg depletion with ONTAK, four studies were published about metastatic melanoma. Taken together, ONTAK showed its efficacy to transiently deplete Tregs in metastatic melanoma patients at 12 μg/kg/day for 4 consecutive days [66, 67], at 5 and 18 μg/kg/day for 3 consecutive days [68], but not at the dose of 18 μg/kg for a single injection [65] nor at 9 and 18 μg/kg/day for 5 consecutive days [59]. Those results show that appropriate dosage of ONTAK is important to effectively deplete Tregs in melanoma patients as well as in other type of cancers [63, 69].

Despite the depletion of Treg populations observed in some studies, ONTAK clinical benefits have not been as strong as expected, leading to no or very limited beneficial outcomes for patients. This leads to the question: What if ONTAK has other undiscovered effects on patients' immune system explaining its lack of efficacy? In a clinical trial, melanoma patients were treated with ONTAK to deplete Tregs before a DC-based vaccination. Interestingly, ONTAK pretreatment failed to induce tumor antigen-specific $CD4^+$ and $CD8^+$ T cells after numerous DC vaccinations, leading to no clinical benefits for the patients [60]. The authors demonstrated that ONTAK treatment acts not only as a Treg-depleting agent but also as a strong immunomodulator, leading to a tolerogenic DC phenotype [70, 71]. ONTAK also failed to induce apoptosis on resting Tregs, which showed increased survival [60].

Very recently, a phase III trial was launched to evaluate the safety and efficacy of E7777 (improved purity ONTAK) in persistent or recurrent CTCL (NCT01871727).

Table 29.2 ONTAK (denileukin diftitox) in cancer clinical trials

Treatment	Cohort	Indication	Trial phase	Start date—status	NCT ID
ONTAK denileukin diftitox + aldesleukin	20	Kidney cancer	Early phase I	04/2005 (completed)	NCT00278369
ONTAK denileukin diftitox	17	Adult T-cell leukemia	Phase II	07/2005 (completed)	NCT00117845
ONTAK denileukin diftitox	15	Advanced breast cancer	Phase I/II	09/2005 (active, not recruiting)	NCT00425672
ONTAK denileukin diftitox + autologous DC-based vaccine	24	Adult solid tumor	Phase I	09/2005 (completed)	NCT00128622
ONTAK denileukin diftitox	69	Stage IV melanoma	Phase II	03/2006 (completed)	NCT00299689
ONTAK denileukin diftitox	19	Epithelial ovarian cancer, extraovarian peritoneal cancer, and fallopian tube carcinoma	Phase II	02/2007 (completed)	NCT00880360
ONTAK denileukin diftitox + rituximab	24	Advanced non-Hodgkin lymphoma	Phase II	04/2008 (completed)	NCT00460109
ONTAK denileukin diftitox	90	Advanced melanoma	Phase II	06/2010 (active, not recruiting)	NCT01127451
ONTAK + allogeneic NK cells + rituximab + chemotherapy	32	Non-Hodgkin lymphoma and chronic lymphocytic leukemia	Phase II	08/2010 (completed)	NCT01181258
E7777 (Eisai Inc.) improved purity ONTAK	90	Cutaneous T-cell lymphoma	Phase III	05/2016 (recruiting)	NCT01871727

A non-exhaustive list of recent clinical trials (since 2005) monitoring ONTAK effect on Treg depletion. The choice of the samples was made as described in Table 29.1. All clinical trials can be found in https://clinicaltrials.gov/

29.3.3 Anti-CD25 Immunotoxin Antibodies

Two CD25-directed immunotoxin antibodies, **IMITOX-25** (RFT5-dgA), a CD25 antibody RFT5 linked to deglycosylated ricin A (after internalization, dgA induces a cell death via protein synthesis inhibition), and **LMB-2**, a fusion protein composed of the Fv portion of CD25 antibody attached to a fragment of *Pseudomonas* exotoxin A (this immunotoxin is known to induce caspase-mediated apoptosis) (Fig. 29.1b), have been developed to reduce anti-CD25 in vivo half-life (<4 h) and have been tested in clinical trials [72–74] (Table 29.1). Also, a transient depletion of $CD25^+$ Tregs among the patients were noted, the $CD25^-$ Treg subset survived, and the depletion of $CD25^+$ Tregs was not sufficient to increase the immune response to peptide vaccination.

In summary, daclizumab, ONTAK, and other $CD25^+$ cell-depleting agents have shown, in some cases, great potential to deplete $CD25^+$ Tregs in cancer patients. However, the ability of these agents to target all $CD25^+$ cells could explain why in some trials, those molecules failed to increase vaccine-induced specific response, as CD25 marker is not specific for Tregs [75]. The persistence of $CD25^{int/low}$ Tregs in those studies also shows the need to develop more Treg-specific therapies or to combine them with other Treg-depleting agents to target a larger population of Tregs.

29.3.4 Emerging New Anti-CD25 Antibody Therapies

Another pitfall of anti-CD25 targeting is the systemic depletion of Tregs leading to profound disruption of peripheral homeostasis and severe side effects. An exciting new generation of anti-CD25 antibodies aims to selectively deplete intratumoral Tregs.

Recently, Sato et al. associated an anti-CD25-F(ab')2 with a photoactivable silicon phthalocyanine dye (IRDye 700DX) to selectively deplete TI Tregs on tumor-bearing mice [76]. After injection of the photoactivable antibody, local exposure of near-infrared (NIR) light on the tumor leads to the activation of the IRDye 700DX, and therefore induces the depletion of Tregs within the TME, without damaging adjacent cells nor NIR-unexposed $CD25^+$ systemic Tregs. After intravenous injection of the labeled antibody and exposure to NIR light, they reported a depletion of 85% of $CD4^+$ $CD25^+$ Tregs at the tumor site within 30 min. They also reported a rapid $CD8^+$ T cells, NK cells, as well as antigen-presenting cells activation after NIR treatment. This local Treg depletion was followed by tumor regression and prolonged survival of the mouse after only one treatment.

FDA-approved human CD25 antibodies such as daclizumab and basiliximab reduce the barrier of translating this new treatment into clinical trial. Moreover, a phase I human study using the anti-epidermal growth factor receptor (EGFR) conjugated with the IRDye 700DX is currently being evaluated for inoperable head and neck cancer (NCT02422679), making these NIR photoimmunotherapy strategies suitable for the treatment of cancer patient in clinical trial.

29.4 Blocking Treg Cell Trafficking into Tumors

29.4.1 CCL22–CCR4 Pathway Blockade

The C-C motif chemokine receptor 4 and C-C motif chemokine ligand 22 (CCR4–CCL22) pathway represent a dominant mechanism responsible for intratumoral Treg recruitment, as described in multiple tumor types [28]. Indeed, Treg trafficking and infiltration into different tumor types appear to be dependent on the expression of CCR4 ligands (CCL17 and CCL22) produced by tumor cells or infiltrating macrophages [32] (Fig. 29.2a). Furthermore, CCR4 is highly expressed by $FoxP3^{high}$ $CD25^{high}$ $CD45RA^-$ cells, designated as effector Tregs (eTregs) [77], which are predominant among TI $FoxP3^+$ T cells [78].

In a CTCL mouse model, Ito et al. used an anti-CCR4 antagonist mAb and showed a significant depletion of TI Tregs mediated by NK-induced antibody-dependent cell-mediated cytotoxicity (ADCC) [79]. Our group has also shown that inhibition of the CCR4 expressing Treg population by means of a CCR4 antagonist was sufficient to break immune tolerance to spontaneous mammary tumors, suggesting a major role for CCR4 in the immunosuppressive activity of Tregs [80]. All together, these observations provide a rationale for the development in clinical trials of CCR4 and CCL22 antagonists in order to block Treg cell trafficking into tumors.

So far, CCR4 antagonists have been mainly evaluated in patients with T-cell lymphoma (adult T-cell leukemia–lymphoma (ATLL), peripheral T-cell lymphoma (PTCL), and CTCL, whose tumor cells also express CCR4 at their surface) [81, 82] (Table 29.3).

Mogamulizumab (KW-0761) is a defucosylated, humanized IgG1 mAb targeting CCR4 (Fig. 29.2a), engineered to exert potent ADCC. It showed promising therapeutic potential in phase I and II clinical trials in treating CTCL which is associated with poor prognosis [81, 83, 84].

The initial phase I/II multicenter study evaluated the efficacy of mogamulizumab (dose escalation study) as a monotherapy, in pretreated patients with PTCL or CTCL. The anti-CCR4 mAb depleted efficiently the circulating $CCR4^+$ eTregs, even at the lowest dose of 0.1 mg/kg, and restored antigen-specific cytotoxic T lymphocyte (CTL) responses [85, 86]. Recently, the same group launched a phase III trial, comparing the progression-free survival of mogamulizumab versus vorinostat (histone deacetylases inhibitor) in the treatment of CTCL (NCT01728805).

Sun et al. showed that monocyte chemoattractant protein-1 (MCP-1), an endogenous CCR4-binding ligand, was specifically upregulated in the head and neck squamous cell carcinoma (HNSCC) patients, compared to the other CCR4-binding ligands. Using a CCR4 antagonist that

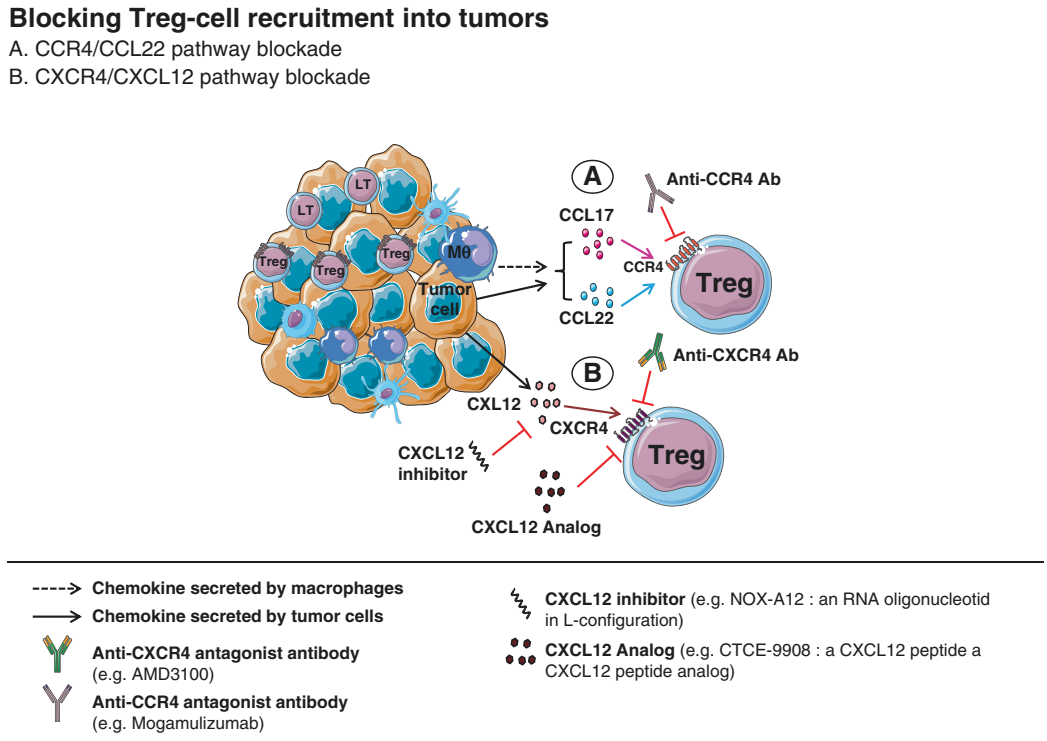

Fig. 29.2 Blocking Treg cell recruitment into tumors. Tregs are recruited into the tumor in response to chemokines secreted by malignant cells and innate immune cells (e.g., macrophages). CCL17/CCL22–CCR4 (**a**) and CXCL12–CXCR4 (**b**) are two key chemokine–chemokine receptor pathways, targeted by specific antagonist monoclonal antibodies and chemokine inhibitors and analogs (which competitively binds to the chemokine receptor). Blocking this axis may impede tumoral homing of Tregs and promote tumor growth regression

blocks eTreg cell recruitment induced through MCP-1/CCR4 signaling, the authors observed an inhibition of tumor growth and prolonged survival [78].

Since 2014, the mogamulizumab efficacy is evaluated in advanced or metastatic solid cancer patients. Most of these trials are using mogamulizumab in combination with other immunotherapies including anti-PD-1, anti-PD-L1 and anti-CTLA-4, anti-4-1BB antibodies, and indoleamine 2,3-dioxygenase (IDO) inhibitor (Table 29.3).

29.4.2 CXCR4–CXCL12 Pathway Blockade

The CXC chemokine ligand 12 (CXCL12), also called stromal cell-derived factor-1 (SDF-1), secreted by bone marrow, lymph node, and inflammatory cells is another chemokine responsible for the trafficking of Tregs expressing CXCR4, the receptor of CXCL12 [33, 87].

The CXCL12–CXCR4 pathway is known to be widely involved in tumor cell progression, angiogenesis, and metastasis in a number of human cancers [88, 89] including melanoma [90], ovarian [91], breast [33], small cell lung [92, 93], and gastric [94] cancers. Therefore, this axis is associated with metastasis induction [95, 96] and poor clinical outcome [33, 97], making it an attractive target for therapeutics that can block the CXCL12/CXCR4 interaction or inhibit downstream intracellular signaling.

Multiple agents are currently being developed to target the CXCL12 pathway in cancer, including **AMD3100**, a highly specific CXCR4

Table 29.3 CCR4-targeting antibody in clinic

Treatment (company)	Cohort	Indication	Trial phase	Start date—status	NCT ID
Mogamulizumab (Kyowa Hakko Kirin Co.) KW-0761 a defucosylated humanized anti-CCR4 antibody	16	Adult T-cell leukemia and lymphoma (ATL), adult peripheral T-cell lymphoma (PTCL)	Phase I	02/2007 (completed)	NCT00355472
Mogamulizumab	42	PTCL or cutaneous T-cell lymphoma (CTCL)	Phase I/II	05/2009 (completed)	NCT00888927
Mogamulizumab vs. vorinostat	372	Previously treated CTCL	Phase III	11/2012 (active, not recruiting)	NCT01728805
Mogamulizumab	72	Advanced solid tumors	Phase I/II	10/2014 (recruiting)	NCT02281409
Mogamulizumab ± MEDI4736 ± tremelimumab	108	Advanced solid tumors	Phase I	11/2014 (recruiting)	NCT02301130
Mogamulizumab + docetaxel	13	Previously treated non-small cell lung cancer	Phase I	01/2015 (completed)	NCT02358473
Mogamulizumab + PF-05082566	70	Advanced solid tumors	Phase Ib	05/2015 (recruiting)	NCT02444793
Mogamulizumab + nivolumab	188	Advanced solid tumors	Phase I/II	12/2015 (recruiting)	NCT02705105
KHK2455 IDO-1 inhibitor ± **mogamulizumab**	50	Solid tumors	Phase I	08/2016 (recruiting)	NCT02867007

A non-exhaustive list of recent clinical trials monitoring anti-CCR4 mAb in cancer patients. The choice of the samples was made based as described in Table 29.1. All clinical trials can be found in https://clinicaltrials.gov/

antagonist [98], also known as plerixafor; **CTCE-9908,** a CXCL12 analog which competitively binds to CXCR4; and a CXCL12 inhibitor, Spiegelmer/**NOX-A12** (Fig. 29.2b). Among these drugs, AMD3100 and CTCE-9908 are approved for clinical use in patients with leukemias and osteosarcoma, respectively [99].

Recent studies have demonstrated the potential involvement of CXCL12–CXCR4 pathway activation in tumor resistance to conventional chemotherapies [100–102] and anti-angiogenic treatments [103]. Therefore, emergent combinatory approaches involving CXCL12 pathway blockade are being developed in order to face these drug resistance [103].

However, the impact of CXCL12–CXCR4 pathway blockade on Treg cell populations has not been fully investigated in human and requires further investigations.

29.5 Anti-angiogenic Therapies Targeting Regulatory T Cells

Vascular endothelial growth factor-A (VEGF-A) has a central role in tumor-induced immunosuppression, especially in the accumulation of Tregs [104]. VEGF-A can block DC maturation [105] or enhance myeloid-derived suppressor cell proportion in tumor-bearing hosts [106]. These two cell types can be involved in the proliferation of Tregs or the conversion of Tconv to Tregs. Moreover VEGF-R2, a VEGF-A receptor, is expressed on Tregs in tumor-bearing mice [107] and in human tumors [108] suggesting a direct role of VEGF-A on Tregs. Indeed, tumor-derived VEGF-A was shown to directly induce the proliferation of Tregs in tumor-bearing mice and in metastatic cancer patients [107].

Anti-angiogenic molecules targeting the VEGF-A/VEGF-R2 pathway are routinely used to treat many cancer patients (e.g., metastatic colorectal cancer, metastatic renal cell cancer, advanced non-small cell lung cancer, ovarian cancer). The administration of **sunitinib**, a tyrosine kinase inhibitor that targets VEGF-R, PDGFR, c-kit, and Flt3, decreases the proportion of Tregs in metastatic renal cell cancer (mRCC) patients [109]. This decrease has been associated with a better OS in another cohort of mRCC [110].

The negative impact of anti-angiogenic molecules on Tregs has been confirmed with another multi-target tyrosine kinase inhibitor which is **sorafenib** (VEGF-R, PDGFR, c-kit, Raf kinase, RET inhibitor) in mRCC patients and in hepatocellular cancer patients [111].

Bevacizumab, a humanized anti-VEGF-A mAb, also decreases the proportion and number of Tregs in tumor-bearing mice and in metastatic colorectal cancer patients [107]. Thus, VEGF-A/VEGF-R2 blockade can restore steady-state Treg proportion but can also modulate other escape mechanisms induced by the tumor (myeloid-derived suppressor cell (MDSC) induction, expression of inhibitory checkpoints on $CD8^+$ T cells, etc.).

Based on these properties, we have shown that VEGF-A antibody can synergize with immunotherapy and especially with checkpoint inhibitors in a mouse model of colorectal cancer [112].

The association of anti-angiogenic molecules with immunotherapies is currently evaluated in different cancer locations (metastatic melanoma NCT02400385, renal cell carcinoma NCT02348008, non-small cell lung cancer NCT02039674).

29.6 Immune Checkpoint Blockade Therapy (Anti-CTLA-4 Antibodies)

CTLA-4, a co-inhibitory receptor, is transiently expressed on activated cytotoxic T lymphocytes and acts as an inhibitory molecule to reduce immune response, IL-2 production, and cell cycle [113, 114]. This negative feedback prevents lymphoproliferative and autoimmune responses in different mouse models [115–118].

In cancer, CTLA-4 expression on Teff induces hyporesponsiveness or anergy against malignant cells [119]. This characteristic of CTLA-4 has led to the development of numerous clinical trials using anti-CTLA-4 antibodies such as **ipilimumab** (IgG1) and **tremelimumab** (IgG2), two full human mAbs [120, 121]. Ipilimumab was approved by the FDA in 2011 and is currently a first-line treatment option for patients with advanced melanoma. Tremelimumab was approved by the FDA in 2015 for patients with malignant mesothelioma.

However CTLA-4 is also constitutively expressed by Tregs, which plays an important role in their regulatory function [122]. This feature led the scientific community to investigate potential effects of this therapy on Tregs, both in preclinical and clinical studies.

Kavanagh and colleagues investigated the effect of different dosage of ipilimumab on metastatic prostate cancer [113]. They observed that low dose of 1.5 mg/kg or more induced a dose-dependent increase of peripheral $CD4^+$ $FoxP3^+$ functional Tregs. Similar results were also found by others, showing that anti-CTLA-4 therapies can induce Treg proliferation in periphery [123–125]. However, the immune profile of the peripheral blood does not necessarily reflect what occurs in the TME.

Indeed, two preclinical studies have shown that anti-CTLA-4 mAbs induce a reduction of TI Tregs, associated with tumor regression. This CTLA-4-mediated Treg depletion was dependent on the anti-CTLA-4 isotype [126] and the Fcγ receptor (FcγR) expressing cells, present in the TME [127]. Similar results were found in human studies, where ipilimumab was able to mediate, through NK cells, an ADCC of $CTLA\text{-}4^+$ TI Tregs (Fig. 29.3a) isolated from head and neck squamous cell carcinoma patients [128]. In melanoma patients also, ipilimumab induced ADCC mediated by $FcγRIII^+$ nonclassical monocytes [129].

Tremelimumab, an IgG2 mAb, is not likely to do ADCC and induce subsequent TI Treg

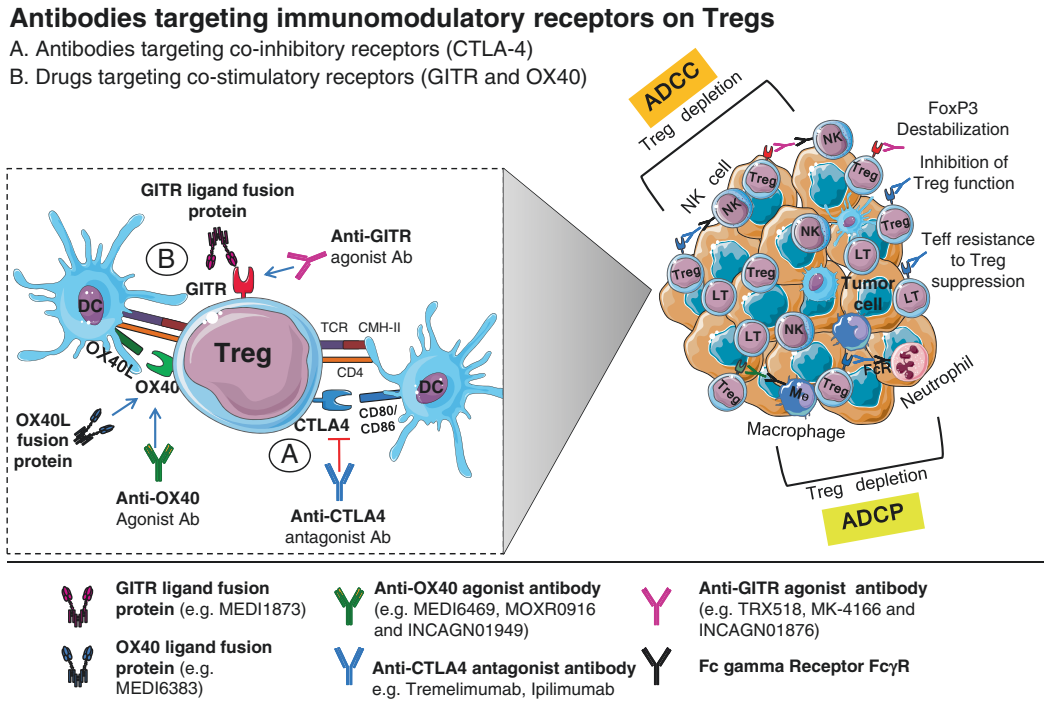

Fig. 29.3 Antibodies targeting immunomodulatory receptors on Tregs. (**a**) Anti-CTLA-4 mAbs may induce a reduction of TI Tregs, associated with tumor regression. Engagement of ipilimumab, an IgG1 mAb, with CTLA-4 on Tregs is able to mediate an ADCC of CTLA-4$^+$ TI Tregs, while CTLA-4 blockade by tremelimumab, an IgG2 mAb, inhibits Treg suppressive functions and induces Teff resistance to Treg suppression. (**b**) Engagement of costimulatory receptors (GITR and OX40) expressed on Tregs by agonist antibodies or by their respective ligand fusion protein bearing an IgG1 Fc antibody that can also bind to activating Fc gamma receptor (on NK cells, neutrophils, and macrophages) leads to Treg depletion via ADCC or ADCP. Anti-GITR antibodies can also induce loss of Treg stability through FoxP3 destabilization

depletion. In phase I and II studies, tremelimumab showed some clinical outcomes in metastatic melanoma patients [130]. However, in a phase III study (NCT00257205), tremelimumab failed to obtain statistically significant survival advantage compared to conventional chemotherapy in first-line treatment of metastatic melanoma patients. Based on these results, the clinical trial has been halted in April 2008 [131].

On the other hand, Ménard et al. showed in advanced melanoma patients that tremelimumab was able to restore circulating effector memory T-cell proliferation and that this population became transiently resistant to Treg immunosuppressive function [119] (Fig. 29.3a).

In summary, although CTLA-4 mAbs have shown promising results in clinical trials, little is known about their effects on Treg cell populations. A better understanding of their mechanisms of action would be of great interest to develop even more potent therapies and maybe limit severe immune-related adverse events observed in various trials [120, 130, 132, 133].

29.7 Agonist Antibodies Affecting Treg Immunosuppressive Activity

Another alternative therapeutic strategy to boost antitumoral immunity is targeting costimulatory molecules involved in Treg cell modulation. We will address here two major costimulatory tumor necrosis factor (TNF) receptor superfamily members: GITR and OX40.

29.7.1 Agonist Anti-GITR Antibody

GITR, a cell surface costimulatory receptor, is highly and constitutively expressed by Tregs [11, 12], whereas naive and memory Tconv express it at low levels [134]. However, on conventional $CD4^+$ and $CD8^+$ T cells, GITR expression is also enhanced following activation and then declines 1–3 days later [135]. Thus, GITR signaling and function are context and cell type dependent [136].

Under suboptimal TCR stimulation, the activation of GITR has opposite effect on Tregs and conventional $CD4^+$ and $CD8^+$ T cells. The use of agonist anti-GITR antibodies such as **DTA-1** [12], recombinant GITR ligand (GITRL), or GITRL transfectants (Fig. 29.3b) abrogates Treg cell-mediated suppression exerted on Teff cells, while it enhances the expansion and the cytokine production of $CD8^+$ T cells [137]. This observation has been shown both in vitro [138] and in vivo in murine tumor models [139–144]. In these preclinical studies, GITR stimulation has been reported to increase Teff-to-Treg ratio [145], leading to a beneficial antitumoral effect and tumor regression in tumor-bearing mice.

Moreover, Coe et al. showed in mice that DTA-1-mediated Treg depletion was more marked in tumors than in tumor-draining lymph nodes [140], as TI Tregs express much higher levels of GITR than circulating Tregs [146]. This means that GITR could be used as a target for selective depletion or inhibition of $CD4^+$ $FoxP3^+$ $GITR^{high}$ Tregs without interfering with systemic maintenance of self-tolerance.

The in vivo activity of GITR-targeting agents is complex and may rely on several suggested mechanisms (Fig. 29.3b), including (1) FcγR-mediated depletion of Tregs [139], (2) activation-induced apoptosis through Fas–FasL signaling [147], (3) inhibition of Treg suppressive functions and induction of Teff resistance to Treg suppression [148], and (4) loss of Treg stability through FoxP3 destabilization [149].

Very recently, a novel hexameric GITRL, **MEDI1873**, based on a fusion protein bearing a human IgG1 Fc domain, has been developed by MedImmune. The assessment of MEDI1873 in vitro and in a CT26 tumor model (a murine colorectal cancer model) revealed an increased binding to FcγRs resulting in the depletion of TI Tregs, likely through Fc-mediated effector functions [150]. MEDI1873 is currently assessed in a phase I clinical study (NCT02583165) in patients with solid tumors.

In light of the preclinical results, a number of drugs, including traditional GITR-targeting antibodies and the GITRL protein fusion, have already entered [136] or have been selected for clinical trials that should be launched soon (Table 29.4).

29.7.2 OX40/OX40L Agonist Agents

OX40 (CD134) and its ligand OX40L (CD252) are members of the TNFR/TNF superfamily and provide a costimulatory signal upon primary activated effector and memory $CD8^+$ lymphocytes [151]; $CD4^+$ T helper cell subtypes including Th1, Th2 [152], and Th17 [153]; as well as $CD4^+$ $Foxp3^+$ Tregs [154]. OX40L is likely expressed on activated antigen-presenting cells (APCs) and in particular DCs [155, 156].

Interestingly, it has been shown that TILs, isolated from murine [157] or human tumor biopsies [158, 159], express higher level of OX40 than their peripheral counterparts, where it increases T-cell activation, proliferation, and survival.

The mechanisms by which OX40 regulates Treg functions are still poorly understood, and data surrounding this question are contradictory [160, 161]. However, the majority of mouse and human studies revealed that OX40 engagement with agonist OX40 antibody alters the differentiation and suppressive activity of Tregs [161–163] and can also indirectly act by making Teff cells resistant to suppression by Tregs [163].

In a recent murine study, agonist antibody targeting OX40 has been reported to be able to selectively deplete TI Tregs that constitutively express OX40 [164]. This depletion seems to be likely mediated by ADCC, which relies on the

Table 29.4 GITR-targeting drugs in ongoing trial

Treatment (company)	Cohort	Indication	Trial phase	Start date—status	NCT ID
TRX518 (GITR, Inc.) humanized nondepleting mAb	40	Malignant melanoma	Phase I	10/2010 (recruiting)	NCT01239134
MK-4166 (Merck) humanized mAb ± pembrolizumab	94	Solid tumors	Phase I	06/2014 (recruiting)	NCT02132754
BMS-986156 (Bristol–Myers Squibb) ± nivolumab	260	Solid tumors	Phase I/IIa	10/2015 (recruiting)	NCT02598960
MK-1248 (Merck) ± pembrolizumab	96	Solid tumors	Phase I	11/2015 (recruiting)	NCT02553499
MEDI1873 (MedImmune LLC) hexameric GITRL protein/human IgG1	47	Solid tumors	Phase I	11/2015 (recruiting)	NCT02583165
TRX518	44	Solid tumors	Phase I	12/2015 (recruiting)	NCT02628574
INCAGN01876 (Incyte/Agenus) humanized IgG1 mAb	146	Solid tumors	Phase I/II	04/2016 (recruiting)	NCT02697591
GWN323 (Novartis Pharma) humanized IgG1 mAb ± **PDR001**	264	Solid tumors	Phase I/Ia	07/2016 (recruiting)	NCT02740270

A non-exhaustive list of ongoing clinical trials monitoring GITR-targeting drugs in cancer patients. The choice of the samples was made as described in Table 29.1. All clinical trials can be found in https://clinicaltrials.gov/

activation of FcγR expressed by myeloid and NK cells [165].

Together, these findings made the OX40/OX40-L axis-targeting an interesting approach for anticancer immunotherapy [166–169].

In many preclinical mouse tumor models, OX40/OX40L targeting agents including **anti-OX40 mAbs** and **OX40L-Fc fusion proteins** (Fig. 29.3b), when used as a monotherapy, gave promising results with a protective antitumor immunity and an improved tumor-free survival [157, 170–174].

Moreover, in several murine studies, the antitumoral effect of anti-OX40 agents has been further improved by combination with other immunomodulatory antibodies, such as immune checkpoint inhibitor antibodies [164, 175] and therapeutic cancer vaccines [171, 176, 177].

Currently, there are at least six anti-OX40 agonist mAbs and one OX40L-Fc which have been developed by numerous companies, undergoing early phase clinical trials testing for the treatment of advanced solid malignancies (Table 29.5).

9B12, a murine agonist antihuman OX40 mAb, was the first antibody to enter the clinic in 2003 and has been tested in a phase I trial in patients with advanced solid cancer (melanoma, renal cancer, urethral cancer, prostate cancer, and cholangiocarcinoma) refractory to conventional therapy (NCT01644968). The anti-OX40 mAb was well tolerated, and in 12 of 30 patients, Curti and colleagues reported a regression of at least one metastatic lesion following just one cycle [178]. Furthermore, the authors reported that TI Tregs expressed more OX40 (50% of TI Tregs) than peripheral Tregs, confirming the observation in mouse studies that the anti-OX40 antibody may modulate Treg function in the tumor. However, they did not report a decrease in the number of Tregs within tumors.

MEDI6469 (a murine agonist antihuman OX40 mAb), **MEDI0562** (developed through humanization of MEDI6469), **MOXR0916** and **GSK3174998** (two humanized IgG1 agonist anti-OX40 mAbs), **PF-04518600** (a fully human IgG2 agonist anti-OX40 mAb), and **INCAGN01949** (a fully human IgG1 agonist anti-OX40 mAb) are currently involved in clinical trials (Table 29.5). They are being tested in several cancer types, including advanced or metastatic prostate, breast, and colorectal cancers, as

Table 29.5 OX40-modulating agents in ongoing clinical trials

Treatment (company)	Cohort	Indication	Trial phase	Start date—status	NCT ID
MEDI6469 (Providence Health & Services/MedImmune LLC) murine antihuman OX40 mAb + radiation + CTX	10	Advanced prostate cancer	Phase I/II	10/2010 (active, not recruiting)	NCT01303705
MEDI6469 + radiotherapy	40	Advanced breast cancer	Phase I/II	02/2013 (recruiting)	NCT01862900
MOXR0916 (Genentech, Inc.) humanized mAb	400	Advanced solid tumors	Phase I	08/2014 (recruiting)	NCT02219724
MEDI6469 (MedImmune LLC) ± rituximab ± MEDI4736	58	Solid tumors, B-cell lymphomas	Phase Ib/II	08/2014 (completed)	NCT02205333
MEDI6383 (MedImmune LLC) human OX40 ligand fusion protein ± MEDI4736	39	Recurrent or metastatic solid tumors	Phase I	09/2014 (active, not recruiting)	NCT02221960
MEDI6469 (prior to definitive surgical resection)	55	Advanced oral head and neck cancer	Phase Ia	10/2014 (recruiting)	NCT02274155
MEDI0562 (MedImmune LLC) humanized mAb	196	Advanced solid tumors	Phase I	03/2015 (recruiting)	NCT02318394
MOXR0916 + atezolizumab ± bevacizumab	762	Advanced solid tumors	Phase Ib	04/2015 (recruiting)	NCT02410512
PF-04518600 (Pfizer) fully human IgG2 ± PF-05082566	190	Advanced solid tumors	Phase I	04/2015 (recruiting)	NCT02315066
MEDI6469	44	Metastatic colorectal cancer	Phase I/Ib	09/2015 (recruiting)	NCT02559024
MEDI0562 ± tremelimumab ± durvalumab	182	Advanced solid tumors	Phase I	03/2016 (recruiting)	NCT02705482
GSK3174998 (GSK) humanized IgG1 ± pembrolizumab	264	Advanced solid tumors	Phase I	09/2015 (recruiting)	NCT02528357
INCAGN01949 (Incyte Europe Sàrl/ Agenus Inc.) fully human IgG1	157	Advanced solid tumors	Phase I/II	10/2016 (recruiting)	NCT02923349

A non-exhaustive list of recent and ongoing clinical trials monitoring OX40-modulating agents in cancer patients. The choice of the samples was made as described in Table 29.1. All clinical trials can be found in https://clinicaltrials.gov/

well as HNSCC and aggressive B lymphomas. These different anti-OX40 mAbs are being assessed as a monotherapy or in combination with other therapies (radiotherapy ± CTX, rituximab (anti-CD20), MEDI4736/atezolizumab/durvalumab (anti-PD-L1), pembrolizumab (anti-PD-1), tremelimumab (anti-CTLA-4), PF-05082566 (4-1BB agonist), and bevacizumab (anti-VEGF)) in different cancer types (Table 29.5).

More recently, in 2014, MedImmune LLC developed a human OX40 ligand fusion protein, **MEDI6383,** that is being tested in recurrent or metastatic solid tumors, either alone or in combination with another immune checkpoint blockade antibody: durvalumab (anti-PD-L1).

29.8 CD39–CD73–A2aR Pathway Blockade: An Emergent Therapy Targeting Tregs

ATP and its metabolites (ADP, AMP, and adenosine), released into the extracellular space in response to tissue damage and cellular stress, play an important role in immune homeostasis. However, to evade the immunosurveillance, tumors can divert the physiological feedback-negative control exerted by these molecules for their own profit, in order to suppress antitumor T-cell responses.

The ectonucleotidases CD39 and CD73 are responsible for the catabolism of extracellular ATP to AMP and AMP to adenosine

(Fig. 29.4), respectively, and are co-expressed on both murine and human Tregs [179–181]. Furthermore, T lymphocytes, including Tregs, mainly express the high-affinity adenosine 2a receptor (A2aR) and the low-affinity adenosine 2b receptor (A2bR) [182]. As a consequence, the activation of A2a and A2b receptors on immune cells induces strong immunosuppressive effects [183].

A2aR engagement on Tregs increases their CTLA-4 and PD-1 expression (shown in vitro) [184, 185], favors their differentiation from naïve T cell, and enhances their immunosuppressive activities [186].

In several types of cancer, adenosine is abundantly released within the tumor site (Fig. 29.4), as a consequence of the hypoxia-induced overexpression of the ectonucleotidase enzymes CD73 and CD39 [187–189]. Moreover, it has been shown that the pro-tumoral effect of adenosine is mediated by (1) inhibiting the anti-tumoral Th1 $CD4^+$ and cytotoxic $CD8^+$ T cells through the A2aR, (2) enhancing the proliferation of the immunosuppressive cells (e.g., Tregs and granulocytic MDSCs), and (3) induction of tolerogenic DCs and type 2 macrophages (Fig. 29.4).

These observations in cancer settings led the researchers to investigate the potential of targeting the adenosine CD73–CD39 pathway to overcome adenosine-mediated immunosuppression, in murine studies and in human patients [186].

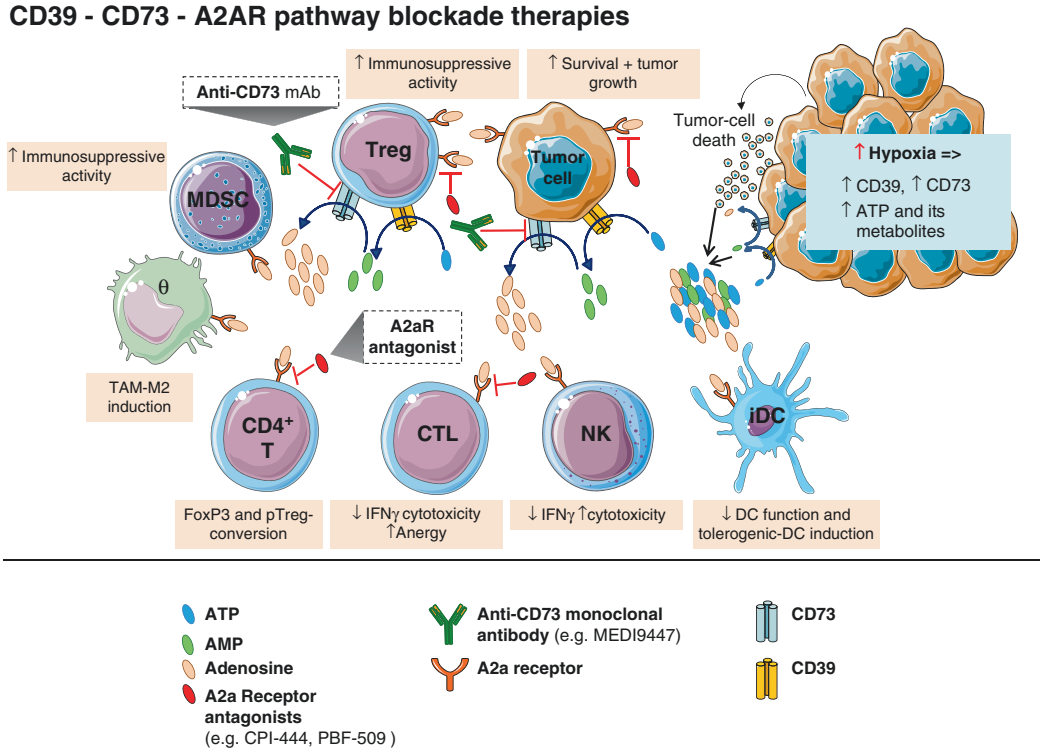

Fig. 29.4 CD39–CD73–A2AR pathway blockade, an emergent therapy targeting Tregs. Immunogenic cell death of tumor cells leads to the release of extracellular ATP and its metabolites (ADP, AMP, and adenosine) into the TME. As a consequence of the hypoxia, the ectonucleotidase enzymes that convert ATP into adenosine (CD73 and CD39), and the receptors that bind adenosine, are overexpressed on tumor and immune cells. Ligation of adenosine to its receptor mediates Th1 $CD4^+$ and CTL inhibition, proliferation of the immunosuppressive cells (e.g., Tregs and granulocytic MDSCs), and induction of tolerogenic DCs and type 2 macrophages. Adenosine plays also a role in survival of cancer cells promoting tumor growth. Antagonists of the adenosine A2aR and monoclonal antibodies targeting the adenosine–CD73–CD39 pathway are currently tested in ongoing clinical trials to overcome adenosine-mediated immunosuppression

Recently, two orally administered antagonists of the adenosine A2aR (Fig. 29.4) have entered the clinic for cancer treatment: (1) **CPI-444** (Corvus Pharmaceuticals and Genentech), tested in various solid tumors alone or combined with an anti-PD-L1 mAb (NCT02655822), and (2) **PBF-509** (Novartis/Palobiofarma) tested as a single agent or in combination with an anti-PD-1 mAb, in patients with advanced non-small cell lung cancer (NCT02403193).

In July 2016, a first-in-human phase I trial started to evaluate the safety and antitumor activity of **MEDI9447** (MedImmune LLC), a human IgG1 mAb targeting CD73 (Fig. 29.4), alone and in combination with MEDI4736 (anti-PD-L1), in advanced solid cancer patients [190] (NCT02503774).

To date, there is no CD39-targeting antibody tested in clinic.

Conclusion

The immunosuppressive activity of intratumoral Tregs represents a major hurdle for effective antitumor immunity, highlighting their potential as an immunotherapeutic target. However, prognostic/predictive significance of tumor infiltration by Tregs remains a matter of debate. Indeed, high levels of intratumoral Tregs have been associated with poor disease outcome in cohorts of patients affected by multiple, but not all, tumor types.

Various subpopulations of TI Tregs have been identified so far and shown to exert relatively distinct functions and hence to be associated with different clinical significance. It has recently been shown that TI Tregs express some molecules (e.g., IL-1R2 and CCR8) not expressed in Tregs from normal tissues which could lead to specific depletion of TI Tregs avoiding risk of autoimmunity [191].Therefore, a more refined phenotypic and functional definition of the Treg subsets as well as a better understanding of their role in the regulation of immune responses remains a major challenge for future Treg-targeting therapies.

Moreover, there is considerable interest in the possible synergistic opportunities of combining Treg-targeted therapies, in addition to currently available therapies, with other modalities such as immune checkpoint blockade or immune agonist therapies. However, the role of costimulatory and co-inhibitory molecules in modulating Treg function and survival remains unclear and warrants further investigation and clarification. Finally, as systemic targeting of Tregs may severely impact peripheral immune homeostasis, there is a clear need to develop more selective approaches to limit intratumoral Treg cell immunosuppression.

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Synergy Between Radiotherapy and Immunotherapy

30

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Contents

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30.1 Introduction

Radiotherapy has been used for over 50 years as an effective modality to kill cancer cells. Much of the efforts to improve its efficacy have been focused on improving dose delivery to the tumor while sparing as much as possible normal tissue around it. This task has inspired generations of biologists and clinical investigators to study the mechanisms of radiation damage and repair of different tissues. A century of preclinical and clinical research has established the basic principle of dose fractionation, with well-established protocols to safely deliver a cytocidal dose to the tumor while enabling recovery of normal tissue often with daily regimens delivered over several weeks [1]. More recently, technological progress has resulted in linear accelerators that very precisely target of the tumor and its movement during dose delivery, enabling safety of regimens with fewer, larger doses of radiation [2]. Noticeably, this approach has demonstrated not only to be more convenient for the patients but often to achieve better results in tumor control. Since hypofractionated radiation has often resulted in

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outcomes comparable to those of surgery, it has gained the naming of "ablative radiotherapy."

In this the new era of immuno-oncology, with the unequivocal demonstration that the immune system is a critical barrier to tumor progression that can be unleashed therapeutically to treat cancer [3], radiation is taking on a new role, that of an "adjuvant" that can increase the response of cancer patients to immunotherapy [4].

In this chapter we will provide an overview of current standard of care in radiotherapy and discuss examples of unexpected effects outside the radiation field (abscopal effects) observed in patients that were concurrently receiving an immunotherapy. The immunological mechanisms underlying abscopal effects will be presented, and the evidence that radiation can synergize with immune checkpoint inhibitors and other immunotherapies will be reviewed. Finally, we will highlight some of the existing barriers and opportunities in developing this new field of immune-radiation oncology.

30.2 Radiation: Current Clinical Use and Local Effects

Historically, the field of radiation oncology developed on tenants of radiobiology related to dose and fractionation schemes required to eradicate tumor cells, taking advantage of their reduced capacity to repair DNA damage. Tumor treatment models were largely based on in vitro modeling, i.e., cell survival curves. In addition, traditional preclinical models and clinical experience provided guidance on how best to protect neighboring critical structures. This led to the widespread use of 1.8–2.0 Gray (Gy) fraction sizes given daily to minimize toxicity, allowing for normal tissues sufficient time to repair DNA damage between doses. Because of the relatively low doses per day, traditional courses require 5–7 weeks to deliver a sufficient cumulative dose to eradicate tumor cells. Most radiation schemes retaining this protracted course remain standard of care for a curative treatment in breast, prostate, rectal, pancreatic, primary brain, and head and neck cancers.

Efforts to delineate patterns of spread for each disease site often inspired from the patterns of recurrence after radiotherapy or surgery gave rise to the idea of "clinical target volumes" or CTVs, which include not only the visible tumor (gross tumor volume or GTV) but also subclinical tridimensional margin around the tumor including regional draining lymph nodes (Fig. 30.1). Treating these larger volumes has the goal of eradicating all tumor cells in the locoregional area, to prevent recurrence and, possibly, reduce distant spread. Typically a second margin is added to the CTV that further increases the treated volume to the "planning target volume" or PTV. The latter margin takes into account potential differences in the daily reproducibility of the patient position. With older imaging techniques, the combined total field as described often includes significantly large anatomic areas and, despite the low dose per day, can result in significant side effects. Moreover, excessive reduction of daily doses compromises tumor control. With the advent and rapid adoption of intensity-modulated radiation therapy (IMRT), higher gradients of dose can be reliably delivered allowing better protection of neighboring normal tissue. In parallel, image-guided radiation therapy (IGRT), including daily on-treatment computed tomography (CT) imaging, has led to a

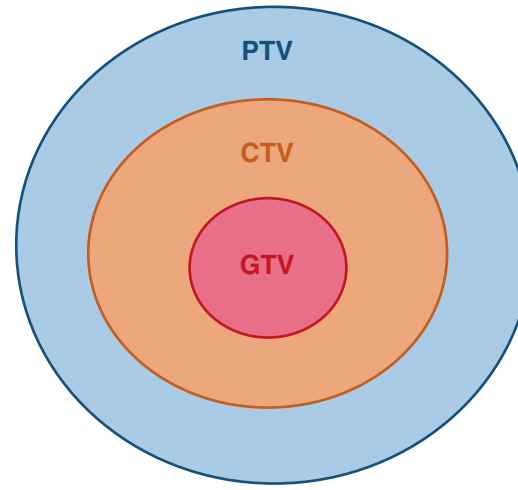

Fig. 30.1 Margins used in radiation treatment. *GTV* gross tumor volume, *CTV* clinical target volume, *PTV* planning target volume

more precise definition of the target and reduction in size of the volume of normal tissue treated, with decreased side effects. While PTVs can be reduced, CTVs generally have not significantly changed but are often better targeted with modern equipment that better controls for physiological movements of the CTV between and during dose delivery.

One rapidly evolving area is in the treatment of patients with metastatic disease. Radiation is commonly delivered to palliate symptoms such as pain, bleeding, or neurological symptoms from mechanical compression of growing tumors. In this setting, shorter regimens have shown similar efficacy with the endpoint of symptom control. For example, the landmark palliative study RTOG 97-14 randomized breast and prostate cancer patients with painful bony metastases to 30 Gy in 10 fractions compared to 8 Gy given in a single fraction and showed equivalent pain relief [5]. This has led the American Society for Radiation Oncology (ASTRO) to recommend this single-fraction treatment in appropriately selected patients for pain relief [6].

In addition to shortening treatment schemes, reduction or elimination of CTV margins is implemented when treating metastases. Improved immobilization and imaging techniques assure adequate coverage of the target with a precision that matches that of surgery and enables much less normal tissue in the field. Safe delivery of doses in the range of 6–30 Gy or higher, over 1–5 fractions, can be achieved, defined as stereotactic radiation treatments (stereotactic radiosurgery or SRS, stereotactic body radiotherapy or SBRT, stereotactic ablative radiotherapy or SABR). For each dose erogation, the patient is immobilized and imaged in the treatment position to ensure the target is treated accurately. Stereotactic treatments are a promising way to deliver a higher dose per fraction with low toxicity rates compared to traditional fields: they are commonly used to treat metastases to the adrenal gland, spine, liver, lung, and brain in appropriately selected patients. One of the most standard applications of this newer technique is treating metastases to the brain, where spearing normal tissue is of particular importance. Prior to stereotactic treatments, patients with brain metastases were treated with radiation to the whole brain, generally with 30 Gy over 10 fractions. Whole brain radiation is associated with significant acute and chronic toxicity, including nausea, vomiting, hair loss, confusion, and cognitive decline. Stereotactic treatments that treat brain metastases result in minimal cognitive decline by sparing the normal brain. They are becoming the standard of care for an increasing number of patients, depending on the number and type of metastatic lesions and life expectancy. Supporting this trend, a recent individual patient data meta-analysis showed that for patients less than 50 years old, stereotactic treatment alone (without the addition of whole brain radiotherapy) was associated with improved survival [7].

The use of a single-fraction dose for stereotactic treatments to brain metastases was studied in RTOG 90-05, a trial of single-fraction dose escalation based on the size of brain metastasis [8]. While higher doses had minimal toxicity for small lesions (less than 2 cm), treatment of larger lesions was associated with neurotoxicity including radionecrosis (necrosis of brain tissue as a result of radiation). Thus, caution needs to be employed when delivering high doses in a single fraction, and many centers split the single dose into 3–5 fractions to reduce toxicity particularly for larger tumors.

30.3 From Local to Abscopal: Radiotherapy and Systemic Tumor Control

While the cytotoxic effects of radiotherapy are exquisitely local, occasional distant tumor regression outside of the irradiated field has been observed and defined by Mole in 1953 as abscopal effect (ab scopus, outside the target) [9]. Abscopal effects have been reported in a number of different malignancies but are rarely seen with radiation alone [10]. Interestingly, abscopal responses have been seen with increased frequency when radiotherapy was used in melanoma patients progressing during treatment with immune checkpoint inhibitors [11–14]. These

clinical observations, together with accumulated experimental evidence that the abscopal effect is mediated by T cells [15, 16], have raised a lot of interest in the possibility of using radiation to increase responses to immunotherapy. As detailed in the following sections, many clinical trials are exploring combinations of radiotherapy with various immunomodulators.

It remains to be established if the ability of radiation to exert an abscopal effect is dependent on the intrinsic immunogenicity of a tumor. Dramatic abscopal effects have been observed in combination with ipilimumab in melanoma and non-small cell lung cancer (NSCLC) [13, 17], two malignancies with a high mutational burden and a higher likelihood of expressing neoantigens recognized by T cells [18, 19]. On the other hand, abscopal responses elicited by radiation alone have also been reported in patients with renal cell carcinoma, a tumor type with low mutational burden but a propensity to respond to immunotherapy [20, 21]. Moreover, abscopal responses elicited by radiation alone or in combination with different immunomodulators have been seen in a variety of other malignancies [22–30]. Overall, these data suggest that abscopal effects can be elicited by radiation across several tumor types. However, it is presently unknown if some degree of pre-existing antitumor immunity is required.

It has also been suggested that the location of the irradiated metastatic site determines the likelihood of achieving abscopal effects [31]. While it is conceivable that, due to tumor heterogeneity and organ-specific features, different metastases generate a more or less immunosuppressive microenvironment, there is currently no evidence to guide the choice of site to be irradiated to achieve abscopal responses [32].

Overall, extensive work in preclinical tumor models has demonstrated that radiation can elicit antitumor T cells that contribute to control of the irradiated tumor [33–35]. However, abscopal effects have been more difficult to achieve, probably because they require a broader and more robust antitumor T cell response, capable of recognizing multiple antigens to account for possible tumor heterogeneity and overcoming the barriers present within the microenvironment of nonirradiated metastases. For a given tumor, it is likely that the strength of the immune response induced by radiotherapy is determined by the immunotherapy agent and radiation regimen used. Experimental evidence in mouse models of breast and colorectal carcinoma unresponsive to immune checkpoint inhibitors showed abscopal responses only when radiation was added and suggested that the radiation regimen is critical: with antibody targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), a single dose of 20 Gy was ineffective, while 6 Gy X 5 and 8 Gy X 3, given in consecutive days, achieved complete and partial abscopal responses [16]. While in the clinic the best radiation regimen to be used in combination with immunotherapy remains undefined, it is intriguing that, at least in combination with anti-CTLA-4, abscopal responses were achieved with the use of radiation regimens similar to the ones effective in mice [13, 17, 36].

30.4 Augmenting Local Control: Radiotherapy Effects that Promote the Effector Phase of the Antitumor Immune Response

The observation that in mice lacking a normal T cell compartment, higher radiation doses were required to achieve cure of irradiated tumors when compared to immunocompetent mice first implicated T cells in the local response to radiation [33]. This early finding was confirmed experimentally three decades later with the use of mouse tumors bearing model antigens [34, 35]. It remains to be determined to which degree T cells contribute to local radiotherapy responses in patients. Model antigens like ovalbumin are convenient for proof-of-principle studies, but their high immunogenicity and levels of expression fail to mimic the reality of cancer patients, with tumors that have gradually evolved to escape the immune system.

In addition, radiotherapy also evokes immunosuppressive signaling. For instance, when two poorly immunogenic mouse carcinomas were

treated with radiotherapy, $CD8^+$ T cell responses to three endogenous antigens were seen only upon neutralization of TGFβ, a strongly immunosuppressive cytokine that is activated by radiation [37]. In patients, evidence of priming of tumor-specific T cells following standard-of-care radiotherapy alone is scant [38]. Thus, it is possible that the priming of T cell by radiation occurs only when radiation is combined with immunotherapy. Noticeably, in patients with a pre-existing antitumor T cell response [39], radiation can reactivate a stalled immune response by countering two key immune escape mechanisms at the effector phase of antitumor immunity: T cell exclusion from the tumor microenvironment and reduced cancer cells antigenicity due to downregulation of major histocompatibility complex class I (MHC-I) antigen-presenting molecules on cancer cells [40, 41] (Fig. 30.2).

Radiation-induced changes that counter T cell exclusion include release of chemokines that attract effector T cell to the tumor [42, 43] and increased expression of adhesion molecules on the vascular endothelium that facilitate T cell infiltration, as demonstrated in mouse breast and melanoma models [34]. In addition, reprogramming of tumor-associated macrophages (TAM) by radiation has been shown to cooperate with adoptive T cell transfer to allow rejection of pancreatic tumors by promoting vascular normalization [44]. Interestingly, increased infiltration of irradiated tumors by T cells activated by peripheral vaccination was also seen in an orthotopic mouse model of brain glioma, suggesting that it can occur in a variety of solid tumor types growing in different anatomical locations [45].

Upregulation of MHC-I expression by radiation has been shown in multiple mouse and human cancer cells in vitro and in vivo [45–48]. In vitro, increased MHC-I improved lysis of the irradiated cancer cells by $CD8^+$ cytotoxic T cells (CTL), and in vivo it improved tumor rejection mediated by adoptively transferred and vaccine-activated T cells [45–47]. In addition to increasing the antigenicity of cancer cells, radiation has also been shown to increase the expression of stress-induced ligands that bind to the NK group 2, member D (NKG2D) receptor expressed by CTL and natural killer (NK) cells and promote killing of the cancer cells by these effectors

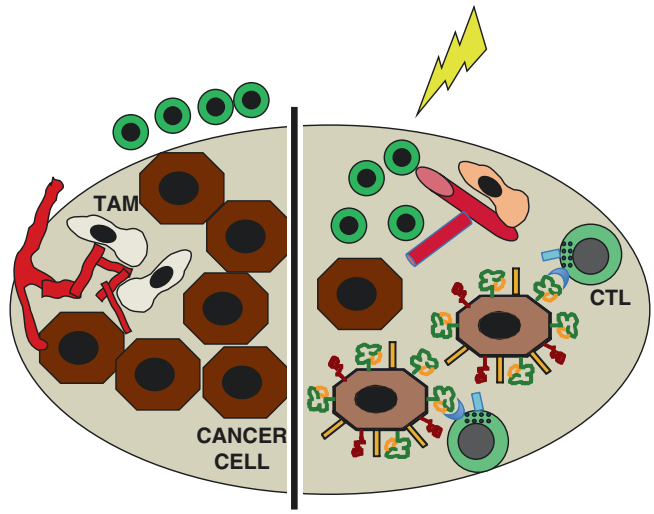

Fig. 30.2 Radiotherapy effects that promote the effector phase of the antitumor immune response. *Left*, cytotoxic T cells (CTL) are present but unable to enter the tumor due to the vascular barrier, which is promoted by pro-angiogenic tumor-associated macrophages (*TAM*). In addition, downregulation of MHC-I on the cancer cells precludes their recognition by CTL. *Right*, radiation reprograms the macrophages leading to vascular normalization, which allows tumor infiltration by CTLs. In addition, radiation upregulates MHC-I, NKD2D ligands, and Fas on the cancer cells, making them good targets for recognition and elimination by CTLs

[49–52]. Another way that radiation increases killing of cancer cells by T cells is by inducing the expression of death receptor Fas/CD95 [53].

Thus, multiple effects of radiation on the cancer cells and tumor microenvironment cooperate to enhance immune-mediated tumor rejection. Adaptive immunity may contribute to local tumor control achieved by radiotherapy if patients have pre-existing antitumor T cells [39]. In addition, tumor-targeted radiotherapy can sensitize resistant metastases to adoptively transferred T cells, as shown in preclinical studies [47, 53]. Radiation may also recover tumor responses to other immunotherapies that activate endogenous antitumor T cells. Downregulation of MHC-I in tumors is one of the mechanisms of resistance to multiple immunotherapies [54] including programmed cell death protein-1 (PD-1) blockade [55], and recent work in a mouse model of PD-1 resistant lung cancer suggests that at least in some cases, radiation-induced upregulation of MHC-I molecules on the cancer cells overcame resistance to anti-PD-1 treatment [56].

30.5 Inducing Abscopal Effects: Radiotherapy Effects that Promote Priming of Antitumor T Cells

Generating antitumor T cells in patients lacking such natural responses remains one of the major challenges in cancer immunotherapy. While presumably all cancers are recognized by the immune system during the course of their development, a majority of tumors that become clinically detectable escape by editing out the antigens recognized by T cells [57]. However, the genetic instability intrinsic to neoplasia fuels the generation of more mutations that can be antigenic and be targets of strong antitumor responses [58]. Rather a major barrier to development of antitumor T cells is exclusion and dysfunction of dendritic cells [59–61].

Radiation has the ability to overcome, at least in part, this major barrier to the priming of antitumor T cells (Fig. 30.3). There is evidence in experimental models that radiation increases the recruitment of DCs to the tumor and their activation, mediated by danger signals that are released during immunogenic cell death (ICD) induced by radiation [62–65]. Well-characterized danger signals released during ICD include calreticulin translocation to the surface of the dying cells that promotes their uptake by DCs, release of high-mobility group box-1 (HMGB-1) that binds to Toll-like receptor 4 (TLR4) on DCs, and ATP that activates the inflammasome in the DCs downstream to P2XR7 receptor [63, 66, 67]. In addition, when DNA from the irradiated cancer cells finds its way to the cytosol of DCs, it activates the production of interferon type I (IFN-I) via the stimulator of interferon genes (STING) pathway providing another signal that acts in autocrine fashion to activate DCs [68].

Generation of the above signals depends on stress response pathways activated, while cancer cells are dying, including the unfolded protein response and autophagy [69]. Cell death after irradiation of cancer cells occurs in a variety of ways, depending on which survival/apoptosis pathways are activated, as well as the type of tumor microenvironment treated and the radiation dose and fractionation used [70]. Importantly, the availability of DCs limits the magnitude of antitumor T cell responses primed by radiation, and the activation of immunosuppressive mechanisms hinders the process [37, 71]. Thus, while radiation can convert the tumor into an immunogenic hub, it tends to provide a suboptimal vaccination. In the clinic most established cancers are poorly immunogenic. To successfully achieve priming of robust antitumor T cell responses, they require immunotherapies that block suppressive pathways or enhance immune stimulation [72]. Successful combinations have been achieved in preclinical studies with immune checkpoint inhibitors targeting CTLA-4, PD-1, and PDL-1, as well as agonistic antibodies to costimulatory receptor CD137 and TLR agonists [73–79].

The issue of whether radiotherapy induces neoantigens remains unsettled. In most studies, priming of T cell responses by radiation used alone or in combination with immunotherapy has

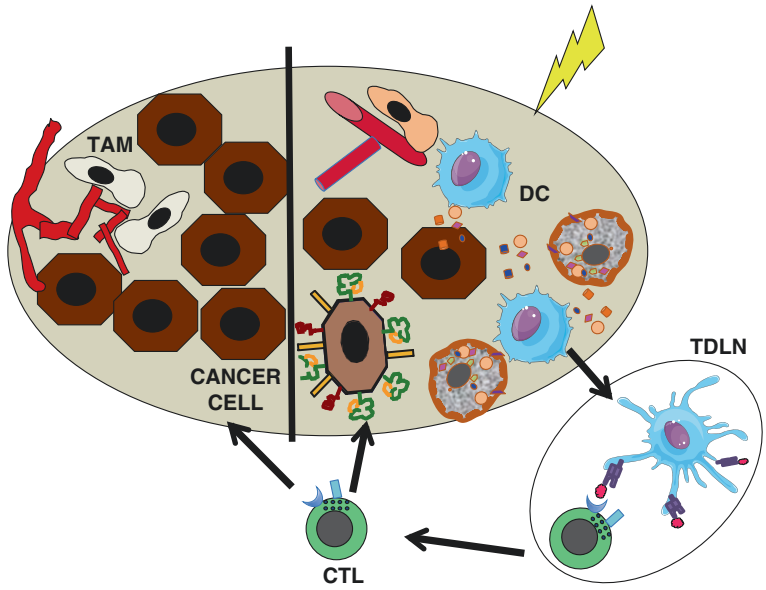

Fig. 30.3 Radiotherapy effects that promote priming of antitumor T cells. *Right*, danger signals generated by irradiated cancer cells drive dendritic cell (DC) recruitment to the tumor, where they uptake dying cancer cells and become activated. Activated DCs carry the antigens to the tumor-draining lymph node (TDLN) where they cross-prime tumor-specific T cells. Primed T cells become cytotoxic T cells (CTL) that go to both the irradiated tumor (*right*) and nonirradiated metastases (*left*) mediating the complete regression of the irradiated tumor and abscopal responses

been measured for a single antigenic epitope, often an immunodominant endogenous antigen or an exogenous reporter antigen introduced into the cancer cells (e.g., ovalbumin, OVA). To obtain a broader view of $CD8^+$ T cell responses elicited by radiation, we have analyzed responses to four distinct epitopes derived from three antigens that are overexpressed in cancer cells, the anti-apoptotic protein survivin, the epithelial-to-mesenchymal transition (EMT) transcription factor Twist, and the envelope of an endogenous retrovirus gp70. Responses to all four epitopes were coordinately elicited by radiation of the poorly immunogenic mouse 4T1 mammary carcinoma when inhibition of radiation-induced DC activation was prevented by TGFβ neutralization [37]. Thus, removal of critical immunosuppressive blocks unleashes the potential of radiation to elicit a broad T cell response. Work is ongoing to comprehensively evaluate the repertoire of T cell clones primed following radiation.

In addition to antigenic specificity, the number and persistence of the antitumor T cells primed by radiation are likely to be critical for abscopal responses. In fact, abscopal responses are rarely seen despite the fact that the irradiated tumor is successfully rejected and represent a more stringent test of effective in situ vaccination by radiation [80]. As mentioned above in Sect. 30.3, the dose of radiation and its fractionation seem to be critical determinants of the ability of radiation to induce abscopal responses in combination with anti-CTLA-4. Doses of 6–8 Gy repeated three to five times achieved abscopal responses but not a single higher radiation dose of 20 Gy [16]. Recent data indicate that the difference between effective and ineffective radiation regimens is due to their differential ability to induce cancer cell-intrinsic activation of IFN-I pathway (Vanpouille-Box, Formenti, and Demaria, manuscript submitted). Overall, the optimal radiation regimens when radiotherapy is harnessed as an adjuvant for immunotherapy may be distinct from those employed in the practice of radiation oncology, an issue of paramount importance for clinical translation [4].

30.6 A Balancing Act: Negative Regulators of Antitumor Immunity Elicited by Radiation in the Tumor Microenvironment

Tumor escape from immune-mediated control that allows tumor progression involves multiple immunosuppressive pathways which are variably utilized by each given tumor [81]. While radiation, as discussed above, can mitigate or counter some of them, it also exacerbates others [82]. Acute activation of latent TGFβ by radiation is due to its dissociation from the latency-associated peptide mediated by a ROS-induced conformational change of the latter [83, 84]. TGFβ is a strongly immunosuppressive cytokine with effects on multiple immune cells [85]. For instance, activation of naïve $CD4^+$ T cells in the presence of TGFβ facilitates their conversion into regulatory T cells [86, 87]. It remains unclear if radiation-induced TGFβ activation contributes to the radiation-induced increase in regulatory T cells that has been reported in some studies [88, 89]. On the other hand, as discussed above, inhibition of DC activation by TGFβ is a clear barrier to radiation-induced priming of antitumor T cells [37].

Increased recruitment of myeloid cells to the tumor has been shown to be driven by radiation-induced upregulation of colony-stimulating factor 1 (CSF1) in prostate cancer [90] and by C-C motif ligand 2 (CCL2) in pancreatic adenocarcinoma [91]. The recruited myeloid cells differentiated into immunosuppressive TAMs to orchestrate a tissue repair program promoting angiogenesis and tumor progression, a functional polarization fostered by hypoxia, which is exacerbated after extensive endothelial cell death caused by high-dose (>10Gy) radiation [92, 93]. Interestingly, while vascular death has been implicated in cures by high-dose radiation [94], recent work using an elegant spontaneous carcinogenesis model with selective deletion of ataxia-telangiectasia mutated (ATM) in either endothelial or sarcoma cells demonstrated that the increased death of ATM-deficient endothelial cells failed to improve tumor eradication by SBRT [95]. While the role of the immune system in tumor eradication was not evaluated in this study, it is intriguing to consider if TAM-mediated immunosuppression played a role in precluding tumor eradication.

Radiation also upregulates hypoxia-inducible factor-1α (HIF1-α), a key transcription factor that, in addition to promoting angiogenesis [96], induces PD-L1 expression on both tumor cells and myeloid cells in the tumor microenvironment, inhibiting T cell-mediated tumor rejection [97, 98].

Thus, the effects of radiation are complex and dependent on several variables, including the tumor type, the pre-existing tumor microenvironment, and the radiation dose used. Improved understanding of treatment protocols and combinations that strike the right balance of immune-activating versus immunosuppressive signals is critical for the optimal use of radiotherapy to generate an in situ tumor vaccine.

30.7 Revisiting Current Treatment Protocols: Lymphopenia Induced by Local Radiotherapy

An important practical consideration for the use of radiation in combination with immunotherapy is the volume of tissue that is irradiated or "field size." As discussed in the beginning of the chapter, traditional techniques use large fields and often include draining lymph nodes and adjacent bones, with hematopoietic marrow. Despite these efforts to include and treat microscopic disease at the margins of a tumor, in many cases such as advanced head and neck cancer, locoregional recurrence rates remain ~40%, even with high total doses, large CTV, and significant short- and long-term toxicity. Traditional radiobiological paradigms explain these failures as inadequacy of radiation dose or the margins. However, with the emerging role of radiation in activating the immune system to control cancer cells, this

paradigm is being reevaluated. One key adverse effect of large field radiation is killing of lymphocytes that reside or are circulating (and exposed when the beam is on, during radiation delivery) within the irradiated region. Since lymphocytes are very radiosensitive, their exposure reduces the availability of cells that might participate in tumor rejection.

Radiation-induced lymphopenia has been modeled in glioblastoma multiforme [99], which also often incorporates large fields to the brain, an organ highly perfused. Severe and persistent treatment-related lymphopenia occurs in 40% of patients undergoing standard treatment. Importantly, lymphopenia predicted for decreased survival: hazard ratio for death attributable to 2-month CD4 count below 200 = 1.66 ($p = 0.03$) [100].

In a modeling effort to predict this effect on blood, the mean radiation dose to circulating lymphocytes was calculated to be 2.2 Gy, and after 30 fractions, 99% of circulating blood had received ≥0.5 Gy, which was dependent on the margins used [99]. To examine the impact of margins on lymphopenia in the setting of locally advanced pancreatic cancer, total lymphocyte counts were recently compared between stereotactic and standard tridimensional fields of treatment [101]. At 2 months, 46% compared to 13.6% of patients were severely lymphopenic with standard compared to stereotactic treatment. Again, higher total lymphocyte counts posttreatment were associated with improved overall survival, further indicating the detrimental effects of large treatment fields.

Irradiation of circulating T cells may have significant effects on maintenance of immunological memory, which requires continuous homeostatic turnover [102]. In addition, functional impairments in the T cell compartment have been reported. T cells harvested during radiotherapy from peripheral blood of patients and restimulated in vitro showed reduced proliferation, with impairment still detectable 4 weeks after completion of radiotherapy [103]. Persistent lymphopenia post-radiotherapy has been linked to a persistent failure of homeostatic cytokine responses [104]. Thus, considerations for the volume of tissue irradiated and the number of fractions used are likely to be critical for successful combinations of radiotherapy and immunotherapy. Preliminary evidence suggests that classical regimens of fractionated radiotherapy protracted over 4–8 weeks of daily fractionation to large target volumes should be avoided.

30.8 Exploiting Radiation Effects to Improve Responses to Immunotherapy

The priming and effector phases of the antitumor immune response are modulated by multiple checkpoints that need to be countered to generate a clinically effective tumor rejection. Immunotherapies currently approved or under investigation target one or a few of the blocks but in many cases have failed to achieve detectable clinical responses [105]. The ability of radiation to affect many processes at both the priming and effector phase makes it an especially attractive partner in combination with different immunotherapies. Examples of preclinical combination studies are listed in Table 30.1. While Table 30.1 is not exhaustive of the large available literature, the data illustrated provides strong support for the hypothesis that radiation can work in concert with different strategies to improve tumor control. Interestingly, the radiation doses tested in different studies vary widely, but in only few studies, different doses and fractionation schema have been tested side by side, precluding in most cases any conclusion about the most effective dose. Moreover, only a few studies carefully examine the effects of the combinations on abscopal tumors. While in-field control is a necessary condition for abscopal responses, enhanced in-field responses don't necessarily translate to abscopal effects. Thus, preclinical data should be used with caution to infer which radiation dose(s) should be tested in clinical studies [4].

Table 30.1 Radiotherapy improves responses to multiple immunotherapies in preclinical tumor models

Main step of antitumor immune response targeted	Agent	Main effect	Radiotherapy tested	Reference(s)
Tumor antigen presentation	Flt3-ligand	Growth factor for DCs, increased systemic availability of DCs	60 Gy X 1 2 Gy and 6 Gy X1	[106] [15]
	Exogenous DC s.c., i.v. Exogenous DC s.c., i.t.	Increase local and systemic availability of DCs Increased availability of DCs in irradiated tumor	10 Gy X 3–5 8.5 Gy X 5 15 Gy X 1	[62] [107] [108]
	CpG s.c. peri-tumorally and i.t.	TLR9 agonist, DCs activation	10–55 Gy X 1	[109]
	Imiquimod, topical R848, i.v.	TLR7 agonist, local DCs activation TLR7 agonist, systemic DCs activation	8 Gy X 3 10 Gy X 1	[78] [110]
	ECI301 (CCL3 variant) i.v. 2′3′-cGAMP, i.t. Vaccinia and avipox recombinants expressing CEA and T cell costimulatory molecules Autologous tumor cell vaccine expressing GM-CSF	Recruitment of DCs, T cells, NK cells STING agonist, IFN-I production Generation of tumor antigen-specific T cells	6 Gy X 1 20 Gy X 1 8 Gy X 1 4 Gy X 2	[111] [68] [46] [45]
T cell priming and activation	Anti-CTLA-4 antibody, i.p.	Immune checkpoint inhibitor	12 Gy X 1–2 6 Gy X 5, 8 Gy X 3, 20 Gy x 1	[73] [16]
	Anti-CD137 antibody, i.v or i.p.	Costimulatory receptor agonist	5 Gy X 1, 10 Gy X 1, 15 Gy X 1 4 Gy X 2	[112] [76]
	Anti-OX40 antibody, i.p. IL-2, i.t. NHS-IL-2 (fusion of antibody to necrotic DNA with modified IL-2), i.v. TGFβ neutralizing antibody, i.p.	Costimulatory receptor agonist T cell growth factor Modified T cell growth factor, targeted to tumor Blocks TGFβ immunosuppressive effects on DC and T cells	20 Gy X 3 2 Gy X 10 3.6 Gy X 5 6 Gy X 5	[113] [114] [115] [37]
Killing of cancer cells	Anti-PD-1 antibody, i.p. Anti-PDL-1 antibody, i.p. Adoptively transferred T cells	Immune checkpoint inhibitor Blocks PD-1 ligand on tumor cells/infiltrating myeloid cells Activated tumor antigen-specific CD8 T cells	12 Gy X 1 10 Gy X 1 12 Gy X 1, 20 Gy X 1 2 Gy X 5 8 Gy X 1 10 Gy X 1 2 Gy X 1	[77] [116] [74] [75] [53] [47] [44]
	CSF1R inhibitor	Reduced post-RT recruitment of MDSC and TAM	3 Gy X 5 5 Gy X 1	[90] [117]

CEA carcinoembryonic antigen, *CpG* C-G enriched, synthetic oligodeoxynucleotide, *CTLA-4* T-lymphocyte-associated antigen 4, *DC* dendritic cells, *GM-CSF* granulocyte-macrophage colony-stimulating factor, *i.p.* intraperitoneally, *i.t.* intratumorally, *i.v.* intravenously, *MDSC* myeloid-derived suppressor cells, *MHC* major histocompatibility complex, *NK* natural killer cells, *PD-1* programmed death-1, *s.c.* subcutaneously, *TAM* tumor-associated macrophages, *TLR* Toll-like receptor

30.9 Clinical Translation of Combinations of Radiotherapy and Immunotherapy: A Work in Progress

Some of the combinations of radiation and immunotherapy that were shown to have additive or synergistic effects in preclinical models have been tested in the clinic. Pioneering studies tested strategies to increase antigen-presenting cell function and/or activation with the use of growth factors (granulocyte-macrophage colony-stimulating factor, GM-CSF) or TLR agonists (CpG) achieving close to 30% abscopal responses in solid tumors and lymphoma, respectively [27, 30].

Recent groundbreaking success of checkpoint inhibitors targeting CTLA-4 and PD-1 signaling has led to rapid FDA approval of multiple agents (nivolumab, pembrolizumab, ipilimumab, and atezolizumab) for metastatic melanoma, lung cancer, renal cell carcinoma, urothelial cancer, head and neck cancer, and Hodgkin lymphoma. However, the response rates of single-agent checkpoint inhibitors remain ~15–30%, depending on the disease, and resistance develops in the majority of cases [118]. Thus, radiation is being investigated in combination with these agents for the ability to overcome resistance and potential to generate a more robust and prolonged response in close to a hundred trials [119]. Most of these studies are in the phase I/II setting; however the combination of radiation and PD1 inhibition is currently being tested in phase III studies in non-small cell lung cancer (NCT02768558), head and neck cancer (NCT03040999), and glioblastoma (NCT02617589).

A few studies have reported results. In a phase III trial, 799 patients with castrate-resistant metastatic prostate cancer that had progressed after docetaxel treatment were randomized to receive a single 8 Gy radiation dose to a bone lesion followed by ipilimumab (10 mg/kg) or placebo every 3 weeks for up to 4 doses. The endpoint of improved survival in ipilimumab versus placebo group was not achieved [120]. However, within the group of patients with better prognostic characteristics and no visceral metastases, ipilimumab improved median survival, suggesting that selection of patients with less advanced disease and possibly a better immune function may be important [121].

Results of two phase I trials testing radiotherapy and ipilimumab in metastatic melanoma have been reported, both enrolling 22 patients. In both studies, the toxicities were similar to what is expected with ipilimumab alone. In the first study, 18% of patients had partial response (best outcome), and 18% showed stable disease in nonirradiated lesions [122], an outcome not significantly different from what would be expected with ipilimumab alone [123]. In the second study, 27.3% of patients had objective responses, including three patients with complete response and three with partial response, and 22.7% had stable disease [124]. Complete responses are rarely seen in melanoma patients treated with ipilimumab alone, suggesting that the addition of radiation can indeed enhance responses. Interestingly, the two studies differed in the sequencing of radiation and ipilimumab: ipilimumab was given 3–5 days after radiation in the first study and within 5 days before radiation in the second study, suggesting the possibility that sequencing is critical. Administration of immune checkpoint inhibitors before or during radiation was also shown to be more effective than administration after radiation in preclinical studies [75, 125].

Results have also been reported for the phase I of a phase I/II trial (NCT02239900) testing SBRT with ipilimumab in advanced solid tumors, including NSCLC (n = 8), colorectal carcinoma (n = 4), sarcoma (n = 3), and renal cell carcinoma (n = 3), with a total of 31 patients evaluable for abscopal responses. SBRT was given at 12.5 Gy X 4 fractions or 6 Gy X 10 fractions to a metastasis in either the liver or the lungs concurrently with ipilimumab or sequentially (1 week after the second dose). Clinical benefit (partial response or stable disease lasting 6 months or longer in nonirradiated lesions) was seen in 23% of patients. This result is encouraging in tumors known to be largely unresponsive to ipilimumab alone but too early to determine whether the radiation dose, site of irradiation, or sequencing with ipilimumab

make a difference [126]. Increased peripheral blood $CD8^+$ T cells and CD8/CD4 T cell ratio was associated with clinical benefit. Interestingly, greater expression of some T cell activation markers was seen in patients receiving radiation to the liver than lung, but this remains to be validated.

Very encouraging results of a phase II clinical trial (NCT02221739) testing radiation and ipilimumab in chemotherapy-refractory metastatic NSCLC were reported by our group at the ASTRO meeting [36]. Of 21 patients that received radiation (either as 6 Gy X 5 or 9.5 Gy X3), with first dose of ipilimumab given during radiation, and completed four cycles of ipilimumab, 33% showed partial or complete abscopal responses. Follow-up for survival is ongoing, but data in this small study indicate that radiation stimulates responses to ipilimumab in a disease refractory to ipilimumab alone and suggest that it may be more effective in combination with ipilimumab than chemotherapy [127].

Blocking other immunosuppressive pathways in combination with radiation has also yielded promising results. TGFβ, in addition to suppressing antitumor immune responses, is also an attractive target due to its role in radiation-induced fibrosis. Clinical trials are evaluating antibody neutralizing TGFβ as well as small molecule inhibitors of TGFβ receptor in combination with radiation in metastatic breast cancer (NCT01401062, closed to accrual), non-small cell lung cancer (NCT02581787), rectal cancer (NCT02688712), and glioma patients (NCT01220271, closed to accrual).

TLR agonists have also been utilized with success to overcome the immunosuppressive tumor microenvironment. For example, the TLR7 agonist imiquimod enhances DC maturation and antigen presentation and promotes T-helper (TH1) skewing and increased homing of T cells. Preclinical data in a mouse model of breast cancer skin metastasis showed that topical imiquimod combined with hypo-fractionated radiation inhibited tumor growth in a CD8-dependent fashion and when combined with cyclophosphamide-induced immunologic memory [78]. This provided the basis of a phase II study for breast cancer patients with chest wall recurrence or skin metastases (NCT01421017).

Building on prior encouraging data with the use of a TLR9 agonist with tumor-targeted radiation in lymphoma [30], a new study aims at improving responses in low-grade B cell lymphoma by adding Flt3L and changing the Toll-like receptor (TLR) agonist to poly-ICLC, an optimal TLR agonist for the DCs recruited by Flt3L (NCT01976585).

High-dose interleukin-2 (IL-2) expands T cells and increases their function and was the first immunotherapy to be approved for the treatment of metastatic melanoma and renal cell carcinoma [128]. A pilot study examined SBRT followed by high-dose IL-2 in patients with metastatic melanoma and renal cell carcinoma. An impressive 8 out of 12 patients achieved a complete or partial response in this small study, a significantly higher percentage than the ~15% responders usually seen with IL-2 alone [129]. There are now multiple phase II trials investigating high-dose IL-2 or a recombinant fusion protein of IL-2 with L19 human vascular-targeting antibody (Darleukin, L19-IL2) with SBRT in metastatic melanoma and renal cell carcinoma [119].

30.10 Toward the Use of Radiation to Induce a Personalized Tumor Vaccine

Mutations in the tumor genome can encode proteins that are immunogenic but largely unique to each given tumor (neoantigens) [130]. While neoantigens are an attractive target because the immune system is not tolerized to them and their selective expression by the tumor avoids the risk of autoimmunity, eliciting responses to neoantigens requires a very personalized approach [131]. In this context, radiotherapy may provide a quicker and more affordable strategy to generate a personalized tumor vaccine than deep sequencing and bioinformatics in silico predictions [4, 58]. However, many outstanding questions remain concerning the best way to use radiation to prime effective tumor-specific T cell responses [4]. The role of histology, radiation dose and

fractionation, site irradiated, sequencing of therapy, and immunotherapy agent used are under active investigation. In addition to these factors, patient-specific characteristics like underlying genetic background and polymorphisms in immunologically relevant genes [132], tumor-specific genetic, epigenetic, and posttranslational pathways, and environmental factors including temperature, metabolism, and microbiome are likely to influence the ability of radiation to generate an in situ tumor vaccine [133, 134].

Identification of predictive biomarkers is urgently needed to help patient selection and treatment tailoring. Currently, the only widely used clinical test to predict response to immunotherapy with pembrolizumab in NSCLC is immunostaining for PD-L1 [135]. Emerging biomarkers include the "immunoscore," i.e., the comprehensive profile of infiltrating immune cells in a tumor specimen prior to treatment [39, 136] that reflects the overall immunogenicity of the tumor microenvironment and also may inform on which immune cells to best target (e.g., tumor suppressive macrophages versus regulatory T cells). In addition to microenvironmental indices, the genomic landscape of tumor cells measured by mutational burden or neoantigen burden has shown predictive value. For example, in NSCLC, mutational and neoantigen burden both predicted response to anti-PD1 immunotherapy [19]. In this vein, tumors with DNA repair defects such as mismatch repair deficiency are also more likely to respond to anti-PD1 immunotherapy [137]. Lastly, a role for single nucleotide polymorphisms (SNPs), or minor germline genetic differences between patients independent of tumor mutations, is emerging. For example, SNPs in PD-L1 have shown to be predictive in the context of chemotherapy [138].

While efforts at understanding the immune and genetic landscape of a patient's cancer at diagnosis are important, it is already clear that even if there is initial response to immunotherapy, most patients progress. Consequently, understanding immune escape mechanism is critical [139]. Lastly, while initial biopsy and blood testing hold the promise to inform the appropriate therapy, given the genetic evolution of tumors in response to immune pressure or "immunoediting," sampling tumor and immune cells at each step of cancer progression is likely necessary to inform intervention. To this end "liquid biopsies" or the use of peripheral blood samples as a surrogate of tumor populations offers the promise of a minimally invasive strategy to monitor patients over time.

In summary, exceptional responses to the combination of radiotherapy and immunotherapy, like our patient with advanced chemotherapy-refractory metastatic NSCLC who remains tumor-free more than 4 years after non-ablative irradiation of one metastasis combined with 4 doses of ipilimumab [17], have generated considerable enthusiasm. However, such responses remain relatively rare. We are only beginning to understand how to best deliver and combine radiation with immunotherapy and address the many factors that should be considered in preclinical and clinical trial design. Ultimately, patient-specific factors will guide therapy delivery.

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Predictors of Response to Immune Checkpoint Blockade

31

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Contents

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31.1 Introduction

The face of cancer treatment has changed markedly over the last decade, undergoing first a molecular revolution with the establishment of the personalized medicine era and now shifting again with the rise of effective medical immunotherapeutic strategies. Not only does the current immuno-oncology revolution challenge notions of patient fitness, treatment expectation, and toxicity management, but the unique mechanism of action of agents targeting immunoregulatory checkpoints mandates accordingly unique methods of disease assessment, monitoring, and harmonization with other—more traditional—treatment modalities.

Central to efforts to optimize the way in which immune checkpoint inhibitor agents are used is the search for appropriate biomarkers of response and toxicity. While the clinical imperative to maximize treatment options drives continued drug development in the field, concerted efforts are being made to understand the basic immunobiology of cancer in order to prevent a widening gap between our understanding of what novel agents do and how they achieve this. A deep understanding of the complex interactions between tumor

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cells, the broader tumor microenvironment, the immune system, and even influences from further afield is essential to the discovery of clinically useful predictors of immunotherapeutic efficacy. Importantly, a greater appreciation is developing for how inter-connected the new (immuno-oncology) and the old (molecular carcinogenesis) truly are, as reflected by the addition of immune evasion to the (emerging) hallmarks of cancer by Hanahan and Weinberg in 2011 [1].

In this chapter, we will describe the molecular and cellular processes that form the hallmarks of response to immune checkpoint blockade, building upon extensive and seminal work delineating the network of dynamic interactions that constitutes the cancer-immunity interface. Additionally, we discuss the current and emerging factors that may be of predictive value for patients receiving immune checkpoint blockade therapies and which are mechanistically expected also to apply to novel immunotherapy agents acting as agonists of immune stimulatory molecular targets. Consideration is given to the necessary symbioses that will arise in future biomarker-driven immunotherapy clinical trial designs, driven by the common aim of integrating immunotherapy into the domain of truly personalized medicine.

31.2 Hallmarks of Response to Immune Checkpoint Blockade

In order to understand how best to monitor and predict responses to immune checkpoint blockade, one must have a fundamental understanding of the mechanisms driving carcinogenesis and disease progression, as well as a working knowledge of systemic and antitumor immune responses. These factors, along with external influences from the broader environment, are critical in shaping therapeutic responses and provide the context for the hallmarks of response to immune checkpoint blockade (Fig. 31.1).

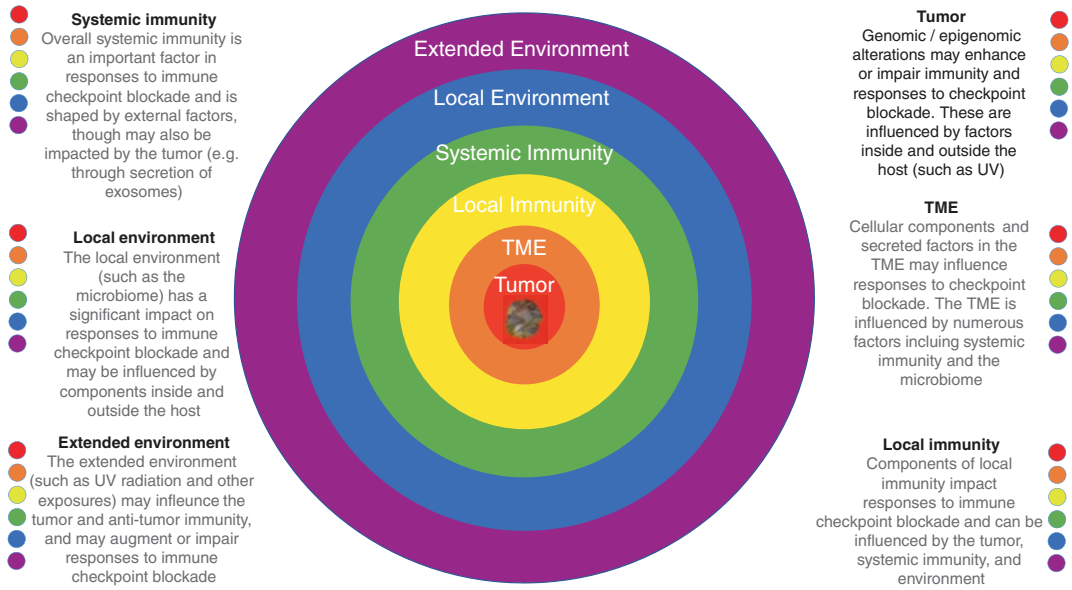

Fig. 31.1 Hallmarks of response to immune checkpoint blockade. Identification of predictors of response to immune checkpoint hinges on an understanding of the influence of numerous factors on cancer development and antitumor immunity, with "concentric spheres of influence" from the tumor itself, the tumor microenvironment, local immunity (such as in the peri-tumoral environment and draining lymph nodes), systemic immunity, and the local and extended environment. Forces affecting each of these areas are listed and are highly interrelated and interdependent. Ultimate responses to immune checkpoint blockade are the result of a complex tally of these positive and negative interactions; thus, all must be taken into account when considering predictive biomarkers for use in these therapies

31.2.1 Tumor and Microenvironment

31.2.1.1 Tumor Genomics and Epigenomics

Significant inroads have been made in the understanding of carcinogenesis and cancer progression through the use of next-generation sequencing techniques, which are now integrated into the fabric of clinical care. Targeted sequencing panels are being used worldwide across multiple cancer types and are helping to inform therapeutic decision-making—particularly to guide treatment with molecularly targeted therapy. However, genomic profiling is also highly relevant when considering responses to immune checkpoint blockade, as there is a growing list of molecular alterations implicated in antitumor immune responses [2–6].

The genomic characterization of tumors through efforts such as The Cancer Genome Atlas (TCGA), has delivered tremendous insight into the types and burden of mutations across different cancer types [7–10]. With regard to genomic mutations, both quality and quantity "matter" and may influence antitumor immunity and subsequent responses to immune checkpoint blockade. Perhaps one of the best illustrations of this is the concept that total mutational load may influence response to immune checkpoint inhibitors; in general, cancers with a higher mutation rate or with defects in DNA mismatch repair genes are much more likely to respond to immune checkpoint blockade [2, 11, 12]. This is likely due to an associated higher burden of neoantigens, defined as tumor-associated antigens arising from somatic mutational events; as these are not expressed by other "normal" tissues, they may be much more immunogenic due to the presence of neoantigen-reactive T cells that have likely not been tolerized. However it is important to note that a relatively small percentage of neoantigens that are predicted using conventional algorithms are actually expressed, and even fewer are likely to be capable of eliciting a meaningful antitumor immune response [13].

Mutations can be a "double-edged sword," as they may also contribute to therapeutic resistance. There is a growing list of examples of genomic mutations and other genomic or epigenomic alterations that weaken responses to immune checkpoint blockade. Aberrant antigen processing and presentation machinery resulting from mutations, copy number alterations, or altered expression patterns of such molecules as HLA and β-2 microglobulin have been described [3, 14, 15]. It is also important to consider that oncogenic genomic or epigenomic alterations may be associated with downregulation of tumor-specific antigens, and therapeutic targeting of these alterations may result in enhanced antigen expression. This is exemplified by the effects of kinase inhibitors targeting V600-mutated BRAF in the case of melanoma [16, 17] and is also seen in the setting of epigenetic regulation of cancer testis antigen expression across cancer types [18, 19].

Other mutations or alterations associated with oncogenic progression may be associated with immunoregulatory effects of relevance to immune checkpoint blockade, for example, increased expression of programmed death receptor ligand-1 (PD-L1) driven by AKT-mTOR [20] or EGFR [21] pathway activation in lung cancers or induction of an immunosuppressive cytokine milieu by functional insufficiency of the tumor suppressor gene PTEN [4], resulting in impaired responses to immune checkpoint blockade. Defects in interferon signaling within tumors may also confer resistance to immune checkpoint blockade [3, 22].

31.2.1.2 Tumor Microenvironment

In addition to considering molecular alterations in tumors, one must consider the tumor microenvironment in modulating responses to therapy. Indeed tumors are complex ecosystems, consisting not only of tumor cells but also blood vessels, tumor-infiltrating immune cells, cytokines, and numerous other stromal components including fibroblasts, myeloid-derived suppressor cells, extracellular matrix, and other cellular entities. Together, these components contribute to carcinogenesis and may ultimately contribute to therapeutic response and resistance to immune checkpoint blockade.

A strong foundation delineating the diverse roles played by distinct functional elements of

the tumor microenvironment arises from the seminal work of Hanahan and Weinberg as well as that of Schreiber, who described the "hallmarks of cancer" [1] and principles of immunoediting [23], respectively. Together, these describe mechanisms by which tumors grow and metastasize, as well as the intimate and dynamic interactions between tumor and immune cells, leading to tumor elimination, equilibrium, or escape [23].

This foundation was subsequently built upon by other investigators, who described the "cancer-immunity cycle" [24], the "cancer-immune set point" [25], and the "cancer immunogram" [26]. Recently, these concepts have been explored in the setting of checkpoint inhibitor therapy, and "hallmarks of response to immune checkpoint blockade" have also been described [27]. Each of these acknowledges the complex relationship between tumor cells and cells within the microenvironment as well as external influences and strives to demonstrate the influence of these on therapeutic responses. These are outstanding models, though our incomplete understanding of the complex processes is certainly acknowledged—and these and other models will be revised using an iterative approach as more data emerges.

A key feature in governing responses to immune checkpoint blockade is the ability (or inability) to exclude T cells from the tumor microenvironment itself. The presence of a T cell infiltrate within tumors has been known to correlate positively with prognosis across multiple tumor types for several years [28], and more recent studies have evaluated the relationship between the density and distribution of CD8+ T cells, including location at the tumor center or invasive margin, and response to immune checkpoint blockade [29]. Additional assessment of the tumor microenvironment using both architecture and gene expression profiling has helped classify tumors into several different phenotypes including "inflamed vs non-inflamed" [30], with later refinement of these phenotypes to "immune-desert, immune-excluded, or inflamed" [25]. Mechanisms of immune exclusion have been described and may originate in tumor cells themselves such as defects in Wnt/β-catenin pathways [5] or MAPK hyperactivation [31, 32]. Alternatively, immune exclusion may arise from other cellular components in the tumor microenvironment, such as myeloid-derived suppressor cells (MDSCs) [33], or from secreted factors such as vascular endothelial growth factor (VEGF) [34–36], interleukin-1 (IL-1), and interleukin-6 (IL-6) [37–39]. An appreciation of the contributions from each of these factors is paramount to designing adequate immune monitoring strategies in the setting of treatment with immune checkpoint blockade and is also important in the rational design of strategies to overcome therapeutic resistance.

31.2.2 Immunity

In addition to the tumor and microenvironment, overall host immunity plays a key role in modulating tumor growth, spread, and response to therapy. Immune responses are dynamic and are shaped by exposure to antigenic stimuli but are also influenced by factors within and outside the host (Fig. 31.1). Debris from dying tumor cells are taken up by host antigen-presenting cells (such as dendritic cells), processed and presented to CD4+ (helper) and CD8+ (cytotoxic) T lymphocytes, but additional signals (co-stimulatory versus co-inhibitory) and secreted factors are critical to determining the fate of this T cell-antigen interaction, resulting in either T cell stimulation and generation of an antitumor immune response or T cell anergy. T cell recognition of presented antigen is, however, only the first of many steps in the process, and immunoregulatory influences at any stage (priming, trafficking, tumor infiltration, T cell-tumor cell engagement), arising locally or from distant sites, may impair an otherwise effective immune response (Fig. 31.2).

It has long been appreciated that overall host immunity may influence cancer development and progression, from reports of spontaneous regressions of cancer [40] to the concept of inducing immune responses against cancer by administering microbial products (Coley's toxins) to patients beginning in the late 1800s [41, 42]. This

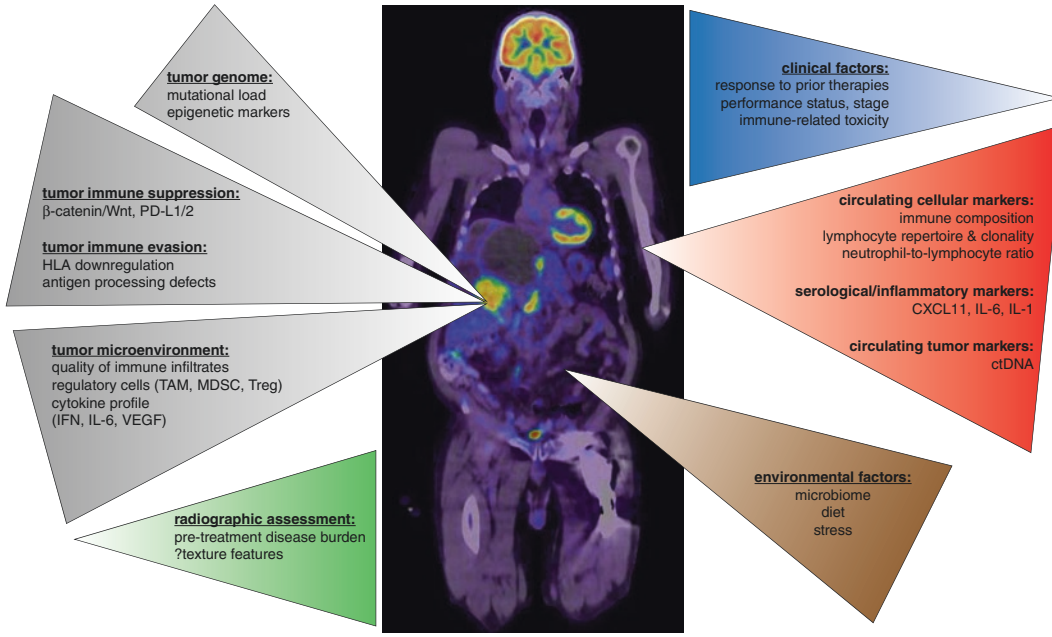

Fig. 31.2 Current and novel predictors of immune checkpoint blockade. Existing predictors of response to immune checkpoint blockade are predominantly focused on tumor-based assessments (*gray*) documenting tumor mutational and associated neoantigen burden, tumor cell-intrinsic immune suppression and evasion mechanisms, and microenvironmental factors, principally the presence or absence of an activated CD8+ T cell infiltrate. Emerging predictors extend upon these factors but are gradually broadening in scope to include historical factors as well as biomarkers sourced from other biocompartments; these include radiographic assessments (*green*), clinical factors (*blue*), blood biomarkers (*red*), and environmental influences, currently dominated by the effect of the gut microbiome (*brown*)

is also substantiated by findings that patients with immunosuppression are at increased risk of developing several forms of cancer. These and other concepts are embodied in the seminal work of Schreiber in which the "three E's" of immunoediting were described [23], highlighting the tight interaction between host immunity and tumor, albeit largely at the level of the tumor microenvironment.

There is now a growing appreciation of the influence of overall systemic immunity on tumor rejection and response to immunotherapy. Recent reports have helped to delineate the complex role of numerous cell types in multiple compartments of the body in generating an antitumor immune response, demonstrating that immune activation in the periphery is required and is heavily dependent on CD4+ T cells and that PD-L1 expression in the periphery is a major driver of immune escape [43]. Additionally, expression of 4-1BB [44] or PD-1 [45] is known to enrich for tumor-reactive T cells within both tumor and circulating lymphocytes, hinting at an as yet poorly understood immune circulation that may be relevant to the establishment and maintenance of the tumor-reactive T cell population. Given these findings, it is critically important to consider more general measures of host immunity when contemplating predictors of response to immune checkpoint blockade. However, ideal monitoring strategies for overall systemic immunity remain elusive and will require extensive development.

31.2.3 Environment

In addition to considering internal features such as the tumor, microenvironment, and general immunity, one must consider the influence of the wider environment in the setting of cancer development,

progression, and treatment. These factors act in parallel with the local, or host immune parameters described above, and include physical influences from the extended environment such as ultraviolet radiation and carcinogen exposures (e.g., tobacco smoke), biological influences from the diet and microbiome, and neurophysiological influences from stress.

Environmental factors can have a significant impact on all domains affecting response to immune checkpoint blockade. One of the strongest examples of this is the influence of carcinogens on mutational load and response to immune checkpoint blockade, with patients suffering damage from UV exposure and smoking noted to have typically higher mutational load and enhanced responses to immune checkpoint blockade [46]. Environmental carcinogens are not the only example, as other factors of potential impact on antitumor immunity and response to immune checkpoint blockade are slowly being identified, including diet, stress [47], and the gastrointestinal microbiome [48, 49]. These have important implications not only for monitoring responses but also have significant potential therapeutic implications. This is highlighted by recent literature demonstrating that modulation of the gut microbiome can actually enhance therapeutic responses [48, 49] with clinical trials now being designed to capitalize on these insights.

31.3 Current and Emerging Predictors of Response, Resistance, and Toxicity

Based on an understanding of the complex influences on antitumor immunity, biomarkers have been and are being developed to predict response, resistance, and potentially even toxicity to therapy. It should be noted that there is currently a paucity of approved highly validated biomarkers at present, with the exception of PD-L1 and CD8—though each of these has limitations on its own. Nonetheless there is a large and growing list of putative predictors of response to immune checkpoint blockade that are currently being explored and tested, and these will be discussed herein.

31.3.1 Clinical Predictors of Response

Early clinical experience with checkpoint inhibitor agents raised concern that higher pretreatment burden of disease and more rapidly progressive disease were associated with a lower likelihood of response and poorer survival outcomes, due in part to the sometimes delayed onset of objective responses and low overall objective response rate observed in the setting of treatment with ipilimumab—the first checkpoint inhibitor to be approved in cancer therapy. This has not been recapitulated in the setting of treatment with immune checkpoint inhibitors targeting the PD-1 pathway or with combined CTLA-4 and PD-1 blockade—where response rates are higher and onset of response more brisk [50]. Nevertheless, disease burden clearly still plays a role, as baseline burden of disease (determined by radiologically assessable lesions), was independently prognostic of poorer overall survival in patients treated with the PD-1 inhibitor pembrolizumab [51], and was strongly inversely associated with survival in a small cohort of patients treated with ipilimumab in combination with bevacizumab [52]. These findings essentially confirm the adverse prognosis conferred by a larger burden of disease prior to checkpoint blockade without specifically identifying a predictive role. Furthermore, patients achieving a response by either RECIST or irRC criteria clearly have superior long-term outcomes to objective non-responders [53]; however, the prognostic and/or predictive value of additional imaging-based metrics such as density- and texture-based features remains to be determined and may ultimately require complex assessment criteria unique to distinct tumor types and sites of disease.

Prior durable response to ipilimumab appears to predict for benefit from subsequent treatment with pembrolizumab [54], but increasing evidence confirms that patients refractory to prior immune checkpoint blockade targeting CTLA-4 may still respond to subsequent challenge with anti-PD-1-based therapy and vice versa [55, 56]. Larger cohort data is required to determine the effects of confounding factors such as the interval

between separate checkpoint blockade agents, the significance of sequential therapy without clear evidence of resistance to the first agent, post-progression fitness and disease burden, as well as subtle differences in toxicity profile arising from treatment sequence [56]. In general, it appears that prior treatment per se may not be predictive of immune checkpoint blockade response, but instead correlates with (and is confounded by) independently prognostic variables such as disease stage and performance status.

31.3.2 Tissue-Based Predictors of Response

The majority of research aiming to identify predictors of checkpoint blockade efficacy has focused on analysis of the tumor itself. Tumor-intrinsic factors that lead to immunogenicity are well described, arising ultimately from the genomic instability characteristic to all cancers and resultant somatic mutational events which may produce altered peptides, protein expression patterns, peptide processing/display machinery, and responses to immune attack.

Conceptually, predicting the antigenicity of a tumor is difficult to assess in the context of checkpoint blockade, given that the underlying T cell repertoire being targeted by therapy, and the antigens to which these T cells may be reactive, is unknown. Furthermore, the T cell repertoire is highly diverse and partially redundant, comprised of innumerable unrelated TCR sequences, many of which may be reactive to the same peptide epitope. Computational methods to predict protein targets from TCR sequence data alone are lacking and additionally hampered by the largely private T cell repertoires between patients. However, given the relationship between neoantigen burden and inherent antigenicity of a tumor, the underlying somatic mutational burden of a tumor has been found to correlate with response to both CTLA-4 and PD-1 blockade. Despite this, profiling of the mutational landscape in large cohorts of tumors reveals that consistently, a large range of mutational burden exists across tumor types [10], and somatic mutational load lacks sufficient negative predictive value as a biomarker of immune checkpoint response.

Unsurprisingly, the most heavily investigated biomarker for PD-1 and PD-L1 inhibitors is PD-L1 expression on the tumor itself. In advanced melanoma, positive PD-L1 expression is clearly predictive of improved response to PD-(L)1 targeted therapy; however, low or absent PD-L1 expression does not entirely preclude therapeutic response, leading to significant reticence toward its widespread strict application as a treatment selection tool [57, 58]. Elements of specific tumor biology likely also impact on the utility of direct target biomarkers like PD-L1. For example, in non-small cell lung cancer, tumoral expression of PD-L1 demonstrated a continuous predictive association with response and survival following nivolumab treatment in non-squamous patients [59] but appeared to lack either prognostic or predictive value in a large cohort of squamous NSCLC patients treated with the same agent [60]. Precise mechanisms for this stark contrast remain to be defined but may reflect etiological differences (e.g., smoking history) and both burden and type of cumulative mutational load.

Direct assessment of the intratumoral immune landscape has also been employed as a biomarker discovery strategy. In anti-CTLA-4-treated patients, higher baseline expression of FOXP3 and IDO was associated with clinical response, as was an increase in TIL from baseline to 3 weeks after treatment initiation. Exploratory gene expression profiling found increases in expression of genes associated with immune response, and decreased cancer-associated genes, consistent with expectation [61]. Multiparameter flow cytometric analysis of TIL from fresh melanoma samples demonstrated that the frequency of PD-1+ CTLA-4+ CD8+ T cells prior to initiation of anti-PD-1 therapy was highly predictive of response and progression-free survival; this was associated with accumulation of an activated, and relief of an exhausted, CD8+ T cell phenotype in TIL populations during treatment [62].

The use of baseline predictors of response is increasingly being supplemented with the use of early on-treatment markers in light of data

demonstrating the superior utility of longitudinal tumor assessment in tumor [14]. In a study of baseline and longitudinal tumor biopsy samples from melanoma patients, pretreatment T cell enrichment in TIL was associated with response to PD-1 inhibitor treatment regardless of prior CTLA-4 inhibitor therapy; however, early on-treatment biopsies displaying enrichment of T cell infiltrate, immunomodulatory marker expression (PD-1, PD-L1, LAG3), and, particularly, FOXP3 and granzyme B expression were highly associated with likelihood of response [14].

Importantly, the tumor-immune interaction likely evolves in parallel with the process of cellular transformation, and as such, a substantial degree of adaptation and evolution has already occurred long before the clinical diagnosis, or scientific interrogation, of a tumor. Implicit in this is the notion that the mechanisms which may lead to failure of any immunotherapy strategy, including checkpoint inhibition, may be selectively driven by that therapy but may also have spontaneously arisen *prior* to therapy, in response to preexisting immune pressure. Thus, much can be considered directly transferrable from our knowledge of immune checkpoint blockade *resistance* mechanisms into the treatment-naïve setting and thus used as predictive factors.

The central role of interferon signaling in shaping the tumor-immune interface is well described, with IFN-γ critically important for the establishment of a suitably inflamed tumor microenvironment but equally important for the emergence of immune checkpoint-driven adaptive resistance. Due to the presence of this high-level negative feedback mechanism, it is not surprising that the role of IFN in antitumor immunity can be confusing, promoting aspects of both tumor cell destruction and survival. Mutations in IFN signaling pathway components have been identified as molecular mechanisms of checkpoint blockade resistance in melanoma; *JAK1* and *JAK2* mutations not identified in pretreatment samples of patients who initially responded, but subsequently progressed while receiving anti-PD-1 therapy, have been shown to directly facilitate tumor cell resistance to IFN-mediated growth suppression and apoptosis [3]. Similar mutations were subsequently demonstrated to result in lack of response in patients previously untreated with anti-PD-1 therapy [63].

General immune exclusion may also predict for a lack of immune checkpoint inhibitor response, in keeping with the necessity of immune effector cells (principally tumor-specific T cells) to physically engage with or enter the tumor in order to facilitate tumor killing. Several mechanisms of tumoral immune exclusion have been identified including tumor cell expression of β-catenin [5]; however, firm evidence of specific molecular mechanisms predicting success or failure of immune checkpoint inhibition in patient samples is lacking.

31.3.3 Blood-Based Predictors of Response

Significant interest is emerging in the use of cell-free circulating DNA as a noninvasive marker that can be used to monitor the response to treatment, typically by quantitating mutated DNA originating uniquely from tumor cells (circulating tumor DNA, ctDNA). In melanoma and colorectal cancer, ctDNA has been shown to vary in accordance with disease burden measured by more conventional means (e.g., radiologically) with the distinct advantage of potentially identifying changes in response status (either response or progression) significantly earlier than clinico-radiological assessment [64–67]. Whether this can be exploited as an early clinical decision-making trigger to improve long-term treatment outcomes is not yet known. Additionally, although preliminary evidence suggests at least a correlation between dynamic ctDNA alterations early during therapy and eventual treatment outcome [64], the potential clinical use of ctDNA as a predictive marker for immunotherapy has not been defined. As highly recurrent tumor mutations are found in a minority of cancer patients overall, future interest lies in the identification of personalized, sequence-independent tumor markers such as aberrantly methylated ctDNA as a means of

noninvasive monitoring. Given the increasingly apparent role of epigenetic changes in modulating the response to immune checkpoint blockade, it will be of significant interest to see whether methylation-specific ctDNA assessment of molecular targets known to influence immunotherapeutic outcomes can be devised as predictive assays in this setting. Other serological markers specifically predictive of checkpoint blockade response have not yet been identified. In one study, independent and clearly clinically useful markers were not identified other than LDH, which is itself more prognostic than predictive [68].

Similarly to tumor biopsy-based analyses, circulating immune markers have been studied prior to and early after initiation of immune checkpoint blockade. In several retrospective studies, pretreatment neutrophil-to-lymphocyte ratio (NLR) has been shown to be an independent predictive factor of ipilimumab benefit (lower NLR predictive of improved PFS and OS) although this would be a difficult metric to apply to patients requiring steroids, and no consistent optimal NLR cutoff to stratify patients into "good" or "bad" responders has yet been agreed upon [69–71]. PD-1 expression on CD4+ T cells in peripheral blood is negatively prognostic in NSCLC and correlated inversely with response to anti-PD-L1 therapy in a small cohort of patients [72]. Conversely, elevated circulating regulatory T cell numbers during treatment are associated with non-response to PD-1 inhibitor treatment [55], while baseline levels of the chemokine CXCL11 and—less robustly—soluble MHC class I-related chain A (sMICA) may be predictive of poor survival in anti-CTLA-4-treated patients [73]. Also of predictive value early after starting therapy, an increased frequency of inducible T cell co-stimulatory (ICOS)-expressing CD4+ T cells in peripheral blood has been shown to be a reproducible biomarker of anti-CTLA-4 therapy efficacy after as few as one or two doses [74]. Early on-treatment elevation in absolute lymphocyte count and eosinophil count may also be predictive of improved overall survival in patients treated with ipilimumab [75].

31.3.4 Noninvasive Predictors of Response

Efforts are underway to establish novel imaging techniques to help predict responses to immunotherapy; however, the use of noninvasive imaging has thus far been limited in routine clinical practice to staging investigations performed at baseline and throughout treatment. Notwithstanding the prognostic and potentially predictive value of imaging-based assessments of burden of disease prior to treatment initiation, standard radiologic assessments based on the response evaluation criteria in solid tumors (RECIST), while extensively validated across tumor types and treatment modalities, were found to be misleading in a small proportion (3–15%) of patients treated with immunotherapy due to the unique mechanism of action and response kinetics inherent to this treatment type [53, 76]. The phenomenon of pseudoprogression, in which therapy-related tumoral inflammation may temporarily lead to an increase in size of evident lesions and appearance of apparently new lesions at sites of previously occult micrometastatic disease, belies the potential for subsequent response in and/or disappearance of these lesions once the antitumor immune response progresses and ultimately subsides. To account for these "atypical" patterns of response, a new set of imaging-based assessment criteria were proposed, dubbed the "immune-related response criteria" (irRC), being also applicable to other forms of immunotherapy based on a common tumoral inflammation mechanism of action [76].

The necessity to derive new criteria in the form of irRC suggests that radiologic assessment is inherently a flawed monitor and worse predictor of response in checkpoint blockade-treated cancers. It must be noted, however, that atypical radiological patterns of response are uncommon. In standard clinical settings, radiologic response is commonly obtained only 10–12 weeks after the initiation of immune checkpoint blockade therapy, and any response at that time may be unreliable until confirmed upon repeat scanning at least 4 weeks later. Indeed, cases of profoundly delayed

responses occurring with an onset of 6–9 months after the initiation of therapy have been noted.

However, novel imaging-based metrics are under development which may afford clinically useful insights for diagnosis and for therapy, either prior to treatment commencement or at early on-treatment time points, potentially facilitating prompt changes in management in patients who appear destined not to respond to their current treatment regimen [77]. In a cohort of patients treated with ipilimumab and the anti-angiogenic agent bevacizumab, tumoral density features were incorporated into radiologic assessment using the Choi criteria; unfortunately changes in tumor density during treatment were not predictive of response [52]. In a modest cohort of patients treated with pembrolizumab on the KEYNOTE-001 protocol, both tumor size and density, or combined metrics (e.g., Choi or modified Choi criteria) were associated with overall survival [78]. Notably, density features suggest yet more complex patterns of response than can be assessed by either RECIST or irRC, thus opening the door wide to future development of radiomic-/texture-based features as putative prognostic or predictive features in the setting of immune checkpoint blockade.

31.3.5 The Gut Microbiome as a Predictive Biomarker

An emerging field of major significance to cancer immunotherapy is the study of commensal microbiota and their influence on checkpoint inhibitor responses, principally driven by the gut microbiome. In a seminal translational study of the gut microbiota using animal models and fecal microbial transplants from CTLA-4 inhibitor-treated patients, three key observations were made: firstly, response to therapy was clearly linked to the abundance of specific *Bacteroides* species in the gut; secondly, CTLA-4 inhibitor treatment in patients altered the gut microbial composition; and thirdly, CTLA-4 inhibitor efficacy was dependent on the presence of microbe-specific T cell responses [49]. In another study, intestinal *Bifidobacterium* was associated with stronger spontaneous antitumor responses in mice, and specific microbial "supplementation" independently phenocopied—or synergized with—PD-L1 checkpoint blockade [48]. Similarly, differences in gut microbial composition and diversity were identified between responders and non-responders to PD-1 inhibitor therapy [79]. Although the gut microbiome presents a clear interface between environment, diet, host immunity, and antitumor immunity, the precise mechanisms by which this interaction acts to modulate immune checkpoint inhibitor response are yet to be fully elucidated and will likely prove to be complex.

31.3.6 Predictors of Toxicity

Based on the imperfect tumor specificity of immune checkpoint blockade therapies, off-target immune stimulation leading to immune-related toxicities (immune-related adverse events, irAEs) is common. Anecdotally, the occurrence of irAEs has frequently been seen as a surrogate for the likelihood of antitumor immune stimulation; however, reported data regarding a putative association between irAE occurrence and antitumor clinical benefit in the setting of CTLA-4- and PD-1-directed therapy are conflicting across tumor types [80–83].

Given the potential severity of many irAEs, identification of reliable predictors of toxicity is equally as important as predictors of response; however, none have been identified thus far. Mechanistically, it is thought that preexisting autoreactive T cell clones, present at likely low frequencies, are subject to checkpoint blockade-induced reactivation leading to autoimmune toxicities. Low pretreatment prevalence of such clones, combined with the current lack of any technology able to reliably predict the antigen target of a given T cell receptor based on sequence information alone (other than a very limited number of previously described sequence-target relationships), makes the prospective identification of autoreactive T cell populations effectively impossible. Furthermore, it is not yet known why some of these preexisting but suppressed/tolerized clones are induced to reactivate, while presumably more numerous other clones do not. In a study of whole blood gene expression profiling in CTLA-4 inhibitor-treated patients, potential

predictive signatures of GI toxicity were identified, primarily in on-treatment samples; however, independently predictive genes were not [84].

31.4 Static Versus Dynamic Assessment of Systemic and Antitumor Immunity

Conventional analysis of biomarkers of response to cancer therapy has traditionally hinged on analysis of static time points in tissue or blood samples—typically taken just before the start of therapy. However, limitations with this approach exist, as analyzed samples may be archived for a significant period of time—thus biomarkers may not necessarily be accurate if measured in temporally distant samples due to evolution of genomic and immune profiles over time. This, together with spatial heterogeneity and sampling error, may account for some of the modest predictive value of pretreatment biomarkers such as PD-L1. Additionally, static assessment of a pretreatment sample does not provide a complete picture given the dynamic nature of the antitumor immune response.

As evidence regarding predictive biomarkers of response to immune checkpoint blockade grows, it is becoming evident that analysis of adaptive immune signatures in early on-treatment samples (or changes from pre- to on-therapy) may be far superior in their predictive power, reflective of the dynamic state of the tumor microenvironment (Fig. 31.3). This is exemplified in several recent studies, including one dem-

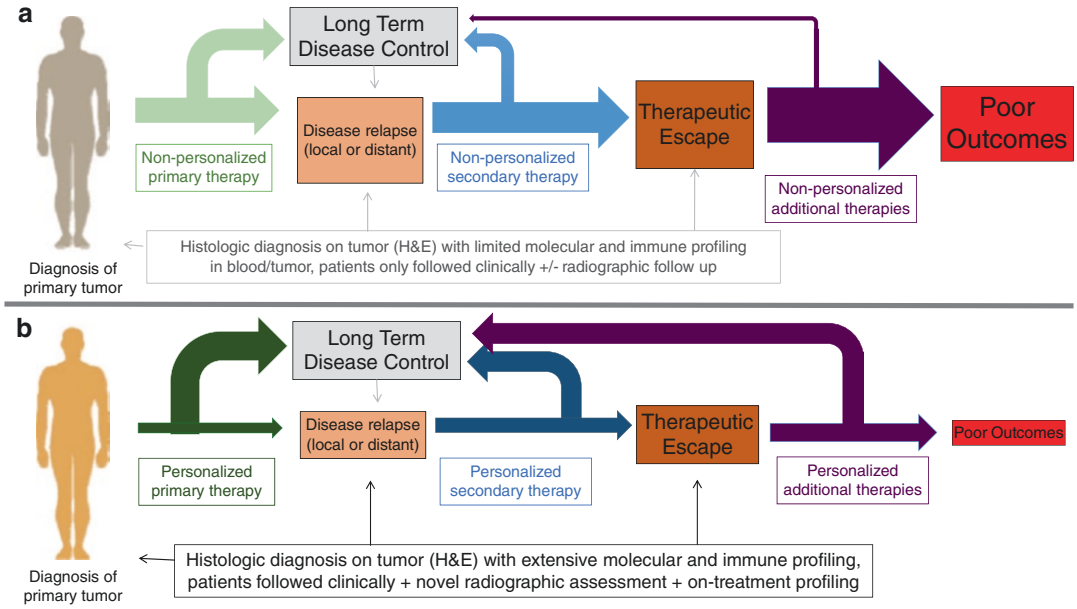

Fig. 31.3 (**a**) Importance of dynamic and static markers for checkpoint inhibitor blockade. In conventional cancer therapy, diagnosis of a primary tumor is followed by an evaluation of the tumor via histologic diagnosis with limited molecular and immune profiling in tumor +/− blood. Non-personalized primary therapy is then chosen empirically, with patients followed clinically and potentially with radiographic follow-up. In this scenario, a significant proportion of patients experience disease relapse, and a secondary form of non-personalized therapy is initiated—again with minimal to no biomarker assessment. Again, few patients respond and the majority experience therapeutic escape with poor outcomes overall. (**b**) In innovative approaches to cancer therapy, histologic diagnosis of a primary tumor is augmented via molecular and immune profiling at baseline. Personalized primary therapy is chosen based on insights gained, and a higher proportion of patients achieve long-term disease control. Disease relapse occurs in a smaller proportion of patients, and iterative approaches to personalized secondary therapy are utilized incorporating molecular and immune profiling of the tumor at relapse as well as on-treatment profiling of tumor and blood. Fewer patients experience disease escape, and when this does occur, responses to additional personalized approaches are higher based on a more customized treatment strategy. With this approach, far fewer patients experience poor outcomes to therapy

onstrating that immune profiling via immunohistochemistry and gene expression profiling in early on-treatment samples revealed robust biomarkers of response, whereas few biomarkers were evident on analysis of pretreatment samples alone [14]. Similar dynamic findings have been noted in several other studies investigating both tissue-based and blood-based profiling [29, 62, 85]. Together, these studies suggest that consideration should be made for a new paradigm in immune monitoring, with routine assessment of early on-treatment responses to help guide therapeutic strategies—at least until better pretreatment biomarkers are identified (Fig. 31.3). Importantly, such analyses should be incorporated into contemporary clinical trial design—and ultimately may even be incorporated into standard of care therapy (Fig. 31.4).

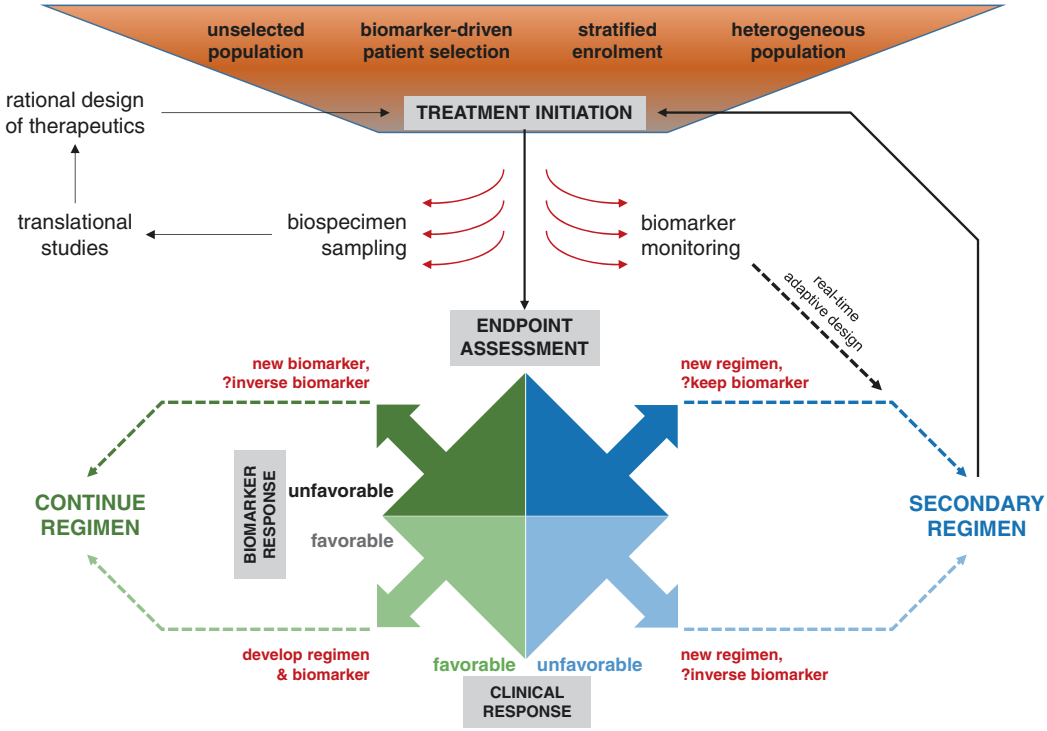

Fig. 31.4 Incorporating biomarkers into clinical trial designs for immuno-oncology. Existing biomarkers may be used to guide patient selection and/or enrolment into clinical trials, with validated biomarkers providing predictive information to inform treatment assignment. Alternatively, prognostic and predictive markers may be used to stratify subject enrolment in order to optimize translational and clinical outcomes. Intentional enrolment of heterogeneous populations may be desirable in order to remain essentially therapeutically "agnostic" and identify biomarkers of response that are independent of tumor histology. Patients being treated on clinical trials may undergo biospecimen collection procedures prior to and during therapy, with significant emphasis on longitudinal sampling to provide vital information about dynamic changes while on therapy, including the development of resistance. Adaptive designs incorporating "on-the-fly" biomarker monitoring may facilitate early, biomarker-directed, therapy changes in patients who appear unlikely to respond. Alternatively, achievement of a relevant clinical endpoint may trigger correlative analysis of clinical and biomarker outcomes, leading to critical decisions regarding acceptance or rejection of the biomarker, the treatment regimen, or both. In discordant cases, the biomarker may be found to be predictive of treatment failure and could be subsequently validated as an *inverse* treatment assignment biomarker. In patients failing to achieve clinical benefit, secondary or subsequent regimens may be chosen ad hoc or adaptively in light of biomarker information

31.5 Building Predictors into Clinical Trials and Standard of Care Therapy

The continued success of immune checkpoint blockade strategies will be realized through the maximization of efficacy and minimization of immune-related toxicity, both of which will require careful focus on biomarker discovery. Due to the current lack of robust or universal predictors of non-response, "real-world" clinical practice is as yet unable to function in the same way that clinical trials do, with their typically "responder-enriched" populations. Substantial efforts are required to ensure that the reach of immunotherapy is extended by ongoing clinical trial research, rather than increasingly focused on patient populations already known to have greater likelihood of benefit.

Numerous novel checkpoint inhibitor molecules are in active preclinical and clinical development, targeting both inhibitory checkpoints—such as LAG3, TIM3, and TIGIT—and activating ("costimulatory") checkpoints such as OX40, 4-1BB, and GITR. Adding further to the complexity of future immunotherapy regimens, numerous combination strategies are being studied, including multi-immunotherapy combinations, as well as cross modality combinations of checkpoint and molecularly targeted therapy. Integrating biomarker discovery into clinical trials of these regimens requires attention to several factors: (1) the types of biospecimens obtained, which must reflect the biological compartments affected by treatment; (2) the timing and frequency of repeated specimen sampling, with attention to the pharmacokinetics and pharmacodynamics of the treatments employed and the different tissue compartments into which these agents must penetrate; (3) the feasibility and patient acceptability of biospecimen collection; and (4) the potential for reciprocal integration of biomarker results into design and adaptation of treatment regimens (Fig. 31.4).

Current biomarker-driven clinical trial designs such as the TITAN-RCC trial in renal cell cancer (NCT02917772) incorporate dynamic clinical outcomes into decisions regarding subsequent per-protocol treatment changes, with associated tissue/blood assessments aiming to identify correlates of response at each therapeutic branch point. Alternatively, molecular tumor signatures hypothesized to predict response to immune or targeted therapy modalities may be used for initial treatment assignment (e.g., BIONIKK renal cell cancer study, NCT02960906). The FRACTION-Lung trial (NCT02750514) considers prior therapy and tumor PD-L1 expression when assigning patients to initial treatment arms, melded with an adaptive trial design to facilitate movement to novel combination treatment arms upon progression for non-small cell lung cancer patients [86]. Two clinical trials aim to identify biomarkers of response to combination ipilimumab and nivolumab therapy through treatment and study of patients with a broad range of rare cancers otherwise unfeasible to study in isolated cohorts (NCT02834013, NCT02923934).

The neoadjuvant setting is well suited to exploratory biomarker studies and is increasingly being employed in immune checkpoint blockade. Trials of this type are currently recruiting in melanoma (NCT02519322, NCT02736123, NCT02977052), colorectal cancer (NCT03026140), glioblastoma (NCT02550249), renal cell cancer (NCT02446860), non-small cell lung cancer (NCT02818920), and others, both to address areas of clinical need and for parallel biomarker discovery.

While the goal will ultimately be to identify robust, pretreatment predictive biomarkers accessible via a noninvasive route, the unique mechanism of action of immune checkpoint blockade suggests that at least for now, biomarker discovery necessitates comparative sampling of tumor (typically invasive) with noninvasive biospecimens. Successful identification of predictors of response will thus hinge upon increased "acceptability" of biopsy procedures to patients already generously donating their involvement in clinical trials; methods to improve this will likely differ according to regional practice and custom.

31.6 Summary and Conclusions

The age of cancer immunotherapy is upon us, and advances continue based on a deep understanding of antitumor and systemic immune responses, genomic and epigenetic alterations in tumors, influences of the tumor microenvironment, as well as the extended environment even outside the host. A working knowledge of this provides the basis for hallmarks of response to immune checkpoint blockade and also provides a framework for current and emerging predictors of response to immune checkpoint blockade. However, complexities exist as perfectly predictive biomarkers in pretreatment biopsies have not been identified, and it is becoming increasingly clear that integrative approaches and predictive models will be needed to optimally guide therapy, as will measurement of adaptive responses to therapy in longitudinal tumor and blood samples. These concepts must be embraced in clinical trials and should also be strongly considered in the setting of treatment with standard of care therapy. It is through such an approach that we will realize optimal therapeutic and monitoring approaches in the setting of treatment with immune checkpoint blockade.

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Part V

Changes in Clinical Practice

Immune Therapies in Phase 1 Trials

32

Sophie Postel-Vinay and Jean-Charles Soria

Contents

32.1 Introduction

Oncologists working in drug development have been facing a revolution in the molecular landscape of agents evaluated in phase 1 over the last few years. If therapies targeting the immune system did represent a minimal fraction of drugs until 2010, the success of immune checkpoint blockers in multiple solid tumor types has led to an exponential development of therapies targeting the immune system, both as monotherapy and combination. Concomitantly, traditional phase 1 trial designs have been deeply challenged, leading to innovative and unprecedented drug development strategies. Although the need to expedite drug development has never been as high, it is critical to ensure that patient's safety remains the highest priority while optimizing early phase trial designs.

This chapter will highlight specificities of phase 1 trials in the era of immune therapies with a particular focus on antibodies targeting immune checkpoints—for which most data are available—and provide practical considerations for early drug development.

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32.2 Antibodies Targeting Immune Checkpoints

32.2.1 Dose-Limiting Toxicities and MTD Definition

Historically, drug development methods and phase 1 designs have been established following the "the more the better" rule, assuming that higher doses of anticancer agents provide better efficacy, even if at the cost of higher toxicities (Fig. 32.1). Therefore, dose-escalation schemes have been established in order to: (1) limit the number of patients treated at low (suspected inefficacious) doses, (2) allow rapid dose-escalation to limit the duration of the trial, and (3) maximize the probability of detecting early severe toxicities that may become harmful for the patients and invert the benefit-risk balance of receiving the anticancer agent. Such intolerable toxicities are also called "dose-limiting toxicities" (DLTs), as they limit the dose-escalation when occurring in more than a pre-specified number of patients. DLTs usually constitute of severe (Grade 3 or above) toxicities according to the National Cancer Institute—Common Terminology Criteria for Adverse Events (NCI-CTCAE v4.0) that occur during the first cycle of therapy, i.e., the first three to four treatment weeks. The traditional 3 + 3 dose-escalation design aims at targeting a dose-limiting toxicity rate between 17 and 33% (one or less than one out of six patients presenting a DLT), corresponding to the maximum tolerated dose (MTD) [1]. In this context, immune therapies challenge this historical drug development method in several ways (reviewed in [2]).

32.2.1.1 Safety and MTD Definition

Toxicities of immune therapies differ in several ways from toxicities observed with cytotoxic therapies or targeted agents. If a dose-toxicity relationship has been observed with anti-CTLA-4 agents, no correlation between dose and safety profile has been observed in phase 1 trials evaluating anti-PD-1 or anti-PD-L1 agents as monotherapy—which was confirmed in later phase studies. For example, nivolumab (Opdivo®, Bristol-Myers Squibb) efficacy and safety profile appears relatively constant at doses ranging between 2 and 20 mg/kg either Q2W or Q3W [3–5]. Also, when

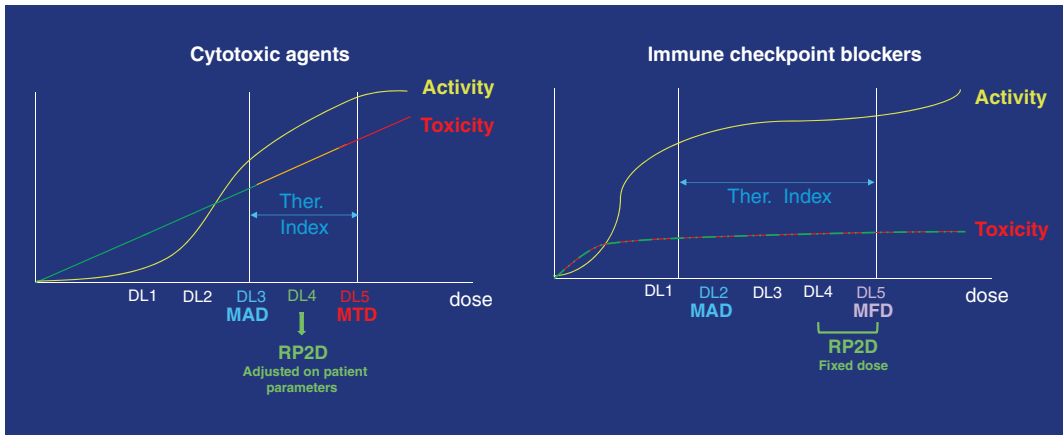

Fig. 32.1 Dose-toxicity and dose-efficacy relationships with immune checkpoint blockers. Contrary to cytotoxic agents (*left panel*), immune checkpoint blockers (*right panel*) do not display any linear dose-toxicity or dose-efficacy relationship. Rare, but severe and sometimes life-threatening, immune-related adverse events (irAEs) can be observed at all dose levels; biological activity is independent of the dose above a certain threshold required for triggering the immune response. The therapeutic index of these therapies is consequently wider than the one of traditional cytotoxic agents. Color code: mild, moderate, and severe toxicities are represented respectively with *green*, *orange*, and *red lines*; *red dots* represent the potential for severe irAEs on a backbone of dose-independent mild AEs with immune therapies; activity is depicted in *yellow*. *DL* dose level, *MAD* minimum active dose, *MFD* maximum feasible dose, *MTD* maximum tolerated dose, *RP2D* recommended phase 2 dose, *Ther Index* therapeutic index

considering the 13 main phase 1 trials evaluating anti-CTLA-4, anti-PD-1, or anti-PD-L1 agents as monotherapy, only one trial identified some per-protocol-defined DLTs, and no MTD could be determined in almost all trials. Therefore, the recommended phase 2 dose (RP2D) was most frequently established as the maximum feasible dose (MFD)—rather than being based on safety data. Interestingly, the nivolumab recommended dose was established based on an integrated analysis of pharmacokinetic (PK, reflecting drug exposure), pharmacodynamic (PD), and safety and efficacy data of a large phase 1b study [3, 6]. Similarly to targeted therapies, most immune checkpoint blockers are eventually administered at fixed doses, which are neither adjusted on body weight, body surface area, nor any patient-specific parameter (Fig. 32.1).

Does this absence of dose-limiting toxicities or MTD mean that these therapies are not toxic? If the number of severe toxicities observed with immune checkpoint blockers is indeed generally lower than with cytotoxic therapies or targeted therapies, this absence of DLTs is mainly due to a difference in kinetics of toxicity appearance. DLTs are traditionally assessed during the first cycle of therapy only, which is relevant for toxicities that are directly resulting from an acute effect of the drug on healthy cells. By contrast, toxicities resulting from immune therapies are mostly indirect: they are caused by an inadequate response of the immune system to the drug—which can be inadequate in nature or in quantity—and inappropriate reprograming of immune cells. This has led to the distinction between adverse events (AEs) and immune-related adverse events (irAEs)—including infusion reactions. A latency period, which duration varies according to the nature of the drug (e.g., 8–10 weeks for ipilimumab [7]), is necessary before irAEs can occur. Therefore, a DLT period limited to the first cycle only does not appropriately capture such drug-related toxicities, and these are not taken into account in the dose-escalation process. If the latter is completely acceptable, considering the frequent absence of dose-toxicity relationship as well as necessity to complete phase 1 trials in a timely manner, such late toxicities should however be carefully reported in phase 1 manuscripts together with their cycle of occurrence. Alternatively, some phase 1 trials assessing anti-PD-L1 agents, such as trials evaluating BMS-936559 [8] and MEDI4736 (durvalumab, AstraZeneca) [9], have made the choice to lengthen the DLT period to two cycles, in order to better take immune-related toxicities into account.

Dose-limiting toxicities may also not be limited to severe (NCI-CTCAE Grade 3) toxicities in phase 1 trials evaluating immune therapies. Indeed, irAEs require rapid and specific management—based mostly on immunosuppressive and anti-inflammatory agents such as steroids, anti-TNF, or anti-IL-6 agents in more severe cases. Therefore, most trials now recommend discontinuing drug administration as soon as moderate (Grade 2) irAEs are observed and starting rapid therapeutic intervention according to dedicated guidelines [10]. Even if some uncertainties remain regarding the pharmacokinetics of elimination of immune checkpoint blockers from their target, drug interruptions are likely leading to decreased drug exposure and should therefore score as DLTs according to the most recent recommendations [11]. Most importantly, such delayed toxicities should deserve specific attention and should be taken into account in the dose-recommendation process as well as in the establishment of guidelines for toxicity management [12].

32.2.1.2 Dosing Schedule

In most cases, immune therapies do not present a clear and linear dose-response relationship (Fig. 32.1). For example, the activity has consistently been observed at all dose levels in the phase 1 trial evaluating several doses of nivolumab [3]; similarly, higher doses of the anti-CTLA-4 agents ipilimumab and tremelimumab did not result into higher response rates in melanoma patients. For example, the dose-response suggested in the phase 2 trial comparing three doses of ipilimumab in metastatic melanoma [13] was not confirmed in subsequent larger phase 3 trials [14–16]. Therefore, aiming at administering the highest tolerable dose might not be

relevant for these agents, and their potential for efficacy at lower dose levels should be explored. In this context, innovative designs, allowing to dose-escalate rapidly (accelerated titration designs or modified toxicity probability interval designs [17]) and to expand at low dose levels as soon as satisfactory pharmacokinetic and pharmacodynamic parameters have been obtained, should systematically be considered (Fig. 32.2).

Beyond the question of the dose, the optimal schedule of administration also remains debated. If most monoclonal antibodies are currently administered every 2 or 3 weeks, no consistent pattern can be found according to the antibody isotype, and there are still several unknown parameters—such as antibody clearance from its target—which require further exploration. Phase 1 trials should endeavor to best explore such questions, as detailed below (see pharmacokinetic and pharmacodynamic parameters section). The total duration of treatment is also the matter of intense debate. For example, if anti-PD-1 and anti-PD-L1 agents are currently administered following a continuous schedule as monotherapy, the anti-CTLA-4 agent ipilimumab is currently approved at 3 mg/kg Q3W for a total of four doses, and some studies evaluated the potential for maintenance therapy with dosing at lower

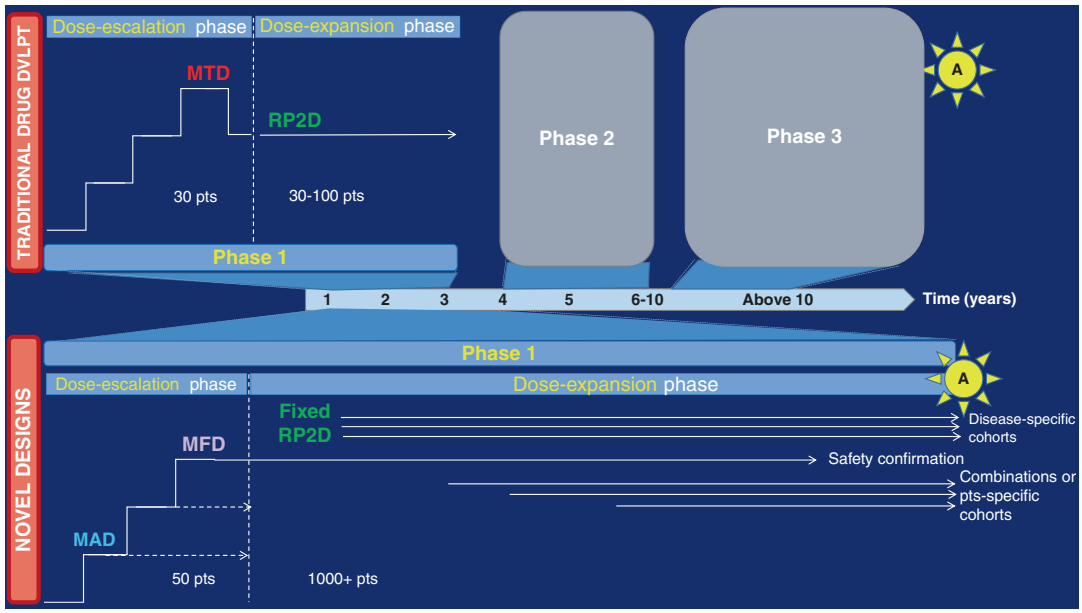

Fig. 32.2 Novel phase 1 designs in the era of immuno-oncology. The traditional drug development process (*top panel*) includes three separate phase 1, 2, and 3 studies prior to reaching drug approval (depicted by "A" in the *sun shape*). Classical phase 1 associates a dose-escalation phase followed by a single-cohort dose-expansion phase primarily looking for safety and confirmation of the recommended phase 2 dose. This approximately 3-year-long phase 1 is, in case of data supporting preliminary activity, followed by subsequent phase 2 and 3 trial, for a total drug development duration of more than 10 years. Novel drug development designs include "drug registration phase 1 trials," in which expansion cohorts do not only look for safety but also activity in disease-specific cohorts that are adequately powered to do so. Further, multiple combination cohorts can be launched as a part of the original phase 1 trial (pending appropriate protocol amendment), to assess safety and preliminary efficacy of various drug associations. These multiple parallel cohorts can include several hundreds of patients and lead to breakthrough designation, conditional, or accelerated approval, thereby significantly shortening the drug development process. Patient-specific cohorts may also be open, in order to allow assessing the drug's safety in patients presenting certain comorbidities. Additional patients could also be enrolled in the dose-escalation phase at dose levels which have proven to be safe and are above the minimum active dose; this allows enriching for safety, PK, PD, and efficacy data at several dose levels while increasing the number of patients who will potentially benefit from the drug, without any delay in dose-escalation. *MAD* minimum active dose, *MFD* maximum feasible dose, *MTD* maximum tolerated dose, *RP2D* recommended phase 2 dose

frequency [18]. The potential for effective reinduction therapy for patients who present sustained tumor response off-treatment but ultimately relapse should also systematically be considered [19]. However, if phase 1 trials are the place for testing the feasibility and safety of such schedules, the formal comparison of their efficacy requires on-purpose adequately powered later phase trials in dedicated tumor types.

32.2.2 Safety Profile and Toxicity Management

A full chapter of this book being dedicated to side effects of I-Os, only key notions and historical developments of interest for phase 1 trials are presented here.

There are several specificities of immune toxicities (immune-related adverse events, irAEs) that need to be considered in phase 1 trials (Fig. 32.3). First, irAEs can affect any organ, even if initial trials with anti-CTLA-4 and anti-PD-1/PD-L1 agents mostly focused on colitis and pneumonitis [10, 20]. Any irAE semiology should therefore be carefully and exhaustively described in the medical records. Second, their potential for rapid worsening and life-threatening consequences contrasts with their low incidence. Therefore, patients should be systematically taught to promptly report any symptom without delay and avoid self-management. Third, as irAEs require specific management, phase 1 protocols should implement clear toxicity management guidelines, and patients should be referred to the local organ specialist as soon as irAE reaches Grade 2 [10]. Phase 1 units should ideally set up a collaborative network with organ specialists in order to optimally explore and manage irAEs. Beyond optimizing treatment, this would allow collecting tissue or blood samples early—while the patient still experiences the toxicity and before any immunosuppressive agent has been administered—in order to study its physiopathology.

If antibodies targeting immune checkpoints are currently seen as "safe nontoxic" drugs, early developments have proven to be sometimes hectic. The most famous example resides in the story of the CD28 superagonist TGN1412, developed in 2006 by TeGenero [21]. All six healthy volunteers who received the first drug injection developed life-threatening cytokine release syndromes requiring transfer to intensive care units within a few hours of the drug administration. Although all six survived, this event put the development of

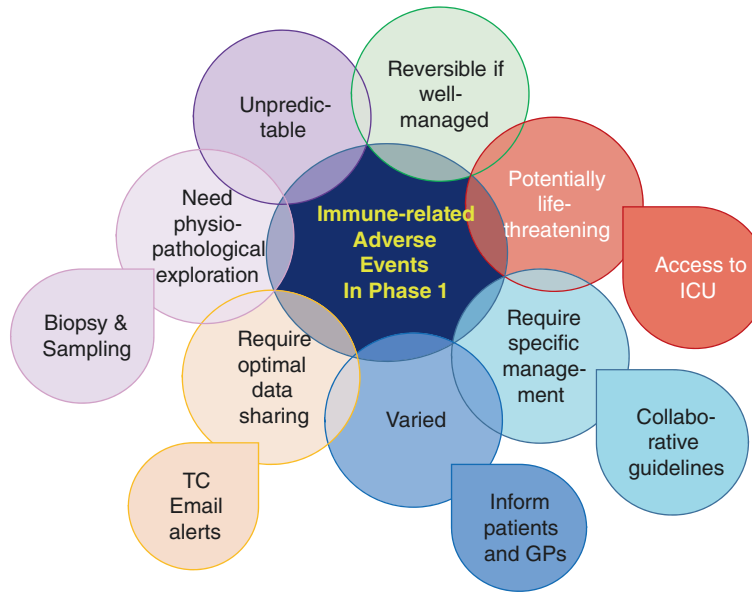

Fig. 32.3 Specificities of irAEs requiring peculiar attention in phase 1 trials. *GP* general practitioner, *ICU* intensive care unit, *TC* teleconference

therapies targeting the immune synapse in the shade for several years. The causes of such severe toxicities were eventually deciphered [22] and included the inadequate choice of the starting dose—which should have been based on the minimum biological effect level (MABEL) instead of the traditional non-observed adverse effect level (NOAEL), as well as differences between the immune systems of humans and animals which had been passed over (including differences in memory T cells in mice and macaques). This example, associated to others that will be discussed below with vaccines and CAR-T cell therapies, illustrates how toxicities of I-O agents are unpredictable and, even if rare, are often severe and require prompt and specific management in specialized units. Therefore, phase 1 trials of immune therapies should, at least for first-in-class or first-in-human drugs, solely be performed in large experienced phase 1 centers with immediate access to intensive care unit and efficient immunosuppressive agents (including anti-TNF and anti-IL-6 agents).

32.2.3 Pharmacokinetic and Pharmacodynamic Assessments

32.2.3.1 Pharmacokinetics

Considering the mechanism of action of immune therapies and the variety of agents currently assessed in phase 1 trials, pharmacokinetic evaluation is becoming increasingly complex. Mechanism of absorption, distribution, metabolism, and elimination is highly variable according to the type of agent [23]. For example, if most immune checkpoint blockers have a common IgG backbone—which accounts for a dose-dependent C_{max} and a median half-life of approximately 15 days—differences exist according to the isotype. IgG1 and IgG3 can cause NK cell-mediated antibody-dependent cell-mediated cytotoxicity (ADCC), whereas IgG4 rather activates the alternative complement pathway. Other factors, such as a concentration-dependent half-life, the ability of the Fc region to bind to the salvage receptor (FcRn) [24], or the presence of circulating soluble forms of the ligand, introduce further variability and complexity in the assessment of pharmacokinetic characteristics of these agents. By contrast, bi-specific antibodies lacking an Fc region traditionally have a very short half-life, which can be of interest notably for the management of drug-related adverse events, such as cytokine release syndrome or appearance of anti-drug antibodies (ADAs), either human anti-human antibodies (HAHAs) or human anti-mouse antibodies (HAMAs) [25, 26]. The bi-specific T-cell engaging (BiTE®) antibody anti-CD19xanti-CD3 blinatumomab (Blincyto®), for example, has a half-life of approximately 2 h [27]. Finally, novel routes of administration, such as intra-tumor administration, lead to novel challenges, with a maximum administrable volume or the requirement to—ideally—assess the drug's pharmacokinetics not only in the peripheral blood but also locally. The optimization of the drug formulation is therefore increasingly important.

32.2.3.2 Pharmacodynamics

The difficulty in designing appropriate pharmacodynamic assays for immune checkpoint blockers resides in their highly variable level and pattern of expression. For example, PD-1 is mostly expressed on T-cell surface, while CTLA-4 only displays transient expression, and biomarker expression can both be constitutive or inducible [28]. Thus, flow cytometry methods assessing receptor occupancy on circulating T cells are feasible and relevant only for a fraction of molecules. Such technique could successfully be used for the phase 1 trial evaluating nivolumab [29], where receptor occupancy appeared to be dose-independent and prolonged (with a decay in occupancy being observed only 3 months after the last drug administration). Importantly, which degree and duration of target modulation should be achieved to trigger the optimal immune modulation, and whether findings observed in circulating lymphocytes reflect what is happening in the tumor bed, remains unknown. Further, data have highlighted the critical role of the interaction of

the Fc portion of monoclonal antibodies with FcγRs expressed on innate immune effector cells in therapeutic activity. Patients displaying activating FcγR alleles (resulting in higher affinity) respond better to therapy, and polymorphisms in the *FcγRIIIα* were associated with response to rituximab in patients with follicular lymphoma [30, 31]. Therefore, strategies have been developed to limit or abrogate the binding of the antibody Fc to cellular FcγRs, which may introduce further variability in drug efficacy and pharmacodynamic parameters [32].

The ability to perform immunomonitoring studies, i.e., assessing the levels of circulating cytokines, chemokines, as well as phenotyping immune cell populations in peripheral blood, represents an attractive pharmacodynamic biomarker for immune therapies. For example, the phase 1 trial assessing the 9B12 anti-OX40 monoclonal antibody evidenced that, following drug infusion, patients displayed a non-Treg ($FoxP3^{neg}$) $CD4^+$ T cell expansion associated with an increase of $CD8^+$ lymphocytes expressing the CD38 and HLA-DR activation markers, together with a transient decrease of follicular helper $CD4^+$ T cells in the peripheral blood [33]. The comparison of OX40 surface expression on Treg from the peripheral blood and on tumor infiltrating Tregs in three patients for which tumor tissue was available revealed that less than 20% of circulating Treg expressed this marker, whereas more than 50% of infiltrating Tregs were OX40 positive. By contrast, anti-CTLA-4 has been reported to increase both FoxP3+ and FoxP3− CD4+ T cells in a dose-dependent manner.

Such extensive and comprehensive pharmacodynamic studies are essential as early as phase 1 trials, even if such studies are not powered to show any statistically significant differences in biomarkers. Indeed, phase 1 trials are the only situation where the effect of the dose schedule and drug exposure on PD parameters will be evaluable. Further, this will allow orientating biomarkers that should be evaluated as a priority in later phase trials that will enroll a larger number of patients, thereby limiting the cost of such analyses.

32.2.4 Patient Eligibility

Considering the peculiar physiopathology of the immune therapy-associated toxicities, and the "phase 1 efficacy and registration trial" development strategy currently chosen by most drug companies (Fig. 32.2), one can wonder whether the traditional phase 1 eligibility criteria should be unwound. Patients included in phase 1 trials are usually required to have (1) life expectancy above 18 weeks and (2) no major baseline organ dysfunction. Life expectancy has traditionally been assessed using objective scores developed on chemotherapy-treated patients, such as the Royal Marsden score based on LDH, albumin, and number of metastatic sites [34]. Recent data have revalidated the relevance of such scores on immune therapy-treated patients and highlighted the potential for adding lymphocyte count in the scoring [35]. Regarding the criteria excluding patients with any baseline organ dysfunction, phase 1 trials evaluating immune therapies represent a major paradox. Indeed, recent data of antibodies targeting immune checkpoints tend to support that immune-related toxicities might be much rarer and varied than chemotherapy- or targeted agent-associated toxicities [10]. However, inclusion criteria of phase 1 trials have become more restrictive. Logically, patients with history of autoimmune disease (psoriasis, type 1 diabetes, inflammatory bowel disease, etc.), chronic viral infection (hepatitis B, hepatitis C, HIV), or history of severe allergic, anaphylactic, or hypersensitivity reactions have been excluded from such trials. However, if such narrowing of the inclusion criteria is legitimate at the very first development steps of a novel drug, i.e., during the dose-escalation phase or for first-in-class/first-in-human compounds, it further decreases the representativeness of phase 1 patients of the all-comer populations. This is contra-intuitive in a context where drug companies aim at obtaining accelerated approval for some compounds based on phase 1 trials results. Responses and clinical benefit have been observed in patients with elevated LDH receiving tremelimumab [36], in patients with performance status of two treated

with BMS-936559 [8], or in patients with brain metastases [37, 38] with no additional toxicity. Provisions should therefore be made to open dedicated cohorts of patients with poorer prognosis in the dose-expansion phase, after unwinding of selected eligibility criteria, chosen based on the drug safety profile that has been observed in the dose-escalation phase and safety dose-expansion cohort. Also, evaluating such "more at risk" patients within the initial phase 1 trial would allow that physicians looking after them are already familiar with the drug, which is ethical and clinically sound. Together with the removal of some limitations put on the number of previous treatment lines, this would allow speeding up recruitment and expediting drug development, while obtaining additional safety information on the evaluated compound. Finally, most phase 1 trials currently require patients being immune therapy-naïve. With the exponential development of immune therapies, this becomes no longer feasible as most patients will have received at least one prior line with an immune modulator. Unless there is a strong rationale and fear of interaction that would put the patient's safety at risk, such criteria should be removed.

32.2.5 Patient Selection and Personalized Immune Therapy

Immune therapies have brought an additional level of complexity in the patient selection and personalized medicine era. Biomarkers are not anymore located only on cancer cells (or molecules released by cancer cells), but immune cells and tumor microenvironment need to be taken into account [39]. Further, some biomarkers—such as PD-1 or PD-L1—have a dynamic expression profile and can be induced under specific circumstances only [28]. The challenges of choosing the best biomarker for predicting efficacy to immune therapies—including checkpoint expression, neoantigen/mutational load, immunoscore, etc.—have been discussed in a previous chapter of this book, but several phase 1 specificities need to be highlighted. First, phase 1 trials need to remain limited in time and able to enroll patients efficiently. Therefore, no molecular selection should be performed in the dose-escalation phase, where the primary objective must remain the determination of the dose-limiting toxicities and maximum tolerated dose. Molecular enrichment could then be performed in the dose-expansion phase in dedicated "companion biomarker" cohorts. The successful development and approval of the 22C3 IHC PD-L1 PharmDx test, together with pembrolizumab, nicely illustrates this concept [40]. Second, none of the current biomarkers used to predict response to immune therapies, notably immune checkpoint blockers, is sufficiently specific or sensitive to exclude patients from receiving such therapies. For example, responses to anti-PD-1 or PD-L1 agents have consistently been observed in PD-L1-negative populations [41, 42] or in tumor types with virtually no mutations and a very low neoantigen load—such as Hodgkin lymphomas [43]. Such observations would have been missed if a too stringent preselection had been applied as early as phase 1 trials. Third, "surprise" responders can allow to learn more on the drug's mechanism of action and target population than a well-selected homogeneous cohort of patients. Phase 1 trials are the only place where eligibility criteria can be flexible enough to enroll a variety of patients, and dedicated cohorts for rare tumor types or unselected patients should therefore be systematically added in the dose-expansion phase. Also, exceptional responders (or resistant patients) should be extensively sampled and studied, both in the tumor and peripheral blood—as well as lymph nodes if relevant—as such patients can be extremely informative. Of note, it is somehow worrying to observe how much the number of companies following the same targets has been increasing over the last 3 years (from 26 to 36%), while the number of targets pursued by only one company has been reduced from 42 to 26% [44]. Although no specific data is currently available for immune therapies in oncology, most immuno-oncology drug companies are currently following the same route and do not only develop agents directed against the same molecules but also target the same patient populations. Beyond making

recruitment more difficult for phase 1 units, secondary to competing trials, this "herd mentality" also constantly reduces accessibility to novel drugs for the same patient populations (such as sarcoma, brain tumors, or rare diseases) and harms real and original innovation.

32.2.6 Response and Efficacy Assessment

Considering their mechanism of action, immune therapies can have unusual profiles of responses, including dissociated responses, delayed responses, hyperprogressions, and pseudoprogressions (initial increase in tumor size followed by tumor shrinkage) [45]. These correspond mostly to the fact that immune activation can take time, leading to a delay in onset of clinical effect, and that immune cells recruited to the tumor bed may cause an increase in tumor volume before it effectively shrinks [46]. Therefore, traditional World Health Organization (WHO) and Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1) criteria do not appropriately apprehend responses observed with immune therapies. Novel criteria, named immune-related response criteria (irRC), have therefore been developed [46] and added to the traditional RECIST criteria in most of the recent phase 1 trials evaluating immune checkpoint blockers. These better allow taking into account the different patterns of atypical responses observed with immune checkpoint blockers, including the potential for appearance of new nontarget lesions, growth of existing lesions preceding tumor shrinkage, and prolonged stable disease—sometimes ultimately followed by delayed response, even after treatment cessation. For example, 7.3% (24/327) of the melanoma patients treated with the anti-PD-L1 agent pembrolizumab in the Keynote-001 study presented atypical responses, including 4.6% (15/327) early pseudoprogressions and 2.8% (9/327) delayed pseudoprogressions [47]. Further, 14% (84/592) of the patients experienced progressive disease per RECIST v1.1 but nonprogressive disease per irRC. Also, the 2-year overall survival rates were 78% in patients with nonprogressive disease per both criteria, 37% in patients with progressive disease per RECIST v1.1 only, and 17% in patients with progressive disease per both criteria. This suggests that, at least in this population, using RECIST v1.1 may cause premature treatment interruption and lead to underestimation of the immune checkpoint blockers' benefit. Although atypical patterns of response appear to be more frequent in melanoma patients and the benefit of using irRC criteria has been poorly validated outside this population, most phase 1 protocols evaluating immune therapies now recommend using (1) RECIST v1.1 criteria for response assessment and (2) irRC for treatment cessation. This means that confirmation of the progression is required by a second imaging assessment 4 weeks after the initial progression has been diagnosed, prior to taking a patient off-study. iRECIST criteria, which should become available soon, aim at merging irRC and RECIST v1.1 criteria in order to offer a widely implementable classification that better takes into account these atypical response profiles. Finally, provisions should be made to allow pursuing treatment beyond confirmed progression in case of clinical benefit (pending written approval from the study promotor, from the patient, and based on a consensus among all co-investigators). In line with these considerations, non-progression rate or clinical benefit rate might represent potential relevant alternatives to assess immune therapy efficacy in phase 1 (Fig. 32.4).

Overall, it is likely that current criteria—either RECIST, irRC, or iRECIST—will be insufficient to recapitulate all peculiar patterns of response observed with immune therapies. Therefore, alternative strategies should be systematically envisioned and implemented in phase 1 trials. These include dynamic assessments (such as tumor growth rate), metabolic imaging, and also peripheral immunomonitoring—which could also be an indicator of response. Finally, whether overall survival should be systematically recorded in phase 1 trials of immune therapies is a current question. This endpoint has traditionally not been registered in early phase trials, as these primarily aim at assessing safety. Preliminary efficacy data,

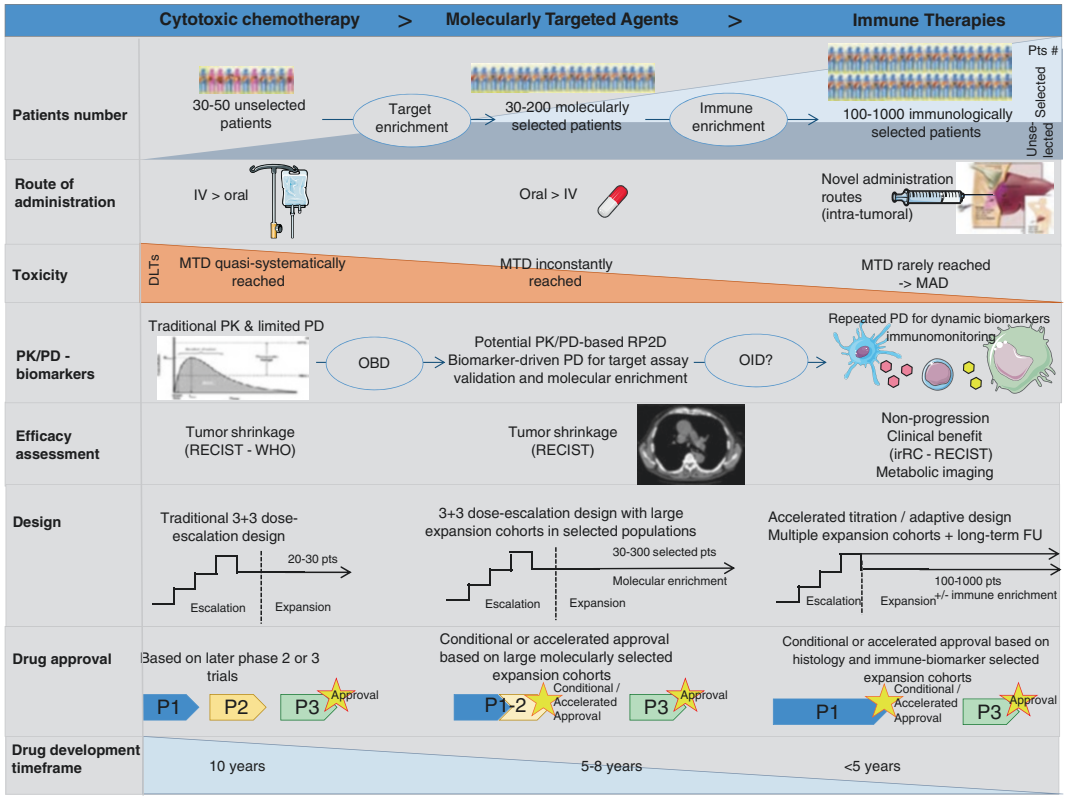

Fig. 32.4 The evolving landscape of phase 1 trials from cytotoxics to the immuno-oncology era (adapted from Postel-Vinay et al., with permission of the Editorial Office from Annals of Oncology). Striking changes have been observed in the landscape of phase 1 trials since the era of cytotoxic chemotherapy. Trials have moved from small-sized trials enrolling molecularly unselected patients to large-scale studies of several hundreds of molecularly—or phenotypically—selected patients enrolled in parallel expansion cohorts. Novel routes of administration have been developed as well as novel methods for dose recommendation, as the MTD is inconstantly reached. Pharmacodynamic and pharmacokinetic parameters play an increasingly important role, notably in helping determining the recommended dose for later phase trials. Whether, by analogy with the optimal biological dose of targeted agents, an optimal immunological dose based on PK and PD data of immunological monitoring could be used for immune stimulatory agents, which warrants further exploration. Early phase trial designs have evolved in parallel (also see Fig. 32.2), in order to maintain a rapid dose-escalation phase and assess efficacy in large selected cohorts. Consequently, the traditional phase 1–phase 2–phase 3 drug development strategy is being replaced by large phase 1/3 trials, which bypass the traditional phase 2 studies. All these evolutions have allowed dramatically reducing the drug development time, for patients' benefit. *#*: number, *IV* intra-venous, *MAD* maximum administered dose, *MTD* maximum tolerated dose, *OBD* optimal biological dose, *OID* optimal immunological dose, *P1* phase 1, *P2* phase 2, *P3* phase 3, *PD* pharmacodynamics, *PK* pharmacokinetics

a secondary endpoint, was mostly assessed by the response rate or progression-free survival (PFS). Considering the effect of immune therapies on "raising the tail of the curve," overall survival is a relevant endpoint to assess their efficacy. However, phase 1 trials, even within their current transformation to "phase 1 registration trials," are not the place to assess overall survival, because: (1) phase 1 trials do not contain any control arm; (2) patients are likely to receive many lines of therapy thereafter, which will represent as many confusion factors; (3) this uselessly increases the workload associated with phase 1 trials, especially as phase 1 patients are often referred from external centers and go back to their hospital of origin after trial completion; and (4) as phase 1 studies arrive more and more early in the patient's treatment lines, this data will very likely only be

available once trial results have already been reported and published and therefore will virtually never be communicated.

32.2.7 Trial Design: The "Phase 1 Registration" Trials

A striking evolution has been observed over the last 5 years in phase 1 trial design (Figs. 32.2 and 32.4). Whereas early phase trials traditionally enrolled approximately 30–60 patients (e.g., the ipilimumab phase 1 study), recent phase 1 studies of immune therapies have included more than 1000 patients in several expansion cohorts, thanks to modular designs and consecutive amendments. The development of the anti-PD-1 pembrolizumab nicely illustrates this paradigm shift in the multiple corresponding Keynote studies [40]. Considering the high level of activity observed in the initial first-in-human phase 1 study in patients with melanoma and NSCLC, dedicated cohorts were added for these tumor types, not only to confirm the safety profile and PK characteristics of the investigational drug but also to confirm efficacy—a traditional phase 2 trial endpoint [48]. After treatment of several hundreds of patients in each of these cohorts, the confirmation of clinical efficacy led to accelerated approval of pembrolizumab for unresectable or metastatic melanoma in September 2013 [49] and, for metastatic PD-L1-positive NSCLC in October 2015 [14], only 3 and 5 years after the first patient had been treated with the drug, respectively. Of note, the immunohistochemistry companion diagnostic 22C3 PharmDx test was approved concomitantly, reinforcing the successfulness of this drug development model [40]. Such adaptive designs are currently almost systematically used in phase 1 trials of immune checkpoint blockers, not only to expand in selected histotypes but also to test in parallel multiple combinations with a common immune therapy backbone (Fig. 32.2). This is fully laudable, as this allows prompt and efficient drug development. The current MASTERKEY-265 phase 1/3 trial evaluating the association of talimogene laherparepvec (T-VEC) with pembrolizumab is another example of this "phase 1 registration trial" evolution [50].

However, it should be remembered that the primary objective of phase 1 trials is to define the optimal dose that needs to be administered safely in later phase trials; once determined, this dose will almost never be reevaluated. Therefore, this objective should not be overlooked too rapidly by opening cohorts designed to look for efficacy, as this may unethically expose a high number of patients to suboptimal doses of an experimental agent. Further, such expansion cohorts should have clear pre-specified stopping rules for non-efficacy once the number of enrolled patients exceeds the one required for obtaining satisfactory safety, PK, and PD data. Finally, results from these cohorts should be optimally exploited to best prepare for drug registration and later phase large randomized trials [2].

32.3 Other Immune Therapies and Their Specificities: Selected Examples

The variety of immune therapies, with more than 1500 phase 1 trials currently open, precludes from drawing a complete landscape of their individual specificities (Fig. 32.5). Here, we therefore highlight three examples of immune therapies—two of which have recently been approved—whose development displays significant particularities and differs from what has been described above regarding antibodies targeting immune checkpoints.

32.3.1 Blinatumomab

Blinatumomab (Blincyto®, Amgen) is a bi-specific antibody that was granted accelerated approval from the Food and Drug Administration in December 2014 for the treatment of Philadelphia chromosome-negative relapsed or refractory B-cell precursor acute lymphoblastic leukemia. Blinatumomab is a bi-specific CD19-directed CD3 T-cell engager that activates endogenous T cells when bound to the CD19-expressing target

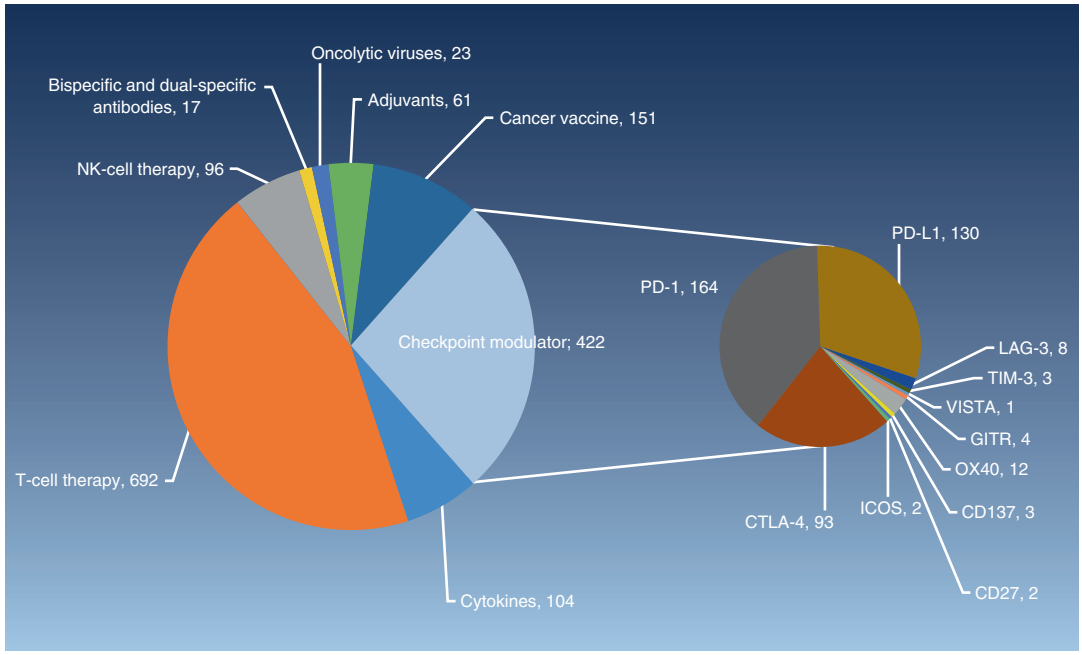

Fig. 32.5 Phase 1 trials of I-O therapies (clinicaltrial.gov, accessed January 10, 2017). Search was performed for phase 1 trials recruiting only, with keywords "anticancer class" and "cancer," where "anticancer class" corresponds to each of the category detailed in this figure

cell. In line with its specific formulation, the efficacy and safety profile of this drug significantly differs from what has been described above with monoclonal antibodies targeting immune checkpoints [51]. First, this drug was associated with frequent severe irAEs resulting from the activation of the immune system, including life-threatening cytokine release syndrome in 11% of the patients at its time of approval. Further, a specific form of central neurological toxicity, which included disorientation, convulsions, tremors, and speech disorders, was observed in half of the patients—all these symptoms proved to be reversible shortly after treatment discontinuation. Therefore, a boxed warning regarding cytokine release syndrome and neurological toxicities was provided in the product labeling, and the drug was approved with a Risk Evaluation and Mitigation Strategy (REMS) (i.e., a communication plan to inform healthcare providers about the serious risks and the potential for preparation and administration errors). Second, mild but frequent irAEs (pyrexia, leukopenia, chills, and CRP increase) were observed during the first weeks of treatment and usually lowered during further treatment [52]. Third, a clear relationship between dose level and antitumor activity was observed [51].

32.3.2 Talimogene Laherparepvec (T-VEC)

The genetically modified oncolytic viral therapy T-VEC (Imlygic®, Amgen) was approved by the FDA in October 2015 for the local treatment of unresectable cutaneous, subcutaneous, and nodal lesions in patients with recurrent melanoma. In the initial phase 1 study [53], OncoVEXGM-CSF was administered at increasing doses, and the local reaction to injection was dose limiting at 10^7 plaque-forming units (pfu)/mL in seronegative patients treated with a single dose. However, this dose could eventually be increased to 10^8 pfu/mL in patients treated with multiple doses, after seroconversion of seronegative patients with 10^6 pfu/mL. In this multidosing group, no MTD could be determined. Apart from injection site reactions, the most frequent adverse events

observed with T-VEC (fatigue, pyrexia, chills, and flu-like symptoms) generally occur during the first 3 months of treatment and are classically mild or moderate and resolve within 3–4 days. This particular example illustrates the need to incorporate severe local toxicities in the DLT definition of immune therapies that are designed to be administered in situ, as these can prove to be dose limiting in the absence of systemic toxicities.

32.3.3 CAR-T Cell Therapy

Chimeric antigen receptor T (CAR-T) cell therapy is a dramatically active cancer immune therapy but has been associated with peculiar safety challenges. Their potential to cause life-threatening cytokine release syndromes, associated with specific toxicities such as macrophage activation syndrome and hemophagocytic lymphohistiocytosis, has led to the development of recommendations from immunology experts [54]. These included the use of nonspecific immunosuppressive agents (such as corticosteroids) as well as the anti-IL-6R antibody tocilizumab (Actemra®, Genentech). Further, some clinical trials performed at the Memorial Sloan-Kettering Cancer Center (MSKCC) in New York were put temporarily on hold in March 2014 after occurrence of several infusion-related patient deaths [55]. Although experts postulated that toxicity was needed to get maximal effects of the drug, this latter seemed to be more related to some patient parameters (e.g., tumor burden) than to the drug itself. The protocol was therefore modified to reduce the dose in patients with large tumor burden and exclude patients with pre-existing comorbidities that would preclude from aggressive supportive care management in case of severe CRS. Other issues, such as off-target effects due to cross-reactivity with normal antigen on healthy tissues, have been reported to be sometimes fatal with CAR-T cell therapy. For example, an affinity-enhanced T-cell receptor for melanoma-associated antigen 3 (MAGE-3) caused deadly cardiogenic shock in the first two treated patients following cross-reactivity with titin, an unrelated peptide on striated muscle [56].

32.4 Combinations

Combination therapy will unavoidably be needed to overcome primary or acquired resistance to immune therapies or increase duration of response. Excellent recent literature exists on this topic [57–60], and we will therefore focus here on key notions for optimizing the development of I-Os in combination.

The immune system being a tightly regulated balance, some "fine-tuning" will be needed to allow efficiently activating the immune system and avoid over-boosting it and eventually causing either chronic autoimmune diseases or deadly acute complications. Therefore, the drug development strategy needs to take into account the nature of agents that are being combined. Combining an immune agent with a nonimmune drug—including cytotoxic agents, targeted therapies, or anti-angiogenic agents—seems intuitively to be feasible at full dose of both agents, as limited overlapping toxicity or PK interaction is anticipated. Maximum caution should however be applied. Indeed, if some combinations have proven to be feasible with acceptable increase in toxicity at full doses of cytotoxic agents [61], small molecules [62] or antibodies targeting angiogenesis [63], others have proven to be intolerable. For example, the phase 1 trial evaluating the combination of vemurafenib plus ipilimumab in melanoma was terminated early owing to hepatotoxicity [64]. By contrast, durvalumab could be safely combined with trametinib and/or dabrafenib at full doses in patients with BRAF-mutant and wild-type melanoma.

The combination of two immune therapies is anticipated to face several toxicity and tolerability challenges—as exemplified by the high rate of severe toxicities observed when combining ipilimumab and nivolumab [65, 66]. However, increased efficacy could be achieved at the cost of lower toxicities using specific routes of administration, including notably in situ immunization strategies [67].

Overall, there are infinite possibilities for combining immune therapies with other immuno-oncology drugs, and several hundreds of combinations are currently tested in early phase trials

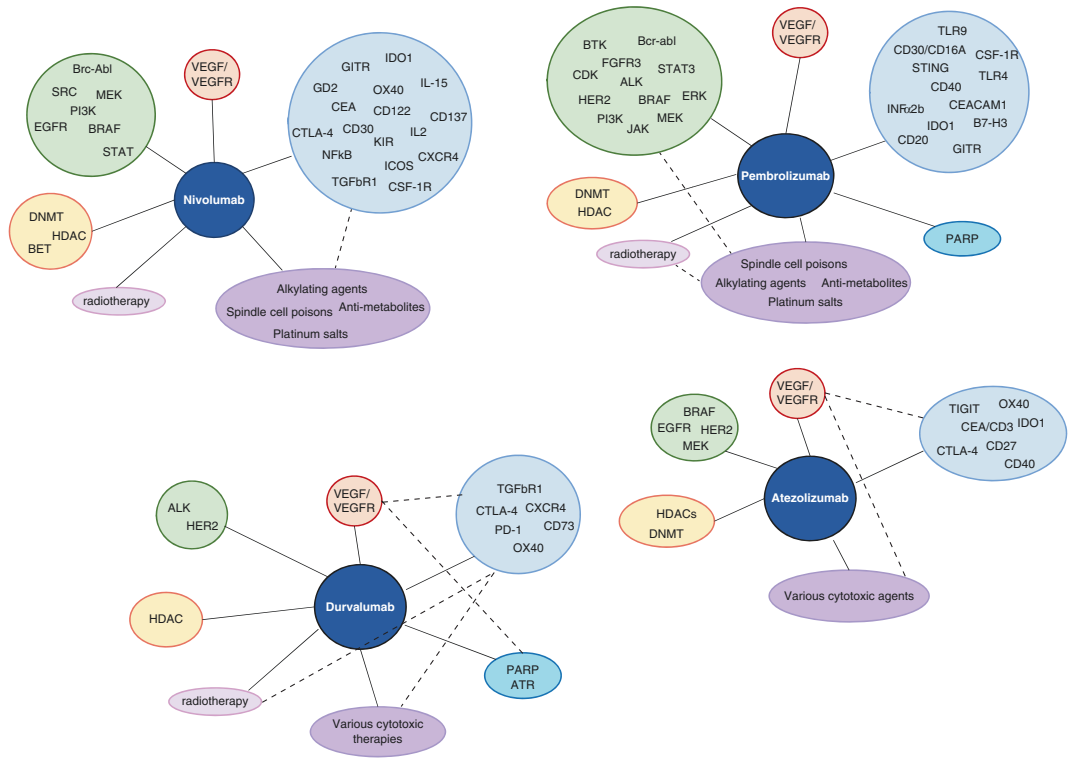

Fig. 32.6 Examples of ongoing phase 1 trials evaluating combinations with anti-PD-1 or anti-PD-L1 therapies. The main target classes that are currently being evaluated in association with some anti-PD-1 or anti-PD-L1 agents are depicted, with targeted therapies in *green*, epigenetic modifiers in *orange*, conventional cytotoxic chemotherapies in *purple*, DNA repair inhibitors in *blue*, immune therapies in *light blue*, and antiantiogenic agents in *red*. *Dotted lines* represent associations of three different therapeutic classes, on the anti-PD-1 or anti-PD-L1 therapy backbone. Only some trials are depicted here, but others may exist. Based on clinicaltrials.gov, accessed 14 January 2017, with phase 1 trials currently recruiting only

(Fig. 32.6). The diversity of such combinations does not only reside in the number of targets that could be modulated but also in the variety of doses, routes of administration, and combination schedules. For example, epigenetic drugs could be used at nontoxic doses to act as immunomodulatory agents [68–70].

32.5 Conclusion and Practical Considerations

The advent of I-O therapies has led to a paradigm shift in drug development. It has become increasingly clear that traditional phase 1 modalities are not well-suited for such agents. Accordingly, early phase trial designs need to adapt and be constantly rethought in order to best target the specific challenges addressed by these therapies. Such challenges include not only traditional phase 1 endpoints—safety, toxicity evaluation and management, dose recommendation, PK/PD characteristics, and preliminary activity—but also companion biomarker development, efficacy assessment, and potentially drug registration. These rapid changes in clinical and scientific aspects of early phase trials are associated with practical challenges that should also be anticipated. Considering the increase in the number of participating centers, optimal communication between all the investigators, medical monitors, and scientists involved in the development of the agent should be ensured, so that relevant safety information is broadly shared in a timely manner.

Further, considering the specific safety profile of I-Os, clear algorithms should be implemented in phase 1 protocols regarding toxicity management, and the quality of the reporting of irAEs should be maximized. This is even more important because some investigators might not be fully familiar with these toxicities—as the wide implementation of I-Os in the therapeutic armamentarium is still recent and has been increasing at an unprecedented speed. Finally, any protocol modification should be supported by robust clinical data or scientific hypotheses. A very exciting time has now started in early drug development in immuno-oncology, and the highest level of clinical, scientific, and ethical rigor will be instrumental in successfully optimizing and expediting drug development.

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Side Effects of Cancer Immunotherapy with Checkpoint Inhibitors

33

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Contents

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33.1 Introduction

Immune checkpoint inhibitors are immunomodulatory monoclonal antibodies that block inhibitory immune pathways that tumor cells use to evade immune suppression. The main targets are the cytotoxic T lymphocyte-associated antigen (CTLA)-4 receptor on T lymphocytes and the programmed cell death (PD)-1 receptor and its PD-1 ligand (PD-L1). The first checkpoint inhibitor to be approved for treating cancer patients was ipilimumab, an anti-CTLA-4 antibody. Ipilimumab resulted in significant improvements in overall survival (OS) when compared with standard chemotherapy in phase III studies and is now approved as frontline therapy for patients with metastatic melanoma [1, 2]. However, another anti-CTLA-4 antibody, tremelimumab, did not show any OS benefit over standard chemotherapy in first-line treatment of patients with metastatic melanoma [3] but is being investigated in combination regimens in other tumor types.

The second class of inhibitors are directed against PD-1 and its PD-L1 ligand, and several antibodies have been developed for the treatment of advanced melanoma as well as other tumors, including non-small-cell lung cancer (NSCLC), renal, bladder, head and neck cancers, and Hodgkin's lymphoma [4, 5]. The PD-1 inhibitors, nivolumab and pembrolizumab, are the most advanced in terms of clinical development and have been approved in several indications.

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Among anti-PD-L1 agents, atezolizumab has been approved by the FDA for the treatment of metastatic NSCLC and advanced urothelial cancer [6], avelumab has been approved for metastatic Merkel cell carcinoma, and durvalumab is undergoing approval for advanced or metastatic urothelial carcinoma.

The use of these immunomodulatory drugs is associated with a spectrum of immune-related adverse events (irAEs) associated with hyperactivation of the immune system consequent to the reduction in immune response inhibition (Fig. 33.1). These include a range of mainly dermatological, gastrointestinal (GI), endocrine and hepatic toxicities, as well as several other less common inflammatory events. All of these toxicities have variable times of onset and require careful monitoring, follow-up, and management. They are usually reversible with appropriate and timely intervention but can become severe and even life-threatening if not recognized early enough. Guidelines for the management of irAEs associated with ipilimumab, which were developed based on clinical experience gained in clinical development studies, have been approved by the FDA [7].

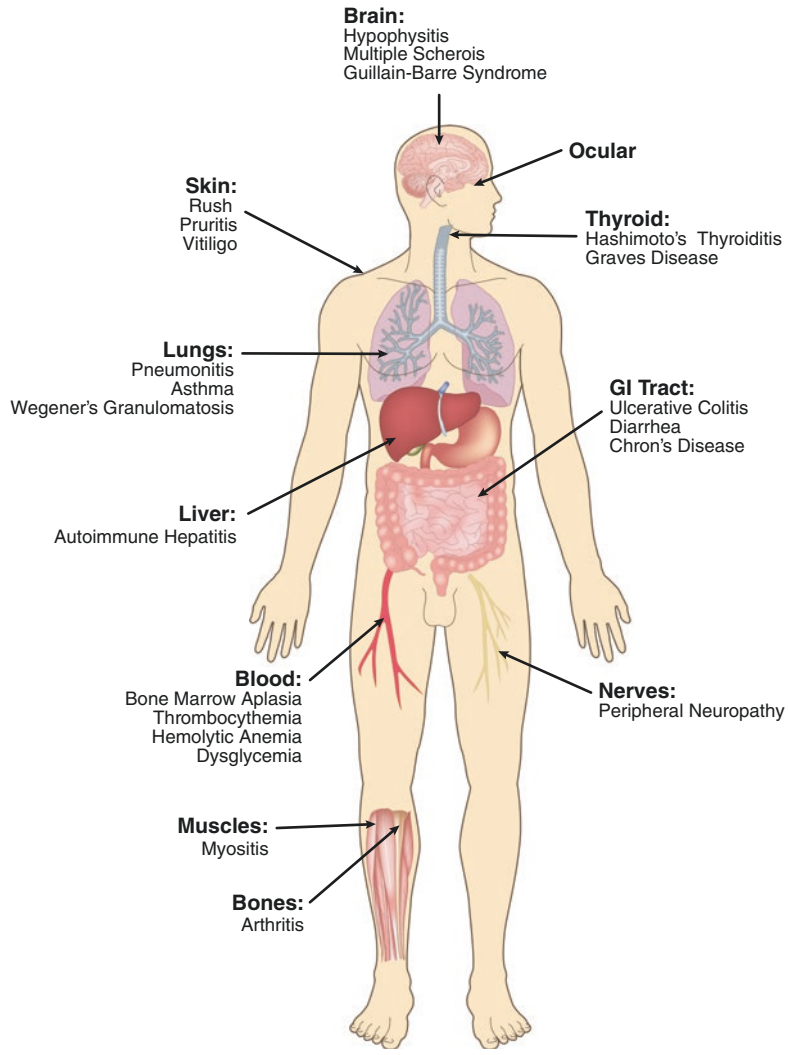

Fig. 33.1 Tissues of the body affected by immune-related adverse events

33.2 Incidence of Immune-Related Adverse Events

Among patients receiving ipilimumab, the most commonly reported adverse events include diarrhea, colitis, fatigue, pruritus, rash, and endocrinopathies [8], while for patients treated with PD-1 inhibitors, common adverse events include fatigue, rash, diarrhea, pruritus, arthralgia, and constipation [9–11]. The incidence of any grade irAE is reported to range from 15–90% [1, 12] in single-agent trials, while the rate of severe irAEs requiring immunosuppression and withdrawal of immunotherapy is estimated to be 0.5–13% [1, 3, 4, 13].

With combined anti-CTLA-4 and anti-PD-1 therapy, grade 3–4 drug-related adverse events occurred in 54% of patients, with the most common being colitis (17%), diarrhea (11%), and elevated alanine aminotransferase levels (11%). Immunosuppressive medications for the management of irAEs, including topical agents for dermatologic adverse events, were used in a higher percentage of patients receiving combination compared with ipilimumab alone (89% vs 59%) [12, 14, 15].

The risk of severe grade adverse events increased from 7 to 25% with an increase in the dose of ipilimumab from 3 to 10 mg/kg, largely due to an increase in the number of episodes of diarrhea. However, this pattern was not observed when nivolumab dosing was increased from 0.3 to 10 mg/kg. Similarly, occurrence of severe grade toxicities with pembrolizumab did not significantly increase with dose, occurring in 20% of patients treated with 2 mg/kg and 25% of patients treated with 10 mg/kg [16, 17]. As such, it appears that toxicities due to anti-CTLA-4 antibodies are dose dependent, whereas toxicities with anti-PD-1/PD-L1 antibodies are independent of dose.

33.3 Timing of Immune-Related Adverse Event Onset

The onset and outcome of irAEs with ipilimumab seem to vary according to the organs involved, and although most occur within the first 3 months of treatment, some specific toxicities are reported months after completion of therapy. The majority of toxicities appear temporally, with skin manifestations the earliest to appear at 2–3 weeks after the first dose of ipilimumab. Immune-mediated colitis and hepatitis appear at approximately 5–10 and 12–16 weeks, after the second and third doses, respectively. Endocrine dysfunctions present from the ninth week onwards following the fourth dose [1] and can take time to resolve or may even be irreversible, as is the case for most occurrences of hypophysitis. Immune-mediated pneumonitis is seen 8–14 weeks after treatment initiation [1], and immune-mediated nephritis appears later, after 14–42 weeks on immunotherapy [18].

33.4 General Considerations

Guidelines for the management of irAEs associated with ipilimumab were approved by the FDA as part of a risk evaluation and mitigation strategy. Anti-PD-1 antibodies, in particular nivolumab and pembrolizumab, seem to be better tolerated than ipilimumab, but adverse events related to these drugs require the same types of treatment. The guidelines provide a specific toxicity approach, depending on the organ or system involved but, in general, involve cessation of immunotherapy and initiation of steroid therapy for immunosuppression, with dosages and timing depending on the severity and type of toxicity.

For grade 1 toxicity, only symptomatic therapy should be used. For grade 2 toxicity, immunotherapy should be interrupted and treatment with oral steroids (prednisone 0.5–1 mg/kg/day or equivalent) should be started within 1 week after onset of symptoms if these persist. Immunotherapy may be resumed only if symptoms and signs of toxicity fall to grade 1 or resolve completely. For grade 3–4 toxicities, high doses of glucocorticoids (prednisone 1–2 mg/kg/day or equivalent) should be given. Doses should be gradually tapered when symptoms subside to grade 1 or less. Treatment with immune checkpoint inhibitors should be permanently discontinued. Finally, in cases of severe toxicity not responsive to high-dose corticosteroid therapy,

administration of infliximab (5 mg/kg), a chimeric monoclonal tumor necrosis factor (TNF)-α inhibitor, should be considered. This may be repeated about 2 weeks after the first dose if symptoms fail to resolve. The use of an anti-TNF agent is based on its use in autoimmune diseases such as Crohn's disease and ulcerative colitis. Histological studies of ipilimumab-induced immune-related colitis demonstrate the colonic mucosa infiltrated with both lymphocytes and neutrophils, similar to idiopathic ulcerative colitis. The early use of these agents allows for a quick resolution of adverse events and a reduction in the use of corticosteroids [19].

The use of corticosteroids for the treatment of irAEs does not seem to affect the efficacy of checkpoint inhibitors. Data derived from a large case series of patients (n = 298) receiving treatment with ipilimumab (3 mg/kg) at the Memorial Sloan Kettering Cancer Center outwith of clinical trials between April 2011 and July 2013 report the use of corticosteroids for the treatment of irAEs in 103 patients (35%) and treatment with anti-TNF in 29 patients (10%) who did not respond promptly to steroid therapy [20]. The OS and time to treatment failure were not affected by treatment with immunosuppressive therapy. There are less data on the effect of immunosuppressive agents on the efficacy of anti-PD-1 treatment, but based on what is currently known, there does not seem to be a reduction in the efficacy of anti-PD-1 therapy due to the use of immunosuppressive therapy for irAEs [21].

The association between irAEs and the efficacy of immune checkpoint inhibitors is controversial [22]. Data from early clinical trials of ipilimumab showed an association between increased irAEs and greater clinical benefit [23]. A pooled analysis of three phase II studies reported that patients with no or grade 1 irAEs achieved a disease control rate (DCR) of 20–24%, while a DCR of 34–43% was observed in patient with irAEs of at least grade 2. The OS in the two groups of patients was 8.2 months compared with 14.8 months [24]. In addition, in two trials of adjuvant ipilimumab in patients with high-risk melanoma, a significant association was found between relapse-free survival and irAEs [25]. In contrast, a retrospective study of 135 non-melanoma patients who received anti-PD-1 treatment found no significant correlation between ORR or OS and incidence of any irAE (p = 0.21) or the use of steroids (p = 0.27) [26]. The mechanism of action of infliximab is to block the ability of TNF to recruit neutrophils to the site of inflammation, which is unlikely to affect the antitumoral activity of immune checkpoint inhibitors.

33.5 Organ-Specific Immune-Related Adverse Events

33.5.1 Cutaneous Toxicity

Skin manifestations including rash/pruritus and mucositis are the most frequent irAEs associated with immune checkpoint inhibitors. Approximately 47–68% of patients treated with anti-CTLA-4 antibodies [23] and 30–40% of patients treated with anti-PD-1/anti-PD-L1 antibodies [27] experience skin toxicities of any grade.

The typical rash is reticular, maculopapular, and slightly erythematosus, especially developed on the trunk and extremities, and may be pruritic (Fig. 33.2). The majority of immune-related rashes can be treated with topical creams based on corticosteroids. Itching, if persistent, can be treated with oral antihistamines. Grade 2 rash, especially if pre-existing, requires treatment with oral steroids. Grade 3–4 requires discontinuation of treatment and therapy with intravenous (IV) corticosteroids. A consultation with a dermatologist is recommended, as is a skin biopsy if possible.

Stevens-Johnson syndrome is a fairly rare occurrence that can be fatal (Fig. 33.3). In these patients, IV treatment with corticosteroids is always recommended. If there is no improvement in symptoms, the use of immunosuppressive agents in combination with corticosteroids should be considered [28]. Vitiligo is a common

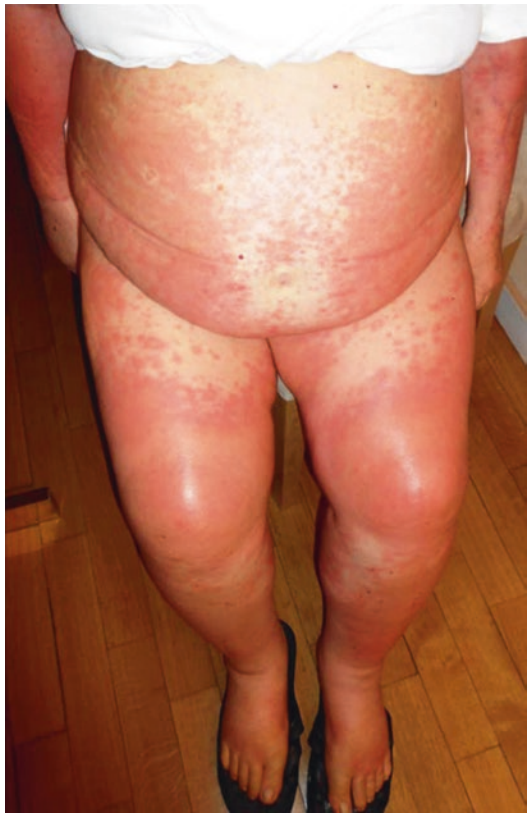

Fig. 33.2 Cutaneous rash in a patient treated with ipilimumab

manifestation and generally occurs late following treatment with immune checkpoint inhibitors. It is characterized by a perivascular lymphocytic infiltrate in the deep dermis layer in proximity to melanocytes and is considered as a marker of response to treatment [29]. Oral mucositis is more common with anti-PD1 antibodies than with ipilimumab and usually requires treatment with topical corticosteroids and lidocaine, to be used after excluding the presence of candidiasis (Fig. 33.4).

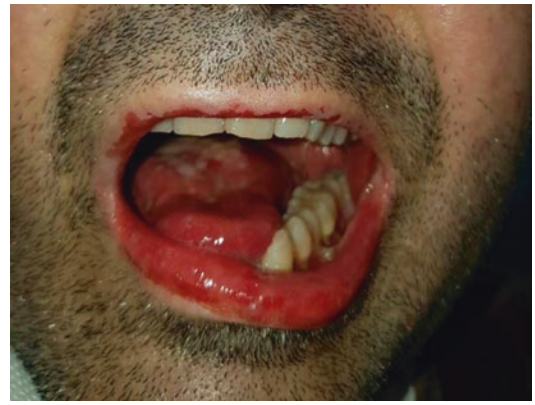

Fig. 33.4 Mucositis induced by anti-PD-1 treatment

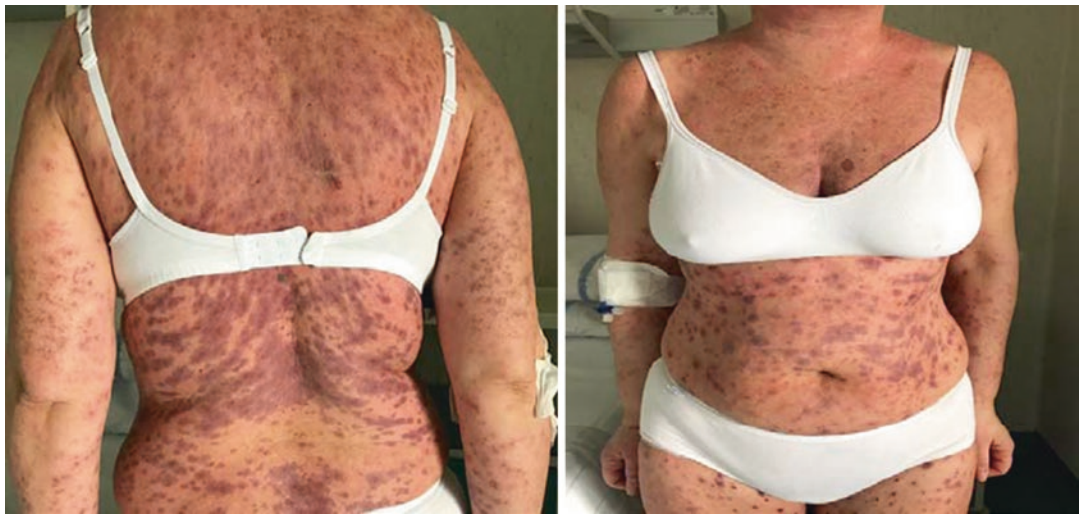

Fig. 33.3 Stevens-Johnson syndrome

33.5.2 Gastrointestinal Toxicity

Diarrhea is one of the most common toxicities in patients receiving immunotherapy and must be diagnosed and treated in an adequate and timely manner to prevent it from developing into a serious adverse event. Generally, diarrhea appears after about 6 weeks of treatment [13] and is more often related to treatment with anti-CTLA-4 than with anti-PD-1 agents. Diarrhea has been reported in about 30% of patients with melanoma treated with ipilimumab, being grade 3–4 in less than 10% [30]. In a phase II study, the rate of severe diarrhea was higher in patients treated with ipilimumab 10 mg/kg than with 3 mg/kg (10% vs 1%) [31]. Similarly, in a phase III trial comparing ipilimumab 3 mg/kg and 10 mg/kg in patients with metastatic melanoma, GI toxicity was higher with the 10 mg/kg dose, with diarrhea reported in 79% of patients treated with 10 mg/kg versus 64% of patients in the 3 mg/kg arm. Grade 3–4 diarrhea was almost twice as common with ipilimumab 10 mg/kg compared with 3 mg/kg (34% vs 18%) [32]. Grade 3–4 diarrhea only occurs in 1–2% of patients treated with anti-PD-1 agents. GI toxicity is the main irAE with the combination of nivolumab plus ipilimumab, with an incidence of about 40%, including 9% of patients experiencing severe diarrhea and colitis [9, 10].

Enteritis, in some cases without diarrhea, can cause obstruction of the small intestine [33]. On biopsy, histology shows an edematous mucosa with rich infiltrate of neutrophils and/or lymphocytes [29, 30]. To date, there are no effective treatments to prevent the development of diarrhea and/or colitis. The use of budesonide to treat enteritis has been assessed but did not show any significant benefit [34].

In cases of diarrhea, the right level of hydration is critical, and the presence of other possible causes of diarrhea (e.g., *Clostridium difficile* infection) must be excluded. For grade 1 diarrhea, symptomatic treatment with loperamide is usually sufficient. For grade 2 diarrhea, suspension of immunotherapy and treatment with oral corticosteroids (e.g., prednisone 0.5–1 mg/kg/day or equivalent) are indicated. Treatment can be resumed in cases which resolve, but cases which do not resolve within 3–5 days should be treated similar to grade 3–4 events, which involves high-dose IV corticosteroids (e.g., methylprednisolone 1–2 mg/kg/day) and prophylactic antibiotic therapy. Treatment with anti-CTLA-4 and/or anti-PD-1 should be permanently discontinued. The patient should be clinically monitored because of the high risk for bowel perforation. In patients not responding to treatment with high-dose corticosteroids after about 3–5 days, treatment with infliximab 5 mg/kg is recommended and should be repeated after 2 weeks if incomplete resolution of symptoms.

33.5.3 Endocrine Toxicity

About 10% of patients treated with anti-CTLA-4 experience clinically significant endocrinopathy [35], while the incidence of endocrine disorders in patients treated with nivolumab is 14%, with 2% of events grade 3–4 in severity [4]. Less than 5% of patients experienced grade 3–4 endocrine toxicity with combination treatment [32]. The main types of endocrine toxicity derive from inflammation of the thyroid, pituitary, or adrenal glands and are often difficult to identify because they typically present with non-specific symptoms, such as fatigue, nausea, headache, and visual changes.

33.5.3.1 Thyroid Toxicity

Thyroid toxicity may involve both hypothyroidism and hyperthyroidism. In patients treated with anti-PD-1, the main toxicity is hyperthyroidism (high values of free thyroid fractions associated with suppressed thyroid-stimulating hormone [TSH]) which frequently occurs with mild symptoms and is diagnosed based on laboratory test results. After subacute onset, the disease can often evolve into Hashimoto's thyroiditis with hypothyroidism. In patients treated with anti-CTLA-4s, the onset of Hashimoto's thyroiditis without preceding hyperthyroidism is more frequent. The disease typically has a slow progression with gradual onset of symptoms; however, cases of acute onset with myxedema crisis

have been reported [36]. Laboratory results show increased values of TSH, free thyroid-reducing fractions, and thyroid autoimmunity (antithyroid peroxidase positivity of autoantibodies and antithyroglobulin). Treatment is based on the use of levothyroxine (L-thyroxine), as a thyroid hormone replacement.

33.5.3.2 Hypophysitis

Hypophysistis has been reported to occur with different frequencies, ranging from 0–25% (average 4%), and is most commonly encountered in men. It occurs almost exclusively in patients treated with anti-CTLA-4 rather than anti-PD-1, possibly because of different distributions of CTLA-4 and PD-1 in the tissue. It has been hypothesized that anti-CTLA-4 antibodies may cause pituitary toxicity if bound to CTLA-4 antigen expressed "ectopically" on pituitary endocrine cells. Typically, it occurs after 6–8 weeks of treatment. The initial symptoms often consist of fatigue and hypotension and in overt forms can present as mass effect-related symptoms (e.g., headache, decreased visual acuity, diplopia, etc.) associated with failure of the pituitary axes (hypocortisolism, hypothyroidism, hypogonadism, panhypopituitarism).

Magnetic resonance imaging (MRI) may show a pituitary gland uniformly increased in volume with intense and homogeneous contrast enhancement. A very serious complication is the advent of an adrenal crisis which can lead to serious negative outcomes including death if not promptly recognized and controlled. Even in these cases, symptoms can be non-specific, e.g., fatigue, asthenia, anorexia, weight loss, nausea, vomiting, and hypotension. Laboratory tests typically show a reduction in circulating cortisol and adrenocorticotropic hormone (ACTH) values and reduced daily urinary excretion of cortisol, often associated with reductions in follicle-stimulating hormone (FSH), luteinizing hormone (LH), and TSH, with central hypothyroidism.

High doses of glucocorticoids are reserved for patients who have serious mass effect-related symptoms, such as severe headache, visual field disturbance, or simultaneously present with other irAEs. Physiological replacement doses should be considered for patients with corticotropic deficiency since pharmacological glucocorticoid therapy is not clearly associated with improved outcomes in such patients [37]. In case of grade 3–4 toxicity, treatment should be discontinuated.

Type I diabetes has been highlighted with both anti-PD-1 and anti-CTLA-4, with an incidence of 8% in patients treated with combination therapy. Treatment with immune checkpoint inhibitors should be deferred in patients with grade 3 hyperglycemia, while treatment should be permanently discontinued with grade 4 toxicity. Corticosteroids are not indicated.

33.5.4 Hepatic Toxicity

Liver toxicity typically occurs in less than 10% of patients [29]. Among patients receiving ipilimumab 3 mg/kg, severe, life-threatening, or fatal hepatotoxicity occurred in 2%, while the incidence of all grade hepatitis was 2.3% with nivolumab monotherapy. However, occurrence was higher (13%) in patients treated with ipilimumab plus nivolumab in combination. Very often, patients are asymptomatic and the only indicator of toxicity is increased transaminases. A worsening of total bilirubin is less often found and usually occurs late. Generally, liver toxicity begins after about 8–12 weeks of treatment.

Although clinical trials have excluded patients with a history of hepatitis B and C, these patients have been treated in clinical practice. Although data are limited, the presence of previous viral hepatitis does not seem to increase the risk of hepatotoxicity [38, 39]. Before starting treatment with immune checkpoint inhibitors, it is mandatory to assess liver function and markers for hepatitis B or C virus. In all patients with HBsAg positivity, early antiviral treatment may be needed in high viral load (HBV DNA).

In patients with grade 1 toxicity (i.e., asymptomatic increases in transaminases and hyperbilirubinemia), treatment can be continued, and liver function tests should be monitored until resolution. Grade 2 events require the interruption of immunotherapy until resolution. Oral prednisone

1 mg/kg/day should be administered. In patients with grade 3–4 events, treatment should be permanently discontinued; high-dose IV glucocorticoids (methylprednisolone 2 mg/kg/day) are recommended. Mycophenolate 500 mg every 12 h should be considered if liver enzymes are still elevated after 48 h of treatment. If no improvement occurs in the following 5–7 days, tacrolimus at the dosage of 0.10–0.15 mg/kg/day is recommended. Infliximab is not recommended because of its hepatotoxicity.

33.5.5 Pulmonary Toxicity

Grade 3 or higher pneumonitis has been reported in 5–7% of patients with NSCLC treated with nivolumab or pembrolizumab [40]. However, the incidence of symptomatic pneumonitis is only 1% with ipilimumab [41]. About 7% of patients treated with nivolumab plus ipilimumab had pneumonitis, with only 1% of grade 3–4 severity [32]. A recent systematic review and meta-analysis suggest that the overall incidence of pneumonitis during PD-1 inhibitor monotherapy was 2.7% (95% CI: 1.9–3.6%) for all grades and 0.8% (95% CI: 0.4–1.2%) for grade 3 or higher pneumonitis. The incidence was higher in patients with NSCLC compared with melanoma for pneumonitis of all grades (4.1% vs 1.6%; $p = 0.002$) and grade 3 or higher (1.8% vs 0.2%; $p < 0.001$). The incidence of pneumonitis was more frequent during combination therapy than monotherapy for all grades (6.6% vs 1.6%; $p < 0.001$) and grade 3 or higher (1.5% vs 0.2%; $p = 0.001$) pneumonitis [42, 43]. Several factors, such as pre-existing lung damage due to tumor burden, smoking, chronic obstructive pulmonary disease, pulmonary fibrosis, and variable expression of PD-L1 on normal lung tissues, may play a role in this although the exact cause of this difference is still unknown.

Pulmonary toxicity should be considered whenever patients present with symptoms of a respiratory infection, new-onset cough, or wheezing. In symptomatic patients, a chest CT scan and a pulmonary consultation are recommended, as is initiation of oral or IV corticosteroids. In patients with moderate or severe symptoms, a diagnostic bronchoscopy that can assess whether there is widespread lymphocytic infiltration is advised. If the toxicity is grade 1 with only asymptomatic radiological signs, treatment suspension of 2–4 weeks until resolution of radiographic findings should be considered. If grade 2, suspension of treatment is mandatory and treatment with oral corticosteroids should be initiated. In severe or recurrent cases, IV treatment with methylprednisolone 2 mg/kg or equivalent may be appropriate, and immune treatment should be permanently discontinued.

Although rare, there have been documented cases of pulmonary sarcoidosis in patients treated with immune checkpoint inhibitors [44].

33.5.6 Renal Toxicity

Renal failure has been reported in patients treated with ipilimumab, anti-PD-1s, and combination therapy. Immune checkpoint inhibitors can cause acute kidney injury that presents similar to other drug-induced tubulointerstitial nephritis. Cortazar et al. analyzed data from published phase II and III trials enrolling 3695 patients and reported an overall incidence of acute kidney failure of 2.2%, with 0.6% grade 3–4 events. The median duration until the appearance of kidney injury was 13 weeks [45]. Acute kidney failure occurred more frequently in patients treated with nivolumab plus ipilimumab (4.9%) than in patients treated with anti-PD-1 monotherapy. The most common signal of acute kidney failure was an increase in serum creatinine, which occurred in all affected patients. Pyuria (68%) and hematuria (16%) were also frequently noted in patients with renal impairment. In patients with suspected immune-related renal failure, a renal consultation should be sought early and a biopsy performed if possible. In patients with grade 2–3 renal toxicity, treatment should be suspended and steroids are recommended. Treatment can be resumed if symptoms improve to grade 1 severity. High doses of corticosteroids and treatment discontinuation are recommended for grade 4 toxicity.

33.5.7 Neurological Toxicity

Based on data in prospective studies, the overall incidence of any grade of neurotoxicity is 3.8% with anti-CTLA-4 antibodies, 6.1% with anti-PD-1 antibodies, and 12.0% with combination therapy [46]. However, most of these neurological adverse events are grades 1–2 and consist of non-specific symptoms such as headache (55%), dysgeusia (13%), or dizziness (10%). The incidence of grade 3–4 events was 1% across all treatments. On the basis of the nervous system area involved, neurotoxicity can be classified as encephalopathy, myelopathy, pure meningitis, meningoradiculitis, Guillain-Barre-like syndrome, peripheral neuropathy, or myasthenic syndrome. The spectrum of neurological symptoms appears to be highly heterogeneous, including headache, fever, tiredness or weakness, confusion, memory problems, sleepiness, hallucinations, seizures, and stiff neck. In the literature, several cases of serious toxicity have been reported, such as reversible posterior encephalitis syndrome [47], Guillain-Barre syndrome [48], myasthenia gravis, transverse myelitis [49], and demyelinating polyneuropathy [50].

All serious neurological irAEs should be treated with high-dose corticosteroids, and a neurologist should be consulted for differential diagnosis and additional therapy.

33.5.8 Rheumatological Toxicity

Arthralgia and arthritis are the most commonly reported rheumatic and musculoskeletal irAEs associated with immune checkpoint treatment. The incidence of arthralgia secondary to nivolumab in phase III trials ranges from 5 to 16% [51], and similar rates have been reported with ipilimumab monotherapy [52]. With combination therapy, incidence of arthralgia was about 10% [32]. Thus, although arthritis is a very common manifestation of autoimmune disease, it has not been reported as frequently as an adverse event in clinical trials of checkpoint inhibitors as might have been expected. One reason for this could be that there are several mutually exclusive ways to code musculoskeletal adverse events in the current system. For example, arthralgia, arthritis, joint effusion, and musculoskeletal pain are all potential options for coding the same event. Furthermore, its incidence could be underrepresented because most clinical trials report only grade 3–4 toxicity and arthritis is often considered to be less severe than other adverse events [53]. No observational studies that monitored patients for inflammatory arthritis with confirmation by a rheumatologist have been reported. However, in a case series of nine patients treated with immune checkpoint inhibitors and who developed inflammatory arthritis, clinical presentation was variable and involved large and small joints, with or without systemic involvement (colitis, urethritis) and autoantibody detection. Patients were treated with corticosteroids and some also required methotrexate and/or anti-TNF treatment [54]. The same series also reported on four patients who developed sicca syndrome while receiving checkpoint inhibition therapy. Symptoms of dry mouth were more severe than dry eyes in all four patients. Three had positive anti-nuclear antibodies. One patient was positive for La/SS-B antibodies and had parotitis treated with 6 weeks of prednisone that resulted in complete resolution of symptoms. All patients were negative for Ro antibodies [54].

Vasculitis is a rare irAE. Two cases of giant cell arteritis after ipilimumab confirmed by temporal artery biopsies and treated with oral corticosteroids were described in one report [55]. Single-organ vasculitis from immune checkpoint inhibitors has also been described, occurring as retinal vasculitis with pembrolizumab [56] and uterine vasculitis with ipilimumab [57].

Myalgia and muscle weakness have been reported as adverse events in clinical trials. Myalgia was seen in 2–18% of participants in trials of nivolumab [58] and ipilimumab [59], whereas muscle weakness was reported in 1–12% of patients.

Cases of inflammatory myositis have been reported. In one patient, this was more consistent with dermatomyositis, with proximal muscle weakness, a heliotrope rash and V-neck sign, and an elevated creatine kinase (CK) level of 1854 U/L [60]. The other case occurred after

nivolumab therapy and involved respiratory muscle along with proximal muscle weakness and an elevated CK of 2812 U/L [61]. Both patients received corticosteroid treatment with complete resolution of the event.

A single case of lupus nephritis after treatment with ipilimumab has also been reported.

33.5.9 Cardiotoxicity

Cardiotoxicity is rarely observed as an irAE in clinical trials with immune checkpoint inhibitors. Nevertheless, several cases have been reported, involving different manifestations of immune-related cardiac syndromes. A recent published case series of 12 patients treated with anti-CTLA-4 and anti-PD-1 documented occurrences of cardiac fibrosis, autoimmune myocarditis, cardiomyopathy, heart failure, and cardiac arrest. Treatment with corticosteroids improved symptoms for all patients, most of whom had a previous history of heart disease. Although rare, it seems a reasonable precaution to closely monitor the heart function of patients with pre-existing cardiac disease even if asymptomatic. Patients with suspected immune-mediated cardiotoxicity should be promptly treated with corticosteroids [62].

33.5.10 Pancreatic Toxicity

Clinical trials of anti-CTLA-4 and anti-PD-1 frequently reported asymptomatic increases in serum amylase and lipase. However, treatment is not indicated unless there are symptoms or signs of pancreatitis.

33.5.11 Ocular Toxicity

Eye toxicity, including conjunctivitis, episcleritis, and uveitis, is rare (<1%) and usually responds well to topical steroid treatment, generally with no long-term sequelae. Oral corticosteroids are reserved for severe events. All patients with ocular irAEs should be referred to an ophthalmologist [63].

33.5.12 Hematological Toxicity

Aplasia, neutropenia, thrombocytopenia, and acquired hemophilia have all been reported with ipilimumab. Corticosteroid therapy is the standard approach, with the addition of other immunosuppressive agents if symptoms do not improve [64–66].

33.6 Pre-Existing Autoimmunity

Checkpoint inhibition leading to potentiation of T cell activity could conceivably exacerbate inflammation and autoimmunity in patients with pre-existing autoimmune disease. An increasing body of evidence supports the role of immune checkpoint regulation, involving both CTLA-4 and PD-1 pathways, in the pathogenesis of inflammatory and autoimmune disorders. For example, in humans, some CTLA-4 alleles and PD-1 polymorphisms are associated with various autoimmune diseases. However, only sporadic case reports of patients treated with immune checkpoint inhibitors with pre-existing autoimmune disease have been reported. A recent multicenter retrospective case series reported on 30 patients with melanoma and prior autoimmune disease treated with ipilimumab. Outcomes varied, ranging from no toxicity (35.3%) to exacerbation of the pre-existing autoimmune disease (25.5%) or de novo irAEs (29.4%), fatal in one patient with psoriasis who developed grade V colitis [67]. Approximately 10% of patients had both disease exacerbation and de novo events. There were two treatment-related deaths, but most adverse events were controlled with glucocorticoids, and few required treatment with infliximab. In more than 50% of patients, adverse events did not require treatment discontinuation. In a report of eight patients with melanoma and preexisting rheumatoid arthritis who received ipilimumab, only two required corticosteroids. Discontinuation was required for the appearance of other more severe irAEs such as colitis [68]. In another report, the safety of pembrolizumab and nivolumab was evaluated in 119 patients with advanced melanoma who either had a pre-existing autoimmune

disease or had experienced severe irAEs with ipilimumab therapy [69]. Exacerbation of the underlying autoimmune disease was reported in 38% of patients while 29% reported other irAEs. Thirty percent of patients did not report exacerbations or irAEs, and there were no treatment-related deaths. Due to the limited data, there are no clear recommendations for patients with cancer and pre-existing autoimmune disease, and the benefits and risks of immunotherapy should be carefully considered before starting treatment with immune checkpoint inhibitors. Data from the CheckMate 172 study, which enrolled patients with a known history of grade 3–4 irAEs during or after anti-CTLA-4 to investigate if pre-existing autoimmunity increased the risk of new immune-mediated toxicities, should be available in the near future.

33.7 Immunologic Biomarkers

Several studies have proposed biomarkers that may predict side effects of immune checkpoint inhibitor therapy, such as eosinophilia, IL-17, or gene profiling, but results have been inconclusive.

An attractive hypothesis that has received attention in recent years is that gut microbiota might be involved in the modulation of the immune response and may also affect the response and toxicity to treatment with immune checkpoint inhibitors. In humans, trillions of bacteria are distributed in complex and site-specific communities on the skin and at mucosal surfaces, and the largest community is found in the distal gut. Crosstalk between an organism and its gut commensal microbiota has both potentiating and detrimental effects on the immune response [70]. In a prospective study of patients with metastatic melanoma undergoing ipilimumab treatment, the presence of bacteria belonging to the *Bacteroidetes* phylum was correlated with resistance to the development of checkpoint blockade-induced colitis limiting inflammation by stimulating T-regulatory cells [71, 72]. Further studies are needed to assess the possibility of assessing the potential toxicity of immune checkpoint inhibitor therapy through a preliminary assessment of individual microbiota.

Conclusions

Immune checkpoint inhibitors are associated with a range of toxicities related to potentiation of the immune response. The most frequent are dermatological, GI, endocrine, and hepatic adverse events, but other less frequent events can also occur. Anti-PD-1 antibodies are associated with a comparable range of adverse events to ipilimumab, with the addition of pneumonitis, and seem to be better tolerated with a lower incidence of grade 3–4 irAEs. The combination of ipilimumab plus an anti-PD-1 has a higher incidence of grade 3–4 irAEs than either treatment alone but is not associated with novel immune-related safety signals.

IrAEs have variable times of onset and require careful monitoring, follow-up, and management. Most are reversible with appropriate and timely intervention but can be severe and even life-threatening if not adequately recognized and treated. Management depends on the organ or system involved and the severity but, in general, involves cessation of immunotherapy and initiation of oral or IV steroid therapy for immunosuppression. Additional immunosuppressive therapy, e.g., infliximab, may also be recommended for severe nonresponsive toxicity. Given the increasing use of various immunomodulatory antibodies across different tumor types, it is important that clinicians are aware of the need for timely diagnosis of irAEs and are familiar with guidelines to manage these toxicities.

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Melanoma: Immunotherapy in Advanced Melanoma and in the Adjuvant Setting

34

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34.1 Cytokines in Melanoma

34.1.1 Interferon-Alpha

Interferon-alpha was the first FDA-approved cytokine for treatment of melanoma. However this was not for advanced melanoma, where randomized controlled trials failed to demonstrate that its use or addition to chemotherapy resulted in improved survival [1]. In 1996 though, interferon-alpha (IFN) was approved as adjuvant therapy for resected high-risk stage IIB–III melanoma [2]. IFN will be discussed further in section on adjuvant immunotherapy of melanoma.

34.1.2 Interleukin-2

Rosenberg and co-workers initiated the development of interleukin-2 alone or in combination with lymphokine-activated killer (LAK) cells [3] and later in combination with IL-2-cultured tumor-infiltrating lymphocytes (TIL) [4]. After confirmatory studies by the Cytokine Working Group and the evidence of long-lasting CRs in 7% of patients that could be considered cured, it leads to the approval in 1998 of interleukin-2 as the first approved immunotherapy for advanced melanoma [5]. Rosenberg and colleagues continued an evolutionary program in adoptive cell therapy to treat cancer, melanoma in particular, that has demonstrated significant progress in insights and results over the years [6]. In highly selected patient

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populations, the combination of lymphodepletion by chemotherapy with or without total body irradiation followed by adoptive transfer of tumor-derived T-cell clones and IL-2 treatment result in 50–70% response rates with CR rates of 10–20% [7]. Current promising approaches use autologous cells genetically engineered to express T-cell receptors [8]. These technologies however are still restricted to a few expert centers.

34.1.3 Tumor Necrosis Factor: Alpha (TNF)

The development of systemic therapy with the cytokine TNF is stranded because of dose-limiting toxicities, in particular hypotension, already at ineffective relatively low doses. Only in the setting of an isolated limb perfusion (ILP), effective concentrations are reached in the clinical setting [9, 10]. In the ILP setting highly significant synergistic activity in combination with melphalan has been observed in melanoma patients with multiple in-transit metastases [11, 12]. For limb threatening soft tissue sarcomas to achieve limb salvage, TNF was approved by EMA in 1998 [13] and is mostly used for STS and multiple in-transit melanoma metastases in some 50 referral centers for limb salvage programs around the world with excellent long-term results [14].

34.1.4 Breaking Tolerance with Immune Checkpoint Inhibitors

Advances in melanoma therapies are at present mainly in the field of immunotherapy and mutation-driven drug development [1]. Breaking tolerance represents a major paradigm shift, and we have entered a new era as the impact of the first checkpoint inhibitors, i.e., anti-CTLA-4 (cytotoxic T lymphocyte antigen-4) and anti-PD1/anti-PDL1 (programmed death-1 receptor and its ligand PD-L1), is unprecedented [1, 15]. In only 5 years advanced melanoma has been transformed from an incurable disease into a curable disease [16, 17], and we are only at the beginning of discovering its transversal impact throughout solid tumor oncology (Table 34.1).

Table 34.1 Key phase III immunotherapy trials with interferons, ipilimumab, nivolumab, and pembrolizumab

Advanced melanoma	Outcome	Publication (Ref)
gp100 vs ipilimumab vs ipilimumab + gp100	Ipilimumab 3 mg/kg approved OS benefit	Hodi et al. [18]
DTIC vs DTIC + ipilimumab	Ipilimumab 10 mg not approved	Robert et al. [19]
Ipilimumab 3 mg vs ipilimumab 10 mg	Ipilimumab 10 mg OS benefit	Ascierto et al. [20]
DTIC vs nivolumab	Nivolumab approved OS benefit	Robert et al. [21]
Ipilimumab vs pembrolizumab	Pembrolizumab approved OS benefit	Robert et al. [22]
Ipilimumab vs ipilimumab + nivolumab	Ipilimumab + nivolumab approved	Hodi et al. [23]
Ipilimumab vs nivolumab vs ipilimumab + nivolumab	Ipilimumab + nivolumab approved PFS benefit in PD-L1 negative tumors	Larkin et al. [24]
Pembrolizumab vs pembrolizumab + epcadostat	Ongoing	
Adjuvant therapy		
Interferon alfa-2b (IFN)	High-dose IFN approved RFS and OS benefit	Kirkwood et al. [2]
Pegylated interferon alfa-2b	Pegylated IFN approved RFS benefit	Eggermont et al. [25, 26]
Ipilimumab 10 mg vs placebo	Ipilimumab 10 mg/kg approved RFS, DMFS, and OS benefit	Eggermont et al. [27, 28]
Ipilimumab 10 mg vs 3 mg vs HD-IFN	Ongoing	
Pembrolizumab vs placebo	Ongoing	
Ipilimumab vs nivolumab	Ongoing	

34.2 Anti-CTLA4

34.2.1 Results in Advanced Melanoma Patients

Monoclonal antibody blocking of cytotoxic T lymphocyte antigen-4 (CTLA4) leads to breaking immune tolerance and can induce tumor regressions. The fully humanized monoclonal anti-CTLA4 antibody ipilimumab was approved in 2011 in the USA in first and second line for patients with advanced melanoma and in second line in Europe at a dose of 3 mg/kg based on randomized controlled trial (RCT) results that showed that the drug either alone or combined with a peptide vaccination provided a significant survival benefit of about 33% compared to vaccination alone [18]. In first-line ipilimumab at 10 mg/kg combined with dacarbazine provided only a small, albeit statistically significant, benefit, and there seems no reason to advocate the use of the combination [19]. This was confirmed by long-term follow-up data demonstrating a plateau ≥5-year survival rate for the combination of dacarbazine and ipilimumab to be around 20% just like what is observed in large data sets with ipilimumab alone in thousands of patients [29, 30]. Of note, also long-term survival with tremelimumab monotherapy was reported to be 20% [31]. Also the efficacy in patients with brain metastases has been established and reported [32]. Since responses can occur after initial tumor progression or appearance of new lesions, immune-related response criteria (irRC) have been developed to avoid premature treatment cessation [33, 34].

At 3 mg/kg adverse events (AE) occur in 40% of patients and are mostly immune-related (irAE), such as skin rashes, colitis, hepatitis, and hypophysitis. Grades 3–4 adverse events occur in about 20% of patients and can, in rare cases, be fatal. Usually they resolve spontaneously or after steroid therapy, except endocrine failure that usually requires permanent hormonal substitution. High-dose steroids are indicated for severe irAEs, but other immunosuppressive agents, like anti-TNF-alpha antibodies, may also be needed [35].

Regarding dose efficacy for ipilimumab, a randomized phase II trial comparing 0.3, 3, and 10 mg/kg suggested 10 mg/kg to be the more effective dose, but associated with more toxicity [36]. In 2016 the results of the randomized phase III trial have clearly demonstrated that the 10 mg/kg dose is more efficacious in patients with advanced melanoma than the 3 mg/kg dose [20]. A significant overall survival benefit was demonstrated (HR, 0.84; $p < 0.04$) with an absolute difference in survival at 3 years of 8% (31% vs 23%). These results come at a price. The 10 mg/kg dose is associated with significantly higher toxicity (grade 3–5 events in 34% vs 19% and irAEs grade 3–5 in 30% vs 14%). The value of four 3 weekly administrations (induction) compared to induction followed by further administrations (maintenance) has not been established.

34.2.2 Biomarker

Good biomarkers for response to ipilimumab therapy still remain to be established. Immune-related adverse events, an increase in lymphocyte counts, an increase in eosinophil counts, the presence of NY-ESO-1 antigen, and the resistance in vitro to T-regulatory cell functions seem to be associated with higher response rates [37–40]. Recently the high levels of soluble CD25 in the serum, especially in the combination with high levels of LDH, were demonstrated to be a very strong prognostic factor for poor outcome [41]. By and large we have only prognostic biomarkers and not clinically important predictive biomarkers.

34.2.3 Adjuvant Therapy in Melanoma and the Recent Approval of Ipilimumab

Approved drugs for adjuvant therapy for stage III melanoma are interferon alfa-2b (USA and EU), based on trial ECOG 1684 [2], and pegylated interferon alfa-2b (USA), based on trial EORTC 18991 [25, 26]. In meta-analyses of adjuvant interferon trials, no dose-effect or duration of treatment effect could be demonstrated, and only a marginal impact on survival of about 3% was

observed. Therefore adjuvant therapy with interferon is not widely accepted or used as standard of care [1].

34.2.3.1 Prolonged Relapse Free and Overall Survival with Ipilimumab

In the EORTC 18071 trial, 951 stage III melanoma patients, after full regional lymph node dissection, were randomized to receive either an intravenous infusion of ipilimumab 10 mg/kg or placebo every 3 weeks for four doses (induction), then every 3 months for up to 3 years (maintenance), or until disease recurrence or unacceptable toxicity. Primary end point was RFS. A significant improvement of RFS by adjuvant ipilimumab (hazard ratio 0.75, $p = 0.0013$) was already reported in 2015 and led to FDA approval. Now in 2016, at a median follow-up of 5.3 years, ipilimumab compared with placebo significantly improved overall survival (hazard ratio for death, 0.72; 95.1% CI, 0.58–0.88; $P = 0.001$) and distant metastasis-free survival (hazard ratio for death or distant metastasis, 0.76; 95.8% CI, 0.64–0.92; $P = 0.002$). Five-year OS rates were 65.4% in the ipilimumab arm and 54.4% in the placebo arm. The 5-year DMFS rates were 48.3% in the ipilimumab arm and 38.9% in the placebo arm. The recurrence-free survival benefit observed previously was maintained (hazard ratio for death or recurrence, 0.76; 95% CI, 0.64–0.89; $P < 0.001$). Treatment benefits were by and large consistent across subgroups with stage IIIC seeming to derive more benefit than stage IIIB and more than stage IIIA, which was the only subgroup that did not seem to benefit (HR 0.98) [27, 28].

34.2.4 Highest Benefit in Ulcerated Melanoma

Post hoc analyses demonstrated a significant impact both in patients with sentinel node-positive disease and palpable node-positive disease. Like in EORTC adjuvant trials 18,952 and 18,991 with IFN and pegylated IFN, patients with sentinel-positive disease derived a greater benefit [25, 26, 42–44]. Patients with an ulcerated primary tumor derived the greatest benefit like in the meta-analysis of the IFN trials 18,952 and 18,991, indicating that ulcerated melanoma is a separate biologic entity [33, 34]. In contrast however to the experience in the adjuvant IFN trials EORTC 18,952 and 18,991, also patients with non-ulcerated melanomas derived a benefit in the adjuvant ipilimumab setting [27, 28]. This is in contrast to the total lack of benefit in IFN trials, which has recently also been confirmed in the IPD meta-analysis of all adjuvant IFN vs observation trials [45].

34.2.4.1 Toxicity and Quality of Life

Global health quality of life scores in the adjuvant ipilimumab trial EORTC 18071 was not significantly different between treatment arms [46]. This is somewhat surprising in the light of significant adverse event rates that resulted in only 42% of patients to receive more than four doses of ipilimumab and only 28.9% of patients to go beyond 1 year of treatment [27]. Grade 3–4 immune-related adverse events (irAEs) occurred in 41.6% of ipilimumab and in 2.7% of placebo-treated patients. The most important grade 3–4 irAEs were diarrhea/colitis in 17.2%, hepatitis in 15.2% and endocrinopathies in 7.8% with hypophysitis in 4.4%, and neurologic events in 1.1%. Five patients died because of drug-related causes, three with colitis, one with myocarditis, and one with a Guillain-Barre syndrome leading eventually to multiple organ failure. The great majority of grade 3–4 irAEs occurred during the induction phase. Median time to resolution after stopping ipilimumab and corticosteroid medication was 6 weeks, except for endocrinopathies (31 weeks). In conclusion, one can state that adjuvant ipilimumab therapy provides consistent improvements in terms of RFS, DMFS, and OS, but that it comes at a price in terms of irAEs that needs expertise and experience to be recognized early and handled with established treatment algorithms. This treatment should be handled by centers with sufficient experience.

Moreover, the trial does not provide evidence that maintenance therapy beyond the four administrations of the induction phase is indicated. The

absence of a significant impact in stage IIIA patients, which have a relatively low risk of recurrence, questions the use of ipilimumab at this dose and with this associated toxicity [47, 48].

34.3 Combination Therapies with Ipilimumab

Various combinations of ipilimumab with other immune modulating, antiangiogenic or chemotherapeutic, or targeted agents have been reported or are ongoing. Guiding principles for combination treatment designs could be to use drugs that lead to immunogenic cell death [49–51]. Since radiotherapy can also induce immunogenic cell death, the reported observations of abscopal antitumor effects after radiotherapy and ipilimumab have led to a number of clinical studies to further investigate this phenomenon [52, 53].

34.4 Chemotherapy

Three studies regarding the combination of chemotherapy with ipilimumab in melanoma patients have been published thus far:

1. **Dacarbazine (DTIC):** A phase III trial comparing DTIC versus DTIC plus ipilimumab at 10 mg/kg in first line in patients with advanced melanoma showed a survival benefit for the patients treated with the combination [19, 29]. The median benefit of only 2.1 months was however disappointing, and the combination is not believed to bring a benefit over ipilimumab alone. Long-term survival in the dacarbazine plus ipilimumab arm was 20% indicating that the combination is not any better than ipilimumab alone, and the combination of this non-immunogenic chemotherapy with ipilimumab is not used in clinical practice [1, 29].
2. **Fotemustine:** In an open-label, single-arm phase II trial, 86 patients with advanced melanoma, 20 of them with asymptomatic brain metastases, received induction treatment of 10 mg/kg intravenous ipilimumab every 3 weeks to a total of four doses, and 100 mg/m^2 intravenous fotemustine, a cytostatic nitrosuree weekly for 3 weeks, and then every 3 weeks from week 9 to 24 [54, 55]. Patients with a confirmed clinical response were eligible for maintenance treatment from week 24, with ipilimumab every 12 weeks and fotemustine every 3 weeks. Forty patients (46.5%) in the study population achieved disease control, as did ten patients with brain metastases (50%). Toxicity was considerable with 47 patients (55%) having grade 3 or 4 treatment-related adverse events. This combination therapy is rarely used because of the arrival of anti-PD1.
3. **Carboplatin/Taxol**: Preliminary results of a randomized phase II trial comparing concurrent carboplatin plus paclitaxel and ipilimumab (4 doses at 3 mg/kg) with sequential treatment of these agents have been reported [56]. Patients with cutaneous melanoma (n = 24), mucosal melanoma [2], ocular melanoma [3], and unknown primary melanoma [1] had entered the study. Response rates (RR) and disease control rates (DCR) for 14 evaluable patients at 24 weeks were 35.7 and 64.3% by irRC, respectively, with grade 3–4 AEs in 63% of patients. No further reports on this study have been published. Overall chemo-immunotherapy combinations have been abandoned in melanoma because of the efficacy of anti-PD1 monotherapy and the efficacy of the combination therapy with anti-CTLA4 and anti-PD1.

34.5 Antiangiogenic Agents

Bevacizumab In a phase II study, 46 patients with metastatic melanoma were treated in four dosing cohorts of ipilimumab (3 or 10 mg/kg) with four doses at 3-week intervals and then every 12 weeks and bevacizumab, an anti-VEGF monoclonal antibody (7.5 or 15 mg/kg), every 3 weeks [57]. Best overall response included eight partial responses, 22 instances of stable disease, and a disease control rate of 67.4%. Median survival was 25.1 months. irAEs included giant cell arteritis (n = 1), hepatitis (n = 2), and uveitis

(n = 2). Extensive CD8(+) and macrophage cell infiltration were observed in on-treatment tumor biopsies. From this initial experience, it appears that the combination of bevacizumab and ipilimumab can be safely administered, and it indicates that VEGF-A blockade influences inflammation, lymphocyte trafficking, and immune regulation that should be explored and understood further.

34.6 Cytokines (IL2, IFN-Alpha, GM-CSF)

Interleukin-2 (IL-2) The most mature data on the combination of IL-2 and ipilimumab are on a 36 patients cohort treated at the NCI Surgery Branch [58]. There were six complete responders (17%) which were higher than the 6% CR rate in 56 patients treated with ipilimumab alone and the 7% CR rate among 85 patients who received ipilimumab by an intra-patient dose-escalation schedule in combination with gp100 peptide vaccination. All CRs except one were ongoing at 54+ to 99+ months at the time of the report. The combination with IL-2 did not seem to increase toxicity and should be explored further.

Interferon-Alpha (IFN) The first phase II trial regards the combination of high dose IFN (HDI) with the anti-CTLA4 drug tremelimumab [59]. In this study 37 stage IV melanoma patients were enrolled to receive tremelimumab 15 mg/kg/course (three cycles [one cycle = 4 weeks]) intravenously every 12 weeks with the concurrent administration of HDI. From course 2 onward, HDI maintenance was administered subcutaneously. Response data in 35 evaluable patients: overall response rate is 24% (4 CRs and 5 PRs). Fourteen patients (38%) had stable disease with a median progression-free survival of 6.4 months and median overall survival was 21 months. These results suggest additive antitumor activity of this combination.

The second phase II trial regards the combination of pegylated IFN and ipilimumab [60]. In this study in 31 patients, ipilimumab was administered at 3 mg/kg for four doses along with concurrent peginterferon alfa-2b at 1–3 mcg/kg weekly for up to 156 weeks. Among 27 evaluable patients, there were 4 CRs, 8 PRs, 4 SDs, and 13 PDs. Peginterferon alfa-2b added to ipilimumab resulted in a response rate of 40% and was associated with a grade 3 toxicities rate of 45%. With the arrival of anti-PD1, this combination will probably be replaced by exploring additive effects with anti-PD1.

GM-CSF The observation that CTLA-4 blockade and granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting tumor vaccine combinations demonstrate therapeutic synergy in some preclinical models evoked the question whether systemic GM-CSF (sargramostim) enhances CTLA-4 blockade. This question was addressed in a randomized phase II trial, conducted by ECOG in 245 patients with unresectable stage III or stage IV melanoma, comparing ipilimumab plus sargramostim treatment with ipilimumab alone [61]. Patients were randomized to receive ipilimumab at 10 mg/kg, intravenously on day 1 plus sargramostim, 250 μg subcutaneously, on days 1–14 of a 21-day cycle vs ipilimumab alone. Ipilimumab treatment included induction for 4 cycles followed by maintenance every fourth cycle. The primary end point of the study was overall survival, with secondary end points progression-free survival, safety, and tolerability. At a rather short median follow-up of 13.3 months, overall survival was superior for the combination treatment (17.5 months versus 12.7 months), and the 1-year survival rates were 68.9% versus 52.9%. Surprisingly no differences for PFS were observed (median PFS of 3.1 months for both treatment arms). Strikingly, and poorly understood, was the observation that the combination treatment was associated with less toxicity. Clearly further studies need to be conducted to elucidate these observations. Which can be said about all cytokines mentioned in this section and various trials are on the way [62].

34.7 Vaccines

gp100 Vaccines Theoretically a combination of a vaccine with anti-CTLA4 is very attractive [63]. Yet the results from the randomized phase

III trial comparing ipilimumab versus ipilimumab plus gp100 vaccine versus gp100 vaccination alone did not show a benefit for the combination of ipilimumab plus the vaccine compared to ipilimumab alone [18]. A similar observation was made in the publication of the mature results of the NCI Surgery Branch experience [58].

TalimogeneLaherparepvec(T-VEC) Talimogene laherparepvec (T-VEC) is a herpes simplex virus type 1-derived oncolytic immunotherapy designed to selectively replicate within tumors and produce granulocyte macrophage colony-stimulating factor (GM-CSF) to enhance systemic antitumor immune responses. Intratumoral administration of T-VEC was compared with GM-CSF in patients with unresected stage IIIB to IV melanoma in a randomized phase III trial [63]. The primary end point was durable response rate (DRR; objective response lasting continuously ≥6 months), with overall survival (OS) as one of the secondary end points. Among 436 patients randomly assigned, DRR was significantly higher with T-VEC (16.3) than GM-CSF (2.1%). Overall response rate was also higher in the T-VEC arm (26.4%) versus 5.7%. Median OS was 23.3 months with T-VEC and 18.9 months with GM-CSF (hazard ratio, 0.79; $P = 0.051$). T-VEC efficacy was most pronounced in patients with stage IIIB, IIIC, or IVM1a disease and in treatment-naïve patients. Treatment was very well tolerated with grade 3–4 events in <2% of patients.

Laherparepvec with Ipilimumab In a phase Ib trial, T-VEC in combination with ipilimumab has been evaluated [64]. Intratumoral administration of T-VEC in week 1, 4, and thereafter every 2 weeks was combined with intravenous administration of ipilimumab (3 mg/kg) every 3 weeks for four infusions, beginning in week 6. The primary end point was incidence of dose-limiting toxicities. Secondary end points were objective response rate by immune-related response criteria and safety. In 19 evaluable patients, grade 3–4 AEs events were observed. The objective response rate was 50%, with 44% of patients having a durable response, indicating that the combination may be better that either agent alone.

34.8 BRAF and MEK Inhibitors

Combinations of BRAF inhibitors and MEK inhibitors with immune checkpoint inhibitors such as anti-CTLA are theoretically attractive, but have in practice proven to be not so simple to develop.

Vemurafenib A phase I trial combining vemurafenib and ipilimumab was stopped early, after only 11 patients, because of several cases of grade 3–4 hepatitis [65].

Dabrafenib + Trametenib A phase I trial with dabrafenib and ipilimumab did not reveal a repeat experience of the phase I vemurafenib plus ipilimumab trial. This phase I study was reported at the 2014 ASCO annual meeting, and no high rate of severe hepatitis cases was reported [66]. An extension cohort study in 30 patients was ongoing. However the combination of dabrafenib plus trametenib with ipilimumab was stopped early, after seven patients, because of severe colitis in three patients. Therefore no extension cohort was planned. Regarding the combination of dabrafenib and trametenib with anti-PD1 experimental data suggests upregulation of PDL-1 expression and potential synergy of this approach [67].

34.9 Anti-PD1 and Anti-PDL1

The PD1 protein is another immune checkpoint expressed in many tumor-infiltrating lymphocytes in response to inflammation. It has two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC). The engagement of PD1 on the lymphocyte surface by PD-L1 on melanoma cells and PDL1 expressing dendritic cells and other components of the tumor infiltrate delivers inhibitory signals downregulating T-cell function [15]. This neutralization of the executive phase of T cells at the tumor site is very powerful, and avoiding this by the use of anti-PD-1 and anti-PDL-1 antibodies has been remarkably successful, both in terms of response rates (30–45% in melanoma and 20–30% in various other tumor types) and in

terms of a very favorable toxicity profile in comparison to anti-CTLA4 antibodies (15–17). Immune-related adverse events (irAEs) are rare and less severe than with anti-CTLA4, especially cumbersome events such as colitis and hypophysitis. In only a few years, phase I trial results launched phase III trials leading to rapid approval of nivolumab and pembrolizumab for advanced melanoma [21, 22, 68–75]. Both pembrolizumab and nivolumab have been reported to induce response rates around 30–45% in advanced melanoma patients, even in patients that previously failed to respond to ipilimumab. Responses tend to be very durable, up to 2–3 years. PDL-1 expression in the tumor is a good biomarker for response to monotherapy with either agent, but even in PDL-1-negative patients, it is more effective than chemotherapy or ipilimumab [76]. Anti-PD1 has been shown in all melanoma patients to be superior to chemotherapy or ipilimumab and to be effective in ipilimumab failures and in patients that have failed targeted therapies. Anti-PD1 is the drug of choice in first line for all metastatic melanoma patients, with the exception of bulky rapidly progressive BRAF-mutant melanoma patients [77]. Now that anti-PD1 is positioned in first line for most melanoma patients, it is an interesting observation that ipilimumab administered after progression on anti-PD1 therapy seems to be more effective than anti-PD1 therapy following ipilimumab failure [78]. The role of combination therapy with anti-PD1 and anti-CTLA4 agents will be discussed later.

Regarding side effects and quality of life outcomes, it is a clear observation from the randomized trials that both nivolumab and pembrolizumab have fewer and less severe side effects and irAEs than ipilimumab and that QoL is superior with these drugs than with various chemotherapies or with ipilimumab [79–83]. In particular lower colities, hepatitis, and hypophysitis rates are associated with anti-PD1 treatment than with ipilimumab. Yet a wide variety of irAEs can be seen at low frequencies. Excellent overview articles deal with diagnosis and treatment of these toxicities [84, 85].

Overall the incredible impact of anti-PD1 and anti-PDL1 monoclonal antibodies lies in its broad transversal impact in oncology with now activity demonstrated against a wide panel of neoplasms other than melanoma, including lung cancer, renal cell cancer, bladder cancer, stomach cancer, head and neck cancer, Merkel cell cancer, Hodgkin lymphoma, and many other to come [86].

34.10 Nivolumab in adjuvant setting for melanoma resected stage IIIB/C-IV

In July 2017 a press release by BMS declared that an interim analysis of the randomized trial Checkmate-238 had demonstrated superiority regarding the primary endpoint (RFS) in this trial. Patient who had received nivolumab (3mg/Kg every 2 weeks) had demonstrated a significant prolongation of RFS compared to the patients who had received ipilimumab (10mg/Kg). [86]

34.11 Anti-PD1 Plus Anti-CTLA4 Combination Therapy

The rational to combine these two checkpoint inhibitors is that they have different mechanisms of action, with anti-CTLA4 mainly acting at the central level in the lymph node compartment by perpetuating and/or restoring the induction and proliferation of activated T cells and with anti-PD1 mainly acting at the peripheral level at the tumor site by preventing the neutralization of cytotoxic T cells by PDL1 expressing tumor cells and PDL2 expressing plasmacytoid dendritic cells in the tumor infiltrate. The first report in 2013 already indicated that the combination is associated with clearly increased response rates up to 50–60%, with an increased CR rate of around 20% and a clear increase in near-complete responses [87].

A randomized phase II trial, nivolumab plus ipilimumab versus ipilimumab (2:1), was conducted, and the results were recently reported in 2016 [23]. One hundred forty-two patients were randomized, assigning 95 patients to nivolumab plus ipilimumab and 47 to ipilimumab alone. At a median follow-up of 24.5 months, the 2-year overall survival was 63.8% for the combination

therapy and 53.6 for those assigned to ipilimumab alone. Treatment-related grade 3–4 adverse events were reported in 51 (54%) of 94 patients who received nivolumab plus ipilimumab compared with 9 (20%) of 46 patients who received ipilimumab alone. Serious grade 3–4 treatment-related adverse events were reported in 36% of patients who received nivolumab plus ipilimumab compared with 9% of patients who received ipilimumab alone [23]. A randomized phase III trial in 945 treatment-naïve patients, ipilimumab versus nivolumab versus ipi + nivo (1:1:1), was also launched, and the first results on the PFS end point were reported in 2015 [24]. Median PFS was superior for ipi + nivo (11.5 months) versus nivolumab alone (6.9 months) versus ipilimumab alone (2.9 months) ($p < 0.001$). PD-L1 expression (≥5%) played a very important role in this trial in the sense that in patients with tumors positive for PD-L1, the median PFS was the same (14 months) for patients treated with nivolumab alone or with the combination. But in patients with PD-L1-negative tumors, PFS was longer for ipi + nivo (11.2 months) vs nivolumab alone (5.3 months). Combination therapy was the most toxic with grade 3 or 4 occurred in 55% versus 16.3% for nivolumab versus 27.3% for ipilimumab. Overall results are expected to be reported in 2017. It seems highly unlikely that the combination ipi + nivo will be superior to nivolumab alone in patients with PD-L1 positive tumors. It is not excluded that in patients with PD-L1-negative tumors, the combination will outperform nivolumab monotherapy.

34.12 Other Combination Therapies: Anti-PD1 Will Be the Backbone

Immunotherapy combinations in general are expected to be perhaps the most dynamic drug development field for years to come. Once breaking tolerance is achieved, or even further improved with candidate molecules such as anti-LAG3 and others, the door seems wide open to combine with agonists such as OX40, CD137, and others. Strategies that primarily address additional immunosuppressive mechanisms in the tumor microenvironment, such as indoleamine 2,3-dioxygenase (IDO) inhibition, TGF-β blockade, regulatory T-cell (Treg) depletion, and angiogenesis inhibition, may be particularly effective to enhance or rescue tumor responses achieved with anti-PD-1/PD-L1 monotherapy. Based on preclinical evidence, several IDO inhibitors are currently in clinical investigation as monotherapies and in combination with CTLA-4 and PD-1 inhibition. Promising response rates in NSCLC and melanoma with pembrolizumab plus the IDO inhibitor epacadostat were recently reported, leading to exploration of this combination in a phase III trial in melanoma (NCT02752074) and a recently announced expansion of this phase III program into NSCLC, renal, bladder, and head and neck cancer [88]. Currently a large number of combination trials are ongoing. It is early times and the next winner has as of yet not been identified.

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Immunotherapy for Prostate Cancer: An Evolving Landscape

35

Wendy Mao and Charles G. Drake

Contents

Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen-presenting cell
BiTE	Bispecific T cell engager
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CAR	Chimeric antigen receptor
cGAS	Cyclic GMP-AMP (cGAMP) synthase
DART	Dual-affinity retargeting
DC	Dendritic cell
FcγRIII	Fc gamma receptor III
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IMPACT	Immunotherapy for prostate adenocarcinoma treatment
LLO	Listeria lysin O
Lm	*Listeria monocytogenes*
mCRPC	Metastatic castration-resistant prostate cancer
MMR	Mismatch repair
MSI	Microsatellite instability
NK	Natural killer
OS	Overall survival
PAP	Prostatic acid phosphatase
PSMA	Prostate-specific membrane antigen
PSA	Prostate-specific antigen
PARP	Poly-ADP-ribose polymerase
PD-L1	Programmed death-ligand 1
PD-1	Programmed Cell Death Protein 1
PFS	Progression-free survival

This chapter discusses the basic principles behind agents aimed at treating prostate tumors with immunotherapy, including a discussion of ongoing clinical trials.

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RT	Radiotherapy
TCR	T cell receptor
TAM	Tumor-associated macrophage
TLR	Toll-like receptor
TLR9	Toll-like receptor 9
Treg	T regulatory (cell)

35.1 Introduction

Worldwide, prostate cancer is the second most common cancer in males, with approximately 1.1 million diagnoses and 307,000 deaths in 2012 [1]. The disease occurs mostly in older men, i.e., those past the age of 65. For clinically localized disease, standard treatment regimens include radical prostatectomy and/or radiation therapy. Androgen ablation, including bilateral orchiectomy or the administration of either LHRH agonists or antiandrogens, decreases tumor burden and improves median progression-free survival by 12–18 months. Despite its initial efficacy in controlling disease, most patients on hormonal therapy will eventually progress to castration-resistant prostate cancer (CRPC) (Fig. 35.1), and about 70% of patients will develop metastases, which are localized to the bone in 80–90% of cases [2]. To date, there is no curative treatment for metastatic castration-resistant prostate cancer (mCRPC), although a number of treatments prolong survival and provide palliative benefit. First-line treatments for mCRPC include the antiandrogens enzalutamide and abiraterone acetate, as well as chemotherapeutic agents such as docetaxel. The prognosis for patients with mCRPC is variable, with median overall survival (OS) ranging from approximately 16–34 months [3–5] (Fig. 35.1).

In the past several years, immunotherapy has emerged as a promising part of the treatment armamentarium for a variety of solid tumors. In 2010, anti-CTLA-4 (ipilimumab, Bristol-Myers Squibb, Princeton, NJ) was approved by the US FDA for the treatment of late-stage melanoma after a pivotal randomized phase III trial showed that CTLA-4 blockade increased overall survival [6]. Anti-CTLA-4 treatment also showed activity in renal cell and non-small cell lung carcinoma, but it is not approved by regulatory agencies for either of those indications. More recently, agents that block the PD-1/PD-L1 axis were shown to prolong survival in patients with non-small cell lung cancer, melanoma, kidney

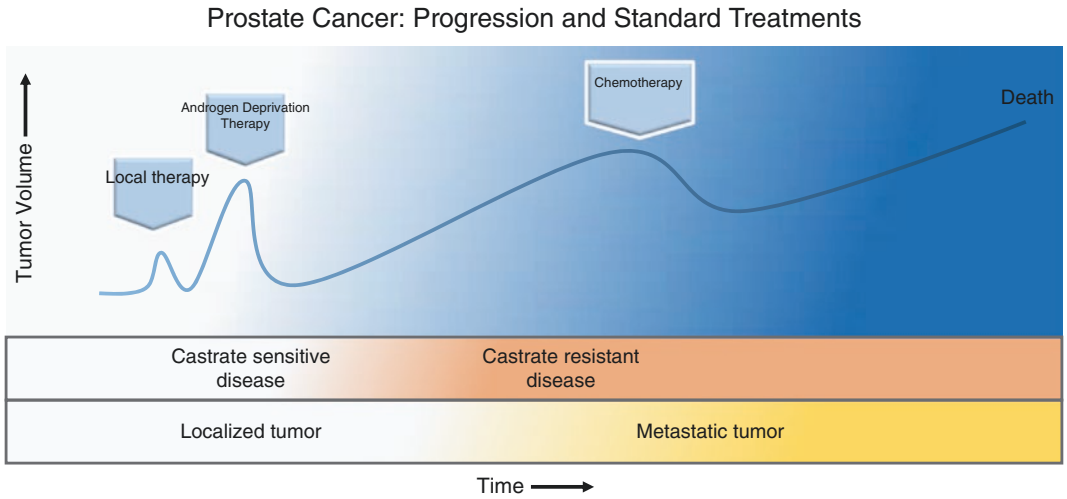

Fig. 35.1 Disease progression of prostate cancer from initial diagnosis until death. Local therapies such as surgery and radiation can control initial disease, and hormone-naïve tumors see clinical benefit from antiandrogen therapy. However, prognosis for advanced metastatic castrate-resistant prostate cancer is poor, and few options exist for treatment

cancer, head and neck cancer, and others—leading to a barrage of regulatory approvals [7]. Across multiple tumor types, objective responses from PD-1/PD-L1 blockade appear to be more durable than those associated with chemotherapy [8]. Additionally, there is one vaccine that is approved for use in a treatment setting for mCRPC—the active cellular therapy Sipuleucel-T (Dendreon, Seattle, WA) [9]. Another notable immunotherapy strategy in late-stage development is adoptive cellular therapy, most notably CAR T cells, which have shown remarkable activity in certain leukemias and lymphomas [10, 11]. T cells engineered to express tumor-specific T cell receptors (TCR) have shown clear activity in melanoma patients [12], although adoptive cellular therapy still faces many challenges in the field of solid tumors [13].

Despite these successes, immunotherapy for advanced prostate cancer still has many challenges to overcome, including lack of tumor immunogenicity as well as the major obstacle that prostate cancer is poorly infiltrated as compared to some of the more responsive tumor types for which agents have been approved (Fig. 35.2). These daunting properties of the prostate cancer tumor microenvironment (TME) may make it a difficult tumor to treat with immunotherapy, and treatments that show activity in other tumor types fail to elicit responses in mCRPC. In terms of anti-PD-1/PD-L1 directed therapies, one hypothesis for this lack of objective response in the clinic is the relative paucity of PD-L1 expression on prostate tumors [14], which may explain why blocking antibodies are generally ineffective as a monotherapy. One potential exception to this lack of immunogenicity is in the context of tumors with microsatellite instability (MSI). Across a wide variety of diseases, tumors with MSI exhibit a more robust response to immunotherapy [15]. Unfortunately, true MSI in mCRPC is quite rare, occurring in less than 5% of patients [16]. Combination immunotherapy with anti-CTLA-4 and anti-PD-1 is active in several tumor types [17, 18], and combination treatment was recently approved by the US FDA for the treatment of melanoma [19].

35.2 Immune Checkpoint Blockade

As highlighted above, immune checkpoint blockade showed impressive activity in several types of solid tumors. During a typical immune response, activated cytotoxic T cells upregulate PD-1, which binds to ligands such as PD-L1 and PD-L2

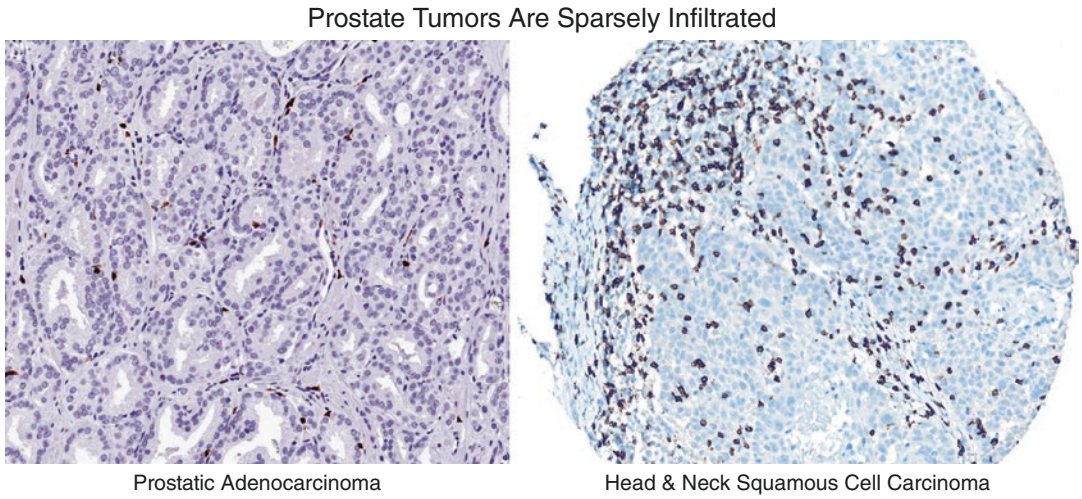

Fig. 35.2 CD8+ T cell infiltration patterns in prostate cancer tissue versus head and neck cancer. IHC staining for CD8 shows poor infiltration of prostate tumor by cytotoxic T Cells as compared to head and neck tumor

(Fig. 35.2) to inhibit T cell proliferation and effector function. This mechanism likely evolved to prevent uncontrolled systemic responses, which could lead to autoimmunity. However, many tumors have co-opted this pathway to evade immune recognition by upregulating immune checkpoint ligands, such as PD-L1 either on the tumor cells themselves or on the myeloid cells that infiltrate the tumor microenvironment. So, interfering with the PD-1/PD-L1 interaction can potentially enhance an anti-tumor response. One further consequence of this mechanism is that PD-L1 expression on tumor cells or on the immune cells infiltrating tumors could serve as a possible biomarker for tumors that would be more sensitive to PD-1/PD-L1 blockade. Extensive evaluation of this hypothesis across a number of trials resulted in sometimes conflicting results. For example, in the pivotal trial of PD-1 blockade in kidney cancer, the PD-L1 status of the tumor showed no association with response [20]. By contrast, the anti-PD-1 agent pembrolizumab (Merck, Kenilworth, NJ) is only US FDA approved for first-line therapy in non-small cell lung cancer but only in patients with PD-L1-positive tumors [21]. With a few exceptions [22], staining of mCRPC specimens has shown them to be mostly negative for PD-L1 [14, 23]. During the second (phase Ib) trial of anti-PD-1 (nivolumab, Bristol-Myers Squibb, Princeton, NJ), none of the 17 mCRPC patients enrolled showed an objective response, and neither of the two tumor specimens taken from that group was PD-L1 positive [23]. This is not particularly surprising, as prostate cancer is typically characterized by low levels of inflammation (Fig. 35.2), a lack of PD-L1 expression, and low mutational burden [24, 25], all of which may make it difficult to target with immunotherapy.

Similar to PD-1, CTLA-4 is another immune checkpoint molecule expressed by T cells upon activation, whose engagement inhibits proliferation and activity. It binds to its ligands, CD80/B7-1 and CD86/B7-2 (Fig. 35.3), with higher affinity than the T cell costimulatory molecule CD28 and thus is able to outcompete CD28 for binding. It is important for maintaining immune tolerance, as CTLA-4 knockout mice quickly succumb to lethal lymphocytic inflammation [26]. However, it also plays a role in immune evasion by tumors, and in early studies, tumor-bearing mice treated with anti-CTLA-4 showed a significant antitumor effect [27]. Two separate clinical trials in late-stage melanoma also showed positive results, with an increased median overall survival [28]. Interestingly, emerging data show that CTLA-4 is likely more highly expressed on tumor-infiltrating Treg than on CD8 T cells, suggesting the possibility that blockade exerts its antitumor effect through inhibiting the function of tumor-infiltrating T regulatory (Treg) [29], which serve to inhibit T cell responses.

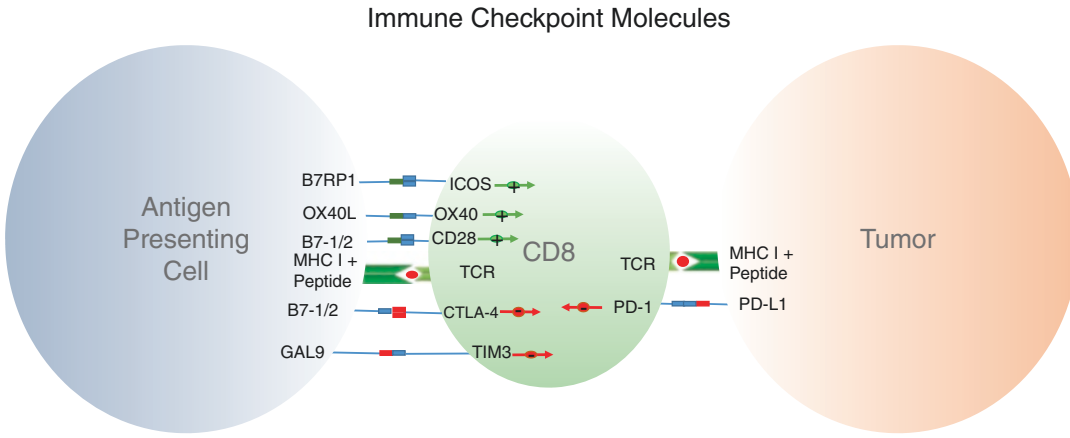

Fig. 35.3 Activating and inhibitory processes compete to mediate tumor response. Antigen-presenting cells can activate CD8 T cells and provide additional costimulation. Tumor cells upregulate inhibitory molecules to dampen CD8 activation and cytotoxic response. Chronic exposure to inhibitory ligands leads to CD8 exhaustion

Early studies of anti-CTLA-4 in mCRPC suggested some degree of activity, with approximately 10–15% of patients experiencing a drop in PSA, and a few objective responses [30]. These data, as well as the fact that few other therapies for advanced mCRPC were available at the time, provided enthusiasm for definitively testing anti-CTLA-4 in men with mCRPC. Two trials were launched, the first of which compared low-dose radiotherapy (RT) plus anti-CTLA-4 to placebo in men who progressed on or after docetaxel chemotherapy [31]. Although the trial failed to meet its primary endpoint of overall survival, there was a small increased in progression-free survival in the treatment arm. This trial was unique among prostate cancer immunotherapy trials, in that it did not exclude men with visceral metastases. This is important, because such patients have a poor overall prognosis [32]. Hypothesis-generating, retrospective analyses showed that patients with visceral metastases appeared to derive no benefit from immunotherapy, whereas men with no visceral metastases treated with anti-CTLA-4 had an OS approximately 4.1 months lower than those in the placebo arm (14.4 vs. 10.3 months) [31]. As above, visceral metastases have been correlated with a worse disease prognosis in mCRPC [32], although second-line hormonal therapies such as abiraterone are active in men with and without visceral metastases [33]. It is possible that metastasis to niches such as the liver fundamentally changes the anti-apoptotic, angiogenic, and immunogenic properties of cells which make them more resistant to immunotherapies [33]. A second phase III trial enrolled men with earlier-stage mCRPC (pre-chemotherapy) and specifically excluded those with visceral metastases. Unfortunately, that trial also failed to reach its OS endpoint [34]. Taken together, the lack of responses in the late phase IB trial of anti-PD-1 and the two failed phase III trials of anti-CTLA-4 provides evidence that metastatic prostate cancer is a relatively nonimmunogenic tumor type and that either combination approaches or other agents may be required.

Two pieces of data suggest that later-stage mCRPC that progresses on enzalutamide might have unique immunological properties. The first of these was a case report of a patient progressing on enzalutamide who was treated with Sipuleucel-T and experienced an unusual complete response [35]. Perhaps more relevant are recent data showing that enzalutamide-resistant prostate cancer cell lines appear to upregulate PD-L1, suggesting that enzalutamide-resistant prostate cancer patients might respond to PD-1/PD-L1 blockade [36]. Based on those data, Graff et al. launched an interesting trial (NCT02312557) in which men with mCRPC who initially responded to enzalutamide and subsequently progressed were treated by adding the anti-PD-1 antibody pembrolizumab to ongoing enzalutamide. Early data on this trial were recently published and showed that three of the first ten patients had objective responses to pembrolizumab [37]. Additional observations included one patient with progression-free survival at up to 55 weeks, resolution of cancer-related pain, and serum PSA levels falling to <0.1 ng/mL. Interestingly, one of the responders also had microsatellite instability positive disease, an indication of defects in DNA mismatch repair (MMR). Previous findings showed that PD-1 blockade has widespread activity in patients with MMR deficiencies [38], possibly because of increased expression of neoantigens serving as targets for a wider repertoire of lymphocytes. Although the incidence of mismatch repair defects in prostate cancer is thought to be relatively low, one study found that in advanced prostate cancer cases, 7 out of 60 men had tumors with MMR defects and microsatellite instability [16]. Specifically, mutations in DNA repair genes MSH2 and MSH6 were found to be prevalent, possibly arising from gene rearrangements generated by androgen receptor itself [16]. Future clinical trials and treatment regimens will take into account the mutational status and nature of mutation of the patient's tumor, with the notion that this could possibly serve as a predictive biomarker for PD-1/PD-L1 blockade-based immunotherapy.

Several phase II and III clinical trials of immune checkpoint blockade, either alone or in combinations, are currently underway (Table 35.1). One particularly notable trial is a phase III trial of the

Table 35.1 Immune checkpoint blockade trials in prostate cancer

Checkpoint inhibitor	Setting	In combination with…	Phase	NCT ID
Pembrolizumab	Enzalutamide progressors		Phase II	NCT02312557
Pembrolizumab	Previous chemotherapy		Phase II	NCT02787005
Pembrolizumab	Previous docetaxel, enzalutamide, or abiraterone	Olaparib, docetaxel + prednisone, or enzalutamide	Phase III	NCT02861573
Pembrolizumab	mCRPC	Cryosurgery	Phase II	NCT02489357
Pembrolizumab	mCRPC	ADXS31-142 (listeria-PSA)	Phase II	NCT02325557
Pembrolizumab	mCRPC	MVI-816 (PAP DNA Vac)	Phase II	NCT02499835
Pembrolizumab	Radiation and ADT	SD-101 (TLR9 agonist)	Phase II	NCT03007732
Atezolizumab	Androgen pathway inhibitor progressor	R-223-D	Phase I	NCT02814669
Atezolizumab	Docetaxel/cabazitaxel naïve	Sipuleucel-T	Phase I	NCT03024216
Atezolizumab	Abiraterone progressors, taxane ineligibility/ progressors	Enzalutamide	Phase III	NCT03016312
Nivolumab	Mismatch repair-deficient tumors		Phase II	NCT03040791
Nivolumab	AR-V7-expressing tumors	Ipilimumab	Phase II	NCT02601014
Nivolumab	mCRPC	Ipilimumab	Phase II	NCT02985957
Nivolumab	mCRPC	Prostvac and/or ipilimumab	Phase I/II	NCT02933255
Ipilimumab	Localized PC	Prostvac	Phase II	NCT02506114

anti-PD-L1 antibody atezolizumab (Genentech, San Francisco, CA). In this trial, men progressing on first-line abiraterone acetate will be randomized 1:1 to treatment with the second-generation antiandrogen enzalutamide either alone or in combination with atezolizumab. The primary endpoint is overall survival. A second trial of note is a fairly large single-armed phase II trial of anti-PD-1 (pembrolizumab) in mCRPC patients previously treated with chemotherapy (Table 35.1), with a primary endpoint of safety and objective response rate. In a more exploratory approach, anti-PD-1 (pembrolizumab) is currently being combined with cryosurgery to induce the release of possible tumor antigens and activate a greater spectrum of CD8+ T cells in a phase II trial (Table 35.1).

As highlighted above, recent studies suggested the possibility that PD-1 and CTLA-4 might be expressed on different cell types in the tumor microenvironment, with CTLA-4 being predominantly expressed on Tregs and PD-1 being strongly expressed on CD8 T cells [39, 40]. Blockade of CTLA-4 inhibits suppressive tumor-infiltrating Treg function [41], while PD-1 blockade enhances CD8 functionality and may also prevent the induction of a tolerogenic program during antigen encounter [42]. These data support the concept that combined blockade of PD-1 and CTLA-4 could show synergistic efficacy in a tumor setting, as was the case in animal models [43, 44]. Clinical data on combined PD-1/CTLA-4 blockade were first reported in advanced melanoma [45] with 65% of patients exhibiting evidence of clinical activity, including a number of responses that occurred quite rapidly, i.e., within the first 8 weeks of treatment. The combination was eventually US FDA approved for melanoma and is currently being evaluated in the first-line setting in NSCLC [18] and in kidney cancer [46]. Interestingly, the phase III trial in melanoma showed that the combination therapy was effective even in patients with PD-L1-negative tumors [19], which is of particular interest in the context of prostate cancer, which typically expresses low levels of PD-L1 [14]. Based on these encouraging results, the Hopkins group recently completed enrollment of a small study of the combination in men with mCRPC who were biomarker selected for resistance to antiandrogens (Table 35.1). The primary end-

point of this trial is safety and tolerability, as well as the rate of objective responses. Additionally, checkpoint blockade can be combined with non-immune agents. Some prostate tumors, especially those with mutations in BRCA1/BRCA2, show a dependence on poly-ADP-ribose polymerase (PARP) [47], and a phase I study is underway combining an anti-PD-L1 antibody (durvalumab, MedImmune, Gaithersburg, MD) with olaparib (AstraZeneca, Wilmington, DE), a PARP inhibitor (Table 35.1).

35.3 Cancer Vaccines

Unlike immune checkpoint blockade, which functions by down-modulating inhibitory immune signaling to augment an existing tumor response, vaccines seek to prime and induce an antitumor immune response. Vaccines function at the level of antigen-presenting cells, i.e., dendritic cells (DCs), which uptake and process antigens before presenting them to cytotoxic CD8 T cells along with costimulatory molecules that enhance the CD8 response. These T cell antigens are small peptide fragments presented in the context of MHC molecules, and when recognized by a cognate T cell receptor (TCR) on a CD8 T cell, activates the T cell. Activated CD8 T cells proliferate, traffic widely, and are capable of specifically lysing targets that express their cognate peptide/MHC ligand. Dendritic cells are the most potent antigen-presenting cells (APC) due to their ability not only to prime naïve T cells but also due to their ability to respond to innate immune signaling and thus orchestrate an immune response. So, in general a cancer vaccine consists of an adjuvant, designed to activate dendritic cells and prime them for presenting antigen and costimulation to T cells, as well as a tumor-specific peptide/protein against which the immune response is mounted [6]. Prostate cancer is an especially good candidate for vaccines due to its expression of fairly specific tumor antigens such as PAP, prostate-specific membrane antigen (PSMA), and prostate-specific antigen (PSA), all of which are expressed relatively more commonly in the prostate [48]. This restricted expression theoretically lowers the risk for unintended targeting of other tissues if T cells are robustly activated via vaccination. In addition, men with metastatic disease typically have undergone primary treatment with either radiation therapy or with surgery; thus the only source of prostate antigens in such men is likely to be the tumor itself.

The only US FDA-approved vaccine for the treatment of a solid tumor is Sipuleucel-T, a dendritic cell vaccine generated from patient-derived cells. To prepare this product, patient monocytes are isolated and co-cultured with a fusion protein consisting of PAP and GM-CSF, which induces the maturation of monocytes to dendritic cells, activates the dendritic cells, and provides PAP as a target antigen [49]. Upon reinfusion of these primed DCs into the patient, the antigen is presented on MHC I and II in order to potentially induce a cytotoxic response as well as aid in the development of a significant humoral effect. The Immunotherapy for Prostate Adenocarcinoma Treatment (IMPACT) study showed that Sipuleucel-T-treated patients derived a 4.1 month average OS benefit, with an OS of 25.8 months for treated patients versus 21.7 months for those receiving placebo. However, only 2.6% of treated patients had a PSA decrease of more than 50% [9]. In these patients, not only was there a significant increase in serum antibodies against the primary antigen, PAP, but also antibodies against "secondary antigens" such as LGALS3 and ECE1 which are known to be expressed in prostate tumors [50]. This phenomenon, known as "antigen spread," likely occurs when immune-mediated attack of cancer cells leads to the release of additional antigens in a pro-immunogenic context [51].

Although Sipuleucel-T was efficacious in phase III trials, the only other vaccine to complete phase III testing in prostate cancer was less encouraging. This reagent, GVAX prostate, is a whole-cell vaccine which consists of two tumor cell lines—PC3 and LnCaP—transduced with a recombinant viral vector to secrete GM-CSF. A randomized phase III trial, comparing GVAX prostate to chemotherapy in men with mCPRC, completed enrollment of approximately 600 patients in 2006. It should be noted that this trial,

unlike the trials of Sipuleucel-T or the ongoing phase III trial of Prostvac-VF (see below), used an active comparator arm—docetaxel chemotherapy—which has clear clinical benefit in mCRPC [52]. The phase III trial for GVAX prostate in mCRPC was terminated early due to a futility analysis showing less than a 30% chance that the target endpoint would be met [53]. Nonetheless, GVAX is now being tested in combination with other treatments, including in a neoadjuvant setting in combination with hormonal therapy in phase II (Table 35.2).

Another vaccine being developed for prostate cancer is Prostvac-VF (Bavarian Nordic, Morrisville, NC), a two-part viral vaccine consisting of a prime (Prostvac-V) and boost (Prostvac-F), targeting the antigen PSA (Table 35.2). In addition, the vaccine includes TRICOM™, which is a triad of costimulatory molecules (B7.1, ICAM-1, and LFA-3), designed to increase APC presentation of the target antigen. A phase II trial with Prostvac-VF did not meet the prespecified study endpoint of enhancing progression-free survival (PFS), but a post hoc analysis showed that the vaccine appeared to improve overall survival by 8.5 months, from 16.6 to 25.1 months, at a 3-year follow-up [54]. A phase III trial of Prostvac with GM-CSF in mCRPC patients is currently underway (Table 35.2). This survival trial randomized 1200 men with chemotherapy-naïve mCRPC 1:1:1 to either placebo, Prostvac-VF plus intradermal GM-CSF, or Prostvac-VF plus placebo (NCT01322490). Patients with visceral disease were excluded. Enrollment has completed, but final results are not yet available. A number of trials combining Prostvac with anti-CTLA-4 (as well as other agents) are currently ongoing, and preliminary results indicate a decline in PSA levels in 14 out of 30 patients [55].

DNA vaccines are another technology undergoing phase I and II testing in prostate cancer. In general, these reagents consist of a bacterial plasmid encoding target antigens; when administered intramuscularly to a patient, encoded proteins are expressed using host cell transcriptional machinery. These antigens are then processed and presented by dendritic cells, potentially leading to the mobilization and activation of lymphocytes. Additionally, the hypomethylated CpG motifs on the bacterial plasmid may activate innate sensors of pathogens, such as TLR9 and cGAS, on multiple immune cell types including DCs, B cells, and natural killer (NK) cells [56]. Signaling cascades downstream of these sensors lead to pro-inflammatory effects, including production of GM-CSF to induce maturation of monocytes, as well as type I interferons which enhance B and T cell activation.

As discussed above, prostate cancer cells express a shared set of tumor antigens, so they are reasonable candidates for DNA vaccines. Several vaccines of this type have already been developed for prostate cancer and are in various stages of clinical trials. The furthest along of these, MVI-816 (Madison Vaccines Incorporated, Madison, WI), is a DNA vaccine encoding PAP, which is undergoing testing in a phase II trial. In this trial, men with nonmetastatic prostate cancer are being randomized to receive either MVI-816 plus GM-CSF or GM-CSF alone, with the primary endpoint being metastasis-free survival (NCT01341652). Since the subset of tumor-specific T cells in patients with advanced cancer may express high levels of PD-1, DNA vaccines alone may not be as effective in activating these exhausted cells. Thus, MVI-816 is also being tested in combination with pembrolizumab in a phase I trial for men with mCRPC (Table 35.1), with primary endpoints of objective response rate, progression-free survival rate, adverse event incidence, time to radiographic progression, and PSA response rate.

A second DNA vaccine approach of note targets PSMA and PSA. This vaccine approach is unique in that it doesn't encode native proteins—rather, it encodes slightly modified versions which are more xenogeneic in nature, in an effort to increase immunity to the natural antigen. This DNA vaccine, INO-5150 (Inovio, Plymouth Meeting, PA), is in a phase I clinical trial either as a monotherapy or in conjunction with their cytokine adjuvant IL-12 plasmid INO-9012 with primary outcomes of safety and antigen-specific response (Table 35.2). The trial targets early-stage patients, i.e., those with a rising PSA postprimary surgery or radiation therapy.

Table 35.2 Selected prostate cancer vaccines in clinical trials

Name	Type	Antigen target(s)	Phase	NCT ID
Sipuleucel-T	Cell-based	PAP	FDA approved	
Sipuleucel-T + radium-223	Cell-based	PAP	Phase II	NCT02463799
Sipuleucel-T + early/late ipilimumab	Cell-based	PAP and CTLA-4	Phase II	NCT01804465
GVAX prostate + cyclophosphamide	Cell-based	PC3 and LNCaP antigens	Phase II	NCT01696877
Prostvac	Viral	PSA	Phase III	NCT02649439
Prostvac + enzalutamide	Viral	PSA	Phase II	NCT01867333
ChAdOx1.5T4-MVA.5T4	Viral/protein	5T4	Phase I	NCT02390063
MVI-816	DNA	PAP	Phase II	NCT00849121
MVI-118	DNA	Androgen receptor (AR)	Phase I	NCT02411786
INO-5150	DNA	PSA + PSMA	Phase I	NCT02514213
ProstAtak	Viral	Oncolytic adenovirus	Phase III	NCT01436968
CureVac	mRNA	PSA, PSMA, PSCA, and STEAP	Phase II	NCT00831467
ADU-741	Bacterial	PSMA, PAP, NKX3.1, and SSX-2	Phase I	NCT02625857
ADXS-PSA	Bacterial	PSA	Phase I/II	NCT02325557

Enrollment has been completed, and immune monitoring data are awaited.

One more direct approach to vaccination involves intratumoral injection of an immunogenic construct to increase immunogenicity of in situ tumors. One such vaccine, ProstAtak (Advantagene Inc., Auburndale, MA), is in late-stage (phase III) development for prostate cancer (Table 35.2). This is a viral vaccine consisting of an attenuated adenovirus which introduces a herpes-derived thymidine kinase (tk) gene injected intratumorally, leading to its expression. This renders the cells vulnerable to valacyclovir (GlaxoSmithKline, Middlesex, UK), a standard antiviral drug. Drug administration results in the death of infected cells with accompanying release of tumor antigens. Although there is one intratumoral vaccine that is FDA approved for melanoma, repeated intratumoral injection into the prostate gland does add a level of complexity not present with other approaches.

Another vaccine method involves inactivating tumor cells with high hydrostatic pressure (HHP), which preserves their antigenicity. When dendritic cells were exposed to these HHP-inactivated cells in a mouse model, production of IL-12 and expression of maturation markers increased as compared to DCs exposed to irradiation-inactivated tumor cells [57]. Additionally, when combined with docetaxel in the poorly immunogenic mouse TRAMP-C2 tumor setting, the combinatorial treatment slowed tumor growth and increased survival [58]. An mRNA-based vaccine, CV9104 (CureVac, Tübingen, Germany), complexes immunostimulatory protamine with mRNA encoding PSA, PSMA, PSCA, and STEAP (Table 35.2). mRNA vaccines reduce the risk of genomic integration of constructs into the host genome, which is a concern with the use of DNA vaccines. Although initial phase I and IIa results were promising, the phase IIb trial failed to improve overall survival and did not improve progression-free survival versus placebo [59].

Inoculation with inactivated bacteria is among the most ancient forms of vaccination and has also been harnessed in the context of antitumor immunity. One especially interesting vaccine vector is the positive bacterium *Listeria monocytogenes*

(Lm). Lm infects antigen-presenting cells directly, leading to the expression of encoded antigens and presentation on class I MHC. Thus, Lm-based vaccines can induce a strong innate and adaptive response [60]. Recombinant strains of Lm can be constructed to express foreign antigens, including tumor antigens. It should be noted that while unmodified Lm is toxic, the vaccines in the clinic include disparate methodologies to attenuate pathogenicity. One method involves the genetic knockout of the ActA and internalin B (InlB) genes, which facilitate cell-to-cell spread and liver homing, respectively [61]. Strains knocked out for ActA and InlB are approximately four logs less lethal, allowing increased dose levels with accompanying increases in antigenicity [62]. That approach has entered phase I testing in prostate cancer, with a live-attenuated double-deleted (LADD) vaccine strain ADU-741, (Aduro Biotech, Berkeley, CA) (Table 35.2). This vaccine strain is unique in that it encodes a total of four prostate-associated antigens—PSMA, PAP, the cancer testis antigen SSX2, and the prostate-associated transcription factor NKX3.1.

A second attenuation approach involves knockout of the listeria lysin O (LLO) gene, which prevents Lm from entering the host cell cytoplasm [63]. That approach (Advaxis, Princeton, NJ) has also entered the clinic in the form of a phase I/II trial in combination with the anti-PD-1 antibody pembrolizumab. Administration of Lm-based vaccines has been shown to decrease the number of regulatory T cells in the tumor environment [64], which impair T cell responses through immunosuppressive cytokines and inhibiting APC function. Additionally, myeloid-derived suppressor cells (MDSCs) also contribute to a highly suppressive tumor environment through production of similar anti-inflammatory cytokines; their numbers and suppressive capabilities were also reduced in models of Lm-based antitumor vaccines [65]. The natural immunostimulatory properties of listeria, along with its ability to be genetically manipulated, show much promise for the development of vaccines for advanced tumors with an established microenvironment, such as mCRPC.

35.4 Adoptive Cellular Therapy

Generalized antitumor treatments may not necessarily be effective in an individual patient, so efforts are underway to develop personalized treatments specific for each patient's tumor. Adoptive cellular therapy (ACT) is a technique in which patient cells, usually lymphocytes, are isolated and genetically manipulated before reinfusion into the patient. Genetic manipulations can involve transducing T cell receptor genes specific for a tumor antigen or introducing chimeric antibody receptors (CARs). CAR T cells express an antibody heavy and light chain linked to downstream activating complexes such as CD3-ζ, allowing them to become activated outside the context of the major histocompatibility complex while retaining specificity [66]. An ongoing phase I trial is examining the effects of CAR T cells with specific for PSMA in mCRPC patients, with safety and tolerability as the primary outcome (NCT01140373). In a relevant recent phase I/II trial, multiple myeloma patients were treated with NY-ESO-1-specific TCR-engineered T cells, with 70% of patients achieving near complete responses or complete responses [67]. This is important, because NY-ESO-1 is a common cancer testis antigen expressed in multiple tumor types [68], including prostate.

35.5 Antitumor Antibodies

Adoptive cellular therapy has the potential downside of providing cells that are relatively long lived, which present certain challenges in terms of long-term toxicity. Antibody-based therapies may offer the same specificity against tumor antigens but with the added benefit of a shorter half-life. The antitumor antibody with the greatest experience in prostate cancer is J591, an anti-PSMA antibody [69]. As a human IgG1 antibody [70], naked or unmodified J591 was hoped to induce antibody-dependent cellular cytotoxicity (ADCC), a cellular process whereby cytotoxic cells, such as macrophages and NK cells, are activated through an interac-

tion between the Fc portion of an antibody and the Fc receptor expressed on their cell surface. In humans, this usually occurs via antibodies of the IgG1 subtype via the interaction with Fc gamma receptor III (FcγRIII) on the surface of NK cells. An uncontrolled (single-armed) phase II trial of J591 and IL-2 in patients with recurrent prostate cancer showed the agent to be well tolerated and found that the survival of the treated cohort exceeded the Halabi-predicted survival, suggestive of a possible long-term survival benefit [71]. Additionally, J591 has also been tested in conjugation with a radioisotope in order to target radiotherapy to prostate tumor cells. A phase II trial of ^{177}Lu-J591 found that eight patients experienced stable disease, with one patient experiencing a partial radiographic response. Additionally, longer survival correlated with higher doses of treatment [72]. Beyond its potential utility as a therapeutic reagent, the ability to conjugate radioisotopes to J591 also offers the ability to image tumors with excellent sensitivity and specificity [73].

A second antitumor antibody in development for prostate cancer targets B7-H3, which is highly expressed on primary tumors and which is markedly associated with a worse prognosis post-surgery [74]. A naked anti-B7-H3 antibody (MGA271, MacroGenics, Gaithersburg, MD) is in phase I development, either alone or in combination with pembrolizumab or ipilimumab. Preclinical studies of this reagent showed interesting responses, fueling enthusiasm for these trials [75].

One interesting approach to immunotherapy involves the use of engineered antibodies to target T cells to tumors. One class of agents like this includes a tumor-specific antibody portion (Fab) and a second portion that is specific for T cells (usually the Fab of anti-CD3). Mechanistically, these reagents are thought to function when the tumor-binding portion binds to its target and the anti-CD3 portion captures passing T cells in a TCR non-specific manner and localizes them to the tumor (Fig. 35.4). Engagement of the CD3 cell surface molecule by antibody may lead to T cell activation and downstream tumor cell death. In the case of prostate cancer, a dual-affinity retargeting (DART™) antibody (MGD009, MacroGenics, Rockville, MD) is in development. This agent targets B7-H3 and CD3, allowing it to traffic to tumor cells expressing B7-H3 and recruit T cells to the site and activate them regardless of antigen specificity (Fig. 35.4). A similar concept using a bispecific T cell engaging (BiTE™) antibody against PSMA and CD3, BAY2010112 (Bayer, Leverkusen, Germany), is also in phase I (NCT01723475) with primary outcomes of maximum tolerated dose and number of adverse events. Antibodies targeting other populations of immune cells also exist, such as AAT-007 (AskAt Inc., Nagoya, Japan), which is an EP4 antagonist in phase II for solid tumors (NCT02538432). EP4 blockade abrogates several suppressive functions of MDSCs, which normally diminish antitumor responses [76].

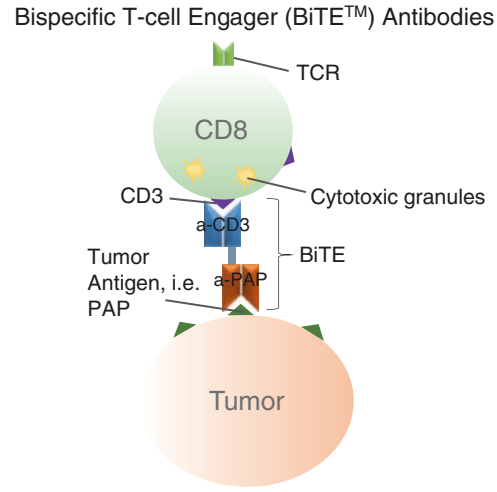

Fig. 35.4 Activation of T cells through BiTE™ antibodies. Engagement of CD3 on T cell by a BiTE™ or DART™ antibody leads activation and cytotoxic activity of the T cell against tumor cell expressing antigen such as PAP, regardless of T cell TCR specificity. A BiTE™ consists of a single polypeptide chain linker, whereas a DART™ has two polypeptide chains and an interchain disulfide bond

35.6 Summary

mCPRC is a deadly disease which is poorly infiltrated by immune cells and for which immunotherapy efforts have generally yielded mixed

results. Therapies that are effective in other solid tumor types, such as the immune checkpoint inhibitors anti-CTLA-4 and anti-PD-1, do not seem to be widely active as a monotherapy in advanced prostate cancer. While low expression of checkpoint ligands on prostate tumors may play an important role in mediating this lack of effectiveness, tumor-intrinsic factors such as a low mutational burden also play a likely role. Conversely, the notion that prostate cancer expresses several key tumor antigens restricted to the organ itself, including PSMA and PSA, may make it a good target for vaccines, which could potentially turn "cold" tumors "hot," and thus susceptible to immune checkpoint blockade.

The suppressive tumor microenvironment in mCRPC poses significant challenges for immunotherapy. Combinatorial treatments, which not only release the "brakes" of immune checkpoints but also improve antigen processing and presentation, may be required. In particular, it may prove necessary to harness the iate immune system through vaccines that toggle pathogen-sensing machinery to create a more inflammatory environment for enhancing activation and function of adaptive immunity. Antibodies can also block interactions required for sustaining the anti-inflammatory tumor environment, including suppressing the function and survival of MDSCs and TAMs. Taken together, these agents may induce a broad spectrum of activation and mobilization of the immune system against prostate tumors. In general, it seems increasingly likely that combinatorial regimens will be required to induce sufficient activity to be clinically meaningful in prostate cancer.

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Challenges of Oncoimmunology for Ovarian and Breast Cancers

36

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36.1 Tumor Microenvironment and Therapeutic Targets of Ovarian and Breast Cancers

The tumor microenvironment (TME) plays an important role in cancer progression and metastasis. Hanahan and colleagues [1] highlighted the contribution of different stromal cells in tumor development and progression, for which metastatic niche formation, metabolic stimulation, stimulation of tumor cell migration, immune modulation, angiogenesis, and matrix remodeling represent major steps in favor of tumor development and maintenance [2]. The TME contains stromal cells, extracellular matrix, and protein factors [3]. The cellular compartment is composed of adipocytes, fibroblasts, and immune and endothelial cells [3]. The extracellular matrix includes hyaluronic acid, proteoglycans, and fibrous proteins (collagen, fibronectin, and laminin) [3]. The protein factors include growth factors, immunoglobulins, cytokines, and chemokines [3]. In breast and ovarian cancer, tumor-associated stromal cells include different subtypes as tumor-associated fibroblasts, adipocytes, endothelial cells, and tumor-associated immune cells (including tumor-associated macrophages) [3]. Here we describe the main cell subtypes that have been characterized in the TME of ovarian and breast cancers (Fig. 36.1).

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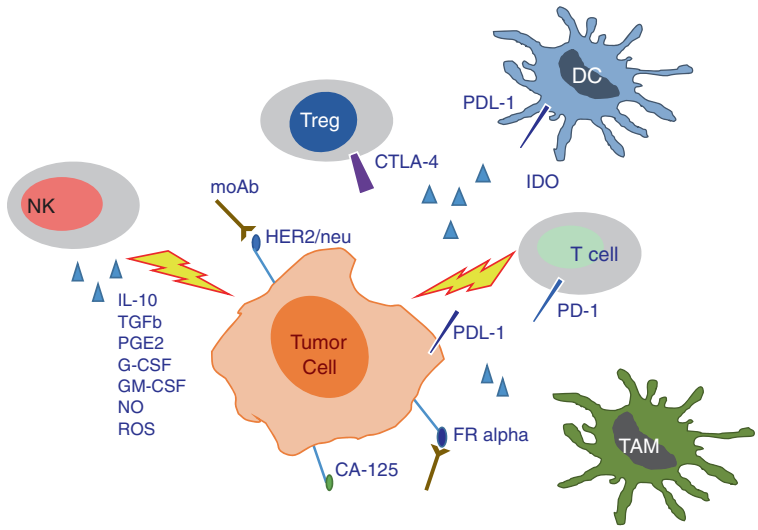

Fig. 36.1 Cellular infiltrates within the tumor microenvironment. Established cancers consist of a wide array of immune cells that contribute to the tumor stroma of a growing malignancy. Tumors possess infiltrating cells of both innate and acquired immunity, such as tumor-associated macrophages (TAM), dendritic cells (DCs), NK cells, lymphocytes, mast cells, eosinophils, and neutrophils. These cells coordinately form a complex regulatory network that fosters tumor growth by creating an environment that enables cancers to evade immune surveillance and destruction. Cell–cell interactions, soluble factors, or enzymes with immunosuppressive properties: *G-CSF* granulocyte colony-stimulating factor, *GM-CSF* granulocyte–macrophage colony-stimulating factor, *NO* nitric oxide, *ROS* reactive oxygen species, *PEG2* prostaglandine E2, *IDO* indoleamine 2,3-dioxygenase, IL-10, TGF, *PD-L1* programmed death 1/programmed death ligand 1, *CTLA-4* cytotoxic T lymphocyte-associated antigen 4. Examples of tumor-associated antigens: HER2/neu, CA-125, folate receptor alpha (FRα) that can be the target of different immunotherapies, including monoclonal antibodies (moAb)

36.1.1 Tumor-Infiltrating Lymphocytes

Tumor-infiltrating lymphocytes (TILs) are frequently found in tumors, suggesting that tumors trigger an immune response in the host. TILs are generally segregated into those that penetrate the tumor islet (intraepithelial) and those that reside in the peritumoral space (stromal) [4]. The immune system, in particular the intraepithelial TIL, is thought to play an extensive role in the control of tumor growth in virtually all solid tumors, and ovarian and breast cancers are no exception. Several reviews have reported the prognostic value of TILs and immune gene signatures in breast cancer. Salgado and colleagues proposed a standardized method of enumerating TILs [5]. In triple-negative breast cancer (TNBC) that does not express the genes for estrogen receptor, progesterone receptor, or HER2/neu, TILs have both a prognostic and a predictive value. In the adjuvant setting, TILs are associated with a better survival regardless of the adjuvant treatment [6, 7]. Several neoadjuvant studies have reported higher pathologic response (pCR) rates among immune-rich breast cancers compared with immune-poor counterparts [8, 9]. Interestingly, each 18% increase in TIL count is associated with an 18% reduction of risk of distant recurrence for patients treated by adjuvant chemotherapy [10, 11]. In HER2-positive breast cancer, TILs are also associated with better prognosis, but results are less concordant regarding their predictive value [12, 13]. The level of immune infiltration is less studied in luminal breast cancers, but the few data available suggest that it may predict for better prognosis in luminal B, highly proliferative subtypes.

The survival benefits of TILs in ovarian cancer have been documented since 1991 [14].

A recent meta-analysis by Hwang et al. sought to evaluate the prognostic value of TILs in ovarian cancer and investigate other factors on prognosis including tumor histology [15]. Using either CD3 or CD8 as identifiable TIL markers, they found ten suitable studies to include in their meta-analysis, which included 1815 subjects. The results demonstrated that lack of intraepithelial TILs is significantly associated with a worse survival among patients (pooled HR: 2.24, 95% CI; 1.71–2.91). Therefore intraepithelial CD8+ TILs appear to be a robust predictor of outcome in ovarian cancer.

36.1.2 Cancer-Associated Fibroblasts

Cancer-associated fibroblasts (CAFs) have emerged as important players in tumor progression and drug resistance [16]. CAFs help the tumor initiation, growth, angiogenesis, invasion, and metastasis. Their pro-tumoral effect is related to the secretion of various extracellular matrix factors such as cytokines, proteases, growth factors, and hormones [17]. CAFs are defined as activated fibroblasts. The difference with normal fibroblasts is on the expression of some molecular markers as α-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), fibroblast-specific protein, and platelet-derived growth factor receptor [18]. CAFs have five different origins in tumor stroma: from normal stromal fibroblast activation, mesenchymal stem cells, pericytes, mammary or ovarian epithelial cells (via the epithelial to mesenchymal transition), or endothelial cells (via the endothelial to mesenchymal transition) [19].

Different therapeutic possibilities have been explored in preclinical studies to decrease CAF number in the TME [20]. Targeting FAP is evaluated in some preclinical and clinical studies. Preclinical studies evaluating FAP blocking alone or in combination with chemotherapy showed decrease in the mice tumor growth in different tumor types [21]. A phase I clinical trial assesses RO6874813, a FAP antibody, in patients with advanced and/or metastatic solid tumors. A phase I study is testing RO6874281 in patients with advanced and/or metastatic solid tumor. RO6874281 is an immunocytokine consisting of anti-FAP/interleukin-2 fusion protein directed against CAF activation. Targeting CAF-derived factors is evaluated in several preclinical and clinical studies. CAFs secrete higher levels of stromal cell-derived factor 1 (SDF-1) than normal fibroblasts. SDF-1 is an important factor in breast tumor progression by binding to its CXCR4 receptor expressed by carcinoma cells. Preclinical studies demonstrated that inhibitors of CXCR4 attenuate breast tumor growth in vivo and in vitro. A phase Ia/Ib study assesses the LY2510924, a CXCR4 peptide antagonist, in patients with advanced refractory solid tumors. LY2510924 is administered in combination with durvalumab, an anti-PD-L1 antibody.

36.1.3 Tumor-Associated Macrophages

Tumor-associated macrophages (TAMs) have emerged as players of tumor progression and metastasis [22]. Human TAMs are broadly divided into two main types: pro-inflammatory M1 macrophages and immunosuppressive M2 macrophages. Breast and ovarian cancer-associated macrophages are mostly infiltrated with the M2 macrophage population [23, 24]. Several studies suggest that circulating monocytes differentiate into M2 macrophages, thanks to different growth factors: colony-stimulating factor 1 (CSF-1/M-CSF), granulocyte–macrophage CSF (GM-CSF/CSF-2), macrophage-stimulating protein (MSP), transforming growth factor-β1 (TGF-β1), chemokines [19], and proangiogenic factors. In addition the pro-tumorigenic effect of TAMs results from their secretion of immunosuppressive cytokines that results in the downregulation of T cell effector functions [24, 25].

Targeting CSF1/CSF1 receptor has been evaluated in preclinical and clinical studies. Pharmacological or genetic deletion of CSF1 or CSF1R inhibits the mammary tumor growth in

mice models [26, 27]. In fact, CSF1R inhibition delays cervical and mammary tumor growth in murine models by the attenuation of TAMs turnover and enhanced infiltration of CD8 T cells. CSF1 inhibition by CSF1/CSF1R blockade or its pathway inhibition allowed to decrease macrophage infiltration of the TME associated with increased CD8 T cell numbers and decreased mammary tumor growth in a mouse model [27]. In several mouse models, CSF1R inhibition in combination with paclitaxel leads to increased survival and to decreased risk of metastases [28].

Therefore, CSF1/CSF1R blocking has been tested in clinical studies. In a clinical phase I study [29], in patients with various advanced solid tumors, inhibition of CSF1R dimerization with RO5509554 decreased macrophage infiltration and induced CD8/CD4 ratio increase with an acceptable safety profile. This humanized anti-huCSF1R IgG1 monoclonal antibody blocks ligand-dependent and ligand-independent receptor activation. Three open clinical phase I trials assess the safety, pharmacokinetics, and activity of RO5509554 in patients with advanced solid tumors. RO5509554 is given as monotherapy and in combination with paclitaxel or atezolizumab, a monoclonal antibody blocking PD-L1. Three clinical phase I trials are open with LY3022855, another CSF1R inhibitor, alone or in association with different molecules. LY3022855 is a human IgG1 monoclonal antibody designed to target the CSF1R. Two studies assess LY3022855 alone in patients with advanced, refractory breast cancer or with advanced solid tumors. One study evaluates LY3022855 in combination with durvalumab or tremelimumab in patients with advanced solid tumors. One phase II study is open, with a humanized monoclonal antibody target CSF-1(MCS110). This randomized study assesses the efficacy of MCS110 given with carboplatin and gemcitabine in advanced triple-negative breast cancer. Another phase Ib/II study assesses MCS110 in combination with PDR001, an anti-PD-1 monoclonal antibody, in patients with advanced malignancies.

In addition, several studies are focusing on CSF1R kinase inhibitors. Two phase I studies assess pexidartinib alone or in combination with paclitaxel in patients with advanced solid tumors. Pexidartinib is an oral, potent multi-targeted receptor tyrosine kinase inhibitor of CSF1R, Kit, and Flt3. A phase I study evaluates BLZ945 alone or in combination with PDR001 in adult patients with advanced solid tumors. BLZ945 is an orally active, potent, and selective CSF1R inhibitor.

36.2 Oncoimmunology Challenges in Ovarian Cancer

Each year almost 90,000 women in the United States are diagnosed with gynecologic malignancies, and over 28,000 will die from their disease [30]. Many women with early-stage disease are cured with a combination of surgery, radiation, and chemotherapy. However, especially in the case of ovarian cancer, the disease is often diagnosed at advanced stage, and many patients relapse despite appropriate management. Even with improved cytoreductive surgery associated with platinum-/taxane-based chemotherapy, the prognosis remains poor with a 5-year survival rate of 38%.

Active therapeutic targets in recurrent ovarian cancer include DNA damage repair and vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) signaling pathways. Bevacizumab, a humanized anti-VEGF monoclonal IgG1 antibody, showed improved progression-free survival in adjuvant setting [31]. Emerging data indicate that the VEGF and VEGFR pathways modulate immune response by increasing DNA damage and tumor mutational load [32, 33]. Mutational load, leading to increased potential neoantigen expression, has been associated with clinical response to immune checkpoint inhibitors in colorectal cancer and melanoma [34, 35]. Approximately 50% of high-grade epithelial ovarian cancers have acquired or inherited dysfunction in homologous recombination, high-fidelity DNA double-strand break repair [36]. All high-grade epithelial ovarian cancers have genomic instability that is partially associated with loss of normal p53 function. Poly(ADP-ribose) polymerase (PARP) 1 has many roles in DNA damage repair,

including repair of single-strand DNA breaks via the base excision repair pathway [37]. Olaparib, a PARP inhibitor, improved survival of women with BRCA-muted tumors [38]. Preclinical studies have shown that PARP inhibitors promote local antigen release, resulting in systemic antitumor response after tumor exposure to radiation or DNA-damaging agents or secondary to spontaneous or heritable defects in DNA [39]. Increased DNA damage resulting from PARP inhibitors or exposure to other DNA repair inhibitors would thus yield greater mutational burden and expand neoantigen diversity.

PARP inhibitors are also associated with immunomodulation. Huang et al. reported that talazoparib (BMN 673) increased the number of peritoneal CD8+ T cells and natural killer cells and increased production of interferon-gamma and tumor necrosis factor-alpha in a *BRCA1*-mutated ovarian cancer xenograft model [40]. These data suggest that PARP inhibitors may be complementary to immune checkpoint modulation in yielding clinical benefit in recurrent ovarian cancer. Currently, a phase I/II study of the programmed death (PD) ligand 1 (PD-L1) inhibitor MEDI4736 (durvalumab) in combination with olaparib is being conducted in patients with solid tumors and recurrent ovarian cancer (ClinicalTrials.gov identifier: NCT02484404). Angiogenesis pathways interact with both immune response and DNA repair mechanisms. Tumor hypoxia induces downregulation and decreased expression of genes and proteins involved in DNA damage repair, leading to further DNA damage, genomic instability, and cell death [41]. VEGF has been shown to reduce the antitumor immune response in preclinical and clinical models, including suppression of dendritic cell maturation, inhibition of T cell responses, and an increase in regulatory T cell proliferation and accumulation of myeloid-derived suppressor cells (MDSCs) [42]. In addition, PD-L1 expression was upregulated under hypoxic conditions in a panel of mouse and human tumor cell lines, as well as in splenic MDSCs, through a hypoxia-inducible factor-1α-dependent mechanism. Angiogenesis inhibitors are active in gynecologic cancers; bevacizumab [43] and the oral VEGFR 1–3 tyrosine kinase inhibitor cediranib [44] have been associated with improved progression-free survival.

While it was originally felt that epithelial ovarian cancer would not respond well to immunotherapy, research has, in fact, demonstrated a key role for the immune system in the control of epithelial ovarian cancer cell growth. This is supported by the observation that increased levels of TILs in ovarian cancer were associated with improved prognosis, with a 5-year survival of 38% in patients whose tumors contained T cells and 4.5% in those whose tumors did not contain T cells [45]. In a separate study focusing on the subtypes of T cells in ovarian cancer, a higher frequency of tumor-infiltrating CD8+ lymphocytes and increased ratios of CD8+ lymphocytes to regulatory T cells (Tregs) were also found to be associated with improved survival [46]. In addition, tumor-reactive antibodies and T cells have been isolated from the peripheral blood of patients with epithelial ovarian cancer, suggesting a spontaneous antitumor immune response [47]. In addition, tumor antigens as CA-125, folate receptor alpha (FRalpha), NY-ESO-1, and MAGE-A3 are overexpressed in ovarian cancer and can be exploited to elicit an adaptive immune response. These studies have provided the rationale for exploring different immunotherapeutic strategies in epithelial ovarian cancer.

36.2.1 Therapeutic Approaches to Enhance Tumor Antigen Recognition

Strategies that aim to enhance tumor recognition by the immune system can be collectively grouped into vaccines and innate immune activators; included in the second group are TLR agonists, type I interferon (IFN), and oncolytic viruses.

36.2.1.1 Vaccines

The identification of unique tissue differentiation antigens expressed in epithelial ovarian cancer has led to the exploration of various vaccination approaches, including simple vaccine preparations consisting of specific peptides and proteins,

as well as more complex strategies, such as engineered, cell-based vaccines, DC vaccines, virus-vectored vaccines, and oncolytic viruses. The majority of studies have explored the cancer–testis antigens (e.g., NY-ESO-1) and proteins known to be overexpressed in epithelial ovarian cancer (e.g., p53, survivin, HER-2, and MUC1). For instance, it has been shown that 43% of advanced ovarian cancer expresses NY-ESO-1. One phase II study published in 2014 evaluated vaccination of 22 patients with epithelial ovarian cancer (82% serous histology and NY-ESO-1 positive) with a HLA-A*0201-restricted NY-ESO-1b peptide after standard treatment (debulking surgery and platinum-based chemotherapy). The median overall survival was 48 months, and the median time of disease progression was 21 months (95% CI, 16–29 months) [48]. Although many studies have demonstrated induction of an immune response to the vaccines, very few have demonstrated clinical benefit. It is likely that these strategies are insufficient to overcome immune tolerance to self-antigens and to result in efficient activation of antigen-specific T cells, although they may prove to be valuable in combination with other therapies.

36.2.1.2 Innate Immune Activators

Another strategy for enhancing tumor antigen presentation by APCs involves agents that target the innate immune response. Antigen processing and presentation by APCs require activation signals, which are provided via activation of pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs) [49]. TLRs recognize signature molecules that are broadly shared by various pathogens and, in addition, sense "danger signals" in the tumor microenvironment, which consist of endogenous molecules produced by dying cells. A phase I study of VTX-2337 (motolimod), a small-molecule agonist of TLR8, in combination with liposomal doxorubicin in patients with advanced epithelial ovarian cancer, demonstrated safety and evidence of immune activation and clinical benefit. Several phase II studies evaluating motolimod in combination with other immunotherapies or liposomal doxorubicin (ClinicalTrials.gov identifiers: NCT02431559, NCT01666444) are ongoing.

Activated APCs produce type I IFN, which plays a role in the antiviral immune response; it has also been demonstrated to be necessary for tumor antigen presentation by APCs [50]. Although type I IFN has been evaluated in various cancer types and is approved for use as adjuvant therapy in patients with resected melanoma, in a study by Alberts et al., systemic or intraperitoneal administration of IFNα had limited activity in patients with epithelial ovarian cancer and was associated with frequent toxicities [51].

Oncolytic viruses have intrinsic properties that allow them to replicate in cancer cells, while sparing normal tissues. While serving as tumor-debulking agents, oncolytic viruses also activate the innate immune response on multiple levels through the release of tumor antigens, PRR ligands, and danger signals, and via production of type I IFN. Several trials using oncolytic viruses in patients with epithelial ovarian cancer have demonstrated safety and durable clinical benefit in some patients [52]. Overall, strategies to enhance tumor antigen presentation by the innate immune system have been demonstrated to be safe, but to date, their efficacy has been marginal. The future of drugs that enhance tumor antigen presentation in patients with epithelial ovarian cancer probably will rely on combination therapies, in which the T cell response primed with a vaccine or an innate immune activator is further strengthened by therapies targeting T cell activation and adaptive immune responses.

36.2.2 Therapeutic Approaches that Enhance T Cell Activation

The survival, proliferation, and activation of T cells are controlled by a variety of factors, including cytokines and a range of immunostimulatory and inhibitory receptors. Several studies have explored agents targeting T cells as immunotherapy in epithelial ovarian cancer, including drugs that target pathways of T cell activation, as well as adoptive T cell strategies.

36.2.2.1 Cytokines

The cytokines interleukin (IL)-2 and IL-12 are potent activators of T cell proliferation and cytotoxicity. Their use as anticancer agents has been explored in multiple types of cancer, including ovarian. The use of both agents, administered systemically, is limited by toxicity. A phase I/II study of intraperitoneal IL-2 in patients with persistent or recurrent epithelial ovarian cancer showed an overall response rate of 25.7%, although the regimen was associated with significant toxicity [53]. A different strategy for delivery of IL-12—the use of IL-12-expressing plasmids—has been explored. In a recent study, 22 patients with recurrent epithelial ovarian cancer who received intraperitoneal EGEN-001, an IL-12 plasmid formulated with lipopolymer, demonstrated a 35% stable disease rate [54].

36.2.2.2 Immune Checkpoint Blockade

Identification of the costimulatory and coinhibitory receptors that regulate T cell activation led to the development of antibodies that target these receptors [55]. In particular, antibodies targeting the inhibitory receptors cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed death 1 (PD-1), and PD-1 ligand (PD-L1) are in advanced clinical development, with the CTLA-4-targeting agent ipilimumab approved in metastatic melanoma and the PD-1-targeting agents nivolumab and pembrolizumab approved in metastatic tumors of multiple histologies.

Based on these findings, therapy with immune checkpoint blockade has been evaluated in trials in patients with epithelial ovarian cancer. Despite its activity in metastatic melanoma, the efficacy of the CTLA-4-targeting antibody in epithelial ovarian cancer as a single agent has so far been limited. In 11 patients with epithelial ovarian cancer who received GVAX, an autologous tumor cell vaccine expressing granulocyte–macrophage colony-stimulating factor, treatment with ipilimumab led to an objective response in one patient that was durable for over 4 years [56]. In contrast, emerging clinical data indicate that targeting of PD-1 and PD-L1 may be a promising strategy in epithelial ovarian cancer. Hamanishi and colleagues published a phase II study of the anti-PD-1 antibody nivolumab in 20 evaluable patients with heavily pretreated, advanced, platinum-resistant, epithelial ovarian cancer [57]. Histology was serous carcinoma in 75%, endometrioid in 15%, and clear cell in 10% of cases. Patients received a dose of 1 or 3 mg/kg every 2 weeks (constituting two ten-patient cohorts). Patients received up to six cycles (four doses per cycle) of nivolumab or until disease progression. Grade 3 or 4 treatment-related adverse events occurred in eight (40%) patients. The best overall response was 15%, which included two patients who had a durable complete response (in the 3-mg/kg cohort). The disease control rate was 45%. The median progression-free survival time was 3.5 months (95% CI, 1.7–3.9 months), and the median overall survival time was 20 months (95% CI, 7.0 months to not reached) at study termination. Similar activity was reported for the PD-L1-blocking antibodies avelumab and pembrolizumab, with response rates ranging from 11 to 17% and disease control rates of up to 65%. Larger studies using these agents are currently underway.

The combination of CTLA-4 and PD-1 blockade has been associated with additive and even synergistic activity in animal models. A recent phase III study evaluating combined CTLA-4 and PD-1 blockade (with ipilimumab and nivolumab, respectively) in patients with melanoma demonstrated enhanced response rate and progression-free survival compared with either agent alone, leading to recent US Food and Drug Administration approval of the combination for the treatment of melanoma [58], although the regimen did result in high rates of grade 3 toxicity. An ongoing NRG Oncology Group randomized phase II study (ClinicalTrials.gov identifier: NCT02498600) is comparing the combination of nivolumab and ipilimumab vs nivolumab alone to determine whether the combination is also active and safe in patients with epithelial ovarian cancer who have relapsed.

36.2.2.3 Adoptive T Cell Therapies

Adoptive cell therapies (ACTs) rely on the infusion of large numbers of autologous tumor-reactive

T cells that have been isolated from tumors and expanded in vitro. Early studies reported significant efficacy for this approach in epithelial ovarian cancer [59], although these studies were necessarily biased by the selection of patients from whom a sufficient quantity of TILs could be isolated. Additional studies using ACT in epithelial ovarian cancer are ongoing (ClinicalTrials.gov identifiers: NCT02482090, NCT01883297). As an alternative strategy, engineered T cell technologies avoid the need for isolation of TILs. Using this strategy, peripheral blood autologous lymphocytes are transduced either with a T cell receptor that recognizes a specific tumor antigen MHC peptide or with a chimeric antigen receptor (CAR) that recognizes a tumor-associated surface antigen. The efficacy of such approaches has been demonstrated in preclinical studies, in which engineered T cells expressing a MUC16-specific CAR were associated with complete eradication of orthotopic ovarian xenografts [60]. A phase I study using this strategy is currently in development (ClinicalTrials.gov identifier: NCT02498912). Several phase I and II trials of adoptive T cell transfer techniques are currently under way targeting other ovarian cancer-associated proteins—such as folate receptor alpha, mesothelin (ClinicalTrials.gov identifier: NCT01583686), MAGE-A3 (ClinicalTrials.gov identifiers: NCT02111850, NCT01567891), and NY-ESO-1 (ClinicalTrials.gov identifiers: NCT01967823, NCT01697527, NCT02457650).

36.2.3 Therapeutic Approaches that Block Other Axes of Immune Inhibition

Despite provocative early clinical data, it is becoming increasingly apparent that the benefit of immune checkpoint blockade in epithelial ovarian cancer is not universal and that development of predictive biomarkers and combination therapies will be necessary. To this end, combination strategies using PD-1- and PD-L1-blocking antibodies together with antibodies targeting other mechanisms of T cell activation (e.g., glucocorticoid-induced tumor necrosis factor receptor-related protein [GITR], OX40, 4-1BB), as well as antibodies targeting other immune checkpoints (e.g., lymphocyte-activation gene [LAG3] and T cell immunoglobulin and mucin domain containing-3 [TIM3] [61]), are already entering clinical trials in multiple tumor types. In addition, several immune inhibitory mechanisms have been associated with poor prognosis in epithelial ovarian cancer, including tumor-infiltrating Tregs, TAMs, myeloid-derived suppressor cells (MDSCs), and the expression of IDO by the tumor or stromal cells [62] (ClinicalTrials.gov identifiers: NCT02327078). There is thus a strong rationale for targeting these mechanisms in combination with PD-1/PD-L1 blockade. Epacadostat, an orally available hydroxyamidine and inhibitor of IDO1, is being currently tested in phase I/II clinical studies in advanced ovarian and triple-negative breast cancer in monotherapy or in combination with checkpoint inhibitors.

36.2.4 Monoclonal Antibodies

Several antigens related to ovarian cancer have been isolated that could be potential target of monoclonal antibodies (mAbs); therefore, different mAbs are emerging as new potential treatments for this disease. Among others, mAbs targeting CA-125 (farletuzumab, ClinicalTrials.gov identifier: NCT02289950), folate receptor alpha (mirvetuximab soravtansine, ClinicalTrials.gov identifiers: NCT02631876, NCT02606305), and delta-like ligand 4 (DLL4) (demcizumab, ClinicalTrials.gov identifier: NCT01952249) are in clinical development.

36.3 Oncoimmunology Challenges in Breast Cancer

Breast cancer is the most commonly diagnosed cancer in women worldwide. During the last decade, we have observed significant progress in its management following the introduction of novel agents such as chemotherapy (eribulin), anti-HER2 compounds (TDM-1, pertuzumab), or cell cycle inhibitors (palbociclib). However,

advanced and metastatic breast cancer remain incurable and a leading cause of cancer deaths with approximately 11,800 deaths in 2012, a median overall survival of 2–3 years, and a 5-year survival of only 25%. There is therefore a real unmet need of new therapeutics. Interest in evaluating immunotherapy in breast cancer has historically been limited based on the belief that breast tumors were nonimmunogenic. However, recent data have shown that breast cancers, particularly HER2-positive and triple-negative tumors, are in fact immunogenic and that the extent of the immune response correlates with prognosis. We will discuss emerging results from clinical trials evaluating immunotherapeutic agents, including vaccines, immune checkpoint agents, and TME-targeting agents, in breast cancer that have shown promise in this disease.

36.3.1 Vaccines

During the last decade, several experimental cancer vaccines have been tested in metastatic solid tumors, but this approach appeared to be ineffective in advanced breast cancer. A paradigm shift has emerged in the recent years, and cancer vaccines are more commonly tested in the adjuvant setting or in the early stages of carcinogenesis [63]. Peptide-based vaccines are the most extensively studied, and they target overexpressed, amplified, or mutated proteins such as HER2, MUC1, CEA, p53, mammaglobin A, and cancer–testis antigens. HER2-derived vaccines have been widely investigated and, in particular, E75 (nelipepimut-S, NeuVax™) consisting of a human leukocyte antigen (HLA)-A2/A3-restricted immunogenic peptide derived from the extracellular domain of HER2 protein is in advanced development. In the adjuvant setting, a phase I/II trial enrolled 195 HER2 positive patients. The 5-year disease-free survival for the vaccinated patients was 89.7% compared to 80.2% for the control group with mild local and systemic toxicities [64]. Based on these encouraging data, the PRESENT study, a phase III in early-stage breast cancer, has been launched (ClinicalTrials.gov identifier: NCT 01479244); however, the trial was discontinued due to futility in accordance with the recommendation from the Independent Data Monitoring Committee in June 2016.

36.3.2 Immune Checkpoints

Preliminary results from the first immune therapy clinical trials suggested that some breast cancers are indeed responsive to immunotherapy with immune checkpoint blockade (Table 36.1).

36.3.2.1 Luminal Breast Cancers

In a phase I, tremelimumab has been evaluated in association with exemestane, an aromatase inhibitor in 26 patients. No objective responses were observed, but interestingly 42% of the patients experienced a disease stability for at least 6 weeks (Vonderheide et al. 2010). In the multicohort phase I trial Keynote-028, 25 patients with PD-L1-positive luminal breast cancer were treated by pembrolizumab, an anti-PD1. The overall response rate was 12% and a clinical benefit rate of 20% was observed in this heavily treated population (SABC 2015 [65]). In the JAVELIN study, patients were treated with avelumab, an anti-PDL-1, irrespectively of their PDL-1 status. In the luminal cohort, only 2.8% of overall response rate (ORR) was observed (SABC 2015 [66]).

36.3.2.2 Triple-Negative Breast Cancer

The Keynote-012 is the first trial to report clinical outcome of anti-PD1 in metastatic triple-negative breast cancer. It is a phase Ib evaluating pembrolizumab in 27 pretreated patients with a PDL-1-positive disease. Pembrolizumab was associated with an overall response rate (ORR) of 18.5% and a progression free survival (PFS) of 23% at 6 months [67]. Atezolizumab has also been evaluated in this population and similar a degree of benefit has been observed in monotherapy. In a PDL-1-positive population, the ORR was 24% and the PFS at 6 months 27% (AACR 2015, Emens et al., #2859). In the JAVELIN study, in a non-selected population, an ORR of 8.6% has been reported with avelumab.

Table 36.1 Main results of immune checkpoints inhibitors in breast cancer

Target	Drug	Trial	Subtype	PD-L1 status	PD-L1+	Evaluable patients	Results	References
PD-1	Pembrolizumab	Keynote-012	TN	PD-L1+	59%	27	ORR: 18.5%	Nanda, JCO (2016)
		Keynote-028	ER+/HER2−	PD-L1+	19%	25	SD: 26% ORR: 12%	Rugo, SABCS (2015)
	Pembrolizumab + eribulin	–	TN	Indifferent	44%	39	ORR: 33% SD: 28%	Tolaney, SABCS (2016)
PD-L1	Atezolizumab	–	TN	PD-L1+	69%	21	ORR: 24%	Emens, AACR 2015
	Atezolizumab + nab-paclitaxel	–	TN	Indifferent	–	24	ORR: 42% SD: 21%	AdamsS, ASCO (2016)
	Avelumab	Javelin	TN ER+, HER2+	Indifferent	–	58 72 26	ORR: 8.6% ORR: 2.8% ORR: 3.8%	Dirix LY, SABCS (2015)
CTLA-4	Tremelimumab + exemestane	–	ER+/HER2−	Indifferent	–	26	BOR: 42%	Vonderheide, CCR (2010)

TN triple negative, *ER* endocrine receptor, *ORR* overall response rate, *SD* stable disease, *BOR* best overall response

Combinations of checkpoint inhibitors with chemotherapy are currently explored. In a phase I trial of atezolizumab in association with nab-paclitaxel, a 38% ORR was observed, irrespectively of the PD-L1 status. It is interesting to note that more than 80% of the population was treated with taxane. Given these encouraging data, IMpassion130, a phase III comparing nab-paclitaxel plus atezolizumab versus nab-paclitaxel plus placebo, is currently ongoing in first line (ClinicalTrials.gov identifier: NCT0242891).

In 2016 during the San Antonio Breast Cancer Symposium (SABCS), Tolaney et al. reported also encouraging results of the association of pembrolizumab with eribulin. In a phase Ib/II, the combination demonstrated 33.3% ORR in pretreated patients. Moreover, in the first line setting, the ORR was 41%, irrespective of PD-L1 status (SABC 2016 [66]). Taken together, these response rates are potentially better than one might expect for a population with very heavily pretreated, chemotherapy-resistant triple-negative disease. Of further note, when responses occurred, they were often durable—occasionally lasting beyond 1 year—a phenomenon that is well described with these strategies in other settings, but would not typically be expected with chemotherapy in a heavily pretreated population.

36.3.2.3 Future Directions in Breast Cancer

It is anticipated that the interest in identifying immunotherapeutic approaches to treat breast cancer will continue to grow and that numerous trials evaluating immunotherapeutic approaches, including monoclonal antibodies, vaccines, and checkpoint blockade, as well as adoptive T cell therapy with genetically engineered T cells or immunomodulatory agents such as cytokines or toll-like receptor (TLR) agonists, will become available for patients with breast cancer. The success of immunotherapy in breast cancer likely will depend on identifying the appropriate immunotherapeutic strategy for the particular disease type and stage. For example, the microenvironment of metastatic lesions is quite hostile to the immune system. Immunosuppressive cytokines and cells inhibit an effective antitumor immune response. To this point, it should be noted that although breast tumors have been shown to be immunogenic, the immune response is not always robust, with one study reporting that the median percentage of stromal area infiltrated with TILs is only 10% in hormone receptor-positive breast cancer, 15% in HER2-positive breast cancer, and 20% in TNBC [10]. Given the lack of immune infiltrate, it is possible that checkpoint blockade may not work as monotherapy. A strategy, therefore, in which an agent such as a vaccine or perhaps a toll-like receptor agonist is given to stimulate an immune response that can be potentiated and maintained with checkpoint blockade may prove more efficacious.

Conclusions

Recent years have seen many advances in immunotherapeutic approaches to various cancer types, and breast and gynecologic malignancies are no exception. Promising early data reported with immune checkpoint inhibitors make it likely that these agents will eventually become part of the treatment arsenal for these cancers. These data also point out, however, that checkpoint inhibitors are not universally effective as single agents, indicating a need for rationally designed treatment combinations. The optimal activation of the antitumor immunity will involve targeting different components of the immune response, which are likely not to be universal, since mechanisms of immune evasion differ from patient to patient. Clinical trials incorporating appropriate biomarkers to identify new immunotherapeutic modalities will allow us to select treatments for the appropriate patients and will inform the development and use of combination therapies that may help overcome current limitations.

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Challenges in Colorectal Cancer: From Vaccines to Macrophage Repolarization

37

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The human body in its entirety as an organism is perceived as the "self," a unit of cells that is functionally connected to each other. The key differentiation between "self" and "nonself" is essentially made by the immune system. This fundamental role in the interaction with other cells and foreign substances is especially important at the interfaces of the body: skin, lung, and intestine. The two central regulatory systems within the immune system are the innate and the acquired immune system. From a developmental standpoint, the innate immune system is the first system with which multicellular organisms defend themselves against influences from outside. The acquired (or adaptive) immune system is evolutionarily younger and offers the advantage of greater flexibility and—quite importantly—of the immunological memory.

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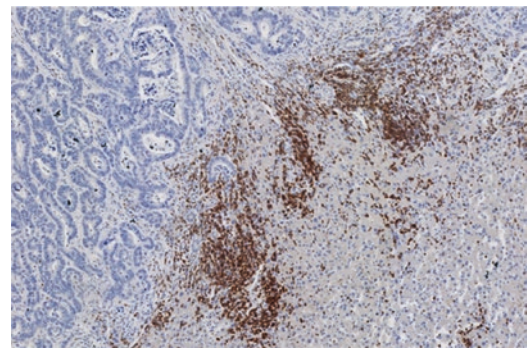
Together these two interlocking systems form the functional immune system. The following paragraphs will present the current view on the local immunological microenvironment and the association between the microenvironment and the course of the disease (see Fig. 37.1 for an overview). Then, clinical data will be discussed together with the prospects of immunotherapy for colorectal cancer.

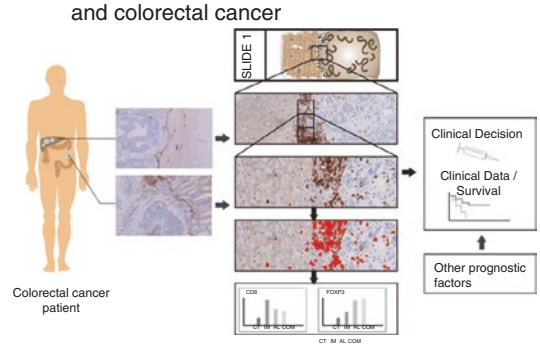
37.1 The Immune System in the Colon and in Colorectal Cancer

The delicate balance between innate and adaptive immune system is of great importance in areas, where there is a continuous challenge of the immune system. This especially holds true for the intestinal tract. As an organ of food intake and resorption, there is a continuous influx of bacteria, viruses, foreign materials of all kinds, and noxious substances. This constant challenge of the immune system requires a finely regulated protection system, in particular on immunological level. Within such a fine tuned and balanced microenvironment, the occurrence of an intestinal tumor is a disaster on various levels. Both the innate and the adaptive immune system can recognize tumor cells as "nonself" and seek out to destroy these. So beyond protection against pathogens, this task is therefore of fundamental importance for our organism, as the detection of malignant cells—or cells that are transformed—is essential for the conservation of the cooperative integrity of the organism. In the event of tumor growth, this function is disturbed and the immune system is not able to fully contain the

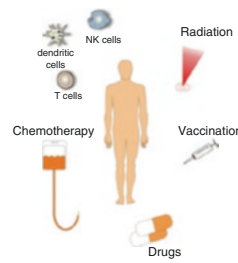
Immunobiology of the colon and colorectal cancer



Immunobiology of colorectal cancer (liver) metastases



Prognostic and predictive aspects of the immunological microenvironment for individual patients



Clinical strategies for immunotherapy of colorectal cancer

Fig. 37.1 Overview

outgrowth of the tumor. In case of the intestine, the distinction between "nonself" and "self" is a highly complex challenge. The intestine as a niche for a huge number of bacteria that are necessary for the homeostasis of the human organism is essential. This leads to the obvious task of the intestinal immune system to keep this microcosm in balance without going to the extreme of an endless process of inflammation or—on the other side of the spectrum—infections. Inflammation is a well-known factor contributing to the neoplastic process. Chronic inflammatory bowel disease induces fundamental alterations in the complex balance in the local microenvironment. This is also reflected in the bacterial species that can be found within the intestines. The occurrence of specific invasive bacterial species [1] or the loss of protective species [2] is frequently observed. This is coupled with a significant dysregulation of the local immune system [3, 4]. It is striking that the resulting inflammatory microenvironment can be ameliorated by stool transplantation [5]. In the event of sustained inflammation, it is not unexpected to see a higher rate of tumor incidence. The precise mechanisms behind the maintenance of the delicate balance and the complexity of specific changes that lead to tumor promotion are hidden behind a complex network of (immune) cells and signaling molecules. Part of these complex networks are, e.g., dietary and lifestyle factors as well as regulatory cytokine networks within the mucosa. On the basis of large epidemiological and scientific studies, evidence suggests that the risk of colorectal cancer is increased by processed and unprocessed meat consumption but suppressed by diets rich in fiber. Moreover food composition affects colonic health and cancer risk via its modulation of colonic microbial metabolism. Gut microbiota can ferment complex dietary residues that are resistant to digestion by enteric enzymes. In this process, the release of short-chain fatty acids (including butyrate) is utilized for the metabolic needs of the colon and the body in its entirety. On a more detailed level, butyrate has a distinct effect of colonic health-promoting and antineoplastic properties. It can promote maintained mucosal integrity, and it suppresses inflammation and carcinogenesis through effects on immunity [6], gene expression, and epigenetic modulation. Only with new integrative analyses do we begin to understand the multitude of interlocking networks [7, 8]. For example, we now begin to understand, why increased iron intake can lead to tumor promotion. But beyond the parameters that influence malignant transformation and tumor initiation, the question of the role of the immune system in the tumor progression and metastatic cascade has many facets.

37.2 Immune Cells in Metastatic Colorectal Cancer: Factors for Prognosis and Therapy

This especially relates from an immunological view the invasion of tumor cells in surrounding tissues, lymph nodes and other organs. In these steps there are numerous interrogations of cellular immunologic interactions between tumor cells and the specific immune cells resident in the respective tissues. The precise interactions and the signals involved are still poorly understood. While passing through radically different immunological microenvironments, tumor cells on their way from the tumor can invade the pericolic fat, pass through the lymph vessels, travel the peripheral blood, and spread to organs like the liver and the lungs. Starting with the primary tumor (see Fig. 37.2), one of the important immunological questions is: what immune cells are implicated in colorectal cancer? The most prominent subgroup of immune cells in colorectal carcinoma are lymphocytes. One of the first descriptions related to immunological processes in colorectal cancer can be attributed to pathologists observing the presence of lymphoid structures in the vicinity of colorectal cancer primary tumors [9]. This finding was observed in addition to the known presence of Peyer's plaques (and other lymphoid structures) in the intestine. Only with the availability of specific staining procedures to better characterize the immunological infiltrate, pathologists, and immunologists began to systematically investigate the presence of immune cells in and around colorectal cancer [10, 11]. Not only was the presence of effector T cells confirmed by multiple groups but also the association between the composition of the local microenvironment and the clinical course of the

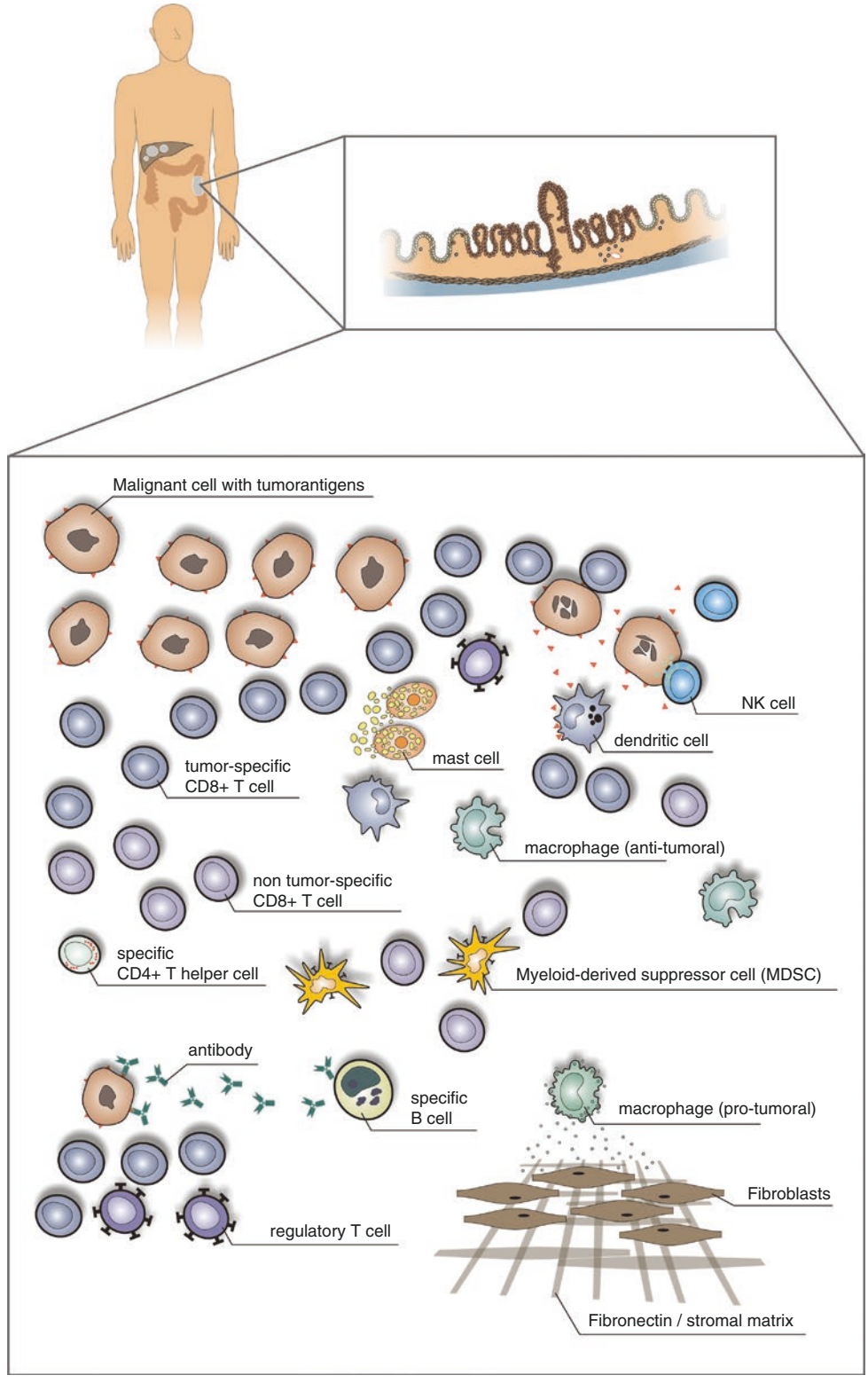


Fig. 37.2 The immunological context of the local microenvironment (from patient to microenvironment and back)

disease across all stages of the primary tumor [11, 12]. Even for the adjuvant situation in stage III colorectal cancer, the relation of T cell density and improved overall survival was shown [13]. The analyses of the primary colorectal tumor showed especially the prognostic role of effector T cell quantities, as characterized by CD3 and CD8 surface markers. These markers, together with granzyme B, have been shown to be of relevance by independent groups and also in other cancer entities [14–22].

37.3 Measuring the Microenvironment: Quantification of Immune Cells in Tissues

So with the step from evaluation of lymphocytic infiltration on H&E sections to automated quantification of specifically stained lymphocyte subsets, the question of the selection of the best markers appeared. This paragraph discusses the general aspects of immune cell quantification as well as two examples that highlight ambiguities and uncertainties in this field. Classical surface markers of effector T cells are CD3, CD4, and CD8, CD3 as a pan-T cell marker and CD4 as a marker for the T-helper cells. Yet there is another T cell marker that has produced conflicting results: FOXP3. This nuclear transcription factor is found mainly in regulatory T cells, a subset of lymphocytic T cells that have inhibitory functions. Initially it was believed that their presence indicates an unfavorable microenvironment with an abrogated immune response. However, the systematic analyses in colorectal cancer revealed a different picture: they showed a positive prognostic relevance not only for CD3, CD8, granzyme B, and CD45R0 positive cells but also for FOXP3-positive lymphocytes [23]. This indicates, that in contrast to the assumptions, a higher density of FOXP3-positive lymphocytes is related to a better prognosis [24]. The explanation for this phenomenon has two components that have been clarified in the meantime. First, the immune response needs a certain level of focusing, a focusing that is driven by regulatory T cells. So a loss of regulatory and focusing immune cells leads to an abrogation of an effective immune response [25]. Second, the further analysis of FOXP3-positive lymphocyte populations revealed relevant functionally diverse subsets. So the use of FOXP3 to identify a functionally inhibitory and for the patient detrimental lymphocyte subset is not specific enough [26, 27]. In addition to the questions around ideal surface markers for quantification, the spatial distribution of these immune cells is another important parameter. The localization of immune cells is not random within the tumor tissue, but the vast majority of lymphocytes is localized within the stromal compartment [15]. Only a minor fraction of these lymphocytes is in direct contact with the tumor epithelium. One of the implications of this observation is that an antitumor effect probably is induced by the cytokines of T cells [28]. Recent data however shows that with a high-resolution analysis of the localization of T cells, the T cells directly in contact with tumor cells are the largest contributors to the prognostic benefit of increased T cell densities [29]. Looking at lymphocytes at the level of the tissue microenvironment, another important group of lymphocytes is natural killer (NK) cells. These innate immune cells with their excellent tumor killing properties were thought to have also an important role in the protection against tumor progression. NK cells have, in contrast to the T cells, a decisive advantage: tumor cell killing is antigen independent. NK cells are capable of activation if inactivating ligands are not present on the surface of target cells [30]. Particularly in the case of colorectal cancer cells, the destruction of tumor cells with loss of HLA class I molecules by NK cells has been very effective in in vitro experiments. Therefore the hypothesis was for NK cells to be contributors to a good prognosis in tumor tissue. Systematic analyses of colorectal cancer specimens with specific identification of localization and density of NK cells within the tissue revealed a different picture [31]. Already at a very early stage, in adenomas the numbers of NK cells in the tumor are massively decreased. This stands in stark contrast to the presence of chemokines and adhesion molecules that would support

the presence and activation of NK cells. This surprising observation was found in all stages from adenoma to colorectal cancer liver metastases. Similar data could also be obtained from breast cancer [32–34]. Another group of lymphocytes that has not been openly implicated in tumor progression in colorectal cancers: B cells. However, antibody responses against tumor-associated antigens have been reported also in colorectal cancer patients [35–40]. B cells and plasma cells are typically found in the microenvironment, and systematic analysis has identified a possible beneficial role [41–43]. It remains to be seen whether and how these cells influence tumor growth.

37.4 Immune Cells in Metastatic Colorectal Cancer: Prognostic and Predictive Implications

With the comparison of lymphocytes across different stages of colorectal cancer, the metastatic stage is tightly linked to chemotherapy and salivatory resection of liver metastases. Prognosis is dire for patients with irresectable metastatic colorectal cancer, and survival rates are showing median 28 months [44]. Again for resected metastases, the quantity of T cells was prognostic for the clinical course after resection [45]. Chemotherapy is the only therapeutic option for patients with irresectable disease. Whole slide imaging quantification of colorectal cancer liver metastases revealed the association between effector T cell density specifically at the invasive margin and response to chemotherapy [46]. This finding was later independently validated by another research team [47]. An interesting aspect of these observations is that this relationship is not limited to irinotecan or oxaliplatinum-based chemotherapy and is not influenced by concomitant antibody therapy. While infiltrate densities could predict chemotherapy responses, from the clinical perspective the robustness of cell infiltration within the tissue is paramount. Heterogeneity of infiltrate density was analyzed with an automated immune cell quantification across complete sections with necrotic areas and artifacts being removed beforehand. In microsatellite stable colorectal cancer, primary tumors showed significant heterogeneity which was also seen in metastatic lesions [46, 48]. Interestingly, systematic analyses in a small number of recurrent metastatic lesions showed that even under prolonged periods of intercurrent chemotherapy, infiltrate densities can remain stable. On the other hand, a recurring lesion after radiofrequency ablation of another liver metastasis showed enhanced immune cell infiltration, suggesting the possibility to convert poorly infiltrated lesions into highly infiltrated lesions [49].

37.5 Primary Tumor and Metastases: Two Different Worlds

Another important aspect is whether the primary tumor situation is reflected in the metastatic situation as well. Little data is available from systematic analyses of colorectal cancer metastases in different metastatic sites (lung versus liver). It is clear however from the available data, that there are organ-specific differences on multiple levels [50]. Data from colorectal and renal cell cancer shows a major prognostic value of the immune pattern of CD8 positive to DC-LAMP positive (for antigen-presenting cells) cell densities in colorectal carcinoma and RCC that was reproducible from primary to metastatic lesions in the lung. The clinical impact was however quite opposite between colorectal and renal cell cancer, which shows the complexities in this area [51]. But is the immune cell infiltration pattern preserved between primary and metastatic sites? In a set of primary colorectal cancer and corresponding liver metastases samples, a ~70% concordance was found for the presence of a “good” or “poor” prognostic immunological signature in the primary and in the metastatic lesion [52]. The factors that drive the heterogeneity in immunologic infiltration in primary and in metastatic lesions have not been elucidated so far and research in this direction is ongoing. Another

pressing question is how a tumor lesion of a patient with no or little immune infiltration can be turned into a highly infiltrated lesion.

37.6 Immune Cells and Therapy: Immunogenic Cell Death and Beyond

But even further: what is the mode of action of these T cells in the primary tumor and in the liver metastasis that leads to an improved clinical course of patients? Especially for the effects in the context of chemotherapy, multiple explanations are possible. Activating effects of chemotherapeutic drugs on immune cells with improved antigen presentation and activation of effector T cells is one of the explanations. This could also result in an additional effect of chemotherapy with reduction of the immunosuppressive microenvironment in the tumor. On the other hand, another elegant concept is showing "immunological cell death" induced by selected chemotherapeutic agents. Chemotherapeutic agents such as oxaliplatin are able to stimulate the immune system by inducing a more antigenic and activating tumor cell death [53, 54]. This activation involves the creation of particularly favorable epitopes for antigen presentation on the relevant cells (e.g., dendritic cells), which in turn renders chemotherapy particularly effective [55]. Chemotherapy has also other effects, which are now investigated on a more translational scale. Several groups have shown independently the relationship between the intestinal microbiome and the success of chemotherapy and immunotherapy [56–61]. The mouse model data shows that chemotherapy induces a translocation of bacteria from the intestine across the mucosa and into the lymph nodes where these lead to an enhanced immune stimulation and recognition of the tumor cells. The composition of the bacteria has a decisive role in this process: certain species are relevant as they either enforce an improved immune response or lead to an inhibition. So the composition or the presence of particularly activating bacterial species in the intestine plays an important role for therapy response [62]. Currently, translation into an interventional trial is ongoing. "Oncomicrobiomics" is the concept of [63–66] microbiome modulation to enhance and optimize therapy response.

37.7 Myeloid Immune Cells in the Microenvironment: Plasticity and Immunosuppression

From a quantitative perspective, myeloid cells are the largest immune cell subgroup present in colorectal cancer, especially in metastatic lesions. Macrophages are a versatile and highly complex subgroup of innate immune cells, and their multiple functions are reflected in their enormous plasticity. Besides tissue-resident macrophages, recruited monocytes differentiate into macrophages within the tissue. It is unclear today, whether tumor cell-associated macrophages are arising from tissue-resident or recruited macrophages. Functionally, macrophages with their phagocytic, immunosuppressive (e.g., arginase and iNOS production), and antitumoral (reactive oxygen species formation) capabilities were initially grouped into tumor-promoting M2 and antitumoral M1 categories [65–67]. This however was soon revised, and the complexity of phenotypes of human myeloid cells is not only different from animal models but also highly diversified [66, 68–70]. Nevertheless, interventional strategies with the aim of ablating or modulating macrophages have reached translation into clinical trials [71, 72]. Another important group are the myeloid-derived suppressor cells (MDSCs). This population is typically defined as an immature myeloid cell from the bone marrow with suppressive effects on the adaptive immune response and modulation of other myeloid cells like macrophages [70]. Another important functional effect of MDSCs is the depletion of L-arginine from the microenvironment. Arginase that is produced by MDSCs is degrading L-arginine to L-ornithine and urea and subsequently

leads to T cell inactivation. In this continuum of functions, precise separations between different cellular myeloid subclasses and the transient functional states that can be found are not yet possible.

37.8 Checkpoint Inhibition and Other Signaling Cascades

Closely related to these cells are the presence and absence of specific signaling molecules. Molecules of the "programmed death" family with the receptors PD-1 and PD-2 and the ligand PD-L1 (B7-H1) are a part of an increasing family of signaling molecules that shape the functional behavior of all classes of immune cells in the microenvironment. PD-1 is a protein (55 kD) with an extracellular domain showing 23% identity to cytotoxic T lymphocyte antigen-4 (CTLA-4), another prominent inhibitory receptor on activated T cells. PD-1 plays a complex role in the selection of T cells in the thymus and in the peripheral blood. Expression levels of PD-1 on naive T and B cells are low, and only with activation an increased expression level on the surface appears. Data from mouse models suggests a protective role of PD-1 against autoreactivity. Interaction of PD-1 with its ligands leads to reduced levels of cytokines of T-helper cells and subsequent suppression of T cell proliferation [73]. This is apparently an important mechanism in viral infections, where a focusing role of PD-1 was also noted. Looking into cancer, the complexity of the presence of these signaling molecules in the local microenvironment is far from being understood. Following the initial descriptions of the functions of the PD-1/PD-L1 axis as a response to interferon [74], still up to date the complexity and regulation of the expression of PD-L1 on a multitude of immune cells are not fully understood [72, 75, 76]. In colorectal cancer liver metastases, practically all lymphocytes are PD-1 positive; tumor cells are negative for PD-L1 with a specific myeloid cell at the invasive margin being positive for PD-L1 [72]. Clinically, inhibition of the PD-1/PD-L1 axis in colorectal cancer has not shown promising results [77]. But there are also other modulatory molecules present (sometimes specifically at the invasive margin): V-domain immunoglobulin (Ig)-containing suppressor of T cell activation (VISTA) [78], cytotoxic T lymphocyte antigen-4 (CTLA-4) [79], tumor necrosis factor receptor superfamily member 4 (TNFRSF4, also known as CD134 and OX40) [80], T cell immunoglobulin and mucin domain containing 3 (TIM3) [81], lymphocyte activation gene 3 protein (LAG3), and others. The modulation of immune responses for the adaptive immune system has been elucidated [82] for the majority of those receptor-ligand pairs. But there are still plenty of uncertainties, whether there are other receptors/ligands involved and how the coordination in the local microenvironment shapes the actual immunological outcome, especially in human patients. The most prominent question currently is how combination therapies can improve clinical responses and what combinations are most likely successful in a clinical setting.

37.9 Cytokines and Chemokines in the Microenvironment

Beyond the quantitative presence of immune cells and modulatory signals, the role of cytokines and chemokines within the microenvironment holds another potential for therapeutic intervention. The composition of the immune cells or cells at all in the local tumor environment is to a large share affected by these specific molecules. In addition to the classical molecules like interferon-gamma and interleukin-2, there is a wide range of proteins altering the behavior of the immune system, and these have subsequent dramatic effects on tumor cells. In this situation there are many complex relationships still completely unclear, and the pioneering work of the research group of Wolf H. Fridman has elucidated clearly: the immunological context is the decisive factor [83]. A particularly interesting example in this context

is the role of IL-10, in particular the work that lead to the discovery of interleukin-10, but also the large number of subsequent publications on the importance of IL-10 for tumor diseases. For a long time, IL-10 was almost uniformly attributed with immunosuppressive and thus tumor-promoting effects. Thus, IL-10 became one of the main target molecules for inhibitory strategies to generate an antitumor activity by the immune system. Several publications recently showed very clear: IL-10 has not only immunosuppressive, but also immune stimulating and focusing functions. A complete absence of IL-10 leads to enhanced metastatic spreading and other pro-tumorigenic effects [84]. This shows not only a high context dependency but also that there is a function of inflammatory cytokines that is also dose-dependent. This dose-dependent functionality in context of other cytokine is difficult to study but shows the urgent need for more integrated analyses. These analyses have to incorporate spatial localization and gradients of chemokines and cytokines together with tumor heterogeneity in terms of tumor cell heterogeneity and immunological heterogeneity as these complex functional relationships allow to dissect the differential effects of the underlying patterns [85, 86]. From a functional perspective, cytokines and chemokines can be grouped into patterns that reflect specific immune activation. This includes the TH1, the TH2, and the TH17 group of cytokines. While the TH1 cytokines indicate an activation of effector T cells favor (with elevated levels of IL-2, etc.), a TH2 environment leads, e.g., to the induction of antibody-producing B cells. The TH17 milieu is typically attributed to autoimmunity and gamma-delta T cell activation. In these different contexts, different groups of cells produce clusters of inflammatory cytokines with differential effects on subsets of immune cells. Cytokines, such as macrophage migration inhibitory factor (MIF), can have wide-ranging pleiotropic effects on multiple different systems (i.e., angiogenesis, cell cycle, etc.) and escape simple classification in one of the above systems.

37.10 Clinical Strategies for Immunotherapy of Colorectal Cancer

The understanding of the immunological mechanisms and the interaction between immune cells, tumor cells, and other cells within the microenvironment has dramatically improved, and the complexity of this interaction has been investigated from many sides [87, 88]. The clinical advances in immunotherapy are doubtless revolutionizing oncology. But has this scientific momentum also affected the clinical development of immunotherapies for colorectal cancer? There is no easy answer, as there are clear benefits for the subgroup of microsatellite instable colorectal cancers [89]. But otherwise, in light of the attempts to utilize immunological interventions, one has to concede that colorectal cancer is a challenging entity for immunotherapy [77]. However, there are promising reports that show clinical effects for advanced stage metastatic colorectal cancer patients, regardless of microsatellite stability or BRAF mutation status.

37.11 Vaccination and Dendritic Cell Therapy: Retargeting the Immune System

From a historical perspective, vaccination has been extensively tried for various cancer entities (see Fig. 37.3). In the year 2006 the summary for vaccination strategies in colorectal cancer looked quite sobering. The review by Nagorsen and Thiel [90] comes to the conclusion that the analysis of 527 patients across a broad variety of specific active immunization strategies showed an overall response rate of 0.9%. Humoral immune responses and cellular responses were seen in approximately half of the patients. Other concepts have been tried, using chemoimmunotherapy in combination with vaccination [91]. This approach resulted in clinical effects, but it was not clarified which effect is contributed by chemoimmunotherapy and which by the vaccination approach.

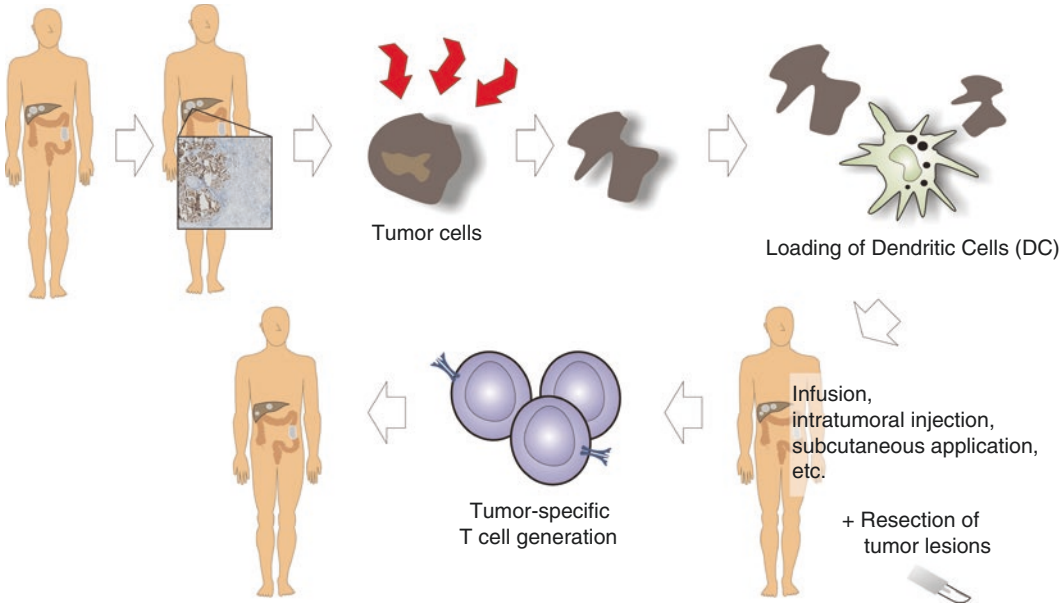


Fig. 37.3 Vaccination strategies for colorectal cancer

Modulation of the immune system with quickly matured dendritic cell vaccines for carcinoembryonic antigen showed no clinical effects [92]. Adjuvant immunization was tried utilizing Newcastle disease modified tumor cells following the resection of the liver metastases in 50 patients [93], but again no clear clinical benefit was identified. An anti-idiotype monoclonal antibody vaccine was also applied to 50 patients following curative resection in the Cancer and Leukemia Group B Study 89,903 [94]. At 2 years, the evaluation showed no benefit for the vaccinated group. A more promising result was seen in a randomized phase II trial, using immunization with dendritic cells modified with poxvectors encoding CEA and MUC1 compared with the same regimen plus GM-CSF in resected metastatic colorectal cancer. Both the dendritic cell and poxvector vaccines showed longer survival for vaccinated patients. A subsequent randomized trial was proposed but no data is available. Interest in vaccination approaches for colorectal cancer has not subsided, and more variations in adjuvants are tried to increase immune cell activation (Garbitsch et al. JCO 32:5 s 2014, Abstract 3093). Given the richness and diversity of approaches in colorectal cancer patients, in summary only for the adjuvant situation, some tangible effects could be observed, no large trials were performed, and for advanced disease the results were disappointing. The Stimuvax trial in colorectal cancer [95] is still ongoing with the trials for lung cancer showing no clinical effects. In 2018 the results for colorectal cancer are expected. In contrast to these "off-the-shelf" vaccines, individualized vaccines seem to be a much more promising new development, and clinical trials are expected to start soon [96].

37.12 Therapeutic Use of Cytokines

Immunomodulation with cytokines has shown interesting results in colorectal cancer. The combination of chemoimmunotherapy with gemcitabine plus FOLFOX-4 followed by subcutaneous GM-CSF and IL-2 (GOLFIG) showed activity in clinical trials. Unfortunately, problems in recruitment for the last trial (for the control arm) led to inconclusive data. Overall, data from this trial series suggests activity of this regimen with objective responses [97, 98]. This combinatorial approach was evaluated with concomitant vaccination [99], utilizing a poly-

epitope-peptide vaccine to thymidylate synthase (TSPP). Adverse events consisted of swelling/erythema at injection sites (17 cases), grade I–II hematological alterations (16 cases), and gastrointestinal events (12). Of note, this regimen induced fever, rhinitis, conjunctivitis, polyarthralgia, and a rise in autoantibodies [ANA, ENA, c-ANCA, p-ANCA]. Further clinical evaluations are planned.

37.13 Adoptive T Cell Therapy and Genetically Modified T Cell Therapy

Adoptive T cell therapy is another field of interest for colorectal cancer. In malignant melanoma, spectacular results could be achieved. The principles of the procedure are shown in Fig. 37.4. For colorectal cancer however, adoptive T cell therapy was largely unsuccessful. An adjuvant trial with tumor-infiltrating lymphocytes plus IL-2 after radical resection of liver metastases showed disappointing results in the 5-year follow-up analysis [100]. This approach has been largely abandoned.

Another concept is modification of T cell receptors for immunotherapy. The exceptional successes in hematologic malignancies have not been replicated in solid tumors and especially not in colorectal cancer. The genetic modification of T cells to implant a T cell receptor specificity is an elegant method, but apparently these modified T cells succumb to other evasion mechanisms in the microenvironment. Another problem for colorectal cancer seems to be the specificity of the available targets. CEA is also expressed on a variety of other cells, which produces a plethora

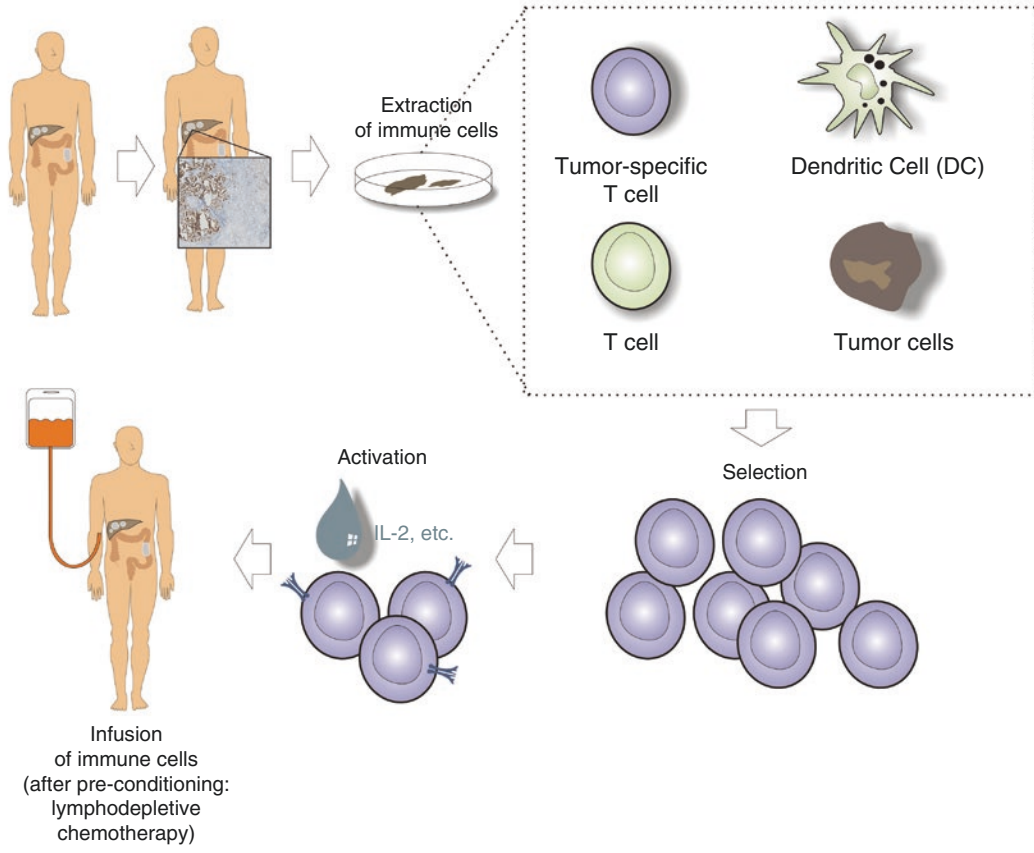


Fig. 37.4 Schematic illustration of adoptive immunotherapy

of unwanted and severe side effects. Severe transient colitis is one of the dominating side effects [101]. The objective regression of metastatic colorectal cancer was followed by unacceptable toxicities. Finding suitable targets and better mechanisms for controlling modified chimeric antigen receptor (CAR) T cells will here allow new opportunities.

37.14 Checkpoint Inhibition in Colorectal Cancer

Immunomodulation with checkpoint inhibition has been very successful for microsatellite instable (MSI) colorectal cancer, but for the vast majority of patients with microsatellite stable disease (MSS), checkpoint inhibition has been unsuccessful. While data for MSI tumors in general show a favorable situation with good responses across different cancer entities, for MSS colorectal cancers, checkpoint inhibition with anti-PD-1, anti-PD-L1 [102], or anti-CTLA-4 has not shown efficacy. The checkmate 142 trial combines anti-PD-1 (nivolumab) with anti-CTLA4 (ipilimumab) inhibition in colorectal cancer patients. Sure enough, patients with MSI tumors showed responses whereas the patients with MSS tumors did not benefit.

Combination of anti-PD-L1 with small molecule inhibition (MEK inhibitor) from an early phase I trial showed promising results (see Fig. 37.5). From the reported 23 CRC patients (22 KRAS mutant, 1 WT), the most common treatment-related side effects included diarrhea (69.6%), fatigue (52.2%), acneiform dermatitis (43.5%), rash (34.8%), maculopapular rash

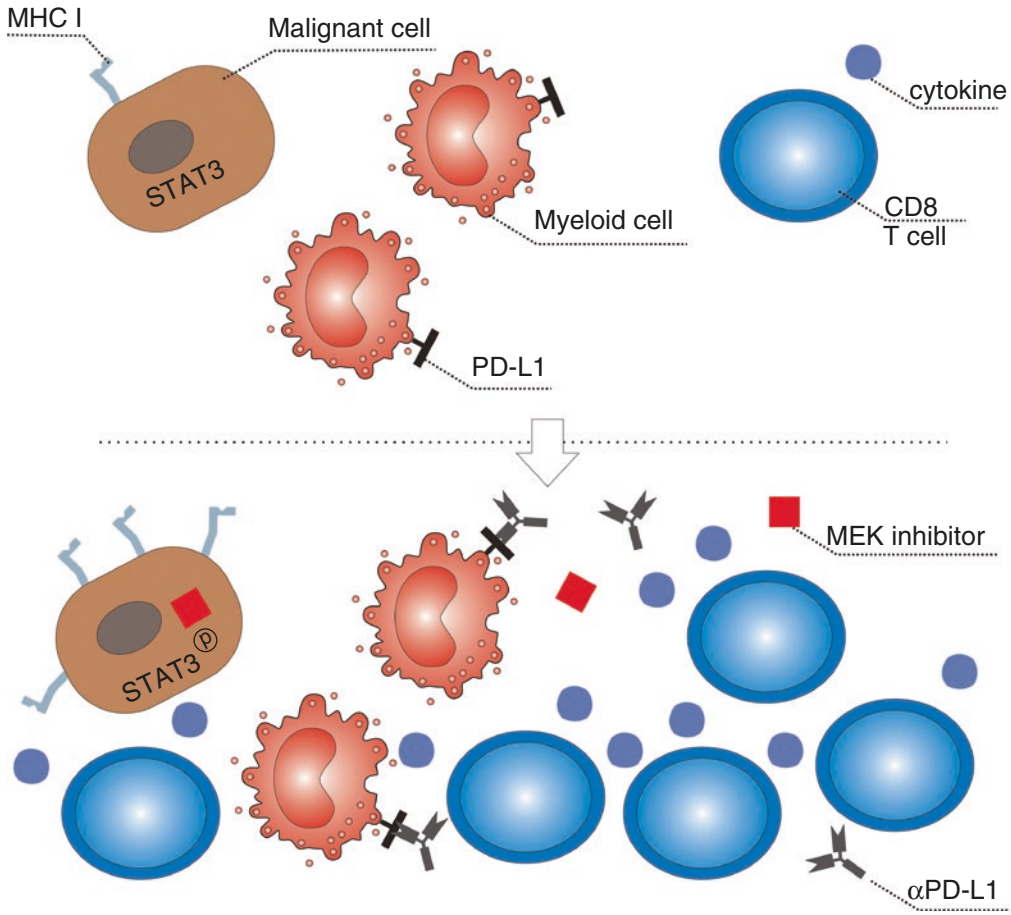


Fig. 37.5 Effects of combined MEK inhibition and anti-PD-L1 therapy in the microenvironment

(26.1%), pruritus (26.1%), and nausea (26.1%). The incidence of treatment-related grade III and IV adverse events was 34.8%, more than two patients developed severe diarrhea (8.7%). However, no grade V adverse events were reported. The ORR was 17% with four partial remissions and five stable diseases. Interestingly, three responses were reported to be still ongoing (range, 4.0–7.7 months at time of data cutoff). As the trial was aiming at microsatellite stable patients, three responders were mismatch repair proficient, and in one patient the status was unknown. In contrast to expectations, response was not associated with baseline PD-L1 expression. To obtain a better image of the effects of therapy on the microenvironment, serial biopsies were taken and showed enhanced PD-L1 upregulation, CD8 T cell infiltration, and enhanced MHC class I expression. This finding is in line with the previous report of the unexpected observation that potent suppression of T cell receptor (TCR) function by MEK inhibition can be largely overcome in the presence of blockade of the inhibitory PD-L1/PD-1 pathway in T cells in vivo. Enhanced antitumor activity was observed combining MEK inhibition with PD-L1 blockade in in vitro experiments, which was likely potentiated by upregulation of tumor MHC class I expression through inhibition of MEK [103]. This therapeutical concept is currently evaluated in a phase III trial (NCT02788279).

37.15 Modulation of the Innate Immune System

In contrast to immunotherapies that aim to activate the adaptive immune system, a new form of immunotherapy aims at immunomodulation of innate immune cells: modulation of macrophage (MOMA) therapy. This modulation is contrasting to depleting approaches like bisphosphonates or depleting antibodies (anti-CSF-1R) [71] and utilizes the innate arm of the immune system against the tumor [104].

A possible therapeutic pathway in this field is the inhibition of the CD47-SIRPalpha interaction. CD47 is a “do not eat me” signal on tumor cells and interacts with SIRPalpha on macrophages to inhibit phagocytosis [105–107]. The initial observation revealed promising effects for hematologic malignancies, but also for solid tumors. Subsequently multiple trials have been initiated that are now evaluating these concepts in the clinical context (NCT02367196, NCT02678338, NCT02890368); results are pending [108].

An alternative way has been uncovered and was already successfully translated into the clinic, based on the analysis of the microenvironment in metastatic colorectal cancer. The role of the CCL5-CCR5 axis in metastatic colorectal cancer liver lesions was investigated (see Fig. 37.6), and the clinically available CCR5 inhibitor maraviroc was used in a phase I trial (NCT01736813). CCR5 inhibition led to antitumoral activation of the innate arm of the immune system, i.e., macrophage repolarization, with little side effects and showed synergism with chemotherapy in a small number of patients (14 patients evaluated) [72]. Patients from the trial had BRAF mutations (7.14%) or KRAS mutations (57.14%), with up to six lines of previous therapy. Patients with previous therapy with regorafenib were included (28.57%). Side effects were generally limited, with a majority of grades I and II adverse events (89.33%). The grade III and IV events were few (10.77%) and in the vast majority consisted of lab value deviations, e.g., gamma-glutamyltransferase elevation. Clinical observations included increased necrosis of metastatic lesions on imaging analyses, which were confirmed by histological analyses from biopsies. Serial biopsies showed stable T cell infiltration at the invasive margin of liver metastases under therapy [109]. Patients from the trial were allowed to subsequently combine the CCR5 inhibitor with a previously (unsuccessful) chemotherapy. The rationale was the assumption that interferon production by myeloid cells would enhance chemotherapy effects [54]. Objective responses to this combination therapy were observed in three out of five patients (one patient not evaluable), and a tumor control rate of 80% was reached. Therefore it was concluded that combination of the activation of the innate arm

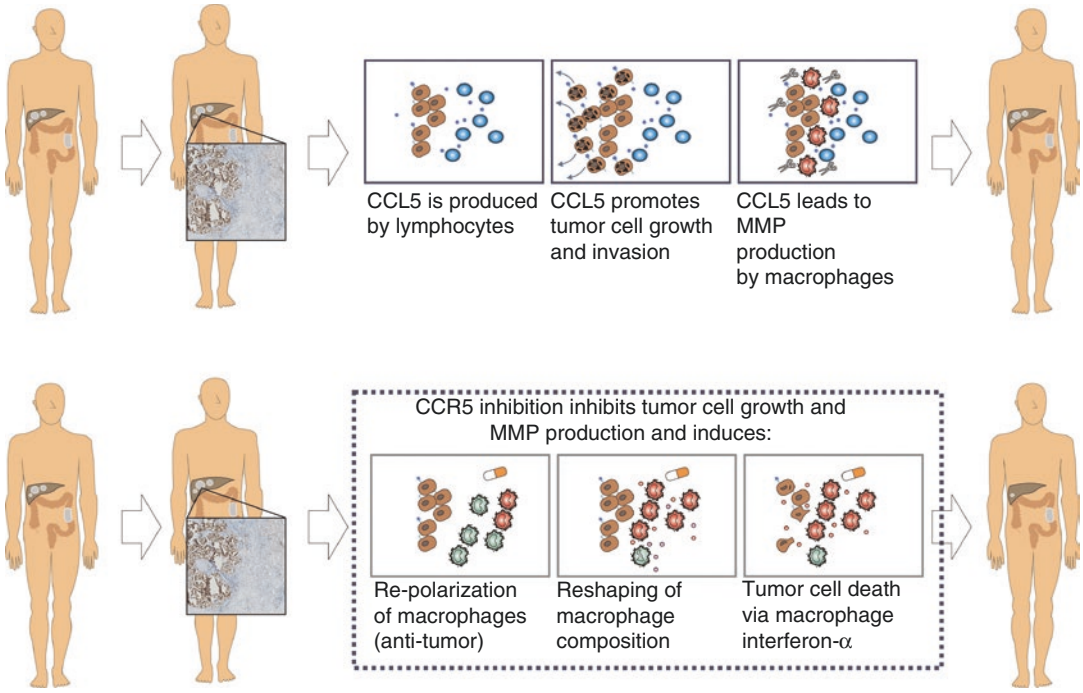


Fig. 37.6 Schematic illustration of the effects on macrophages and tumor cells induced by CCR5 inhibition in colorectal cancer

with other immunomodulatory approaches is promising and this concept is currently being investigated in further trials.

37.16 Summary

Immunotherapy for colorectal cancer has been challenging, and results have largely shown little or no effect until recently. The prospects for patients with microsatellite instable tumors are especially promising, as these tumors show very good response rates to checkpoint blockade. Combinatorial checkpoint inhibition is currently being tested for this selected subgroup of patients, but the effects are very encouraging. The situation for the large group of patients with microsatellite stable tumors is also changing. The hopes for KRAS, BRAF, and PI3K mutation-independent therapy options are high, and immunotherapy has still to deliver here significant response rates. While for checkpoint inhibition the data is unconvincing so far, the efforts to alter the microenvironment in order to utilize the innate immune system together with the adaptive immune system seem to be promising signs of new therapeutic options.

37.17 Outlook and Future Directions

For the challenges of immunotherapy of colorectal cancer, the following topics need to be addressed in the future. Sequences and combinations of chemotherapy, radiation, and immunotherapy with the optimal activation of the immune system are important for the medical decision making and patient selection. Biomarkers for this selection process are not fully developed, and their identification and validation are clear aims for future developments. For patients with colorectal cancer, the selection of individually effective immuno-therapeutical strategies is one of the most difficult but also most promising possibilities of the near future.

The success of checkpoint inhibition in microsatellite instable (MSI-H) colorectal cancer seems now also extendable to the large group of microsatellite stable (MSS) colorectal cancer patients. The role of combinatorial approaches for these questions is clear, and new strategies have to be envisioned for these patients. These strategies will also help in the identification of effective regimens in adjuvant therapy or after curative resection of colorectal cancer liver metastases.

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Current Status of Immuno-Oncology in Hematologic Cancers

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Contents

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38.1 Introduction

Harnessing the immune system in order to eliminate malignant cells has been a long-standing dream for all hematologists and oncologists. Hematologists paved the way with allogeneic hematopoietic stem cell transplantation (HSCT), which was the first successful demonstration of the potential of immunotherapy in cancer, when the benefit of graft versus leukemia was clearly demonstrated. Though allogeneic HSCT is now routinely used in the clinic, major limitations include short- and long-term toxicities, the difficulty to obtain a stem cell graft, and the limited number of hematological cancers that can benefit from this therapy. It took several decades of intensive research to develop and validate other strategies that could expand the field of immunotherapies. Targeting cellular antigens expressed at the surface of malignant cells was the first step, as illustrated by the quintessential example of the anti-CD20 monoclonal antibody (mAb) rituximab. New generations of mAbs targeting novel receptors, bispecific antibodies (BsAbs) that direct cytotoxic T cells toward tumor cells, and genetically modified T cells and the development of immune checkpoint blockers (ICB) recently demonstrated very promising activity in several clinical trials and are expected to be at the forefront of cancer therapy. These new treatments have the capacity to shift the "cancer-immune set point" barrier and overcome cancer-induced immune escape

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[1]. In this chapter, we shall review the most recent clinical trials investigating the impact of immuno-oncology in hematology in the hope to stimulate further exciting work in this field to significantly improve the life of patients living with cancer.

38.2 Direct Targeting of Cellular Surface Antigens

The potential antitumor activity of humoral immunity has been recognized for a long time in hematological malignancies and has led to the development of mAb targeting surface molecules expressed by malignant cells. The mechanisms of action of mAbs include direct induction of cell death, antibody-dependent cell-mediated cytotoxicity/phagocytosis (ADCC/ADCP) through engagement of Fc receptor on immune cells, and complement-dependent cytotoxicity. Cytotoxicity may be enhanced by the coupling of a cytotoxic or toxin which triggers cell death after internalization. The engineering and development of rituximab, the first therapeutic anti-CD20 mAb, constitute a success story in hematology. For more than a decade thereafter, most attempts to target other cellular antigens using mAbs failed to demonstrate potent efficacy and favorable safety profile [2]. Following a better understanding of immunoediting and engineering mAbs, things are starting to change as several mAbs have recently demonstrated extremely interesting activity in several clinical trials (Fig. 38.1).

38.2.1 The Famous CD20 Story

Overcoming the barriers of xenogeneic immunogenicity through humanization of murine antibodies led to the development of rituximab, the first humanized mAb directed against CD20, which showed unprecedented clinical activity in relapsed CD20 diffuse large B-cell lymphoma [3–5]. In the landmark randomized clinical trial published in 2002, the French GELA group demonstrated that the addition of rituximab to the standard CHOP regimen was able to increase the response rate and prolong event-free and overall survival (OS) up to 10 years (44% vs 28%, respectively) in elderly patients with diffuse large B-cell lymphoma [6]. Since that pivotal trial, rituximab has proven its efficacy in nearly all types of CD20-positive B-cell malignancies and now represents the standard of care in indolent and aggressive non-Hodgkin lymphomas (NHL) as well as chronic lymphocytic leukemia (CLL). Recently, the results of two large randomized clinical trials demonstrated the superiority of rituximab-based strategies in Burkitt's lymphoma

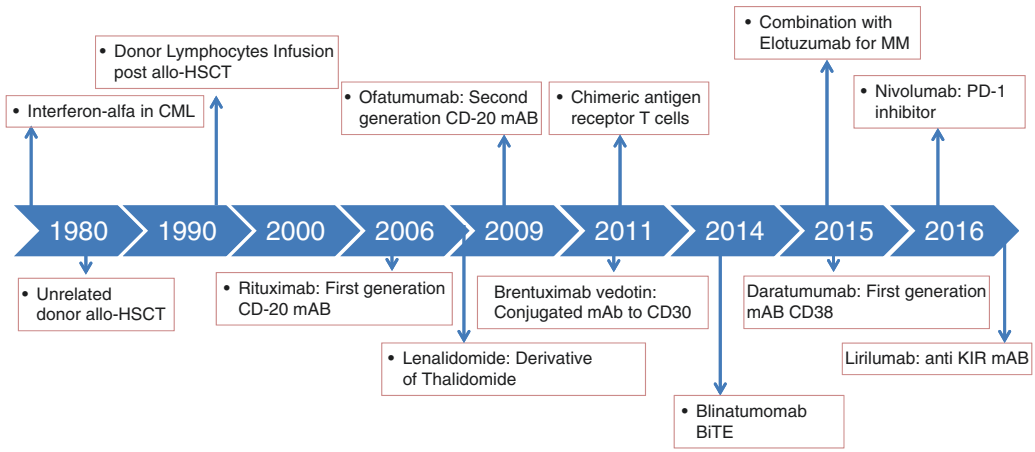

Fig. 38.1 Key time points in the discovery and development of immuno-oncology drugs for the treatment of hematologic cancers

and B-lineage adult acute lymphoblastic leukemia (ALL) [7, 8].

Second-generation mAb against CD20, obinutuzumab (GA101), is a glycoengineered type II humanized mAb that was designed to have a stronger affinity to the FcγRIII on immune cells and potentially being superior to rituximab in vitro. As compared to rituximab, obinutuzumab demonstrated more direct cell death and enhanced ADCC/ADCP but lower CDC. In addition, obinutuzumab, which binds to a single CD20 tetramer, is believed to get less internalized than rituximab, which might increase ADCC/ADCP. Obinutuzumab was approved for the treatment of CLL in elderly or unfit patients following the results of a large randomized trial that demonstrated the superiority of chlorambucil and obinutuzumab over chlorambucil and rituximab in terms of response rate and complete response [9]. It should be noted that obinutuzumab plus chlorambucil was associated with more toxicities. In CLL, a combination of obinutuzumab with novel agents such as the bcl-2 antagonist venetoclax and the btk inhibitor ibrutinib is being evaluated. Promising preliminary results presented in conferences make it likely that obinutuzumab will be widely used in CLL in the near future.

Furthermore, obinutuzumab has been recently approved in relapsed follicular lymphoma in combination with bendamustine based on longer progression-free survival (PFS) when compared to bendamustine monotherapy in the GADOLIN phase 3 clinical trial [10]. Similarly, the primary results of the GALLIUM study showed that obinutuzumab-based induction and maintenance increased PFS in patients with previously untreated follicular lymphoma [11]. However, the success observed in follicular lymphoma and CLL might not be valid in more aggressive lymphomas. Indeed, the GOYA study, a large randomized phase III trial that compared head-to-head rituximab or obinutuzumab plus CHOP in 1418 patients with untreated diffuse large B-cell lymphoma, failed to meet its primary endpoint (investigator-assessed PFS) [12].

38.2.2 Targeting Molecules Other than CD20

38.2.2.1 Alemtuzumab

Alemtuzumab is a mAb directed against CD52, a cell surface antigen expressed by B cells, T cells, and monocytes. It was originally approved as a single agent for the treatment of fludarabine-refractory and del17p CLL [13]. Although alemtuzumab appeared effective in one-third of patients with fludarabine-refractory disease, its broad immunosuppressive activity was associated with severe and lethal opportunistic infections [14]. In CLL and other subtypes of lymphomas, the results of most clinical trials evaluating the association of alemtuzumab with chemotherapy failed to demonstrate a clear advantage over standard therapy, with some studies being prematurely interrupted because of an alarmingly higher death rate related to infections. For the same reason, its use in conditioning regimen in non-myeloablative allogeneic HSCT has been limited by an increased risk of life-threatening infectious complications and a higher rate of disease relapse [15]. Commercialization of alemtuzumab for hematological malignancies was interrupted in 2012, because the manufacturer wished to rebrand the product under a different name for multiple sclerosis. It remains accessible through specific distribution program for CLL in different countries.

38.2.2.2 Gemtuzumab Ozogamicin

Gemtuzumab ozogamicin (GO) is a humanized mAb directed against the CD33 surface antigen coupled to calicheamicin, a potent antitumor anthracycline antibiotic. In AML, CD33 represents an interesting target because it is expressed by the majority of AML cells. Following its approval by the FDA after the promising phase 2 data in relapsed older adults with AML, GO has been the subject of controversies and was even withdrawn following the results of a large randomized trial (recently reviewed by Rowe and Lowenberg) [16, 17]. However, aggregated data from four randomized trials have renewed the interest of using GO in newly diagnosed AML, particularly in patients with more favorable cyto-

genetic. In addition, GO seems particularly active in newly diagnosed and relapsed acute promyelocytic leukemia, which expresses high level of CD33 [18]. Given the absence of novel effective agents in AML over the last three decades, the use of GO should probably be reappraised in the light of these results.

38.2.3 Newer Target of Interest

38.2.3.1 Brentuximab Vedotin

Brentuximab vedotin (BV) is an antibody drug conjugate consisting in a mAb directed against CD30 conjugated to the microtubule-disrupting agent MMAE via a protease-cleavable linker. CD30 is a cell surface molecule belonging to the tumor necrosis factor (TNF) receptor superfamily that is mainly expressed by subpopulations of B and T cells upon activation [19]. The expression of CD30 has been reported in various hematologic and solid cancers. In hematology, the principal malignancies expressing CD30 are classical Hodgkin lymphoma (cHL), anaplastic large cell lymphoma (ALCL) anaplastic large cell lymphoma, primary mediastinal B-cell lymphoma, T-cell lymphomas, and EBV-induced posttransplant lymphoproliferative disorders.

BV demonstrated clinical activity in relapsed/refractory CD30-positive lymphomas [20] and is approved as a single agent for this condition and in ALCL. In HL, administered in monotherapy, BV induces an overall response rate (ORR) of 75% and is associated with durable responses in nearly half the patients relapsing after autologous SCT [21–23]. A phase 2 trial evaluating frontline BV as a monotherapy in older patients unfit for conventional chemotherapy showed a 92% overall response rate including 73% of complete remission [24].

Combination of BV with chemotherapy and radiotherapy was feasible and well tolerated in numerous phase I and phase II trials. A word of caution: BV should not be used in combination with bleomycin-containing regimen because of a high rate of pulmonary complications [25]. In a phase II pilot study evaluating BV and AVD followed by involved-site radiotherapy in early-stage unfavorable risk HL, 90% (26/29) and 93% (27/29) of patients achieved a negative PET scan after two and four cycles, respectively. A large phase III trial is currently evaluating BV plus AVD versus ABVD in advanced classical HL (NCT01712490). The AETHERA study examined the potential of BV administered as consolidation therapy post-autologous SCT in relapsed HL [26]. Median PFS was 42.9 months for patients in the brentuximab vedotin group compared with 24.1 months for those in the placebo group, supporting its efficacy in the posttransplant setting. Updated data confirm the superiority of the BV arm in the AETHERA study [27]. A limited number of studies have shown BV to control disease before [28] or in patients relapsing after allogeneic HSCT [29–31]. Altogether, BV has demonstrated a very promising activity in HL. The results of several ongoing phase III randomized trials, which are evaluating BV in addition with chemotherapy in either untreated or relapsed HL, might lead to the incorporation of BV in standard-of-care regimen for cHL.

BV has been prescribed for other CD30-positive lymphoid malignancies in small trials or case reports; therefore, data are less robust than with cHL. BV induced an 85% ORR with a median duration of 12.6 months in 58 patients with relapsed or refractory systemic anaplastic large cell lymphoma [32]. A phase I/II study of frontline BV in combination with chemotherapy in CD30-positive primary mediastinal B-cell lymphomas (PMBCL), diffuse large B-cell lymphomas (DLBCL), and gray-zone lymphomas is recruiting patients (NCT01994850). Further studies are needed to define its efficacy, especially in T-cell lymphomas where improvement is eagerly awaited given their usual resistance to standard chemotherapy.

38.2.3.2 Daratumumab

Daratumumab is a human IgG1 monoclonal antibody that targets CD38, a transmembrane glycoprotein that is expressed by a large array of cell types including T cells, B cells, monocytes, and NK and NK/T cells. CD38 is ubiquitously expressed on multiple myeloma (MM)

cells, which makes it an interesting therapeutic target. Recent experimental evidence suggests that the biological effect of daratumumab expands beyond the classical properties of therapeutic mAbs and may involve immunomodulation [33]. Daratumumab can deplete regulatory immune cells like myeloid-derived suppressor cells (MDSC), regulatory B cells, and a newly identified subset of $CD38^{hi}$ regulatory T cells, which allows expansion of CD4+ Th cells and CD8+ cytotoxic T cells and increased IFN-γ. Daratumumab as a single agent demonstrated very promising activity in two clinical studies in heavily pretreated patients with relapsed/refractory MM [34, 35]. Daratumumab induced remarkable response rates, including stringent complete responses, and prolonged clinical responses. These results led to its approval in MM with ≥3 lines of treatment including a proteasome inhibitor and an IMID or refractory to both.

Early data from the CASTOR and POLLUX trials, which evaluated the association of daratumumab with bortezomib and dexamethasone or lenalidomide and dexamethasone, respectively, in previously treated MM patients, have been published [36, 37]. Although the results of these studies are somehow limited by their short follow-up (7.5 and 13.5 months), both already showed a significant improvement in terms of response and PFS. The 12-month rates of PFS were 60.7% in the daratumumab group versus 26.9% in the control group (CASTOR) and 83.2% in the daratumumab group, as compared with 60.1% in the control group (POLLUX). Daratumumab has been rapidly moved to the frontline and is being evaluated in combination with other anti-myeloma agents. In HSCT-eligible MM patients, the large randomized phase III CASSIOPEA trial from the IFM is comparing the standard-of-care regimen VTD (bortezomib, thalidomide, and dexamethasone) to daratumumab plus VTD (NCT02252172). In older patients, VMP (bortezomib, melphalan, and prednisone) is challenged against daratumumab plus VMP (NCT02195479). If the results of these studies confirm its efficacy in MM, daratumumab might well become "the rituximab of myeloma."

Besides MM, there is a rationale to try daratumumab in other hematological malignancies that express CD38. Preclinical data support evaluating daratumumab in high-risk CLL, in which the malignant B-cell population frequently expresses CD38 [38]. Recently, daratumumab was used with success in a case of refractory CD38+ NK/T-cell lymphoma [39].

38.2.3.3 Elotuzumab

Elotuzumab is another mAb directed against CS1/SLAMF7 receptor. SLAMF7 is uniformly expressed at a high level by both normal and malignant plasma cells, whereas other cell types to the exception of NK cells do not express it. Elotuzumab's mechanism of action is different than other mAbs like daratumumab. Elotuzumab binds to SLAMF7 expressed by NK cells leading to their activation. Activated NK cells then kill elotuzumab-tagged plasma cells through engagement of CD16 with its Fc region.

Elotuzumab has little or no effect as a single agent in patients with relapsed/refractory MM [40]. However, it demonstrated activity in combination with other anti-myeloma drugs in phase I trials [35, 41]. In a randomized open-label phase 2 study, the addition of elotuzumab to bortezomib slightly increased the PFS in 150 previously treated MM patients [42]. The 1-year PFS rate was 39% vs 33% in the control arm, and in the updated analysis, the 2-year PFS rate was 18% vs 11%. The large randomized multicenter phase III ELOQUENT-2 trial compared the efficacy and safety of lenalidomide and dexamethasone with or without elotuzumab in 646 relapsed/refractory MM patients after ≤3 lines of treatment [43]. The ORR was 79% vs 66% in the control group, and median PFS was 19.4 months vs 14.9 months in the elotuzumab group and the control group, respectively. These results led to its FDA approval in 2015. In conclusion, elotuzumab in combination with lenalidomide has demonstrated clinical activity in MM, although its efficacy appears less impressive than daratumumab. Further studies are needed to clearly define the place of elotuzumab in the armamentarium against MM, in particular what should be the optimal combination.

38.2.3.4 Bispecific T-Cell Engagers

The previous sections revealed that compared to standard chemotherapy, mAbs offer a more selective and less toxic therapeutic approach. However, the major drawback of mAbs is the inability to recruit cytotoxic T lymphocytes to the tumor bed and transform non-inflamed tumor to inflamed one. Several lines of evidence have demonstrated that the number of tumor-infiltrating T cells directly correlates with the clinical outcome in different cancers [44]. It took more than one decade from the development of rituximab to envision methods capable of directly increasing and priming specific CTL within the tumor microenvironment. Two distinct strategies that illustrate this paradigm shift and successfully translate into the clinic are:

1. Unleashing the priming and effector phase of T lymphocyte-mediated immune responses by suppressing the interaction of inhibition receptors and ligands with ICBs such as PD-1 and CTLA-4 blockades.
2. Bypassing the exhausted T cells with chimeric antigen receptor T cell (CAR T cells): adoptive injection of transduced autologous T cells with a specific antigen-binding domain coupled to intracellular receptor on T cell thereby redirects cytotoxic T lymphocytes to specifically recognize cancer cells.

To overcome the cost, complexity, and delay of manufacturing personalized CAR T cells for each patient in a good manufacturing practice (GMP) facility, a different approach to redirect T cells was needed. From this perspective, the development of BsAbs represents a strategy of paramount interest. BsAbs also called dual-targeting antibodies have the capability to bind two different targets bringing two cells in contact. This technology allows engaging a patient's own cytotoxic T cells to cancer cells and promotes a sustained tumor lysis. Following the clinical benefit in ALL, BsAbs represent one of the fastest-growing class of anticancer therapeutics, and more than 50 BsAbs are currently in clinical development [45, 46].

Bispecific T-cell engagers (BiTe) like blinatumomab consists in two scFv separated by a flexible and short linker (Fig. 38.2). The N-terminal

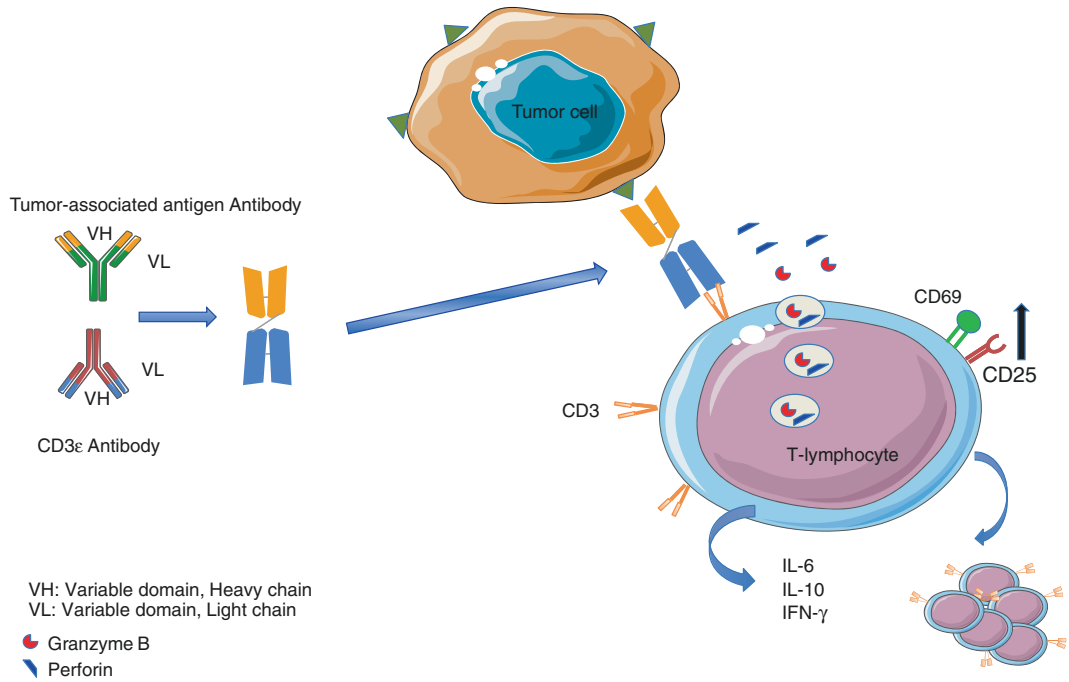

Fig. 38.2 Blinatumomab architecture and mechanism of action

scFv recognizes the tumor-associated antigens (TAA), and the C-terminal scFv binds to invariant CD3ε monoclonal antibody. The two arms are connected by a non-immunological linker and provide a remarkable flexibility. This therapeutic architecture is independent from TCR specificity and from peptide antigen presentation. Therefore, by bypassing the MHC I TCR axis on CTL, polyclonal T cells can be recruited to the tumor microenvironment and overcome the downregulation of MHC molecules that is often found on cancer cells. In vitro experiments revealed that BsAb like blinatumomab (CD3/CD19) coculture triggers immunological synapse between effector T and leukemic cells. These synapses allow the release of perforin and granzyme (pore-forming protein) by T cells further contributing to the cell nuclear condensation and membrane blebbing. The immunological synapses also lead to the upregulation of surrogate immune-activating receptors like CD25 and CD69 and production of pro-inflammatory cytokines such as IL-2, IFN-γ, and IL-6. Importantly, neither CD4+ nor CD8+ T-cell subset proliferation and activation post-blinatumomab require exogenous IL-2 administration.

38.3 Blinatumomab: A New Hope in Relapsed/Refractory ALL

Despite the remarkable advances in the treatment of pediatric ALL over the last three decades, standard therapy still fails in 10–20% of the cases. In adult ALL, only half of the patients remain tumor-free at 5 years, and prognosis post-relapse remains grim. B-ALL leukemic cells express several surface antigens amenable to mAb such as CD19, CD20, and CD22. Despite being expressed on normal B-cell lineage from the late pro-B-cell stage to plasma cell differentiation, these receptors are absent on hematopoietic stem cells. Furthermore, we learned from rituximab that CD20 inhibition was associated with only transient B-cell depletion and rare immunosuppressive complications.

The initial BsAbs clinical trial using standard mAb infusion schedule was negative and poorly tolerated. Interestingly, for the clinical development of these molecules, the initial in vivo proof of concept in macaques revealed a unique pharmacokinetics. BsAbs have an extremely short half-life of 2 h, which is in stark contrast to a 21-day half-life of a mAb like rituximab [47]. Based on these preclinical pharmacokinetics data, blinatumomab was subsequently administered in continuous infusion over 28 days using a mini pump to maintain a steady drug concentration.

In adults with relapsed/refractory ALL, two phase II trials led to the FDA approval of blinatumomab in December 2014 and EMA in November 2015. In the MT103-206 trial, 36 patients received continuous blinatumomab and 69% achieved CR or CR with partial hematological recovery [48]. Of note, as reported in prior phase 1 trial, CNS adverse events appeared to be the most relevant complication and required discontinuation of the treatment. These unprecedented results in a heavily pretreated population led to a larger confirmatory phase II trial with 189 patients [49]. CR or CRh occurred in similar proportion of patients without previous (42%) or post (45%)-allogeneic HSCT. It is important to mention that 32 out of the 52 patients who achieved CR or CRh proceeded to allogeneic HSCT.

More recently, the phase 3 TOWER trial confirmed the superiority of blinatumomab to standard care in adult patients with relapsed/refractory ALL. Of the 405 patients included, the median OS was 7.7 months in the blinatumomab group compared to 4.0 months in the chemotherapy group. Treatment with the BiTe also resulted in higher complete remission (34% vs 16% $p = 0.01$). With respect to toxicity, the investigators reported equal incidence of grade 3 adverse event in both arms [50].

In pediatric relapse/refractory ALL, Arend von Stackelberg published a phase I/II study including more than 90 children. Among the 70 patients who received the optimal dose, 39% achieved complete response, and 52% of them achieved complete minimal residual response. Based on 2-year follow-up, the median OS was 7.5 months and more than 20% were still alive [51].

Safety Profile

Despite the very potent anticancer effect of blinatumomab, unique adverse events have been reported in the majority of clinical trials. From a hematological perspective, thrombocytopenia, neutropenia, and hypogammaglobulinemia were relatively common. Cytokine release syndrome (CRS) was anticipated from the published CAR-T trials. CRS is caused by a transient release of inflammatory cytokines including IL-6 and IL-2 and IFN-γ from T cells after being engaged to tumor cells. Fever, chills, hypotension, and respiratory distress are characteristic. Life-threatening CRS was reported during the first trials of CAR-T, and IL-6 inhibitor tocilizumab was required [52]. To prevent severe CRS, premedication with dexamethasone was recommended especially for patients with high blast count over 15×10^9/L. Neurological events were the most challenging and unexpected toxicity reported for blinatumomab. These included seizure, absence, and confusion. The underlying mechanism is still unclear but might be secondary to a transient neuroinflammatory irritation of the CNS. Activated T cells can cross the blood-brain barrier and trigger toxic inflammation. Guidelines were published in order to adequately prevent and treat neurological toxicity with dexamethasone prophylaxis and dose adjustment.

38.4 BiTe for Non-ALL Hematological Malignancies

The proof of concept on the efficacy of BiTe has been demonstrated in ALL. Similar compounds are being tested for other indications:

1. AMG330 targeting CD3/CD33 is a promising antibody. Preclinical studies demonstrated that targeting CD33 in AML led to T-cell recruitment and expansion [53]. Phase 1 trials are currently being conducted (NCT02520427).
2. Blinatumomab appeared to be effective in 21 heavily pretreated DLBCL patients. After 1 cycle, the overall response rate was 43% and 19 were in CR [54]. More studies are being conducted.
3. Multiple myeloma—multiple epitopes specific to malignant cells are being tested in preclinical studies in phase I. These include BCMA transmembrane activator and calcium modulator exclusively expressed on B-cell lineage or Wue-1 [55].

Blinatumumab are emerging as immunomodulating drugs with significant therapeutic value. However, despite the promising results for patients with ALL relapsed/refractory disease, many patients only experience transient benefit, and the drugs remaine often a bridge to allogeneic HSCT. Clinical trials are already underway to administer BsAbs in less heavily treated patients and in combination with other immunotherapeutic drugs. These approaches might increase the anticancer effects and the cure rate and potentially change the way we treat hematological malignancies.

38.5 Immune Checkpoint Inhibitors (or Activators)

Defective immune response caused by T-cell exhaustion is a common feature of immune escape for many types of cancers and will be extendedly reviewed in this book. The unprecedented positive clinical trials in solid tumors demonstrated the therapeutic potential of blocking the inhibitory receptors CTLA-4 and PD-1. In this section, we will review the most important clinical trials of ICB in hematological malignancies.

Anti-PD-1 Blockade as a Proof of Concept in cHL cHL was one of the first hematological malignancies in which immune checkpoint blockade was attempted as it represented an interesting model for therapeutic inhibition of the PD-1/PD-L1 axis. Recurrent amplification of the chromosome 9p24.1 locus which includes PD-L1, PD-L2, and JAK2 or infection with EBV all led to hyperexpression of PD-L1 and PD-L2 at the surface of Hodgkin and Reed-Sternberg cells

[56]. The pivotal phase 1 trial with nivolumab, a fully human IgG4 directed against PD-1, in 23 patients with relapsed/refractory cHL was a clinical success that led to its approval in 2016 [57] (Table 38.1). With an acceptable safety profile, nivolumab demonstrated impressive clinical activity in heavily pretreated patients, with an objective response rate of 87% (20/23), including 17% with a complete response and 70% with a partial response, and a 24-week PFS rate of 86%. These results have been confirmed in a published phase II trial that enrolled 80 patients after failure

Table 38.1 Clinical trials of Immune checkpoint inhibitors in hematological malignancies

	Study	Patients	Molecule/dosing	Outcome	Toxicity
Hodgkin lymphoma	Ansell et al. [57] Phase I	23 patients relapse/refractory cHL (78%—Brentuximab), (78%—autologous HSCT)	Nivolumab 3 mg/kg every 2 weeks	Overall and complete response rates were 87 and 17%	2 patients discontinued the drug
	Younes et al. [58] Phase II	80 patients who failed to respond to autologous stem-cell transplantation	Nivolumab 3 mg/kg every 2 weeks	Overall and complete response rate 66 and 9%	Grade 3 or 4 adverse events were neutropenia 5% patients) increased lipase concentrations 5%
	Armand et al. [59] Phase Ib	31 patients: disease progressed on or after treatment with brentuximab vedotin	Pembrolizumab, 10 mg/kg every 2 weeks	PFS at 1 year 46%	No grade 4 toxicity or death related to immune side effects
	Herbaux et al. [60] Retrospective	20 patients who relapsed after allogeneic transplantation	3 mg/kg every 2 weeks	Overall response rate 95%	GVHD occurred in 6 patients and 2 died—all of them had prior history of aGVHD
Other hematological malignancies	Ansell et al. [63] Phase I	18 patients refractory follicular, DLBCL, Mantle	Ipilimumab 3 mg/kg and then monthly at 1 mg/kg × 3 & 3 mg/kg monthly × 4 months	2 patients had clinical response (CR & PR)	Colitis grade 3 in 56% but no grade 4 toxicity
	Lesokhin et al. [66] Phase I	Follicular, $n = 10$; DLBCL, $n = 11$; other B-cell lymphomas, $n = 10$; mycosis fungoides, $n = 13$; T lymphoma $n = 10$; multiple myeloma, $n = 27$	Nivolumab 1 or 3 mg/kg every 2 weeks	ORR 40%—Follicular 36%—DLBCL and 40%—Peripheral T cell No objective response in MM	Mostly grade 1–2 toxicity
	Westin et al. [65] Phase II	32 patients with follicular lymphoma	Pidilizumab 3 mg/kg every 4 weeks for 4 infusion + Rituximab 375 mg/m^2 weekly for 4 weeks	ORR 66%, CR 52%	Absence of grade 3–4 toxicity

of both autologous SCT and BV [58]. The phase Ib KEYNOTE-013 trial with pembrolizumab, another anti-PD-1 mAb, demonstrated similar efficacy though the ORR (65%) was slightly lower than with nivolumab [59]. Nivolumab was tried in cHL after failure of allogeneic SCT with some efficacy despite a risk of triggering acute GVHD [60, 61]. The occurrence in patients who received nivolumab prior to allogeneic SCT of a higher-than-expected rate of early severe transplant-related complications, including several fatal cases of acute GVHD, warrants further scrutiny [62]. To date, PD-1 inhibitors are prescribed continuously for as long as they remain beneficial. In melanoma, some patients were on PD-1 inhibitors for 2 years. The duration of PD-1 treatment in responders remains an open question. Indeed, recent clinical observations suggest that a shorter course or intermittent prescriptions of PD-1 might be as potent as continuous injections. Several investigators are now assessing different PD-1 injection schedule and earlier discontinuation for selected patients.

38.6 Checkpoint Blockade in Other Hematological Malignancies

Can we expect the success obtained in cHL with anti-PD-1 to other types of hematologic malignancies? The first results of early-phase clinical trials indicated that single-agent blockade of PD-1 or CTLA-4 was less active, and the underlying reasons remain unknown.

38.6.1 Non-Hodgkin Lymphomas (NHL)

The first study on checkpoint blockade in NHL was a phase I trial with the anti-CTLA-4 mAb ipilimumab [63]. The ORR rate was a disappointing 11%. However, the fact that one patient with DLBCL had complete response >31 months and one with follicular lymphoma a partial response lasting 19 months suggested that checkpoint blockade might have therapeutic relevance even in aggressive diseases.

Following the results of a phase I study [64], pidilizumab, a humanized anti-PD-1 IgG1 mAb, was tried after autologous SCT in 66 very high-risk DLBCL or PMBCL patients to test the hypothesis that PD-1 blockade could help eradicate residual disease and improve PFS. PFS was 72% at 16 months meeting the primary endpoint. Interestingly, pidilizumab led to apparent CR in 34% of patients with a measurable disease after transplant. Furthermore, the 16-month PFS was 70% in patients with a posttransplant positive PET scan, which compared favorably with the results observed in this very high-risk subgroup. Pidilizumab in combination with rituximab in relapsing rituximab-sensitive follicular lymphoma (FL) was associated with an ORR and CR rate of 60% and 52%, respectively [65] (Table 38.1). Although these figures seem higher than one could expect from rituximab alone in this population, the use of combination therapy makes interpretation of these results challenging. Nivolumab also demonstrated antitumor activity in various types of NHL when given in monotherapy, providing an ORR of 40% and 36% in follicular lymphoma and DLBCL, respectively, which lasted for >12 months in some patients who reached CR [66].

In conclusion, NHL seems to be less sensitive to PD-1 or CTLA-4 inhibition than HL, but more patients are currently being enrolled in trials combining different ICBs. One exception might be the PMBCL subtype. PMBCL shares common biological features with cHL including frequent overexpression of PD-L1 due to either amplification of the 9p24.1 locus or other mechanisms, which suggests that PD-1/PD-L1 blockade might also be effective. Owing to the rarity of the disease, clinical data are scarce. Two patients with PMBCL enrolled in a phase Ib trial had clinical responses to nivolumab [66]. Preliminary results in the PMBCL independent cohort of the KEYNOTE-013 trial reported an intent-to-treat ORR of 40% in heavily pretreated patients [66]. Results of pembrolizumab in patients with relapsed PMBCL (NCT02576990) will clarify whether PMBCL are comparable to cHL in terms of response to PD-1 blockade.

38.6.2 Multiple Myeloma

Though PD-L1 is harbored by plasma cells, preliminary results with nivolumab in multiple myeloma (MM) were disappointing with no patient achieving objective responses, though stabilization of the disease was observed in 63% of cases [66]. The one patient with MM enrolled in a phase I trial with pembrolizumab had a stable disease that lasted more than a year. Thus, solely blocking the PD-1 axis may not be effective in MM. There is a growing interest in PD-L1 that is expressed by plasma cells and on plasmacytoid DCs and myeloid-derived suppressor cells (MDSC) which play a role in MM pathogenesis. Several trials are currently evaluating the anti-PD-L1 mAb atezolizumab and durvalumab alone or in combination. Furthermore, promising combinations of pembrolizumab with Revlimid and dexamethasone or pomalidomide are currently being investigated. Phase 1 trials already demonstrated that these combinations were well tolerated.

38.6.3 Myeloid Malignancies

Preclinical data suggest that myelodysplastic syndrome or acute myeloid leukemia may be sensitive to inhibition of the PD-1/PD-L1 or CTLA-4 pathways [67]. Interestingly, several lines of evidence demonstrated that exposure to the hypomethylating agent decitabine resulted in dose-dependent upregulation of PD-L1 and PD-L2. PD-1 inhibition demonstrated limited activity in AML [64]. Data from clinical trials have been published mainly in abstract forms in conferences. Results are still immature to draw conclusions, but preliminary results suggest some activity, at least in some patients. In a phase I/Ib trial after allogeneic SCT, ipilimumab yielded objective responses, including CR in four patients with extramedullary AML and one patient with secondary AML following MDS [68]. As with NHL, it is unlikely that immune checkpoint inhibition will demonstrate major efficacy in myeloid malignancies when used as a single agent. This provides a rationale for trying combination therapy in order to enhance the anti-tumor and immunogenic effects of PD-1 or other immune checkpoint inhibitors.

38.7 Future Directions

Immune-based therapies have been at the forefront of hematology for more than 30 years with the pioneer works describing the anticancer activity of graft versus leukemia (GVL) effect and consequently the development of allogeneic transplantation (HSCT). However, graft-versus-host disease (GVHD) still remains the principle hurdle in HSCT for favorable patient outcome. Subsequently, rituximab confirmed the proof of concept that mAb alone or in combination with standard chemotherapy provided unparalleled clinical response and revolutionized our approach to treat cancer. Despite this success story, the role of the immune system in cancer remained underestimated for many decades. It is only, recently, after unveiling that tumors have the unique ability to downregulate infiltrating T cells by activating negative regulatory pathways that the field of immuno-oncology has led to an unprecedented approval of immune-modifier drugs.

Despite numerous positive trials, the field of immune checkpoint modulation is still in its infancy. Although the results obtained with anti-PD-1 mAb were a breakthrough in the treatment of relapsed/refractory cHL, it should be kept in mind that ICBs only offer a prolonged disease control. Therefore, in order to continue to improve patients' outcome, it is of upmost importance to determine the optimal timing for anti-PD-1 therapy in cHL and whether combinations with chemotherapy of BV can increase its potency. In this sense, clinical trials are now evaluating the benefits of PD-1 blockade in association with chemotherapy in the first line or, similarly to the AETHERA trial, the KEYNOTE-204 study comparing a maintenance treatment with either pembrolizumab or BV in relapsing cHL (NCT02684292) after autologous HSCT. For now, less impressive than in cHL, the activity of PD-1 or CTLA-4 blockade observed in some patients with other hematological diseases justifies further evaluations, in particular in combina-

tions. Furthermore, preclinical data suggest that novel agents such as immunomodulatory drugs (IMIDs) or small-molecule inhibitors, which have an impact on the immune system homeostasis, might augment their therapeutic efficacy [69]. A combination of either IMIDs or ibrutinib and PD-1 blockade is the subject of several clinical trials in MM and NHL.

Experimental data provides a rationale for combining 5-azacitidine and PD-1 blockade [67]. Finally, new mAbs are being developed against other inhibitory receptors such as Lag-3, TIGIT, Tim-3, or OX40.

In addition, for more powerful and targeted engagement of cancer and cytotoxic T cells, bispecific antibodies linking CD3/CD19 were developed, and blinatumomab was the first BiTE approved by the FDA for relapse/refractory ALL. The development of highly active compound in these heavily pretreated patients is an expanding field, and preclinical data also support the combination of BiTE antibodies and blockade of the PD-1/PD-L1 axis in leukemia [70].

For all immuno-oncology compounds being developed, two challenging questions remain to be answered to improve their potency: what may be the optimal sequence/timing and what robust biomarkers may be used to predict efficacy. Multiple clinical trials are now enrolling patients to move these molecules into frontline regimens, and scientists are testing new biomarkers (immunohistology, genetic mutations, and gut microbiota) to better predict response and toxicity.

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Immunotherapy of Gliomas

39

Michael Platten

Contents

39.1 Introduction

Gliomas are intrinsic incurable brain tumors with diffusely infiltrative growth and resistance to genotoxic treatments. Despite this, standard therapy consists of surgery radiotherapy and alkylating chemotherapy. Gliomas are among the molecularly best characterized tumors, yet numerous clinical trials aiming at treating this challenging disease using targeted agents have been unsuccessful, which may not be solely attributed to poor blood-brain barrier penetrance of many targeted compounds. Thus, there is a profound medical need for the development of innovative therapies. The dogma that the central nervous system (CNS) is an immune-privileged site inaccessible for peripheral immune responses has been largely refuted. The CNS is constantly surveyed by antigen-specific T cells preventing opportunistic infections such as progressive multifocal leukoencephalopathy (PML) caused by a CNS-specific activation of the JC virus in immunocompromised individuals [1]. In addition, devastating autoimmune diseases of the CNS such as multiple sclerosis (MS) are initiated by peripheral activation of CNS antigen-specific immune responses infiltrating the CNS. Finally, the recent proof of lymphatic drainage of the CNS supports the notion that there is intense immunological communication between the CNS and the peripheral immune system [2, 3]. Thus, there is renewed interest in glioma immunotherapy, but many concepts and opportunities

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of immunooncology such as checkpoint inhibition are readily applied to gliomas often without taking into account the particular immunological challenges associated with this disease. These challenges are (1) the definition of appropriate target antigens, (2) the translation of an effective immune response through the CNS barrier into the tumor microenvironment, and (3) the prevention of immune deactivation by the glioma immune microenvironment [4]. Conceptually, addressing these challenges requires innovative early clinical trial concepts assessing intratumoral immune responses, which is particularly challenging in gliomas. In the following sections, the current status of glioma immunotherapy will be discussed with a particular focus on these challenges and innovative trial concepts.

39.2 Defining Appropriate Antigens

Targets for vaccines in glioma immunotherapy are in the transition from classic **self-antigens** to private neoepitopes. Melanoma antigens such as AGE-A1/3, TRP-2, or gp100 are also expressed in gliomas albeit at variable levels and are used for vaccination protocols in low- and high-grade gliomas [5]. Recent efforts have focused on antigens expressed by glioma stem cells [6]. ICT-107 a dendritic cell vaccine encompassing antigens believed to be enriched in glioma stem cells: HER2, TRP-2, gp100, MAGE-1, IL13Rα2, and AIM-2 [7]. A randomized, double-blind phase 2 clinical trial indicated a survival advantage in the ICT-107-treated group compared to the control group and also showed an association between immune response and survival, especially in HLA-A2-positive (HLA-A2+) patients. In HLA-A2+ patients, a randomized, double-blind, placebo-controlled phase 3 registrational trial of ICT-107 encompassing 500 HLA-A2+ patients with newly diagnosed glioblastoma is underway (EORTC1587, NCT02546102). This trial as other multipeptide vaccine trial will not be able to address the relevant glioma antigen in a given patient nor provide evidence that an effective intratumoral immune response is generated. In principle, however, self-antigens may be a meaningful target in gliomas. A recent report on a patient with a disseminated glioma demonstrated an objective response after intracranial infusion of IL13Rα2-targeted CAR T cells without overt toxicity [8, 9].

There are two major determinants for the induction of an effective antitumor immune response targeting self-antigens in general. First, many self-antigens are expressed in the thymus, resulting in central T cell tolerance and the development of antigen-specific suppressive T-regulatory cells [10]. Thymic tolerance to tumor-associated antigens in an individual patient, however, is not generally tested. Second, the suitability of patients for vaccination against tumor-associated antigens of tumor antigens is usually assessed based on the expression profile, and the HLA type predicted to present the respective epitope with high affinity. Methods to **test the presentation of antigens in tumor tissue**, however, have only recently been developed and subsequently implemented into clinical trial designs. IMA950 is a multipeptide self-antigen peptide cocktail vaccine for HLA-A2+ glioma patients encompassing tumor-associated peptides (TUMAP) extracted from an HLA ligandome by mass spectroscopy evaluation of peptides eluted from HLA-A2 in glioma tissue [11]. IMA950 has been investigated in a first-in-man study (NCT01222221) in 45 patients with newly diagnosed glioblastoma. Of 40 evaluable patients, 36 were TUMAP responders and 20 were multi-TUMAP responders. PFS rates were 74% at 6 months and 31% at 9 months. More recently, this approach has been extended to discover the presentation of mutated antigens [12]. The multicenter Glioma Actively Personalized Vaccine Consortium (GAPVAC) trial aims at assessing the safety and feasibility of a personalized vaccine in patients with newly diagnosed glioblastoma (GAPVAC-101, NCT02149225). Here, the selection and production of the personalized peptide vaccine are based not only on whole exome sequencing (WES) but also on **HLA ligandome** analyses providing additional information of the actual presentation of the relevant epitopes on HLA molecules in the tumor tissue. Another

method to analyze antigen presentation in tumor tissue comprises the adaptation of an in situ **proximity ligation assay** (PLA). Two prerequisites have to be fulfilled for this method to be applied to detection of antigen presentation: (1) a neoepitope-specific antibody has to be available, and (2) this antibody must recognize the neoepitope in the context of MHC. This method has been developed to assess the presentation of the glioma neoepitope IDH1R132H in situ in paraffin-embedded glioma tissue [13]. Here, proximity of the IDH1R132H neoepitope and MHC class II was detected in 10/20 patients with IDH1R132H-mutant gliomas and 0/19 patients with IDH1 wild-type gliomas indicating high specificity of the assay. The advantage of this assay in contrast to HLA ligandome analyses is the low amount of tissue necessary, the applicability to archival paraffin-embedded tissue, and the cellular resolution, which allows for differentiation of the cell type presenting the antigen by applying co-immunostainings. In gliomas, the neoepitope may be presented by MHC class II-positive glioma cells [13].

39.3 Neoepitopes in Gliomas

As in other tumor diseases, most neoepitopes (mutated or variant epitopes) in gliomas are private epitopes. There are few examples of shared neoepitopes. The variant III of the epidermal growth factor receptor [EGFR] is a tumor-specific antigen generated by alternative splicing of exons two through seven and fusion of exon 1 with exon 8 generating a novel amino acid sequence. **EGFRvIII** is detectable at varying mRNA expression levels in about 25% of glioblastoma. Following preclinical studies in syngeneic mouse models, a peptide vaccine using the neoepitope sequence conjugated to the adjuvant keyhole limpet hemocyanin (KLH) was developed. This vaccine induces anti-EGFRvIII antibody responses in humans, whereas robust T cell responses have not been described [14]. In phase I/II studies (NCT01920191, NCT01222221), the vaccine was safe and resulted in a survival benefit compared with historic controls. Initial studies also suggested proof of biological efficacy based on the observation that in the majority of patients with recurrence EGFRvIII was no longer detectable [15]. Subsequently, EGFRvIII Pep-KLH (Rindopepimut®) was tested in a placebo-controlled, double-blind, multicenter phase III registrational trial in patients with newly diagnosed EGFRvIII-positive glioblastoma as an adjunct to combined radiochemotherapy with temozolomide (ACT-IV, NCT01480479). This trial has missed the primary endpoint and failed to demonstrate efficacy of the vaccine with an OS of 20.4 months in the Rindopepimut® group compared with an OS of 21.1 months in the control group. Potential explanations of this negative result include (1) an insufficient immune response due to simultaneous chemotherapy, (2) the potential lack of EGFRvIII-specific T cell response, and (3) immune escape through antigen loss. This antigen loss is rather due to primary clonal heterogeneity and subsequent selection of neoepitope-negative clones than due to secondary antigen loss as a result of an effective neoepitope-specific immune response. Indeed, on a single-cell level, EGFRvIII is expressed in newly diagnosed glioblastomas only in a fraction of tumor cells and generally co-expressed with wild-type EGFR.

These observations lead to an important topic in immunooncology, which is the **clonal representation of neoepitopes**. Conceptually, a vaccine targeting a subclonal neoepitope will be ineffective unless the initial neoepitope-specific immune response is potent enough to induce antigen spreading. Hence, an ideal neoepitope should not only be strongly expressed and presented with high affinity and generate a neoepitope-specific immune response but also be present in all tumor cells, thus representing a driver mutation. Targeting a true driver mutation will circumvent heterogeneity-driven immune escape. In gliomas one such driver mutation is **IDH1R132H**, a point mutation in the gene for isocitrate dehydrogenase type 1 (IDH1), which occurs in 70–80% of diffuse and anaplastic gliomas. It is the earliest mutation known in these tumors and affects all tumor cells even during malignant progression [16, 17]. The amino acid exchange at position

132 (Arg to His) affects the catalytic center of the enzyme and results in a neomorphic enzyme function leading to the production of the oncometabolite 2-hydroxyglutarate in excess amounts [18]. This, in turn, results in genetic instability via epigenetic modifications and hence tumorigenicity [19]. The presence of IDH1R132H is confirmed in routine diagnostic of gliomas using a mutation-specific antibody [20]. Patients with IDH1R132H-mutated gliomas may harbor spontaneous mutation-specific CD4+ T helper cells and antibodies [21, 22]. IDH1R132H is presented on MHC class II and vaccination of MHC-humanized mice, but also C57BL6 wild-type mice with a mutant IDH1 peptide result in a mutation-specific CD4 immune response effective in controlling IDH1R132H-expressing tumors [22, 23]. A multicenter phase I trial has completed accrual (NCT02454634) after enrolling a planned population of 30 evaluable patients with newly diagnosed grade 3 or grade 4 astrocytomas at eight German sites. Patients receive eight vaccines of a 20-mer peptide emulsified in Montanide-ISA51® integrated into the primary therapy. Primary endpoints are safety and immunogenicity as evidenced by T cell and antibody responses. Important points to be addressed for further development include the mechanism of action of CD4+ IDH1R132H-specific T cells, the degree (if any) of antigen spreading to CD8 epitopes, and the phenotype of intratumoral T cells in the immunosuppressive microenvironment.

IDH1R132H, H3.3K27M, and EGFRvIII are examples of recurrent or shared neoepitopes in gliomas. Also in glioma, most neoepitopes are private. Therefore, **personalized approaches** with the aim of individually targeting patient-specific neoantigens are implemented in glioma. Based on a computational pipeline predicting HLA binding of mutated epitopes [24], a phase I study testing a personalized peptide vaccine (NeoVax) encompassing neoepitopes relevant for the individual patient in patients with newly diagnosed MGMT-unmethylated glioblastoma is currently conducted (NCT02287428). Based on WES and HLA ligandome analyses, the European Glioma Actively Personalized Vaccine Consortium (GAPVAC) currently conducts a multicenter phase I clinical trial in patients with newly diagnosed glioblastoma (GAPVAC-101, NCT02149225). This effort increases the complexity of epitope discovery but maintains a turnaround time for vaccine production sufficient for integration of the vaccine into primary therapy. These personalized concepts are a regulatory challenge necessitating approval of a personalized integrated molecular pathology (IMP) prior to its discovery based on the proposed workflow.

39.4 Checkpoint Inhibitors in Glioma

Preclinical studies demonstrate the efficacy of monotherapy or combination therapy with checkpoint blockers in eradicating established orthotopic syngeneic chemically induced gliomas [25]. So in principle, the CNS is not an unsurmountable barrier for a peripherally induced antitumor T cell response. More than 20 phase I–III trials employ checkpoint inhibitors in patients with glioma worldwide [6]. While available data indicate an acceptable toxicity profile not considerably different from other tumor entities, efficacy data is largely lacking. Anti-CTLA4 as a single agent has been deprioritized, and most clinical studies employ anti-PD1 or anti-PD-L1 antibodies. CheckMate 143 (NCT02017717), a phase III study comparing the efficacy of the PD1 antibody nivolumab to bevacizumab in patients with recurrent glioblastoma, has not shown efficacy of nivolumab in this patient population. Safety and efficacy of the anti-PD1 antibody pembrolizumab are tested in an 80-patient, randomized, phase II trial in patients with recurrent glioblastoma. Patient accrual has completed for the randomized phase II trial (NCT02337491) which will compare the pembrolizumab/bevacizumab combination versus bevacizumab monotherapy. The ongoing CheckMate 498 (NCT02617589) and 548 (NCT02667587) analyze the efficacy of nivolumab in patients with newly diagnosed glioblastoma added to standard therapy (radiotherapy or radiochemotherapy with TMZ) in phase II and III designs, respectively. The PD-L1 antibody durvalumab is tested in a randomized

phase II clinical trial in patients with recurrent glioblastoma.

While it is too early to assess the efficacy of these approaches in glioma, the main argument against the effectiveness of checkpoint inhibitor monotherapy in unselected glioma patients is the comparatively low mutational load. Gliomas—on average—contain 40–80 non-synonymous mutations, which is an order of magnitude lower than in melanoma or small-cell lung cancer. The therapeutic efficacy, in turn, in preclinical animal models can thus be attributed to the comparatively high mutational load in chemically induced experimental murine gliomas, such as the GL261 model.

These observations argue against further trials with checkpoint inhibition alone in unselected glioma patients. Rather, efforts should be focused on patients with hypermutation either as a consequence of germ line mismatch-repair deficiency or of prolonged alkylating chemotherapy [26, 27]. Here, case series in patients with hypermutated gliomas demonstrate remarkable efficacy of checkpoint blockade with partial remission of large lesions [28, 29]. In any case, whether checkpoint inhibitors are combined with irradiation, virotherapy, or specific vaccines, future clinical trials ought to be performed in carefully selected patient populations and backed by a meaningful translational program for hypothesis testing. This translational approach will require posttreatment tumor tissue to analyze intratumoral immune responses, which is a particular challenge in gliomas. Here, innovative neoadjuvant trial concepts may circumvent in part the dilemma of challenging biopsy-treat-biopsy concept feasible in other tumor entities such as melanoma or gastrointestinal cancer.

The type of checkpoint inhibitor to be chosen for glioma therapy will depend on the compartment of target cells. PD-L1 inhibitors may not necessarily require brain penetrance either, as PD-L1 is expressed not only in the tumor microenvironment of gliomas [30] but also elevated in circulating antigen-presenting cells (APC) in glioma patients [31]. As in other tumor entities, intratumoral or peripheral PD-L1 expression may serve as a biomarker predicting response. These measures will hopefully result in proof-of-concept evidence for therapeutic efficacy in glioma. A premature rush into randomized phase III studies in unselected patients may prove to be a disservice for the therapeutic concepts as seen in efforts with antiangiogenic treatments in gliomas, which are largely terminated after negative phase III studies also in unselected glioblastoma patients.

39.5 T Cell Therapy

As with other solid tumors, T cell therapy is still at an experimental stage in gliomas. Early studies have employed unspecifically activated lymphocytes (termed lymphokine-activated killer cells, LAK) isolated from the peripheral circulation and applied locally in the resection cavity with limited success. With the recent discovery of immunogenic (neo)epitopes in gliomas and following similar approaches in lymphomas and solid tumors, the focus has shifted toward adoptive transfer of TCR-transgenic T cells. CAR T cells targeting EGFRvIII [32] are being studied in patients with glioblastoma, but the success is questionable based on the negative results of the EGFRvIII vaccine trial. More recently local adoptive transfer of T cells transduced with a modified CAR-containing 4-1BB and a mutant IgG4-Fc linker recognizing the tumor-associated antigen interleukin-13 receptor alpha 2 (IL13Rα2, ClinicalTrials.gov Identifier: NCT02208362; ClinicalTrials.gov Identifier: NCT00730613; ClinicalTrials.gov Identifier: NCT01082926) was associated with a durable partial remission of distant CNS manifestations after local delivery in a patient with recurrent glioblastoma and leptomeningeal and CSF spread. Eventually, there was tumor progression suggesting immune escape possibly related to antigen loss [8]. In principle, this report illustrates that (a) targeting tumor-associated antigens is relevant and that (b) local (i.e., CSF) delivery is sufficient to deliver therapeutically active antigen-specific T cells to gliomas. Whether systemic delivery is equally effective or whether local delivery is necessary to overcome the BBB or blood-CSF barrier remains to be proven.

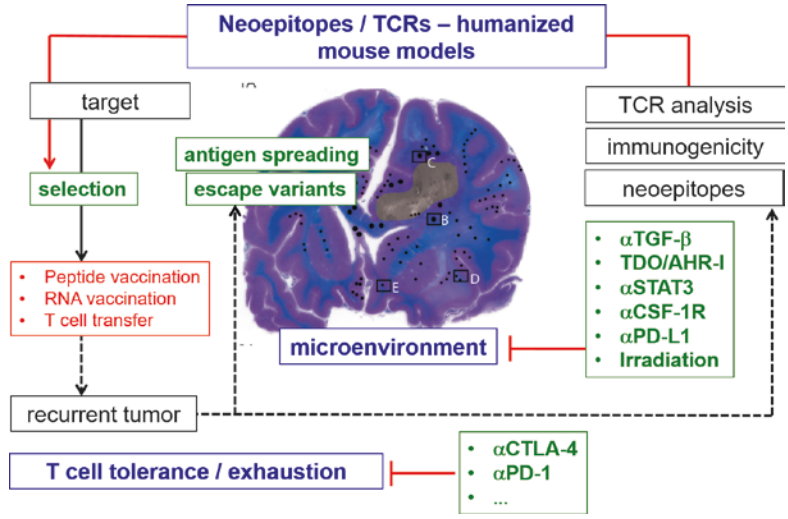

Fig. 39.1 A workflow of immunotherapy discovery and combinatorial treatment approaches in neurooncology

39.6 Targeting the Immune Microenvironment in Gliomas

Once an effective antitumor T cell response has been translated into the glioma microenvironment, its immunosuppressive nature represents the ultimate challenge. Here, physical parameters such as low pH and hypoxia are prevalent particularly in necrotic and perinecrotic areas of glioblastomas. These factors can only be meaningfully met by removal of large necrotic tumors prior to immunotherapy. Chemotherapy-induced cell death does not play a relevant role in inducing immunogenicity in gliomas largely due to the resistance toward agents, which induce immunogenic cell death. Other measures to increase the immunogenicity are radiation therapy and virotherapy. Mechanistically these approaches may increase the portfolio of immunogenic antigens by enhancing antigen presentation, inducing epitope spread, and/or exploiting antiviral immune responses. Other factors shaping the immunosuppressive microenvironment include cytokines such as IL-10, growth factors such as TGF-beta, and metabolites such as kynurenines [33]. Inhibitors of these immunosuppressive factors are available or in development and unlikely to be active as monotherapies. Here, appropriate combinatorial trials in carefully selected patient populations are mandatory (Fig. 39.1).

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40 Assessing T Cell Receptor Affinity and Avidity Against Tumor Antigens

Mathilde Allard, Michael Hebeisen, and Nathalie Rufer

Contents

Abbreviations

pMHC	Peptide-major histocompatibility complex
SPR	Surface plasmon resonance
TCR	T cell receptors

40.1 T Cell-Based Therapies Against Malignant Disease

Cytotoxic CD8 T cells mediate immune protection against a large number of infectious diseases, and recent developments in oncology indicate that they are also able to eliminate tumor cells. Immune responses against cancer rely mostly on T cells characterized by the expression of tumor-specific T cell receptors (TCRs) that allow them to specifically recognize and destroy malignant cells. Immunotherapy aims at mobilizing the body's immune cells to fight against tumor cells in a highly specific manner. During recent years, this approach has become clinically successful for cancer patients with several strategies now capable of exploiting the therapeutic potential of T cells [1]. Firstly, active immunization (or therapeutic vaccination) aims at generating and/or boosting immune responses to destroy tumor cells and preventing tumor progression. This method relies on the knowledge of tumor-associated antigens expressed by a particular type of cancer and is commonly delivered as peptide and protein vaccines or via viral and DNA-

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based vectors. Secondly, passive immunization (or adoptive cell transfer) uses autologous peripheral blood mononuclear cells (PBMCs) or tumor-infiltrating lymphocytes (TILs) isolated from cancer patients, which are then expanded in vitro, selected for tumor reactivity, and infused back into the patient [2]. Genetic modification of T cells before adoptive cell transfer may increase the clinical efficacy, such as inserting TCRs [3] or chimeric antigen receptors (CARs) [4]. Finally, immune modulation can be achieved by therapeutically targeting co-receptors known to inhibit T cell functions [5]. For instance, ipilimumab (Yervoy®), a monoclonal antibody blocking the inhibitory receptor CTLA-4 expressed on T cells, was shown to significantly improve the clinical outcome of metastatic melanoma patients [6]. More recently, remarkable benefit for patients with melanoma, kidney, and lung cancer was demonstrated following treatment with antibodies against the inhibitory receptor PD-1 [7] or its ligand PD-L1 [8], alone or combined with ipilimumab [9, 10]. Together, these recent advancements have shown that T cells are essential players in generating protective and durable immune responses against malignant cells. At present, it is becoming important to determine which T cell properties are essential to achieve clinical benefit. In this chapter, we will specifically focus on TCR-pMHC binding interactions, which are key parameters for protective T cell-mediated immunity, and review the different technologies used to assess such interactions, enabling to identify the most functionally relevant T cells.

40.2 Defining T Cell Functional Avidity and TCR Binding Affinity and Avidity

T cell functional avidity has been repeatedly associated with T cell protection and is a biological measure that describes how well T cells sense their cognate antigens. It is determined in vitro by the quantitative assessment of a given T cell functional response (such as cytotoxic activity, IFN-γ production or proliferation) upon exposure to increasing doses of antigenic peptide and is quantified by the peptide concentration needed to induce half of the maximal responses (i.e., EC_{50}) (Fig. 40.1a). Pioneering the field more than 20 years ago, Speiser and colleagues demonstrated that low T cell functional avidity is sufficient for in vitro proliferation or cytotoxicity but not for in vivo efficacy in responses to viral infections [11]. Meanwhile, there is a general consensus that CD8 T cell responses with increased

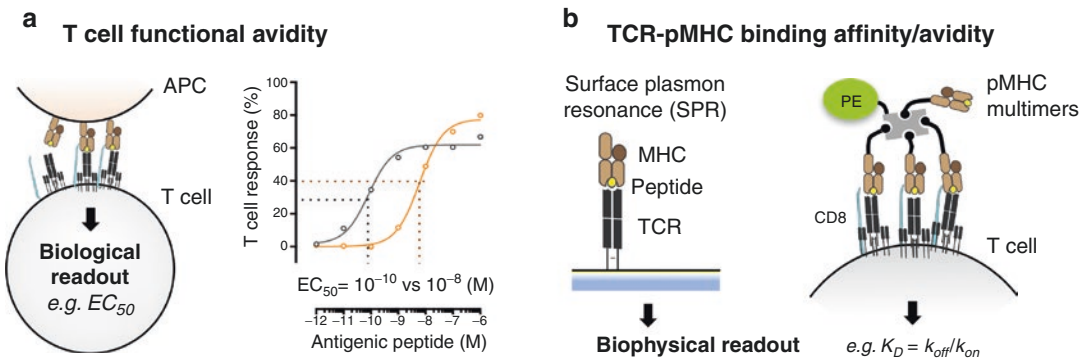

Fig. 40.1 Definition and assessment of T cell functional avidity and TCR-pMHC binding affinity/avidity. (**a**) T cell functional avidity is a biological readout that reflects T cell responsiveness upon antigen-specific stimulation and is assessed by quantifying T cell responses (i.e., cytotoxic activity, IFN-γ production or proliferation) when exposed to titrated doses of antigenic peptide. EC_{50}, defined as the peptide concentration producing half-maximal response. (**b**) TCR-pMHC binding affinity and avidity are biophysical readouts that describe the strength of monomeric/multimeric binding interaction(s) between the TCRs and their antigens and are typically measured using surface plasmon resonance (SPR) and pMHC multimers, respectively. Adapted from [31]

functional avidity provide a better control of viral replication [12–17] and tumor growth [18–22] than T cells of lower avidities (reviewed in [23]). However, the ex vivo assessment of T cell functional avidity remains technically laborious and time consuming. It is also not well suitable for standardization because of the inherent variability of biological/functional metrics. Hence, the ex vivo appraisal of T cell functionality is still mostly limited to assays performed with fixed stimulation doses and to the lack of universal standards of T cell assessment (reviewed in [24, 25]). Based on these considerations, there is a strong need to improve our knowledge regarding the contribution of the different aspects of T cell function to clinical efficacy and to identify additional T cell-based parameters that overcome the major limitations associated to functional assays.

CD8 T cell functional avidity is predominantly guided by the strength of TCR binding and kinetic interactions to antigenic peptides presented by MHC class I molecules (i.e., pMHC). Importantly, the binding and kinetic attributes of this interaction are determinant parameters that influence almost every aspect of T cell biology, including thymic selection (reviewed in [26]), differentiation into effector and/or memory T cells ([27], reviewed in [28]), and functional efficacy (reviewed in [29–31]), and may offer superior metrics to evaluate the quality of T cell responses. However, due to technical limitations, the assessment of TCR-pMHC binding avidity or kinetics is still infrequently determined in research or patient immunomonitoring or in the selection of tumor-infiltrating T cells used for adoptive cell therapy. TCR-pMHC binding interactions can be measured in terms of affinity or avidity (Fig. 40.1b), both of which can directly impact the overall functional T cell response. The TCR binding affinity refers to the physical strength by which a single TCR binds to a single pMHC complex and is typically measured by surface plasmon resonance (SPR). Conversely, the TCR avidity describes the binding strength of TCR-pMHC interactions when assessed in the cellular context, includes the contribution of the co-receptors (e.g., CD8), and has often been estimated using recombinant soluble pMHC multimers. TCR-pMHC binding affinity/avidity is inversely proportional to the dissociation equilibrium constant K_D, defined as the ratio of the dissociation rate (i.e., k_{off}) and association rate (i.e., k_{on}) under equilibrium conditions (reviewed in [29–31]).

40.3 Tumor-Specific T Cell Responses Are Mediated by TCRs of Low Binding Affinity/Avidity

Peripheral T cell repertoire diversity is achieved through random somatic gene segment rearrangements of the TCR alpha and beta chains during thymic development. The TCR repertoire is then shaped by positive intrathymic selection events allowing generating a peripheral T cell pool that can interact with the host pMHC complexes. However, to limit the development and/or activation of potentially harmful self-reactive T cells, the ones displaying TCRs of relative high affinity/avidity toward self-pMHC are deleted or anergized by mechanisms of central (also defined as negative thymic selection) and/or peripheral tolerance (reviewed in [32, 33]). As such it is now commonly accepted that the peripheral T cell repertoires targeting self versus nonself antigens may vary dramatically in terms of TCR-pMHC binding affinity/avidity (Fig. 40.2).

Many tumor-associated antigens that are shared among cancer patients, such as overexpressed (e.g., Her2/neu, WT1, or telomerase), differentiation (e.g., Melan-A/MART-1, gp100 or tyrosinase), cancer-testis (e.g., NY-ESO-1 or MAGEs), or oncofetal (e.g., AFP or CEA) antigens, are not truly cancer specific but are also expressed by healthy tissues (reviewed in [34, 35]). As a consequence, the natural T cell repertoire targeting self/tumor antigens is thought to express TCRs of relative low affinity/avidity, whereas high-avidity T cells are very rare since they are eliminated through mechanisms of central and/or peripheral tolerance. Aleksic and colleagues [36] demonstrated that the K_D values of these interactions are typically in the

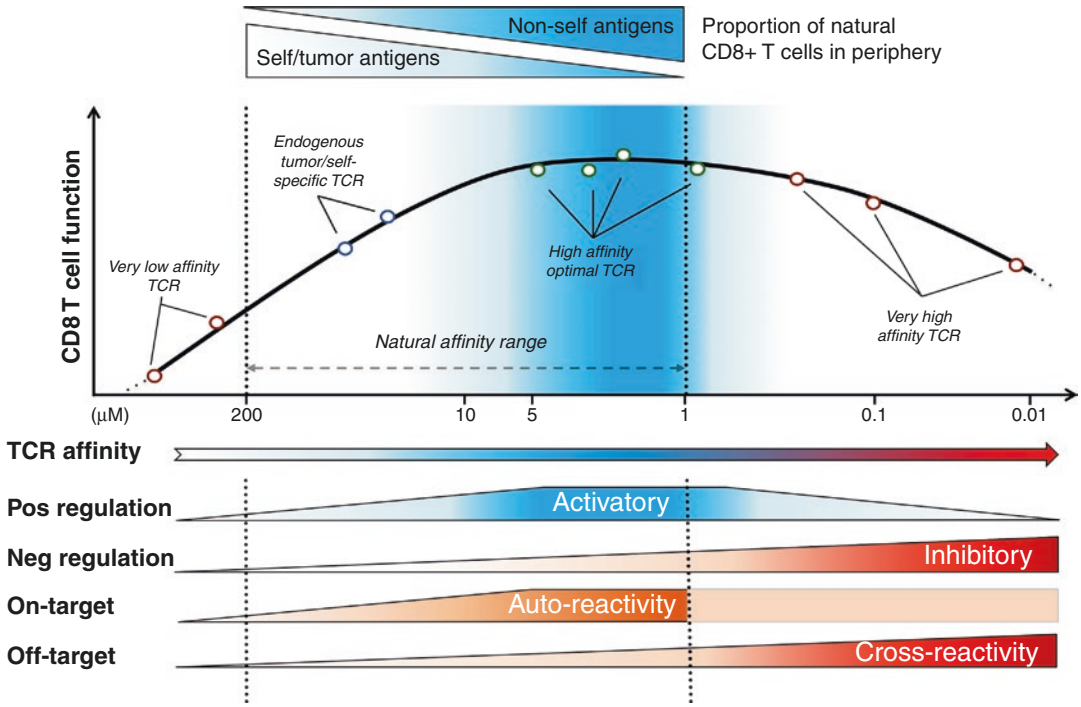

Fig. 40.2 Model integrating the relationship between T cell function, TCR affinity, and positive/negative regulators modulating cell responsiveness. A panel of CD8 T lymphocytes engineered to express antitumoral TCRs with incremental affinities toward the HLA-A2/NY-ESO-1 epitope (*x*-axis) was assessed for functional capacity (*y*-axis). An optimal window of TCR-pMHC affinity can be detected in the upper natural range (K_D from 5 to 1 μM), corresponding to the range where most peripheral nonself-/virus-specific T cells can be detected [36]. We recently described how positive regulators of T cell function including CD28 and TNFR cofactors are enriched in T cells lying within the optimal affinity window and how the inhibitory receptor PD-1 and SHP-1 phosphatase are involved in restricting T cell activation and responsiveness in TCR-engineered CD8 T cells of very high supraphysiological affinities [47]. On-target toxicity is known to follow the functional and activatory signatures of CD8 T cells, while off-target responsiveness and toxicity are described to increase along TCR-pMHC affinity, within the supranatural affinity range (reviewed in [58]). Adapted from [48]

range of 200 μM to 10 μM (with the mean around 100 μM), while virus-specific TCRs interact with K_D ranging down to 1 μM (with the mean value around 10 μM). Nonetheless, these tolerance mechanisms spare T cells that can react to self/tumor antigens with relative low TCR-pMHC binding affinity/avidity [37–40]. Thus, although cancer patients may have many self-/tumor-specific T cells, their TCR affinity/avidity remains relatively low and in fact eventually too low to mediate an effective antitumor immunity. Therefore, increasing the TCR-pMHC affinity and/or avidity of tumor-specific T cells is of particular interest in the context of cancer immunotherapies.

40.4 Optimization of TCR-pMHC Binding Affinity/Avidity Against Cancer Cells

Adoptive transfer of TCR-engineered T cells is a recent type of immunotherapy, which aims to establish and boost immune reactivity toward poorly immunogenic tumors. This strategy relies on the optimization of the TCR sequence to increase its affinity/avidity for cognate tumor antigens with the aim to adoptively transfer them back to patients. Indeed, adoptive cell transfer of engineered T cells augments the functional and protective capacity of tumor-antigen reactive CD8 T cells [41, 42]. During recent years, we estab-

lished a unique panel of human CD8 T cells engineered with TCRs of progressive increasing affinities against the tumor antigen NY-ESO-1 presented in the context of the HLA-A2, obtained through structure-based rational predictions [43, 44]. We observed that T cells expressing TCRs with affinities in the upper natural range (K_D from 5 to 1 μM) displayed greater biological responses when compared to those expressing intermediate affinity wild-type TCR (K_D at 21.4 μM) or very low affinity ($K_D > 100$ μM) ([45, 46]; Fig. 40.2). Strikingly, further increase within the supraphysiological TCR affinity range ($K_D < 1$ μM) led to drastic functional decline, with impairment in global gene expression, signaling, and surface expression of activatory/costimulatory receptors [46, 47]. Major findings revealed that maximal T cell effectiveness was limited by at least two mechanisms (Fig. 40.2). First, we observed the preferential expression of the inhibitory receptor PD-1 within T cells expressing very high TCR affinities, and this correlated in those cells with functional recovery upon PD-1 ligand 1 (PD-L1) blockade [47]. Second, contrasting to PD-1 expression, we observed a gradual up-regulation of SHP-1 phosphatase in CD8 T cells with increasing TCR affinities. Consequently, pharmacological inhibition allowed further incremental gaining of cell function in all engineered T cells, according to their TCR-binding affinities [47]. Our observations fit nicely with other studies performed both in mice and human models (reviewed in [48]) and provide strong evidence that T cell activation and signaling can be increased up to a given affinity threshold for the TCR-pMHC interaction and that above this threshold, T cells may not develop productive functions.

The importance of TCR-pMHC binding parameters has also been demonstrated in several clinical trials (reviewed in [49–52]) (Table 40.1). Initial reports have shown that in contrast to T cells of low avidity, high-avidity tumor-specific T cell responses were often associated with autoimmunity [53–55]. On-target reactivity was more recently observed in a clinical trial whereby melanoma patients received autologous blood mononuclear cells transduced with affinity-optimized TCRs against the differentiation tumor antigen Melan-A/MART-1 ([41]; Table 40.1). Compared to the native low TCR avidity (referred as DMF4), the DMF5 TCR of higher binding avidity toward HLA-A2/Melan-A showed improved clinical efficacy. Importantly, T lymphocytes expressing increased avidity self-/tumor-specific TCRs also targeted normal tissues expressing the cognate antigen (e.g., melanocytes in the skin, eye, and ear for both Melan-A and gp-100-specific T cells), thus mounting harmful cytotoxic immune responses in vivo [41]. Similar on-target reactivity was observed when using a TCR engineered against the human carcinoembryonic antigen (CEA) in patients with refractory metastatic colon carcinoma [56]. Whereas all three patients experienced decline in serum CEA levels, severe transient inflammatory colitis was further reported in those patients [56]. Interestingly, studies based on the A*0201/NY-ESO-1 cancer-testis antigen model showed that genetically optimized T cells were those displaying maximal functionality, leading to objective clinical responses, but without in vivo cross-reactivity or major adverse events [42, 57]. These apparently contradictory results could be explained by the differences in tissue distribution of NY-ESO-1 antigen being only expressed in testis cells when compared to Melan-A or CEA antigens, both widely found in normal melanocytes or epithelial cells of the gastrointestinal tract, respectively. Consequently, the choice of antigen specificity for adoptive cell transfer of affinity-improved T cells is of crucial importance (reviewed in [52]).

Another parameter to be considered is the risk of increased cross-reactivity to structurally related self-peptides, resulting in off-target toxicities (reviewed in [58]; Table 40.1). Using experimental models, it has been demonstrated that T cells, whose TCR binds to pMHC complexes with very high avidities ($K_D < 1$ nM) lose antigen specificity and can become cross-reactive [59–61]. Similarly, critical results from recent clinical trials revealed that affinity-enhanced TCRs engineered above a certain threshold could lead to severe off-target side effects in patients (reviewed in [62]). Patients treated with TCRs engineered for enhanced affinity toward the cancer-testis HLA-A1/MAGE-A3 tumor antigen

Table 40.1 Clinical trials performed with T cells of optimized TCR binding affinity/avidity

Type of cancer	Targeted antigen	TCR origin (name)	OR[a,b] (%)	CR[b,c] (%)	On-/off-target toxicity (%)[b]		Reference
Melanoma	A*0201/ Melan-A	Human (DMF5)	6/20 (30)	0/20 (0)	8/20 (40)	On-target: vitiligo, uveitis, and hearing loss	[41]
Melanoma	A*0201/ gp100	Mouse (154)	3/16 (19)	0/16 (0)	1/16 (6)	On-target: vitiligo, uveitis, and hearing loss	[41]
Colorectal carcinoma	A*0201/ CEA	Mouse	1/3 (33)	0/3 (33)	3/3 (100)	On-target: colitis	[56]
Melanoma and synovial sarcoma	A*0201/ NY-ESO-1	Human (1G4)	22/38 (58)	5/38 (13)	0/38 (0)	NA[d]	[42, 57]
Melanoma and multiple myeloma	A*01/ MAGE-A3	Human (3A3)	0/2 (0)	0/2 (0)	2/2 (100) 2 deaths	Off-target: cardiogenic shock	[63, 64]
Melanoma, synovial sarcoma, and esophageal cancer	A*0201/ MAGE-A3	Mouse (118AT)	5/9 (55)	1/9 (11)	4/9 (44) 2 deaths	Off-target: mental status changes, seizure, and coma	[65]
Melanoma	A*0201/ Melan-A	Human (DMF5)	0/14 (0)	0/14 (0)	2/14 (14)	AE[e]	[105]
Multiple myeloma	A*0201/ NY-ESO-1	Human (NY-ESO^{c259})	16/20 (80)	14/20 (70)	0/20 (0)	NA[d]	[106]

[a]OR, objective response (partial or complete responses), according to Response Evaluation Criteria for Solid Tumors (RECIST) or the International Uniform Response Criteria for myeloma assessment

[b]Number of patients with responses/total number of patients; (percentage of responses)

[c]CR, complete response or near complete response, according to the Response Evaluation Criteria for Solid Tumors (RECIST) or to the uniform response criteria for multiple myeloma of the International Myeloma Working Group (IMWG)

[d]NA, not applicable

[e]Adverse events that were not related to the on-/off-target toxicity of the infused T cells

developed off-target recognition toward a similar but not identical peptide from the cardiac muscle-specific protein titin [63], resulting in a serious adverse effect (SAE) and fatal toxicity against cardiac tissue [64]. Moreover, two patients infused with autologous anti-HLA-A2/MAGE-A3 TCR-engineered T cells experienced mental changes, leading to neurologic toxicities and death, possibly due to cross-reactivity toward the MAGE-A12 self-antigen expressed in the human brain [65].

These results demonstrate not only the functional potency of affinity-improved TCRs with substantial antitumor effector functions in vivo but also highlight the potential safety concerns for those TCR-engineered T cells (reviewed in [49–52, 58, 62]) (Table 40.1). Altogether, we and others propose that the rational design of improved self-specific TCRs for adoptive T cell therapy may not need to be optimized beyond the natural TCR affinity range to achieve optimal T cell function and avoidance of unpredictable risk of cross-reactivity (reviewed in [48, 66]; Fig. 40.2). Thus, there is an urgent need to withstand for better preclinical evaluations allowing assessing for precise antigen specificity of the engineered TCRs and identifying the best suitable tumor antigens for adoptive cell transfer with affinity-improved TCRs. Importantly, TCR optimization through affinity alteration must include the evaluation of optimal T cell responsiveness and lack of on-target and off-target side effects due to self-reactivity to ensure the safety of TCR-engineered T cells in future clinical trials

(reviewed in [49–52, 58, 62]). In that regard, new strategies are required, allowing for the identification and selection of those naturally occurring but rare self/tumor antigen-specific T lymphocytes of highest TCR binding avidities and functional capacities within the physiological TCR affinity range, and will be further described thereafter.

40.5 TCR-pMHC Binding and Kinetic Measurements

Several technologies have been employed to characterize in great detail the TCR-pMHC binding affinity/avidity and to dissect the kinetic contributions of association k_{on} and dissociation k_{off} rates to such molecular interactions. In this section, we will summarize the different methods currently used, focusing on their respective advantages and disadvantages.

Early comparative crystallography analysis revealed a lack of correlation between TCR-pMHC structure and T cell function, suggesting that T cell activation must rely on dynamic processes [67]. Quantitative measurements of the kinetic and binding parameters of TCR-pMHC interactions were thus undertaken, mostly using SPR biosensors. This technology allows for the simultaneous detection of association and dissociation kinetic rates as well as the molecular affinity ($1/K_D$), providing the so-called "3D affinity" parameters, since one of the two molecules is flowed in solution across a sensor chip, on which the potential interacting target is attached (Fig. 40.1b). Data collected from SPR studies revealed that natural human TCR-pMHC binding interactions were of relative weak affinities (K_D ranging from 500 μM to 1 μM) compared to other biomolecular interactions, with rapid off- and slow on-rates [68–70]. Yet, an inherent caveat of the SPR technology is that it requires the laborious and time-consuming production of soluble TCR and pMHC molecules and ignores the binding contribution of CD8 co-receptors and/or other membrane molecules to the overall TCR-pMHC interaction. Recently, new generations of imaging technologies, coupling microscopy to SPR affinity measurements [71, 72], as well as quartz crystal microbalance (QCM) biosensors [73, 74] have been engineered to allow label-free detection of real-time interactions and kinetics between membrane proteins directly at the surface of living cells. These new technologies could potentially be exploited in the context of low affinity TCR-pMHC interactions at the surface of living self/tumor antigen-specific T cells.

Besides SPR, other technologies based on mechanical or fluorescent assays have also been recently developed that enable the deduction of k_{on} and k_{off} kinetics directly at the interface between a living T cell and a surrogate APC, or between a T cell and a supported planar lipid bilayer. As such, these surface-based membrane interactions are designed as "2D interactions" [75–77]. 2D analyses showed good correlations with T cell activation and function, but revealed faster k_{on} and k_{off} kinetics than those found with 3D SPR technology [78–80]. Although 2D approaches allow for the measurements of TCR-pMHC binding parameters in a more physiological way than the 3D technology, both approaches should be viewed as highly complementary. Indeed, 2D analyses require specialized equipment and are time consuming, precluding for the rapid and high-throughput screen of living antigen-specific T cells that could be useful for adoptive cell immunotherapy (reviewed in [81, 82]). It should be noted that a very recent study revealed the successful measurement of single-cell 2D TCR affinity and subsequent TCR sequencing directly from human primary CD8 T cells [83], opening the way to the rapid selection of individual therapeutic TCRs for adoptive cell transfer immunotherapy.

Alternative technologies based on the direct detection and rapid analysis of live antigen-specific CD8 T cells led in the early 1990s to the development of soluble pMHC monomers [84]. However, the low affinity characterizing TCR binding to pMHC precluded any direct detection of antigen-specific CD8 T cells by flow cytometry and/or microscopy using these soluble pMHC monomers [85]. Increasing the molecular valency

and binding avidity of pMHC monomers through controlled multimerization (e.g., dimers, tetramers, decamers) was then performed, enabling direct binding of multimers on live antigen-specific T cells [86] and initiating a whole new area of T cell detection and analysis [81, 87]. Yet, multivalency favors TCR-pMHC clustering and augments the likelihood that a dissociating pMHC rapidly rebinds to another juxtaposed TCR. As such, evaluation of TCR-pMHC affinity using multimer-based saturation binding experiments, as well as association and dissociation kinetic assays, generated biased results, which often did not correlate to the monomeric SPR-based affinity and kinetic data [88]. Consequently, the brightness of multimer staining to cell membrane of TCRs (i.e., the mean fluorescence intensity or MFI), which reflects the total binding capacity of the multimeric molecules at equilibrium, failed to consistently correlate with in vitro functional activity or in vivo protection [20, 89–91].

A major technological improvement was achieved with the development of reversible multimers (reviewed in [31]). Reversible multimers are structurally similar to multimers, but they are engineered in such a way that pMHC monomers can be disrupted from the multimeric scaffold upon addition of a stimulus. Reversible *Strep*tamers consist of a fluorescent scaffold molecule (*Strep*Tactin) coupled to several pMHC monomers carrying a streptag, a linear peptide optimized to bind to *Strep*Tactin [92]. D-biotin binds *Strep*Tactin with higher affinity than streptag and is therefore able to compete for the same binding site, disrupting the multimeric complex and releasing the pMHC monomers at the cell surface. Since pMHC monomers do not stably bind to TCRs, they rapidly dissociate from the TCRs, allowing for the identification and isolation of practically "untouched" antigen-specific CD8 T cells, while preserving their phenotypical and functional status [93]. Based on the same principle, the reversible multimers called NTAmers are made of His-tagged pMHC linked to fluorescent streptavidin carrying an engineered nitrilotriacetic acid (NTA) linker [94] (Fig. 40.3a). Upon addition of imidazole at low and nontoxic concentration, the NTA complexes rapidly decay into pMHC monomers allowing FACS sorting of antigen-specific CD8 T cells without inducing adverse effects on the cell integrity such as activation-induced cell death [81, 94].

Thanks to the fluorescent labeling of the individual pMHC monomers contained in the reversible multimeric complexes, it has recently become possible to monitor and quantify the monomeric dissociation of pMHC from the TCRs directly at the surface of antigen-specific T cells [95–98]. Reversible two-color *Strep*tamers successfully allowed determining monomeric dissociation kinetics of nonself, virus-specific TCRs on human and mouse T cells using a real-time microscopic-based strategy [95]. Specifically, Nauerth and coworkers [95] reported that virus-specific CD8 T cells with longer half-lives (low k_{off}) exhibited increased functional avidity and better in vivo protective capacity than T cells of shorter half-lives (high k_{off}). Moreover, the *Strep*tamer technology allowed for the simultaneous measurement of k_{off} on hundreds of antigen-specific CD8 T cells, which represents a great advantage since it is not limited to the generation of clonal T cell populations. However, the significant lag time required for the *Strep*tamer to decay into monomeric pMHC molecules (around 60 s) as well as the photobleaching effect associated with the microscopic assay prevented the detection of rapid TCR-pMHC off-rates, which are typically found within the self-/tumor-specific CD8 T cell repertoires of lower TCR affinities. Recently, the *Strep*tamer-based experimental settings were further optimized for k_{off} measurements of ex vivo unsorted, polyclonal antigen-specific CD8 T cells using a conventional flow cytometer and may now become suited for high-throughput applications [98].

In parallel, our group used a two-color version of the reversible NTAmers [94] to assess TCR-pMHC dissociation kinetics directly on living CD8 T cells by flow cytometer [96] (Fig. 40.3a, b). The accuracy of the NTAmer approach was validated by finding strong correlations between NTAmer-based monomeric dissociation rates and those obtained by SPR measurements [96]. In contrast to the *Strep*tamer technology, the rapid

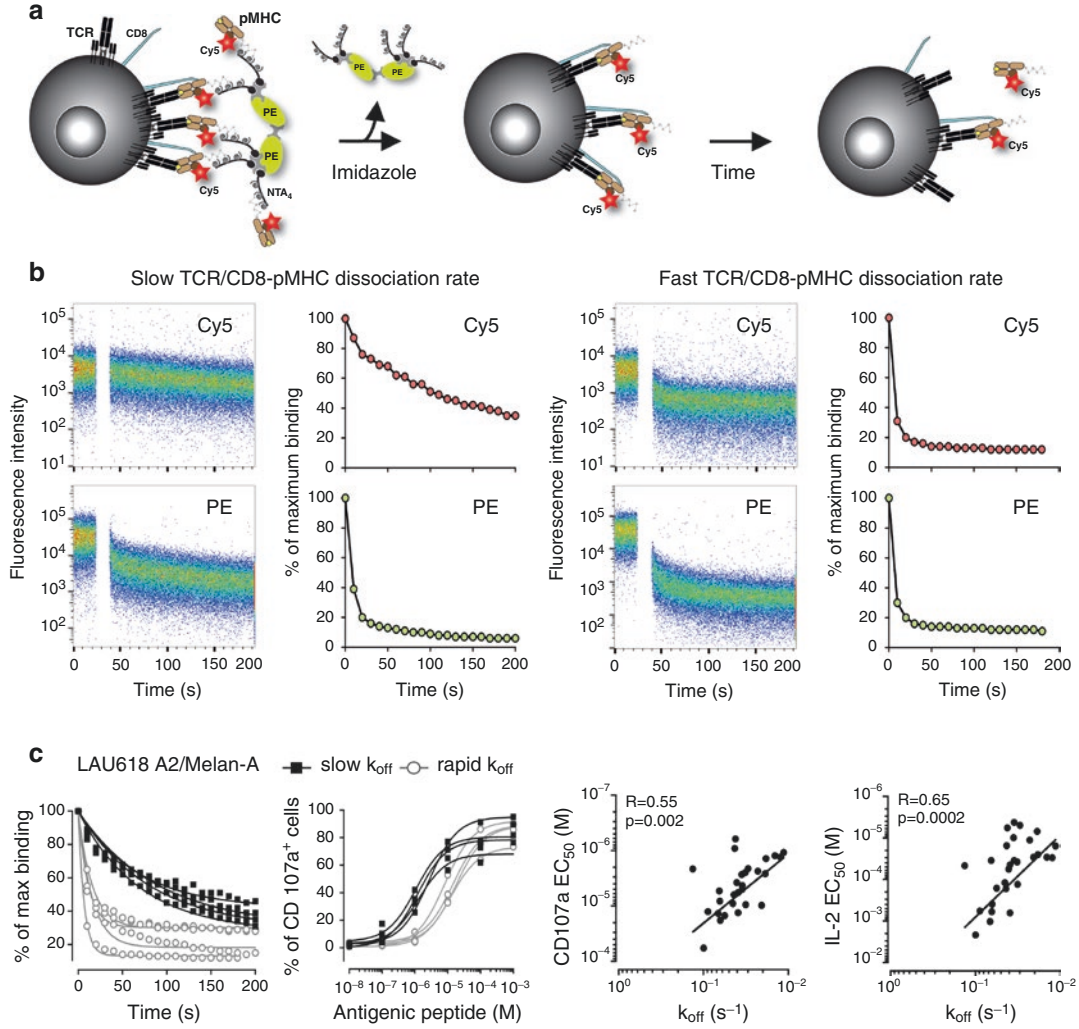

Fig. 40.3 NTAmer-based monomeric dissociation assay allows the identification of antitumor T cells of higher TCR-pMHC avidity and functional potency. (**a**) Schematic representation of the NTAmer-based monomeric dissociation assay. CD8 T cells are stably stained with multimeric NTAmers composed of streptavidin-PE (*green*)-NTA_4 (*gray*) and peptide-MHC (*brown*) monomers containing Cy5-labeled β2m (*red*). Upon addition of imidazole, NTAmers rapidly decay in Cy5-labeled pMHC monomers (*middle panel*). Cy5-labeled pMHC monomers subsequently dissociate from cell-associated TCRs (*black*) and CD8 (*blue*) according to the intrinsic TCR/CD8-pMHC dissociation rate (k_{off}) (*right panel*). Adapted from [31]. (**b**) Representative NTAmer-dissociation staining (*left panels*) and corresponding fitting curves (*right panels*) obtained from CD8 T cell clones defined as slow or fast TCR-pMHC dissociation rate (k_{off}). Imidazole is added after 1 min of baseline recording (*white gap*), and dissociation curves are followed over time within the Cy5 (pMHC monomers) and PE (NTA_4 scaffold) channels by flow cytometry. The corresponding monomeric dissociation rates (k_{off}) are analyzed in Prism (GraphPad Software, Inc.). (**c**) Relationship between TCR-pMHC binding avidity and T cell functional avidity. Representative NTAmer-based monomeric dissociation curves (*first panel from the left*) and CD107a-based titration curves (*second panel*) obtained for HLA-A2/Melan-A-specific CD8 T cell clones isolated from a melanoma patient (LAU618) and arbitrarily separated into rapid (*white circle*) and slow (*black squares*) dissociation rates (k_{off}). Correlations (Spearman coefficient *r* and *P* value) obtained between TCR-pMHC avidity (monomeric TCR-pMHC k_{off}) and functional avidity (CD107a degranulation EC50, *third panel,* and IL-2 production EC50, *fourth panel*)

decay of the NTAmer scaffold into its pMHC monomeric constituents (2–3 s) made it possible to precisely analyze the dissociation kinetics of a wide spectrum of TCR affinities, with a special emphasis for self-/tumor-specific CD8 T cells, which are known to be of low affinities and rapid k_{off} [96, 97] (Fig. 40.3c). Collectively, the two-color reversible multimer technology (i.e., *Strep*tamer and NTAmer) enables the real-time quantification of monomeric TCR-pMHC dissociation kinetics directly at the surface of living primary CD8 T cells within large numbers of nonself/virus and self/tumor antigen-specific CD8 T cell clones. For the time being, no monomeric-based pMHC assays have emerged, that would allow the precise quantification of the k_{on} association kinetics or the K_D affinity directly on living T cells.

40.6 Relationship Between TCR-pMHC Binding Parameters and CD8 T Cell Potency

Productive interactions between TCRs and their antigens are key for the successful initiation and development of potent adaptive immune responses. However, large-scale assessment of endogenous TCR-pMHC binding parameters has remained technically challenging up to now, limiting the available information on the overall impact and clinical relevance of TCR-pMHC affinity/avidity in the context of natural self-/tumor-specific CD8 T cell responses. Specifically, the questions whether T cells of high TCR-pMHC affinity/avidity can be found in the endogenous tumor-specific repertoire of cancer patients and whether the TCR-pMHC affinity/avidity parameter represents a determining factor contributing to a robust antitumor T cell response still remain open.

Many studies conducted within the past two decades have used experimental variations of the TCR-pMHC affinity (i.e., using panels of altered peptide ligands or affinity-optimized TCR variants) to address the mechanisms that relate TCR-pMHC binding parameters to CD8 T cell functions. As such, these reports demonstrated that within the range of physiological interactions (K_D 200 μM–1 μM), the TCR-pMHC affinity (as determined by SPR) strongly correlates with T cell functional avidity [36, 45–47, 60, 99–104]. Functional readouts included T cell potency for target cell conjugation, phosphorylation of downstream molecules of the TCR-signaling complex, intracellular Ca^{2+} mobilization, lytic granule polarization, target cell killing, cytokine production, cell proliferation, polyfunctionality, and in vivo tumor infiltration and protection/survival (reviewed in [31]). Collectively, those investigations provided strong evidence that the functional potency of tumor-specific T cells can be tailored according to the TCR-pMHC binding parameters. However, these artificial models may bypass numerous other molecular and cellular parameters that dampen the impact of the TCR-pMHC affinity/avidity on the overall T cell functionality. Thus, it remains to be proven whether the TCR/pMHC affinity/avidity represents a determining factor when considering natural antitumor CD8 T cell responses.

Taking advantage of the novel NTAmer technology, our group recently dissected the impact of the TCR-pMHC binding parameters (i.e., k_{off} or off-rates) within spontaneous or therapeutically induced tumor-specific T cell responses in cancer patients [96, 97]. Using large panels of Melan-A- and NY-ESO-1-specific CD8 T cell clones that were isolated prospectively from melanoma patients, we showed that TCR-pMHC dissociation rates strongly correlated with functional avidity, with tumor-specific T cell clones having long half-lives (slow k_{off}) exhibiting increased target cell killing [96]. Importantly, these correlations were observed independently of the functional readout used, including production of Th1/Th2-related cytokine, polyfunctionality, or proliferation, indicating that the TCR-ligand k_{off} rate is a reliable predictor of CD8 T cell potency (own unpublished observations [107]; Fig. 40.3c). Furthermore, our results show that NTAmers are effective tools to isolate those rare CD8 T cells of higher antitumor potency within the endogenous repertoire of cancer patients for use in adoptive cell therapies (reviewed in [31]; own unpublished observations [107]).

Finally, using the novel NTAmer-based technology, we could also quantify the potency of an immunotherapy intervention in melanoma patients [97]. Indeed, we confirmed that the type of peptide used for vaccination of cancer patients profoundly influenced the TCR-pMHC binding avidity of tumor-specific T cells, which in turn correlated with T cell functions. Patients vaccinated repetitively with the natural Melan-$A_{26–35}$ peptide generated tumor-specific CD8 T cells with increased TCR-pMHC binding avidities and killing potency as compared to vaccinations with the analog Melan-$A_{26–35}$ A27L peptide, even if the latter binds more strongly and stably to MHC as compared to the natural peptide. Thus, vaccination with peptides with weak/natural MHC binding favors an enrichment of T cells with higher functional competence [97]. Consequently, the assessment of the TCR-pMHC binding avidity enabled to address which therapeutic vaccine protocol triggered the most potent tumor-specific T cell responses within comparative experimental cohorts, providing precious insights into the choice of peptide to be employed for future cancer vaccines.

40.7 Conclusive Remarks

Cancer immunotherapy has made significant progress with the recent introduction of novel therapeutic reagents such as anti-CTLA4 and anti-PD1 antibodies. Yet, we still need robust techniques allowing the fast identification and isolation of self/tumor antigen-specific CD8 T cells with highest TCR-pMHC avidities within the natural repertoires of cancer patients. Ongoing efforts are currently made to design novel technologies that would enable the rapid and high-throughput assessment of TCR-pMHC binding affinities and kinetic rates directly at the single cell level. Ideally, such technics should retrieve the living cell for further amplification and functional characterization and/or parallel cloning of the TCR alpha and beta chains for subsequent therapeutic usage in adoptive T cell transfer. Optimization of flow cytometry approaches that would allow single cell sorting based on kinetic or affinity parameters as well as novel microscopic, microbalance, and plasmon resonance devices with possibilities to recover the tested cells will hopefully enable such advances in a near future. Understanding the correlates of immune protection together with the development of novel technologies allowing selecting for the best (i.e., high-avidity and high functional potency) tumor-specific CD8 T cells should support the progress of T cell-based therapies against cancer.

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Immune Monitoring of Blood and Tumor Microenvironment

41

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41.1 Introduction

Immune responses in cancer patients involve immune cells and soluble factors that can be measured by an increasing number of laboratory techniques, often combined with bioinformatics data processing and interpretation. This is called "immune monitoring." The results often allow to follow disease evolution and therapy effects more directly and more rapidly than the standard clinical assessment tools and outcome measures (which are nevertheless fully exploited in order to maximally monitor cancer patients and provide them with the best clinical care). Immune monitoring methods take advantage of a variety of techniques (e.g., cellular and molecular biology, biochemistry, micro-imaging) based on sophisticated technology and engineering, allowing increasing resolution and precision. The vast majority of approaches, however, are not (yet) used for standardized routine patient analysis. Rather, they are subject of regular optimization and refinement and used in research and to some degree in the context of clinical trials. Extended validation for broad clinical use is ongoing for a small number of methods, particularly those that appear promising for eventual future routine application. The aim is to establish biomarkers, i.e., laboratory benchmarks useful for disease prognosis or predicting treatment outcome, thereby contributing to tomorrows "precision medicine." Figure 41.1 illustrates various techniques that are applied for the analysis of patients'

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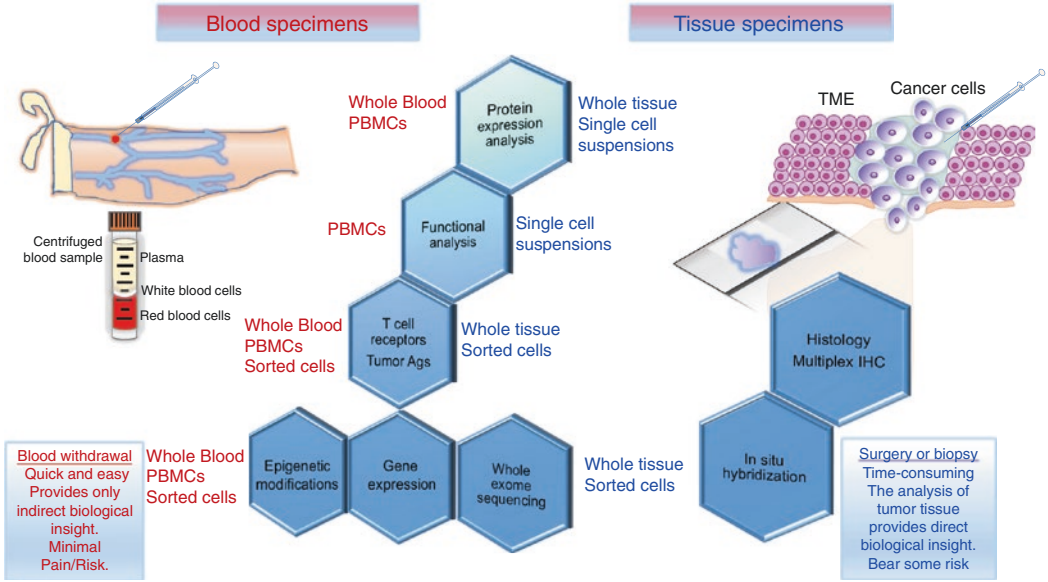

Fig. 41.1 Schematic representation of analyses of patients' blood and tissue specimens. Various methods are used to analyze blood, other body fluids, or solid specimens obtained by surgery or biopsy procedures

blood and tissue specimens. The subsequent paragraphs of this chapter are structured accordingly. By following the journey of cancer patients through diagnosis, treatment and follow-up, researchers carefully investigate the dynamic nature of malignant diseases, providing overwhelming insights into the biology of human cancers.

41.2 Flow Cytometry and Protein Expression Analysis

During the last two decades, characterization of immune cells has gained importance, providing key information in research and clinical studies. A variety of techniques is used to study the patient's T cells (Fig. 41.2). The enormous technical progress during the last years in the field helped to build reliable instruments and to standardize technology and methods for analyzing cells and liquids from different origins, e.g., blood, other body fluids, and various tissues including tumors and lymphoid structures. The big variety and diversity of human samples, and the multiplicity of different cells, factors, cytokines, and chemokines, makes an imperative need for different techniques and dedicated procedures, in order to obtain maximum information from a limited amount of biological material.

The profiling and monitoring of immune responses are key elements in the development of new therapies against cancer and the evaluation of their efficacy. To monitor and quantify an immune response against tumors, it is important to use procedures and techniques (Fig. 41.2) that can reliably detect and characterize tumor antigen-specific T cells, provide information about their phenotype, their specificity, and their proliferation, as well as directly measure their various functions [1].

41.2.1 Flow Cytometry

Flow cytometry is one of the most broadly used technologies for the analysis of cells from blood and the tumor microenvironment (TME). This technology is used since about 30 years and is now widely adopted. Combinations of highly specific, fluorescent-labeled antibodies are used to identify various different cell types such as T, B, and NK cells, dendritic cells (DCs), and monocytes from a few microliters of blood

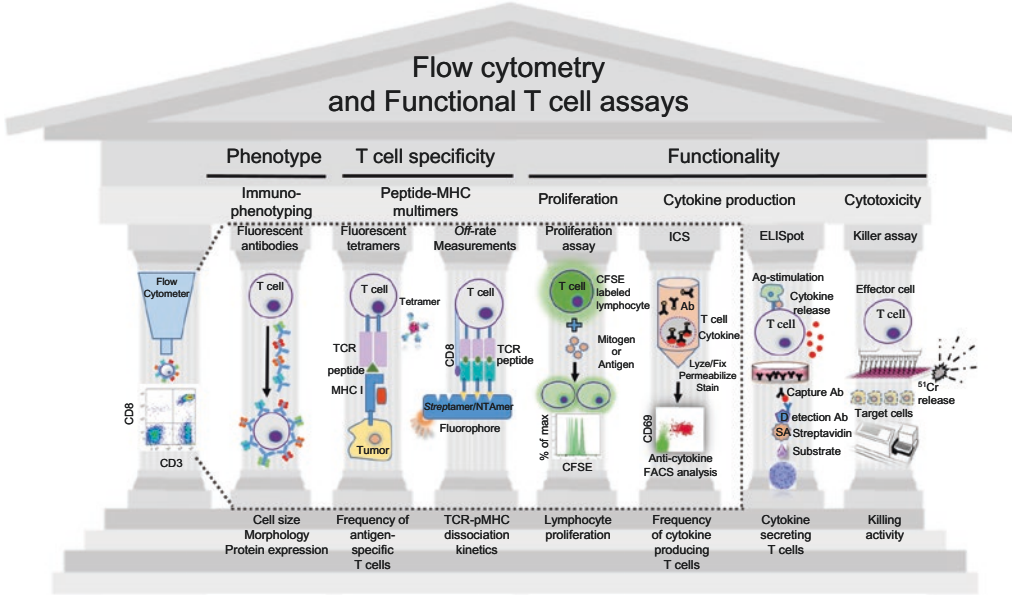

Fig. 41.2 Overview of the T cell assays. The illustrated techniques allow to profile and monitor T cell responses, providing information about phenotype, specificity, proliferation, and functionality of tumor-specific T cells. From the left to the right, flow cytometry enables rapid analysis of physical properties (e.g., size) and multiple molecular characteristics of individual cells, isolated from patient's blood or the tumor microenvironment (TME). Antibodies conjugated to fluorescent dyes are used to label specific proteins on the cell membranes or inside cells. Antigen-specific T cells can be visualized using fluorescently labeled tetrameric peptide-MHC (pMHC) complexes that bind specific T cell receptors (TCRs). Besides determining the frequency of T cells, they can also be phenotypically characterized when combining tetramers with various antibodies. Novel approaches also allow the direct and precise quantification of TCR:pMHC dissociation rates (k_{off}) on living CD8 T cells by real-time flow cytometry (using NTAmers) and/or microscopy (using *Strep*tamers). Fluorescent CSFE labeling is used to assess T cell proliferation in vitro. Intracellular cytokine staining (ICS) is a very useful and widely used flow cytometry-based assay which detects the production and accumulation of intracellular cytokines after cell stimulation, as well as the frequency of the cytokine-producing T cells. Important non-cytometry-based assays are depicted to the right of the immune monitoring "Parthenon." The IFN-γ ELISpot assay, a highly sensitive immunoassay, reveals the frequency of tumor-specific lymphocytes by forming one spot per cell upon antigen stimulation. Cytotoxicity and lytic capacity of lymphocytes is measured by the killer assay, whereby radioactive ^{51}Cr is released from labeled target cells killed by cytotoxic T cells

[2]. The technique allows to enumerate the frequencies of the different cell types. Besides, one can determine many different qualities, for example, the cell's activation and differentiation stages [3], or expression of various receptors by highly defined cell subsets [4]. It has been published that the two surface receptors CCR7 and CD45RA are differentially expressed in human T cells, depending on whether they are naïve cells (CD45RA+ CCR7+), memory cells (CD45RA− CCR7+), or effector cells (CD45RA−/+ CCR7−) [5]. Fluorescent labeling of additional markers such as the co-stimulatory receptors CD27 and CD28 allows to further classify T cells into distinct differentiation stages (Fig. 41.3). Modern flow cytometers have multiple (usually 12–18) fluorescent channels, allowing to simultaneously detect many further parameters such as the expression of activatory and inhibitory receptors, cytokines and chemokines, and (cytotoxic) proteases (Fig. 41.3).

Most practical is the analysis of peripheral blood mononuclear cells (PBMC) which are obtained by gradient centrifugation of freshly obtained venous blood. This technique eliminates red blood cells and granulocytes, giving the advantage to analyze a purified cell population that can also be frozen in aliquots and thawed

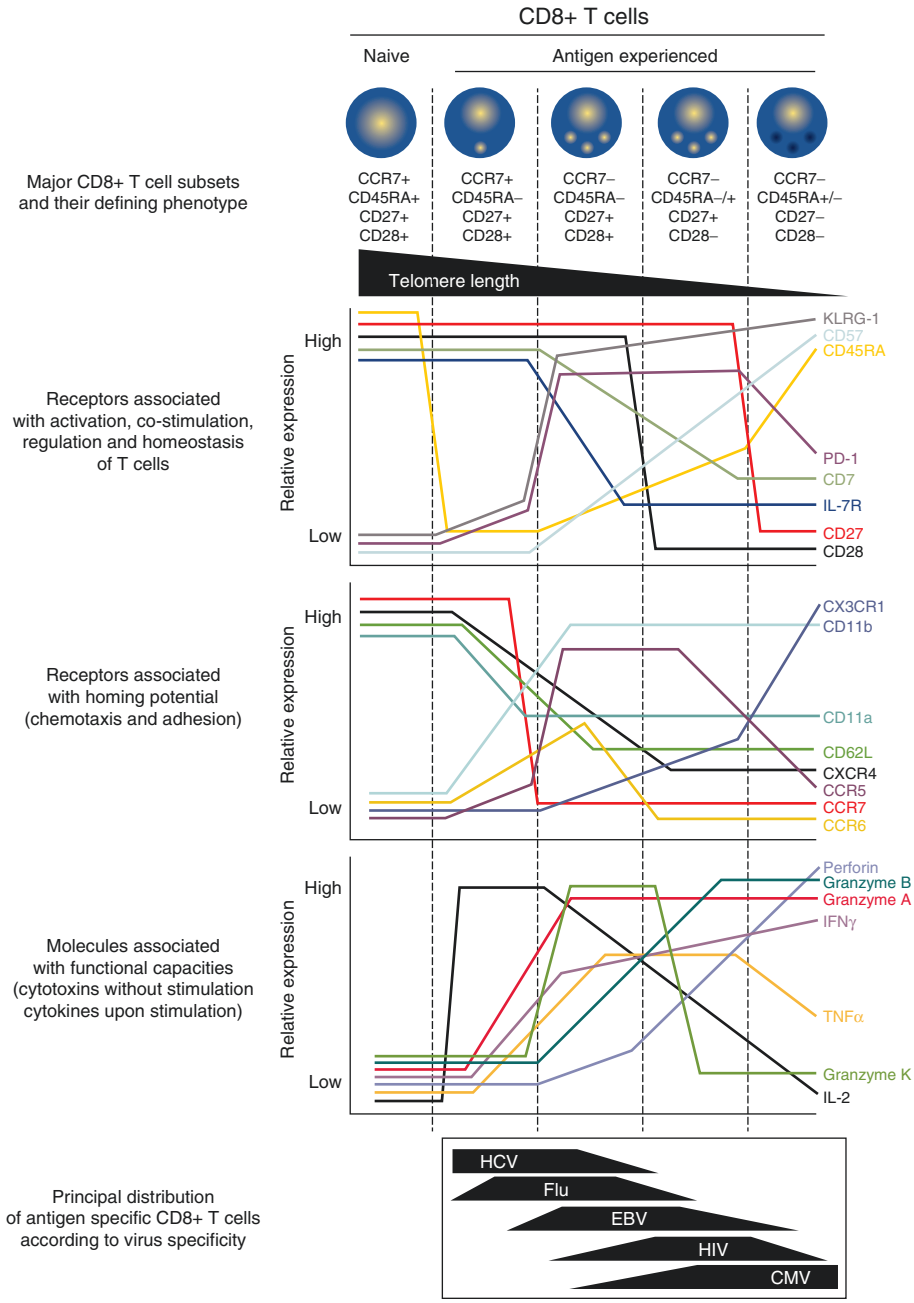

Fig. 41.3 Phenotypic associations within CD8 T cell subsets in humans and relationship with functional attributes. Five distinct subsets of circulating CD8 T cells are defined according to the expression of CD27, CD28, CCR7, and CD45RA. Relative telomere length and expression of a variety of cell surface receptors and intracellular molecules (related to T cell activation, costimulation, regulation, homeostasis, homing potential, and functional capacities) are illustrated in these subsets in a "resting" state according to data from the literature. The common phenotypic distribution of virus-specific CD8 T cells is also depicted, after clearance of the virus (Flu) or in latent infection stages (for HCV, EBV, HIV, and CMV). *Flu* influenza, *HCV* hepatitis C virus, *EBV* Epstein-Barr virus, *HIV* human immunodeficiency virus, *CMV* cytomegalovirus. The figure is taken from the paper by V. Appay et al. [3], *Phenotype and function of human T lymphocyte subsets: consensus and issues.* Cytometry A, 2008 vol 73 (11):975

later on for further investigation. But researchers become more and more aware of the fact that this strategy not only leads to the loss of potentially important cell types but also introduces small artifacts, meaning that some cell populations change their characteristics and thus give rise to misleading results. Therefore, many cell types should rather be analyzed using fresh whole blood, as, for example, shown for myeloid-derived suppressor cells (MDSCs) [6].

Huge efforts have been made in developing new technologies to increase the information, particularly when patients' specimens are small and/or rare. One strategy is flow cytometry with mass spectrometry where antibodies are labeled with heavy metal ion tags, rather than fluorochromes. Here the readout is done by time-of-flight mass spectrometry (Cytof/Fluidigm) [7]. Another strategy is to combine flow cytometry with microscopy. The Image Stream machine (Amnis) is one example of an instrument where fluorescent-labeled cells can be analyzed and identified by their fluorescent molecules like with flow cytometry at the cell surface, and at the same time, a camera is taking pictures of the cells. This allows determining whether the distribution of the fluorescent-labeled molecules is homogeneously distributed over the surface of the cells or polarized in distinct areas or compartments of the cells. The fact that the cells pass with a lower speed through the instrument allows even to observe when cells are in contact to each other and to identify eventual exchange of molecules in their contact zones [8].

Furthermore, new live imaging systems like, e.g., IncuCyte (Essen BioScience), CytoSMART (Lonza), and Operetta (Perkin Elmer), allow to observe cells and their activity in vitro under "online" in culture conditions. In these technologies cameras are placed in incubators and take photos at defined time points over several days under optimal conditions. This allows, for example, observing movements of cells in presence of target cells, e.g., tumor cells or chemotaxis movements of cells due to external stimulation [9]. One of the latest technical developments is the Chip-Cytometer (ZellkraftWerk). This technique allows fluorescent microscope analysis on cells in suspension and on tissues fixed on microscope devices which can be re-stained multiple times with different markers on different cells or on the same cells [10]. The technique still requires optimization but could eventually replace cell consuming flow cytometry with time-consuming immunohistochemistry (IHC). The advantage of this technology is the analysis of rare material/cells for different cell surface and intracellular molecules with the strength of fluorescence detection well known from fluorescent analysis by flow cytometry.

41.2.2 Protein Arrays

Another possibility to obtain information about the differential states of cells and to compare samples from healthy donors with those from patients at different time points is to perform protein arrays, which can be done of bulk material or of distinct cell types previously sorted by flow cytometry or magnetic beads. This technology allows qualitative protein profiling and can also characterize protein phosphorylation revealing intracellular signaling activities. The technique is promising for screening for key factors and eventually identifies candidate biomarkers [11].

41.2.3 Cytokine Analysis

T cells have the capacity to secrete one or even multiple cytokines. The detection of soluble molecules secreted by different cell types from in vitro experiments or directly from the serum of blood samples is possible by standard ELISA assays. This technique is simple and efficient but has a relatively low sensitivity and can only analyze one specific molecule at the time. New sophisticated technologies have been developed for very sensitive detection of multiple cytokines, chemokines, or other molecules, based on either bead capture assays with liquid-based systems (Flow cytometry, Luminex) or on imaging systems, where the secreted molecules are captured on specifically coated membranes, e.g., MSD (Meso Scale Discovery). These techniques also have the advantage that they can detect multiple molecules simultaneously from a small amount of sample [12].

41.2.4 Bioinformatics Support for Data Analysis

Conclusive analysis of huge datasets coming from one or even multiple analytical approaches is challenging. Analyzing data from flow cytometry is tainted even when respecting commonly established and firm rules. For example, subjective gating decisions can strongly influence the results and their interpretation. A more objective way to analyze such data is by using novel dedicated software programs. Mathematic algorithms are capable to analyze and compare datasets in an unbiased way. By combining all different parameters, deep analysis of datasets is possible. Typically, cell populations can be rapidly identified, and even unconventional cells are found that might have been ignored with conventional cell gating.

41.3 Further Assays to Monitor T Cell Functions

Besides flow cytometry, there are various other techniques to analyze T cell functions. Several standard functional T cell assays exist for determining cytotoxicity [13], cytokine production, and proliferation [14], as overviewed in Fig. 41.2. Moreover, assays that can simultaneously detect T cell frequency and function, such as the interferon-γ (IFN-γ) enzyme-linked immunospot assay (ELISpot), have gained increasing popularity for monitoring T cell responses in clinical trials and in basic research.

41.3.1 Analyzing Cell-Mediated Cytotoxicity

T cell-mediated cytotoxicity represents a key mechanism in the immune response to tumors. Therefore, monitoring methods for the appropriate assessment of cytotoxic immune reactivity is thought to be crucial [15]. A classical and popular assay for evaluating cell-mediated cytotoxicity by T lymphocytes (CTLs) and by natural killer cells is the [51]Chromium (^{51}Cr) release assay or cytotoxicity assay, schematically depicted in Fig. 41.2. First developed in 1968 by Brunner et al. [16], it continues to be widely used for testing killing activity in immune monitoring laboratories. It is based on the passive internalization and binding of the soluble radioactive chromium from sodium chromate by target cells in single-cell suspensions. Subsequent lysis of the target cells by added effector lymphocytes results in ^{51}Cr release into the cell culture supernatant, which is then detected by a gamma counter. Although several modified alternatives have been introduced [17], this assay remains a "gold standard" to measure cell-mediated cytotoxicity [18].

However, there are several disadvantages that create a need for more accurate methods: this assay has a relatively low level of sensitivity, often one needs to stimulate cytotoxic lymphocytes several times before they reach detectable levels of lytic activity, which unfortunately alters the composition and activity of the original T cell populations, and it does not provide direct information about the in vivo function and the behavior of single effector cells. Furthermore, there is evidence that the killing activity may largely differ between individual T cells and that "super killers" may dominate in cytotoxicity assays [19], meaning that many other T cells do not contribute significantly to target cell killing. Some target cells label poorly with ^{51}Cr, and other target cells show high spontaneous ^{51}Cr release. Since autologous tumor cells are difficult to obtain, surrogate targets must be used, but they may not reveal the actual ability of lymphocytes to lyse autologous tumor cells in vivo. Finally, the inter-assay variability is considerable, and there are biohazard and disposal problems associated with radioisotope usage.

41.3.2 The ELISpot Technique for Assaying Cytokine- and Interferon-Producing Cells

The IFN-γ ELISpot assay is a highly sensitive immunoassay that reveals tumor-specific lymphocytes by forming one spot per cell provided that it secretes IFN-γ in response to antigen stim-

ulation [15]. This assay is well suited to determine the frequency of functional cells. Versteegen et al. [20] were the first to use the ELISpot to detect human cells secreting IFN-γ. Subsequently, an important modification of the ELISpot assay was made by employing nitrocellulose membranes and specific monoclonal antibodies, increasing reproducibility and sensitivity of the assay [21]. With the recommendation of the 13th International AIDS Congress in the year 2000 to use the ELISpot technique due to its performance for immune monitoring purposes, it became one of the most important direct ex vivo methods in cellular immunology [22, 23].

Clinical trials and immune monitoring require comparability and reproducibility of results. The ELISpot assay is well suited, because it is a relatively simple procedure and accurate for testing any T cell, including those directly withdrawn from patients without any in vitro amplification or modification, thus best reflecting their in vivo state. Therefore, the ELISpot assay has become a standardized and validated method. The first steps to harmonize the process for widespread applications included standardization of protocols, materials, and reagents [24], for instance, by using pre-coated 96-well plates [25]. Consequently, the inter-assay and intra-assay variability was minimized, and precision and reproducibility were increased.

Schematic illustration of the principle of the ELISpot assay is summarized in Fig. 41.2. Each spot corresponds to an individual cytokine-secreting cell. The spots are counted by an ELISpot Bioreader, allowing to calculate the frequency of functional T cells. The ELISpot assay captures the presence of cytokines of interest, immediately after secretion from the "spotting" cells, in contrast to measurements that are skewed by receptor binding or protease degradation. The assay is considered as one of the most sensitive T cellular function assays available. The limit of detection typically achieved can be 1 in 100,000 cells with standard ELISpot assays and with an average of less than 1 in 200,000 PBMC in modified assays [26]. The assay is particularly useful when only small cell numbers are available.

Classically, this assay is performed with a single (high) peptide dose, usually at the concentration of 1 micromolar. Alternatively, or in addition, one can use titrated amounts of peptide to generate a characteristic sigmoid dose response curve (Fig. 41.4), used to determine the functional avidity of T cells. The mean "functional

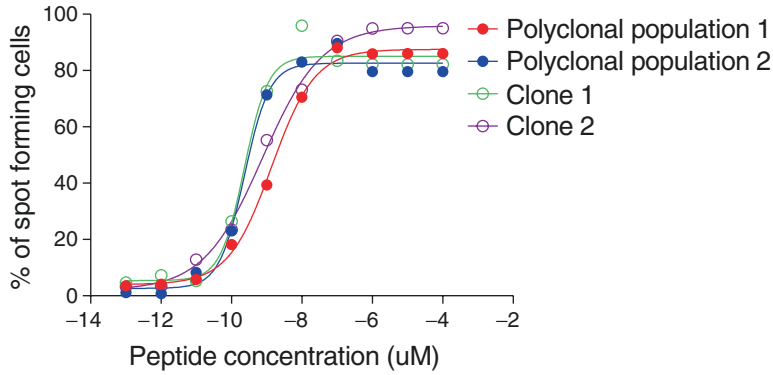

Fig. 41.4 Examples of ELISpot titration curves from two individual CD8 T cell clones and polyclonal T cell populations from two patients. Each microculture was prepared with 300 antigen (Melan-A)-specific T cell clones or polyclonal T cell populations that contained 300 antigen-specific cells. The percentages of antigen-specific T cells in the polyclonal populations were previously determined with fluorescent tetramers by flow cytometry, allowing to calculate how many cells had to be plated to have 300 antigen-specific T cells per microculture. For stimulation, all microcultures were supplemented with 20,000 T2 cells and the indicated concentration of antigenic (Melan-A) peptide. Ten concentrations of peptide were tested, each in triplicates, thus requiring 30 microcultures (in 96-well plates). Spots per well were counted with the ELISpot Bioreader 5000, and *curves* were plotted using Prism software

avidity" corresponds to the peptide concentration that confers 50% maximal activity (EC_{50}), i.e., the amount of antigenic peptide required for half-maximal IFN-γ ELIspots. Because antigen is often rate limiting in vivo, the functional avidity is a parameter that is particularly important for evaluating the functional potency of antitumor T cells. The functional avidity depends on TCR-peptide-MHC interactions on the T cell surface, where the TCR clustering, the involvement of the CD8 co-receptor, the local concentration of adhesion molecules, and the involvement of co-activating and co-inhibitory receptors are all important parameters that modulate the strength of the intercellular interaction and thus T cell activity [27–29]. The functional avidity refers to the accumulated "dynamic strength" of multiple affinities of individual non-covalent binding interactions [30]. An additional parameter is the functional avidity heterogeneity, revealed by the slope of the curve (Ioannidou K, Baumgaertner P, Gannon PO, Speiser MF, Allard M, Hebeisen M, Rufer N, Speiser DE. Heterogeneity assessment of functional T cell avidity. Scientific Reports. 2017 Mar 13;7:44320. doi: 10.1038/srep44320).

41.3.3 Intracellular Cytokine Staining

Intracellular cytokine staining (ICS) revealed by flow cytometry is one of the most popular assays in the immunologists' toolbox, designed to assess complex T cell responses. One of the specific advantages of ICS is that it enables the simultaneous assessment of multiple phenotypic, differentiation, and functional parameters, including the expression of multiple cytokines [31]. ICS is used in combination with other flow cytometry protocols for immunophenotyping using fluorescent antibodies specific for cell surface markers and/or with fluorescent MHC multimers to detect antigen-specific T cells, making it an extremely flexible and versatile method. The main experimental steps of ICS are that the cells of interest are activated using either a specific peptide or non-antigen-specific activation by, e.g., anti-CD3 antibody or a mitogen. In the next step, an inhibitor of protein transport (e.g. brefeldin A) is added to retain the cytokines within the cells. After washing, the antibodies specific for cellular markers are added. The cells are then fixed (e.g., with paraformaldehyde) and permeabilized, and the anti-cytokine antibodies are added. Finally, the cells are analyzed by flow cytometry (Fig. 41.2). Many technical advances have been achieved, e.g., the polychromatic assays, designed to detect five or more separate functions of T lymphocytes (e.g., the production of cytokines and chemokines and the detection of degranulation as surrogate for cytotoxicity) while simultaneously identifying multiple surface markers [32].

41.4 Multiplexed Immunohistochemistry (IHC) Assays

Histology and IHC are excellent methods to approach the great complexity of biological processes in various tissues. For cancer patients, major challenges are to understand the disease driving biological mechanisms in the TME. Microscope-based tissue analysis allows the identification of structural and functional molecules and their intra- or extracellular localization. Analyses can be done with freshly frozen (FF) or formalin-fixed paraffin-embedded (FFPE) tissue samples. Their appropriate and timely handling is critical [33, 34] in order to avoid unnecessary protein and nucleic acid damage. Careful measures must be taken in order to optimally handle tissues such that the integrity of biomolecules is maintained, and detailed knowledge is obtained about eventual procedural artifacts. Also, reference tissues are required for quality control, including the verification whether the staining is capable to reveal the targeted markers.

IHC techniques use antibodies for specific labeling and staining of molecules in tissue sections. Antibody binding can be detected with an enzymatic reaction that induces chromogenic precipitation (to stain for maximally three to four markers on a single tissue section), or by fluorescent dyes (up to about eight markers). Dedicated signal amplification techniques are used to covalently link antibodies with dyes, minimizing

cross-reactions upon the required subsequent rounds of staining with additional antibodies [35]. The markers can be identified with a multispectral camera. Considerably, larger numbers of markers (possibly >100) can be applied with novel techniques based on successive cycles of staining, scanning, and then removal (or bleaching) of the fluorophore, which however also requires specific software that can subsequently overlay the serial images [36, 37].

IHC is usually done on slices carrying conventional tissue sections. An elegant alternative technique is the so-called tissue microarray, which consists of punching out cylinders (of 0.6–2 mm diameter) of tissue paraffin blocks of microscopic regions of special/specific interest, customized according to hypothesis and experimental design. A large number of tissue cylinders are then used to produce a new paraffin block, in which these tissues from up to >100 patients are placed. Slices of such tissue microarray blocks enable to stain and analyze large numbers of tissues much more efficiently, favoring large-scale quantitative investigations [38, 39].

Traditionally, analysis and interpretation of histological images are evaluated qualitatively and semiquantitatively with the microscope by the eyes of a trained pathologist. Novel automated image analysis software is increasingly used to automate and accelerate this process, enabling the evaluation of large tissue regions in a fully quantitative manner [40]. However, this still requires the supervision by pathologists, as the interpretation of tissue complexity must always be quality controlled.

Overall, the use of efficient and precise methods for the investigation of tissues represents a central pillar for both research and patient assessment. For example, this is needed for the quantification of tumor-infiltrating T lymphocytes (TILs), which are often of high prognostic and predictive value and can even be more important than the traditional TNM (tumor, node, metastasis) classification of tumors. These techniques allow quantifications in defined areas, e.g., in tumor cell nests, invasive margins, or peritumoral regions, each of which may have different causes and consequences [41, 42].

41.5 High-Throughput Techniques

Several techniques can determine thousands of parameters and are therefore called high-throughput techniques (Fig. 41.5), particularly

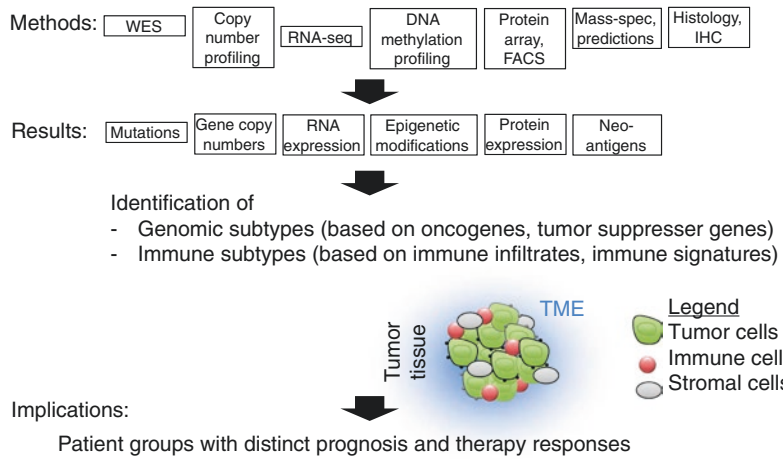

Fig. 41.5 High-throughput approaches to characterize cancer cells and their microenvironments for improving patient diagnosis and treatment, toward precision medicine. An increasing number of techniques allow comprehensive characterization of the various tissues and cells from cancer patients, enabling detailed characterization of tumor biology and immune parameters in primary and metastatic cancers. *TME* tumor microenvironment, *WES* whole exome sequencing, *RNA-seq* RNA-sequencing, *IHC* immunohistochemistry

the approaches that are sequencing based. Whole exome sequencing (WES) can be used to identify gene abnormalities in tumor cells, revealing specific mutations and overall mutational load. A comprehensive overview of gene alterations in tumor cells is obtained when WES is combined with DNA copy number profiling [43], revealing gain and loss of function alterations. In addition, an increasingly important technique is RNA sequencing (RNA-seq), enabling quantitative assessment of the expression of mRNA or microRNA. RNA-seq can be used for very many different purposes, for example, for gene signature studies, or the characterization of chemokines, cytokines, and other immune factors [43–48]. Furthermore, DNA methylation profiling is used to study epigenetic mechanisms that regulate immune gene expression, or cell lineage-specific epigenetic modifications, also permitting estimations of the percentages of the different cell populations present in the microenvironment [49–51].

Due to the large data amounts, data storing, processing, statistical analysis, and interpretations of results have become very demanding and must be supported by bioinformatics tools, requiring powerful hardware and expert knowledge. DNA microarray data can, for example, be analyzed by BRB-Array Tools, a software providing extensive tools for predictive classifier development and cross validation [52]. Dedicated software is also used to study epigenetic programming. These tools must implement mathematical and statistical rules, for example, for multiple testing [49], respecting general principles for the analysis, and statistical assessments of mass data.

Most biological specimens contain multiple different cell types in various compositions. For example, mRNA-Seq data from heterogeneous tissues are confounded by unknown relative proportions of cell types. Therefore, several algorithms have been developed for computational decomposition ("deconvolution"), with the aim to estimate the percentages of distinct cell populations present in the analyzed tissues [53, 54]. Finally, tumor antigens with somatic mutations ("neoantigens") can be predicted with various software tools that attempt foreseeing various factors such as antigen processing and MHC binding [46, 47, 55]. Neoantigens may be recognized by tumor-specific T cells with superior efficacy as compared to self-antigens associated with immune tolerance. However, great caution must be taken knowing that the majority of predicted antigens are irrelevant, because they may not (sufficiently) be presented to T cells or corresponding T cells may not exist. For these and many more reasons, bioinformatics results should always be complemented with wet-bench data, preferable at large scale beyond proof of principles.

41.6 Validation of Assays, Biomarkers for Clinical Use, and Integration of Big Data

Since the very beginning of laboratory medicine, it has become clear that great care must be given to avoid errors and mis- and overinterpretation. Today, this notion is increasingly important, in view of the enormous developments in biomedical laboratories and the massive data production. Considerable efforts are required to reach satisfactory levels of precision and standardization. Importantly, results must be meaningful for the patient's well-being, which is only the case for a small fraction of data, since most have minimal significant relevance to diagnostic or treatment decisions. Numerous experts and a broad literature provide extended basis for responsible development of laboratory approaches, illustrated, for example, in a two-volume paper focusing on validation of biomarkers to predict response to immunotherapy in cancer, published in in the Journal of Immunotherapy of Cancer [34, 56, 57]. All procedures, assays, and results must be validated pre-clinically and clinically, and routine clinical use must be approved by regulatory agencies. So far this is only the case for three assays, all quantifying PD-L1 expression (further outlined below).

Quality criteria apply to all steps, beginning with the withdrawal and handling of blood and

tissue specimens. For example, time delays and unfavorable storage conditions can alter and reduce their quality. Furthermore, modern laboratory methods have large numbers of variables which all need to be defined and standardized, in order to reach high reproducibility of results obtained in different institutions. Also, the distribution of reference samples is important for assay development and subsequent regular proficiency testing. Finally, clinical studies are required to determine clinical relevance, preferably in randomized prospective trials, rather than only retrospectively.

A few prospective studies have been performed, particularly for the assessment of PD-L1 expression in patient's tumors, with the aim to predict outcome of "checkpoint blockade" treatment with anti-PD-1 or anti-PD-L1 antibodies [58–61]. Also, great efforts of international multicenter standardization of TILs' quantification by IHC are being made for patients with colon cancer [41, 42], likely forming the basis for future routine histological assessments of increasing numbers of cancer types. This will hopefully soon lead to the integration of immune parameters in disease stage and treatment assignment. But this must be based on continued efforts to standardize methodology for evaluating and implementing parameters that are likely important in many clinical situations, beyond the initial and essential steps of quantifying TILs and PD-L1 expression.

In addition to the analytical complexity of experiments producing large amounts of data, there is the great challenge to integrate all meaningful laboratory results with all clinical information from each patient, to assure optimal clinical decisions. In order to reach this goal of big data integration, computational technologies are rapidly advancing, as, for example, the IBM Watson system [62, 63]. A version customized for cancer medicine (Watson Oncology) is currently being developed. Such systems have high potential, but their implementation depends on intense multidisciplinary collaboration to assure that patients are managed not only to the best of novel approaches but also by taking into account all the current knowledge and quality criteria.

Conclusions

Immune monitoring can be done in many laboratories, but the challenge remains to develop and apply established and standardized methods for the analysis of samples. Working with cellular material is delicate and needs personnel with defined training. Small deviations from a standard workflow can strongly affect the quality of the material and can influence the results of the different techniques. The characterization and comparison of immune responses in different clinical trials are facilitated by guidelines that standardize both methodologies and the reporting of results. The Minimum Information for Biological and Biomedical Investigations (MIBBI) program [64] has been used as basis, to which several projects have been added, including minimum information about a flow cytometry experiment (MIFlowCyt), minimum information about a cellular assay (MIACA), and minimum information about a T cell assay (MIATA) [65, 66]. Many of the suggestions are based on the NCI's REMARK (REporting recommendations for tumor MARKer prognostic studies) criteria [67] and the projects mentioned above. Immune monitoring of multi-institutional trials may be done by a central laboratory and large-scale banking of clinical specimens allowing future analysis of sera, viable cells, RNA, and DNA. Clinical and laboratory personnel must do their best to implement the existing recommendations and establish even better principles, with the aim to capitalize on clinical trials and support the development of more effective therapies.

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42 Toward Engineered Cells as Transformational and Broadly Available Medicines for the Treatment of Cancer

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42.1 Vision for and History of CAR T Cell and TCR T Cell Medicines

The authors of this book chapter share the common vision to establish engineered cells as broadly available and transformational medicines for patients suffering from multiple hematological and solid cancers. The first examples of the enormous clinical potential of engineered cells for patients have recently been described. In the rare disease field, 2016 saw the first ever marketing authorization for an engineered stem cell gene therapy product, *Strimvelis®,* to treat the rare monogenetic disease ADA-SCID ("bubble boy" disease), following unparalleled efficacy in clinical trials [1]. Meanwhile in the oncology field, unprecedented clinical benefit has been reported from clinical trials in which patients with B cell malignancies such as acute lymphoblastic leukemia (ALL), diffuse large B cell lymphoma (DLBCL), and chronic lymphocytic leukemia (CLL) received engineered T cell targeting CD19 [2–4] using viral transfer of chimeric antigen receptors (CARs). Novartis has submitted a biological license application (BLA) for CTL019 for the treatment of pediatric ALL in March 2017, and Kite Pharma Inc. has recently presented interim efficacy data from their ZUMA-1 trial with 6-month follow-up in DLBCL making highly likely that a filing in

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this indication may soon follow. There has also been progress with an alternative approach to engineer T cells to selectively recognize and kill tumor cells via the introduction of T cell receptors (TCR). With this approach, the most promising clinical data has been reported from smaller cohorts of patients with multiple myeloma [5] or the solid tumors melanoma and sarcoma [6] expressing the cancer germline antigens NY-ESO-1 and LAGE-1a. The main characteristic for both engineered stem cells and immune receptor-engineered T cells is that a one off treatment bears the potential to lead to pharmacological activity that may persist and may therefore potentially lead to long-term disease control or even prove curative. While these initial successes provide reasons to believe in the potential of engineered cells to become broadly available and game-changing treatments for various diseases, there are still major challenges that inhibit full exploitation of the potential for a broad number of patients, namely, (1) the challenge to supply these medicines cost-efficiently to maximize access, (2) to overcome efficacy limitations that may prohibit transformational benefits in many solid cancers where most of the medical need exists, and (3) the challenge to identify novel targets that are suitable for cell therapy approaches. This chapter will address these three key challenges and describe a strategy to open a path for broad success of engineered cells beyond rare diseases and B cell malignancies.

Before we address the open challenges for engineered cell therapies in oncology, we would like to revisit the history for CAR- and TCR-engineered T cells, which goes back more than 25 years. The first molecules to redirect T cells to cancer antigens, called T-bodies at that time, were developed in 1989 [7]. These first engineered T cells expressed simple "first-generation" constructs that were composed of a single-chain variable fragment of IgG (scFv) for binding to a cognate antigen, a transmembrane domain, and a CD3ζ chain to activate the receptor-bearing lymphocyte. The first generation of CAR-engineered T lymphocytes in oncology was introduced to clinical trials more than 15 years ago and targeted folate receptor in patients with ovarian cancer [8], carbonic anhydrase IX (CAIX) in patients with renal cancer [9], and CD171/L1-CAM in pediatric patients with neuroblastoma [10]. The antitumor activity for engineered cell products expressing these first-generation CARs was limited, and the CAR T cells did not persist long term in the patients. The advent of "second-generation" CAR constructs that included co-stimulatory domains such as CD28 or 4-1BB and their use in ALL and CLL showed transformational efficacy [3, 11] changing the dynamics of the field and igniting a massive increase in R&D efforts in both academia and industry. This has included expansion to numerous targets and the development of additional generations of CAR designs, though it is yet to be determined which CAR designs will prove optimal. Reflecting this expansion, at this stage more than 40 commercial entities are developing CAR T-cell products and more than 100 engineered cell products are in nonclinical or clinical development.

Engineered T cells expressing recombinant TCR for human malignancies and chronic viral diseases were pioneered at the University of Washington in Seattle [12]. Their use in oncology was explored following pioneering clinical trials at the NIH in the USA and Niigata University School of Medicine in Japan that showed promising objective response rates when tumor-infiltrating lymphocytes (TILs) were expanded ex vivo and then reinfused to patients with malignant melanoma [13, 14] or epithelial ovarian cancer [15]. With refinements to the cell process and patient management, the objective response rate in patients with melanoma treated with TILs at the NIH reached 72% [16]. These trials delivered the proof of concept that the pharmacological activity of this treatment was mainly conferred via endogenous TCRs recognizing tumor-associated antigens (TAAs). It took only a few years to move from the process of extracting TILs from tumor samples to an approach making use of recombinant TCRs with successful pioneering studies utilizing MART-1 TCR alpha and beta chains [17]. While TCR T cell approaches lag a bit behind the CAR T cell approach, we have recently witnessed a steep increase both in academic and industry-sponsored clinical trials with TCR T cell products.

At this stage, it is too early to predict whether it will be CARs or TCRs that prove optimal to direct T cells to cancer in the most efficient, safe, effective, and sustainable fashion. Each approach has distinct pros and cons, and therefore they might ultimately coexist serving different cancer types or populations. The key pro of the TCR approach versus the CAR approach is that all proteins are processed and presented via MHC to T cells; thus, all potential antigens are in scope for TCRs, whereas for CARs, only antigens expressed on the cell surface are available for binding. This represents no more than 10% of potential targets even before factors such as surface accessibility are considered. The key pro for the CAR approach is that antigen recognition is direct and does not require MHC; thus, a single CAR is suitable across the patient population, whereas TCRs recognize target peptide only in complex with a specific MHC molecule. Since MHC are highly polymorphic, the MHC dependence of TCRs limits the treatable population to only those who express a given MHC molecule. Moreover, immune escape by loss or downregulation of the antigen processing pathway or MHC is an issue for TCRs but not for CARs.

42.2 Sources for TCRs/CARs

Thus far, the vast majority of CARs have utilized scFv binders as their antigen-targeting fragment, with many of these coming from the repurposing of monoclonal antibodies considered as therapeutics either alone or as antibody-drug conjugates. However, any binding element suitable for use in a modular CAR construct that has a sufficiently high affinity and specificity for the target antigen could be considered. Indeed, when targeting a receptor, ligands may be utilized, as in the case of the IL-13 "zetakine" CAR, which incorporates a recombinant IL-13 mutated to improve affinity for tumor-associated IL13Rα2 and reduce binding to the more widely expressed IL13Rα1 [18]. The use of murine-derived scFv in CARs may limit their persistence due to formation of human anti-mouse antibodies (HAMA) and occasionally may pose a safety risk if HAMA of the IgE isotype develop, which can result in anaphylactic shock [19]. Humanized or fully human binders reduce the risks of HAMA and might be preferred in some contexts [20, 21].

There are multiple sources from which TCRs have been derived. Initial TCR gene-engineered immunotherapy utilized TCR from naturally occurring patient or donor T cells. In the case of melanoma antigen recognized by T cells 1 (MART-1) TCRs used clinically to treat patients with metastatic melanoma, both the DMF4 TCR [17, 22] and the higher-affinity DMF5 TCR [23, 24] were obtained from patients who experienced objective clinical response to their TIL therapy. In theory, because of the extensive coverage provided by natural TCR recombination, any individual can have T cells with TCR capable of recognizing virtually any antigen on their MHC. However, TCR with high affinity to normal self-proteins are deleted from the repertoire by negative thymic selection [25, 26] making it difficult to find naturally occurring TCR with sufficient affinity to eliminate antigens shared between normal tissues and tumor, such as so-called developmental antigens including carcinoembryonic antigen (CEA) or differentiation antigens like glycoprotein 100 (gp100) or other tumor-associated antigens including overexpressed growth factor receptors including the ERB/HER antigens. In general, these "self" TCRs are in the high μM binding affinity range, in comparison with mAbs, which tend to fall in the nM to pM range [27]. While occasionally a high-affinity TCR can be isolated from "elite responder" patients whose TIL elicited an effective response against shared tumor antigens, such as MART-1, most of the time these TCRs provide an initial framework from which the specific antigen-binding regions can be affinity matured ex vivo by stepwise amino acid replacement and empirical testing against target antigen [28, 29]. One such example is the c259 TCR which recognizes the NY-ESO-1 (157–165) SLLMWITQC peptide presented by HLA-A*02 [30]. TCR T cells expressing this TCR have achieved 50 and 80% objective response rates in patients with malignant melanoma and spindle-cell sarcoma, respectively [6, 31].

When a sufficiently high-affinity TCR cannot be generated from normal human T cells, higher-affinity variants can be obtained from HLA-A*02 transgenic or HLA-A*02/human TCR chromosomal double transgenic mice [32]. For this method to succeed, the target antigen must not match the murine homolog, to produce an MHC-peptide complex that is considered "nonself" in mouse. This way, high-affinity TCRs binding the target peptide-MHC complex can be generated without being deleted from the repertoire during thymic selection. Transgenic mice can be immunized with the desired protein or peptide, and then T lymphocytes are isolated from lymphoid organs and restimulated in vitro to expand the antigen-positive T cells [24, 33].

42.3 The Challenge of Identifying Suitable Targets for CAR T Cell and TCR T Cell Approaches

The challenge of identifying suitable targets for CAR T cell and TCR T cell products has proven a significant one. Indeed, even antigens that were widely explored by cancer vaccine researchers or as targets for monoclonal antibodies and antibody-drug conjugate therapy such as MAGE-A3 and Her2neu, respectively, have shown new challenges in the context of CARs or TCRs. This is likely due to the potency of directly activating T cells via the numerous antigen receptors present on each cell and unexpected cross-reactivity patterns that had been introduced by the synthetic immune receptors [34–36]. The work to identify suitable antigens has been further complicated by the fact that animal models have proven particularly poorly suited for safety screening of CAR and TCR approaches. This is because the antigens are frequently not shared between mice and humans, the MHC context is completely different, and even when a similar or analogous antigen can be identified or a humanized mouse model utilized, the expression profile of the antigen and cross-reactivity profile of the analogous CAR/TCR poorly reflect the human situation. Indeed, the emergence of neurotoxicity and severe cytokine release syndrome and a host of other toxicities observed in human trials (see below) were not predicted by mouse studies.

The first wave of successful CAR T cell therapies has avoided the challenges inherent in identifying tumor-specific antigens by targeting the CD19 antigen, which is common to an entire lineage of cells enabling transformational efficacy in late-phase trials in a number of B cell malignancies. This efficacy is enabled by the fact that B cell aplasia, even when prolonged, need not result in profound immunodeficiency since humoral immunity can be replaced by infusion of intravenous immunoglobulin purified from the plasma of healthy donors. The next wave of CAR T cell therapies follows this paradigm by targeting additional antigens restricted to the B cell lineage (e.g., BCMA, CD20, CD22, and kappa light chain) to provide additional coverage of B cell malignancies. Unfortunately, no such equivalent has yet been found for non-B cell hematological malignancies or for solid cancers. Indeed, experience with the melanocyte differentiation antigens MART-1 and gp100 serves as a cautionary tale. These antigens are often highly overexpressed in melanoma and are well-characterized tumor-rejection antigens. Indeed, a high proportion of TILs in melanoma patients recognize these antigens [37, 38]. However, TCR T cell approaches targeting these antigens have resulted in lower objective response rates and greater toxicities than observed with autologous TILs [16, 24, 39].

Thus far, the TCR T cell field has borrowed heavily from the cancer vaccine field for target antigens. Table 42.1 summarizes the antigens targeted by TCR T cells that have entered clinical trials, while Fig. 42.1 summarizes the number of distinct assets and their phase of development. It is evident that three classes of antigen have dominated thus far: cancer germline antigens, viral antigens, and differentiation antigens that are overexpressed in tumors but also present on essential normal tissues. While viral antigens ought to be among the safest targets, the indications are limited to those cancers driven by viral infections, i.e., HPV- or HBV-related cancers. EBV- and CMV-specific CTL have been used to prevent posttransplant lymphoproliferative dis-

Table 42.1 Antigens targeted thus far by TCR T cells in clinical trials

Antigen	Antigen class	Phase	Comment
Alpha fetoprotein (αFP)	Differentiation	Preclinical	
Carcinoembryonic antigen (CEA)	Differentiation	Ph1	Terminated—poor accrual
Glycoprotein 100 (Gp100)	Differentiation	Ph2	
Hepatitis B virus (HBV) antigen	Viral antigen	Ph1	
Human papilloma virus (HPV) early protein 6 (E6)	Viral antigen	Ph1	
HPV E7	Viral antigen	Ph1	
Melanoma-associated antigen (MAGE) A3	CGA	Ph1	TCR assets in development cover multiple HLA types: HLA-A*01, A*02, A*24, and DP4. Different TCRs have varying degrees of cross-reactivity to MAGE A6, A9, and A12. One candidate caused patient deaths due to cross-reactivity to MAGE A12 in the brain. Another asset caused patient deaths due to cross-reactivity with titin in heart
MAGE A4	CGA	Ph1	TCR assets in development cover two HLA types: HLA-A*02 and A*24
MAGE A10	CGA	Ph1/2	
Melanoma antigen recognized by T cells (MART)-1/melan-A	Differentiation	Ph2	
New York esophageal antigen-1 (NY-ESO-1)	CGA	Ph1/2	
Preferentially expressed antigen in melanoma (PRAME)	CGA	Preclinical	
p53	Overexpressed mutant form acts as an oncogene	Ph2	
Thyroglobulin	Differentiation	Ph1	Suspended
Tyrosinase	Differentiation	Ph1	
Wilms tumor 1 (WT-1)	Oncogene/TAA	Ph1/2	

Sources: Pharmaprojects® | Pharma Intelligence, 2017, and clinicaltrials.gov
All target peptides have been in the context of HLA-A*02 except where noted. "Phase" denotes the furthest a target has reached in clinical trials to date. The development of some TCR assets/targets has been stopped thus not all are currently progressing
CGA cancer germline antigen, *TAA* tumor-associated antigen

eases, but these cell therapies involve expansion of natural CTL rather than gene transfer with a TCR. The cancer germline antigens are potentially promising targets since their expression is typically limited to fetal development and to germ cells which lack HLA class I expression and thus cannot present antigen to T cells. However, across this class of antigens, there are some examples (e.g., PRAME) that show low to modest expression in healthy tissues [40]. In addition, in some cases, cancer germline antigen-specific TCR T cells have caused fatalities in the

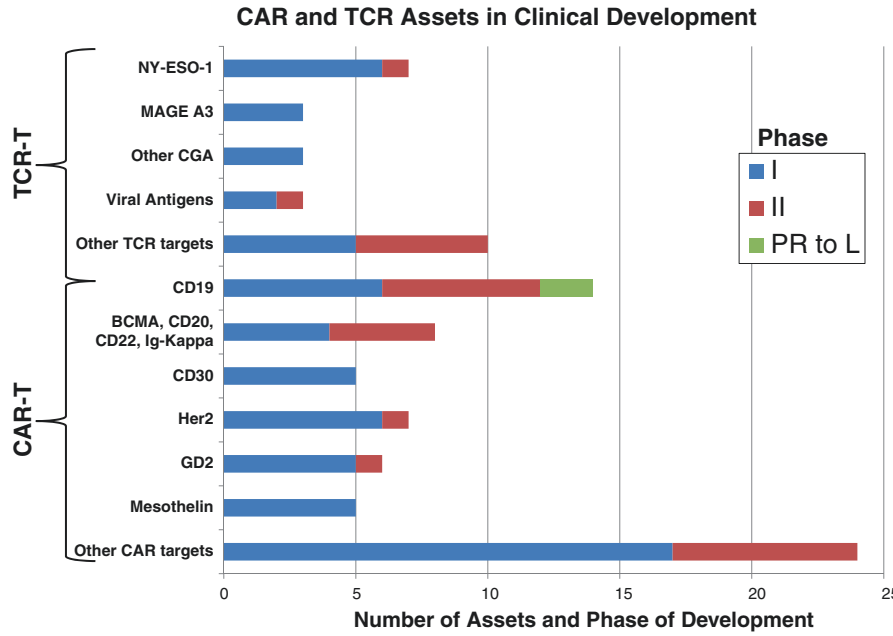

Fig. 42.1 The pipeline of CAR T cell and TCR T cell therapies. Thus far the CAR T cell pipeline is both broader and more mature than the TCR T cell pipeline. Following the success of CD19, other B cell lineage antigens (BCMA, CD20, CD22, and immunoglobulin kappa chain) dominate, and CD30, while strictly a TAA, is being used to target the B cell malignancies Hodgkin's and non-Hodgkin's lymphomas. The cancer germline antigens dominate the TCR T cell pipeline. *PR* to *L* pre-registration to launch. Sources: clinicaltrials.gov and Pharmaprojects® | Pharma Intelligence, 2017

clinic due to cross-reactivity with related [35] and unrelated proteins [41]. The third big group of antigens includes differentiation antigens, which display tumor-associated overexpression compared to the healthy tissues from which the tumors originate. The most heavily studied antigens from this class include gene products that are overexpressed in NSCLC (e.g., EGFR), breast cancer (e.g., HER2neu), prostate cancer (e.g., PSMA and PSCA), or in malignant melanoma (e.g., gp100 and melan-A/MART-1). For the latter two, expression in the healthy skin has led to considerable toxicity indicating that the window of expression between healthy and tumor tissues will not always be wide enough to allow safe targeting with current technologies.

Looking beyond the classes of antigen that have been exploited for TCR T cell therapies, thus far, there are a couple of potential target classes that seem especially promising: patient-specific neoepitopes, including aberrant tumor-associated posttranslational modifications, and T cell epitopes associated with impaired peptide processing (TEIPP).

With the evolution of deep sequencing, mass spec, and bioinformatic technologies, it is becoming feasible to identify the neoepitopes unique to a given patient's tumor and to expand autologous T cells recognizing these private tumor mutations from patient blood [42–44]. This may result in an effective method to implement a polyclonal TIL-like approach and/or identify some neoepitopes that are shared with larger groups of patients for a TCR T cell approach. While many neoepitopes result from amino acid substitutions, frameshifts, or alternate/cryptic open reading frames, altered posttranslational modification resulting in phosphopeptides and arginine (di)methylated peptides have been recently identified as promising target classes [42, 45, 46] and should be shared across a greater proportion of patients as they are not reliant on specific mutations but upon altered post-

translational modifications, which frequently occur in tumors. Indeed, aberrant protein phosphorylation is a hallmark of tumor cells and creates numerous phosphopeptide antigens that can be recognized as distinct from their non-phosphorylated counterparts by conventional αβ T cells.

Under selective pressure from TILs or therapeutic TCR T cells, tumor variants that have lost or downregulated antigen presentation by epigenetic or genetic mutations may prevail. While HLA deletion renders tumors refractory to T cell therapy, it may make them more vulnerable to NK cells. Downregulation or loss of antigen processing pathway components other than MHC is frequently observed and may represent a means to escape both T and NK cells. However, in the setting of MHC expression without antigen processing, tumors may present a unique and broadly shared set of target peptides—the TEIPP [47]. So far suitable epitopes have only been identified in mice, where it has been shown that cells defective for the transporter associated with antigen processing (TAP) present low levels of MHC class I molecules complexed with a peptide from the TRH4 protein. In the presence of TAP, the binding of the TRH4 peptide to the MHC class I molecule D^b is easily outcompeted by numerous more suitable peptides from the proteome of the cell, but without TAP to transport these peptides into the ER, sufficient TRH4-D^b peptide complexes are displayed on the cell surface where they can activate high avidity T cell clones. Such clones exist because they escaped negative thymic selection due to inability of TAP-replete cells to present such peptide-MHC complexes [48].

While the CAR T cell field has pursued a number of the antigens shared with the cancer vaccine field, a key limitation has been that only cell surface targets are tractable for CARs. As described above, lineage-restricted targets have been exploited to target B cell malignancies. There is intense competition around relatively few targets that have attractive efficacy and acceptable safety profiles for B cell malignancies (Fig. 42.1). Beyond these, there are numerous tumor-associated antigens and differentiation antigens that are being explored with more clinical data becoming available in the coming years (summarized in Table 42.2 and Fig. 42.1).

In summary, a couple of suitable targets have already been identified in clinical trials; current nonclinical and clinical work is actively addressing a significantly expanded target space, and there are still areas or untapped potential that come from new and less studies target classes as well as from the potential to utilize mutated neoantigens in the future. This makes us confident that an increasing number of suitable targets covering multiple cancers will become available over the next years and enable fill and flow of future drug development pipelines. Before we revisit the next two key challenges that limit broad use of engineered T cells in oncology, we would like to highlight the key characteristics of the nonclinical and clinical development of CAR T cell and TCR T cell approaches.

42.4 Nonclinical Development

Preclinical development of gene-engineered CAR- and TCR-modified T cells is truly a "first in class" living medicine. Traditional evaluations applied to pharmacologic small molecule inhibitors or even biologics including vaccines and mAb therapies do not apply. Neither do usual evaluations of cellular therapies such as blood transfusions or bone marrow transplants. A third class of medicines that have fairly recently made an impact include gene therapies, generally used to treat single-gene defects in patients with rare diseases. Immunotherapy with CAR and TCR T cells involves aspects of all of these, brought together for the first time. Biologic pharmaceuticals provide the mAb binding region, while gene engineering borrows the use of transforming vectors such as gamma-retro or lentivirus to combine targeting receptor with functional genes. All of these come together to engineer patient T lymphocytes ex vivo to generate a new "living drug" that relies upon millennia of built-in evolution to combat disease inside the patient, in this case, cancer.

There are several steps in common to developing any preclinical CAR or TCR therapy. First

Table 42.2 Antigens targeted thus far by CAR T cells in clinical trials

Antigen	Antigen class	Phase of development	Comment
B cell maturation antigen (BCMA)	Differentiation	Ph2	
Carbonic anhydrase IX (CAIX)	TAA	Ph1	
CEA	Differentiation	Ph1	
CD7	TAA	Ph1/2	
CD19	Lineage-specific antigen	Ph3–launch	
CD20	Lineage-specific antigen	Ph2	
CD22	Lineage-specific antigen	Ph1	
CD30	TAA	Ph1/2	
CD33	TAA	Ph1/2	
CD70	TAA	Ph1/2	
CD123	TAA	Ph1	Expression on pluripotent stem cells poses risks
CD171 (L1-cam)	TAA	Ph1	
c-MET (tyrosine protein kinase MET)	TAA	Ph1	
Disialoganglioside (GD)2	TAA	Ph1	
Epidermal growth factor receptor (EFGR)	TAA	Ph1	
EGFR variant III (EGFRvIII)	Neoepitope	Ph1/2	
Ephrin type-A receptor 2 (EphA2)	TAA	Ph1	
Fibroblast activation protein (FAP)	TAA	Ph1	Expressed by fibroblasts in tumor microenvironment rather than tumor cells
Folate receptor alpha (FRA)	TAA	Ph1	
Human epidermal growth factor receptor 2 (Her2Neu)	TAA	Ph1/2	One asset terminated due to patient death
Glypican 3 (GCP3)	TAA	Ph1/2	
Interleukin 13 receptor α2	TAA	Ph1	
Kappa immunoglobulin	TAA	Ph1	
Mesothelin	TAA	Ph1	
Mucin (Muc) 1	Glycoantigen	Ph1/2	
Prostate stem cell antigen (PSCS)	Differentiation	Ph1	
Prostate-specific membrane antigen (PSMA)	Differentiation	Ph1	
Receptor tyrosine kinase orphan receptor (ROR) 1R	TAA	Ph1	
Vascular endothelial growth factor receptor (VEGFR) 2	TAA	Ph1/2	

Sources: Pharmaprojects® | Pharma Intelligence, 2017, and clinicaltrials.gov
TAA tumor-associated antigen

consideration is the target. Engineered T cell targets should be expressed on a reasonable proportion of tumors and should not be expressed on critical normal tissues. This became frankly apparent in the first-in-human treatment of a 39-year-old woman with metastatic melanoma who received a high number (10^{10}) of autologous HER2 CAR T cells [36]. Although to date, over 400,000 women with breast cancer have received trastuzumab anti-HER2 mAb in the adjuvant setting, with minimal side effects, the first patient to receive the same HER2 mAb-redirected CAR T

cells succumbed to severe respiratory distress, followed by multiple organ failure starting 15 minutes after infusion [36]. This is a clear demonstration of the difference in impact of a naked antibody compared with an antibody tied to the signaling region of a T cell. While there may be mitigating circumstances, whereby utilizing a limited route of administration [49], lowering the affinity of a CAR receptor may allow for selective destruction of high-antigen expressing tumors while sparing low-antigen bearing normal tissues [50, 51], the safest approach is to target truly tumor-specific antigens.

Several methods have been employed to identify preclinical targets on tumors. Initially, many institutions had to rely on their own available tumor banks for antigen detection by immunohistochemical (IHC) or RT-PCR for gene expression. More recently, however, numerous in silico bioinformatic resources have become widely accessible, in particular evaluation through publicly available Human Protein Atlas (proteinatlas.org) or via The Cancer Genome Atlas (TCGA) (cancergenome.nih.gov).

Upon selecting a target, a binder is required, usually in the form of the scFv of a mAb. Once selected, the binder/scFv is engineered into a DNA construct including a linker, hinge/transmembrane domain (typically derived from CD4, CD8, or an IgG), and followed by a co-stimulatory molecule and CD3ζ at minimum, though new additions are continually being produced. The difference between a CAR and a TCR is that a TCR requires expression of a matching full-length CD3 α and β chain comprising the constant and variable regions of each. Only upon appropriate α/β pairing is antigen recognition conferred, implementing the induction of the full CD3 signaling mechanism.

In vitro, recombinant TCR or CAR are expressed in donor T cells, and upon confirming surface expression, these cells undergo specificity recognition and functional testing. At minimum, known antigen-positive and antigen-negative target cells are needed, though in practice, often several panels of different tumor types are assessed for targeting. While there are many variations of experimental methods to determine function, they fall in one of two forms: (1) effector T cell stimulation or (2) target cell destruction. Once they have passed this initial specificity test, they must be evaluated for safety. Two types of safety predominate, specifically (1) on-target, off-tumor, whereby the antigen being targeted is also expressed on normal tissues, or (2) off-target toxicity, in this case the receptor recognizes something other than the specific target. Each type of safety testing poses its own set of challenges, including the need to profile each cell/tissue type with regard to gene expression. In the case of "on-target, off-tumor," essentially a screening panel of live normal human cell types and tissues are needed for use as experimental targets for the CAR in question. Some groups have utilized human-derived primary cell lines for this [52]; while others have obtained direct primary cell and tissue grafts from donors for this purpose [20, 53].

Off-target toxicity is more difficult to predict and has been observed more for TCRs than for CARs. Part of the reason for this may be (1) the potential for more than one gene/protein to share sequences that can be presented in the context of MHC in a similar conformation to tumor-derived epitopes and (2) the act of "affinity tuning" TCR toward higher affinity for a specific peptide-MHC complex which may introduce unforeseen cross-reactive binding to peptide-MHC complexes from other proteins expressed in normal tissues that would otherwise have been screened out biologically during thymic selection. One such example of this was observed in the affinity maturation of a TCR to a MAGE-A3 peptide-HLA-A*01 complex [41]. Subsequent analyses suggested that the fatalities were likely resulted from recognition of an off-target peptide from the muscle protein titin, highly expressed in cardiac muscle [34]. New methods to predict cross-reactivity profiles for new TCRs such as the regular use of 3D culture models for toxicity-relevant tissues and systematic scans of permutated peptides and use of in silico predictions to predict cross-reactivity profiles for novel TCRs have now become a standard requirement of preclinical development programs. While normal cell/tissue toxicity detection methods still continue to

evolve, the progress in developing new models and safety technologies for this type of predictive evaluation will prevent unforeseen adverse events in patients in the future.

In vivo models have some, albeit limited, benefit for translation of CAR and TCR therapies, in that immune-compromised inbred mouse models such as the NOD-SCID-common gamma chain k/o (NSG) or NOG mouse can engraft both human tumors and human TCR or CAR T cells. These animals act as tiny incubators complete with functioning cardio-vasculature and an extensive network of blood vessels to transport the T cells to the tumors. Generally, however, these models are limited to showing antitumor efficacy and homing to tumor sites, and most recently have shown some promise in modeling T cell exhaustion and checkpoint blockade at the tumor site. Unfortunately, few tumor target epitopes are shared between mouse and human, making it difficult to determine information on safety or potential normal tissue toxicity [54]. Syngeneic mouse models with congenic tumors may provide a unique ability to evaluate both scientific questions about the efficacy of CAR therapy, and the specific mechanism of action of downstream cancer "cures," in the context of an animal model with an intact immune system and physiologic expression of tumor antigen in both tumors and normal tissues, if present [55–58]. However, these models are far from perfect: (1) they need separate binders that likely have different affinity for target than human counterparts; (2) they require completely different vector constructs encoding murine genes for CD3ζ, 4-1BB, and CD28 in place of their human orthologs; and last but not least, (3) mice are not people. There has been much divergent evolution in the development of their immunity and physiology, so results in a mouse model are by no means a guarantee of what will work for patients. To date the use of nonhuman primates (NHP) has been of minimal/negligible benefit in determining safety or toxicity to this field, primarily due to the lack of similarity of action and reagent comparability between human and NHP and also due to the lack of any NHP tumor models. Intriguingly, according to the US Food and Drug Administration (oncology), they currently do not require any animal modeling to support CAR or TCR first time in human phase I clinical trials.

42.5 Gene-Engineered T Cell Therapy: Clinical Development

42.5.1 Chimeric Antigen Receptor T Cell Therapy

The most basic first-generation CARs that were introduced to clinical trials more than 15 years ago comprised a scFv fused to the intracellular CD3ζ TCR signaling chain via a short transmembrane domain. CAR T cells built that way exhibited cytotoxic activity upon antigen recognition but lacked persistence. CARs currently being tested in clinical trials are second-generation constructs that result from the addition of a co-stimulatory signal to the constructs. The vast majority of current clinical trials are assessing the activity of second-generation CARs bearing either 4-1BB or CD28 motifs. Third-generation CARs, including more than one co-stimulatory domain, and fourth-generation CARs, including additional choices of co-stimulatory domain such as OX40 or CD27, are beginning to be tested in clinical trials, though it is too soon to know whether they will show any real benefits over the second-generation designs.

CD19 is expressed on the surface of most B cell leukemias and lymphomas and has emerged as the most attractive for adoptive T cell strategies as it is expressed in normal B cells but no other normal tissues. A number of academic centers are developing CD19-directed CAR T cell clinical programs. Exciting preliminary results have resulted in multiple academic/pharmaceutical industry partnerships that have facilitated the launching of registration studies (Table 42.3).

The striking clinical activity of CD19 targeted CAR T-cells containing 4-1BB costimulation (CTL019) was first shown in three patients with heavily pretreated chronic lymphocytic leukemia (CLL) by investigators at the University of Pennsylvania [11]. Long-term follow-up study

Table 42.3 Selection of the most clinically advanced CD19-directed CAR T cell programs

Company	Novartis	Juno	Juno	Kite
Academic partner	University of Pennsylvania	Memorial Sloan Kettering Cancer Center	Fred Hutchinson and Seattle Children's Hospital	National Cancer Institute
CD19-targeted CAR	CTL019	JCAR015	JCAR017	KTE-C19
Vector platform	Lentivirus	Retrovirus	Lentivirus	Retrovirus
Anti-CD19 scFv	FMC63	SJ25C1	FMC63	FMC63
Hinge/transmembrane domain	CD8-CD8	CD28	IgG4-CD28	CD28
Co-stimulatory motif	4-1BB	CD28	4-1BB or CD28	CD28
T cell source	Autologous	Autologous	Autologous	Autologous
Suicide capability	None	None	EGFRt	None

EGFRt truncated epidermal growth factor receptor

showed that 8 of 14 patients responded (overall response rate [ORR] 57%), including 4 patients with complete response (CR) with no evidence of minimal residual disease (MRD), and that CAR T cells persisted for years [4]. In this study, no patient in CR has yet relapsed, hinting at the curative potential of this cell therapy approach. More than 45 patients with relapsed or refractory CLL have been treated at the University of Pennsylvania, with an ORR of 45% [59]. Other groups have shown similar activity in CLL in smaller patient cohorts [60–62].

Several groups using different CAR T cell designs have reported very high CR rates in both pediatric and adult patients with relapsed/refractory acute lymphoblastic leukemia (ALL). The Children's Hospital of Philadelphia and the University of Pennsylvania have reported on 30 patients (25 pediatric and 5 adult) with ALL treated with CTL019 [3]. CR was achieved by 27 (90%) patients, and the probability of survival at 6 months was 78%. Durable remissions up to 24 months were observed and correlated with persistent CAR T cells. The National Cancer Institute treated 45 children and young adults with 19–28z CAR T cells, reporting a CR rate of 60% [63]. At the time of the analysis, all patients remained alive and 89% remained disease-free (range 5–28 months). The Memorial Sloan Kettering Cancer Center group has reported on 45 adult patients with relapsed ALL treated with JCAR015, a CD19-targeted CAR containing the co-stimulatory molecule CD28. A CR was achieved by 82% of patients, although CAR persistence was limited to a few months [64]. At Fred Hutchinson Research Cancer Center, 27 of 29 (93%) patients with relapsed ALL achieved CR after infusion of 4-1BB containing CAR T cells given at a 1:1 ratio of $CD8^+$:$CD4^+$, and 25 of them (86%) had no evidence of MRD [65].

CD19-directed CAR T cell therapy is also active in heavily pretreated patients with non-Hodgkin's lymphoma (NHL). At the National Cancer Institute, the ORR among nine patients with relapsed/refractory diffuse large B cell lymphoma (DLBCL) was 67% [61], whereas at the University of Pennsylvania, it was 47% among 15 patients with DLBCL and 73% among eight evaluable patients with relapsed/refractory follicular lymphoma [66]. In the latter study, no patient achieving CR has yet relapsed.

Results from the pivotal study ZUMA-1, a multicenter pivotal study sponsored by Kite, testing the CD28 containing CAR T cell KTE-C19 (axicabtagene ciloleucel, Axi-cel), have been reported [67]. A total of 101 patients with aggressive NHL (77 with DLBCL and 24 with primary mediastinal B cell lymphoma or transformed follicular lymphoma) received lymphodepletion with fludarabine and cyclophosphamide followed by Axi-cel at 2×10^6 cells/kg. The ORR was 82%, including a CR rate of 54%, with an OS rate at 6 months of 80%. At the time of data cutoff, 44% of patients remained in remission [67]. The ZUMA-1 findings have been submitted to the FDA to support a biologics license application for Axi-cel for the

treatment of transplant-ineligible patients with relapsed or refractory aggressive NHL.

Collectively, these results demonstrate the successful application of CAR T cell therapy in CD19+ B cell malignancies but also raise a red flag regarding potential life-threatening toxicities. Nonetheless, the risk/benefit ratio greatly favors the use of CAR T cells in malignancies such as relapsed/refractory ALL or DLBCL for which effective therapies are desperately needed. Likely, the available clinical trial results with CD19-directed CAR T cells will lead to the first regulatory approval of a gene-engineered adoptively transferred T cell therapy in patients with ALL and DLBCL in 2017.

A series of preclinical and early clinical results suggest that CARs targeting B cell maturation antigen (BCMA), which is expressed in multiple myeloma, may emerge as the next successful clinical application of CAR T cell technology. Several groups, both academic and in the pharmaceutical industry, are developing BCMA-targeted CAR T cells, including the Nanjing Legend Biotech NCI, Kite, bluebird, and Novartis. Similarly, multiple antigens expressed by solid cancers are currently being targeted by CAR T cell approaches (Table 42.2 and Fig. 42.1). Early results from first-in-human studies targeting mesothelin (mesothelioma, pancreatic cancer, ovarian cancer) or EGFRvIII (glioblastoma) expressing malignancies have shown the safety of this approach. While available clinical efficacy results are far from those observed in B cell malignancies, the limited number of patients treated so far at the predicted clinically efficacious doses precludes drawing conclusions regarding the potential of CAR T cell therapies in solid tumors.

42.5.2 T Cell Receptor-Transduced T Cells

As mentioned earlier, investigators at the National Cancer Institute (NCI) demonstrated more than a decade ago that the adoptive transfer of in vitro expanded melanoma-reactive tumor-infiltrating lymphocytes (TILs) extracted from autologous fresh tumor samples induced tumor regression in 49–72% of patients with metastatic melanoma [16, 68]. However, this approach was only applicable to half of the patients with melanoma from whom TILs could be generated, with very limited success in other malignancies. This realization prompted the development of cell transfer studies in which T cells were genetically engineered to express TCRs specific against antigens expressed in a variety of human cancers. Early evidence that TCR-based gene therapy could induce significantly deep and durable responses in cancer was first provided by a clinical trial in which 2 of 13 patients experienced tumor regression upon adoptive transfer of autologous T cells engineered to express a MART-1-reactive TCR [17]. A follow-up study utilizing a higher avidity MART-1 TCR reported objective responses in 6 of 20 (30%) and in 3 of 16 (19%) patients treated with a murine-derived high-affinity TCR against human gp100 [24]. Unfortunately, severe on-target off-tumor toxicity, mostly affecting normal melanocytes in the skin, eye, and ear, highlighted the need to target antigens (nearly) absent in critical normal tissues. As mentioned earlier in this chapter, the adoptive transfer of T cells transduced with a MAGE-A3-reactive TCR led to the deaths due to unexpected of target cross-reactivity for two different TCRs and improved in vitro and in silico methods to predict cross-reactivity profiles for novel TCR lead structures are now available and will increase patient safety for novel TCR-engineered products. Thus far, only a limited number of studies exploring TCR T cell technology have shown consistent clinical activity. The most promising data have been generated by studies using TCR-engineered peripheral T cells targeting the cancer antigen New York esophageal squamous cell carcinoma 1 (NY-ESO-1). These trials therefore require special consideration.

42.5.2.1 Targeting NY-ESO-1

NY-ESO-1 is a CGA expressed in multiple tumors including in 10–50% of metastatic melanomas, lung, breast, and ovarian cancer as well as in 70–80% of synovial cell sarcomas [69, 70]. The first clinical study, using *Gammaretrovirus*

to deliver a high-affinity TCR directed against an HLA-A*02-restricted NY-ESO-1 nonapeptide (residues 157–165) to autologous T cells, was published by investigators at the NCI in 2011 [6, 31]. Seventeen patients (6 with synovial cell sarcoma and 11 with melanoma) bearing tumors that stained strongly for NY-ESO-1 antigen expression (2 to 4+, >50% cells) were treated. Treatment consisted of lymphodepleting chemotherapy consisting of cyclophosphamide (60 mg/kg/d for 2 days) and fludarabine (25 mg/m^2/d for 5 days) followed by NY-ESO-1 TCR-transduced T cells (median 5×10^{10}; range, 1.6 to 130×10^9) and systemic IL-2. Nine patients responded, including two with melanoma that achieved a CR durable beyond 1 year and one with synovial sarcoma achieving a partial response (PR) that lasted 18 months. A recent update of this study provided data on additional 21 patients (12 with synovial sarcoma and 9 with melanoma) [6]. The ORR was 61% in synovial sarcoma, with estimated 3- and 5-year survival rates of 38% and 14%, respectively. In melanoma, the ORR was 55%, and the estimated 3- and 5-year survival rates were both 33% [6]. Overall, the toxicities observed in the trial were those expected from the lymphodepleting regimen and IL-2 therapy. These results validate NY-ESO-1 as an interesting cancer antigen for adoptively transferred T cell immunotherapy. Adaptimmune Therapeutics, a biotechnology company utilizing a proprietary SPEAR® (Specific Peptide Engineered Affinity Receptor) T cell engineering platform, has launched six clinical studies in HLA-A*02-positive patients with NY-ESO-1 expressing tumors. Fifty-three patients have been treated with NY-ESO-1^{c259}SPEAR® as of January 2016, including 27 with multiple myeloma and 26 with a variety of solid tumors, including synovial sarcoma, melanoma, ovarian cancer, and NSCLC [71].

Of the trials in solid tumors, thus far, the most data is available from synovial sarcoma. Cohort 1 in the synovial study has completed accrual. Of the 12 patients with unresectable, metastatic, or recurrent synovial sarcoma, 6 achieved an objective response (ORR 50%). In the ovarian cancer and melanoma studies, six and four patients have been treated, respectively, but no objectives have been observed to date. It is worth noting that lymphodepletion in both these latter indications consisted exclusively of cyclophosphamide (i.e., no fludarabine), which might have contributed at least in part to the lack of objective responses. Both trials will continue treating patients using standardized NY-ESO-1 screening and fludarabine containing lymphodepleting regimens. Notably, NY-ESO-1^{c259} SPEAR™ T cells are able to persist over time, being detectable beyond 3 years post-infusion [71].

The TCR NY-ESO-1 paradigm was extended to multiple myeloma by investigators at the University of Pennsylvania using a lentivirus platform to force the expression on T cells of a TCR recognizing NY-ESO-1 [5]. Twenty patients with NY-ESO-1 expressing multiple myeloma received genetically engineered T cells 2 days after having undergone autologous stem cell transplant (SCT). NY-ESO-1 TCR-engineered T cells were safe, consistently trafficked to the bone marrow, and displayed extended persistence that correlated with clinical activity against multiple myeloma. The median progression-free survival (PFS) was 19.1 months, which suggests a potential role of this approach in patients with relapsed/refractory multiple myeloma. Moving forward, it will be important to determine the exact activity of NY-ESO-1 TCR T cells without the confounding effect of autologous SCT.

A recent publication reported that NY-ESO-1-engineered T cells show efficacy against disseminated neuroblastoma in xenograft mouse studies [72]. These nonclinical supportive data sets suggest that NY-ESO-1 may be a target with suitability beyond synovial sarcoma, malignant melanoma, and multiple myeloma.

Other Clinical TCR Targets

The activity of TCR T cell therapies beyond tumors expressing NY-ESO-1 has been infrequent and hampered by the limited persistence of TCR T cells, which may preclude the achievement of meaningful sustained responses, and fundamentally by tissue liabilities leading to untoward toxicity. Multiple cancer-associated antigens expressed at low levels in normal tissues such as CG antigens, CEA, and Her2 are currently being targeted by TCR-engineered T cells

(Table 42.1). More clinical data from a next wave of TCR-engineered T cells targeting a variety of different antigens will become available in the next 2–3 years and contribute much to our understanding about the best targets and TCRs for the treatment of patients with cancer (Table 42.4).

Table 42.4 TCR T cell product in the clinic

TCR target[a] (notes)	Tumor histology	NCT #	Sponsor
NY-ESO-1	Myxoid/round cell liposarcoma	NCT02992743	Adaptimmune
	Synovial cell sarcoma	NCT01343043	Adaptimmune
	Metastatic melanoma	NCT01350401	Adaptimmune
	Multiple myeloma	NCT01892293	Adaptimmune
	Non-small cell lung cancer	NCT02588612	Adaptimmune
	Ovarian	NCT01567891	Adaptimmune
NY-ESO-1 (armed with dominant negative TGF-β receptor II)	Locally advanced or metastatic solid tumors	NCT02650986	Roswell Park Cancer Institute
NY-ESO-1 (aldesleukin + dendritic cell vaccine + ipilimumab)	Locally advanced or metastatic solid tumors	NCT02070406	Jonsson Cancer Center
NY-ESO-1 (+ vaccine)	Malignant neoplasm	NCT01697527	Jonsson Cancer Center
NY-ESO-1	Bladder/breast/esophagus/lung/other solid tumors	NCT02457650	Shenzhen Second People's Hospital (China)
NY-ESO-1 (murine TCR)	Metastatic non-melanoma cancers	NCT01967823	NCI
NY-ESO-1	Solid tumors	NCT02366546	Mie University (Japan)
MAGE-A3 (HLA-DP4)	Esophageal/melanoma/urothelial/cervical/other solid tumors	NCT02111850	NCI
MAGE-A3 (HLA-A*01)	Esophageal/melanoma/urothelial/cervical/other solid tumors	NCT02153905	NCI
MAGE-A4 (HLA-A*24)	Solid tumors	NCT02096614	Japan
MAGE-A10	Urothelial, melanoma, head, and neck	NCT02989064	Adaptimmune
MAGE-A10	Non-small cell lung cancer	NCT02592577	Adaptimmune
p53.IL2	Bladder	NCT01625260	Altor Bioscience
HBV (HLA allele not disclosed)	Recurrent hepatocellular carcinoma posttransplant	NCT02719782	Sun Yat-Sen University (China)
HPV E6	Vaginal/cervical/anal/penile/oropharyngeal	NCT02280811	NCI
MART-1	Metastatic melanoma	NCT02654821	Netherlands NKI
MART-1 (dendritic cell vaccine + IL-2)	Metastatic melanoma	NCT00910650	Jonsson Cancer Center
Thyroglobulin	Metastatic thyroid	NCT02390739	NCI
Tyrosinase	Metastatic melanoma	NCT01586403	Loyola University
WT1	Mesothelioma/non-small cell lung cancer	NCT02408016	Fred Hutchinson Cancer Center
WT1	Acute myeloid leukemia	NCT02550535	Cell Therapy Catapult (UK)
WT1	Acute myeloid leukemia/chronic myeloid leukemia	NCT01621724	Cell Therapy Catapult (UK)

Source: clinicaltrials.gov
Non-comprehensive selection of ongoing TCR T cell clinical trials
[a]HLA-A*02 restricted unless otherwise stated

42.6 Clinical Safety Considerations

The most frequent and important toxicities derived from the clinical application of gene-engineered T cell therapies are those derived from on-target off-tumor T cell-mediated destruction of normal tissues, cytokine release syndrome (CRS), and neurologic toxic events.

B cell aplasia: The induction of B cell aplasia that results from the recognition of CD19 on the surface of normal B cells by the CAR T cells is an expected and unavoidable on-target off-tumor toxicity of CD19-specific CAR-modified T cells [11, 73, 74]. B cell aplasia has been used as pharmacodynamic marker of CAR T cell function and persistence. In clinical trials, B cell aplasia is customarily managed by intravenous immunoglobulins (IVIg) supplementation [3], as a means to ameliorate the risk of infectious complications associated with B cell depletion. However, available data are insufficient to determine whether this increased risk is clinically significant or whether IVIg replacement therapy is absolutely necessary to prevent infectious complications in patients with CAR T cell-induced B cell aplasia.

Cytokine release syndrome: CRS is a frequent complication associated with both CAR and TCR T cell therapy. Patients with CRS typically present with high fever, hypotension, and hypoxia, which may result in end-organ failure. CRS onset ranges from a few hours to, more frequently, several days post-T cell infusion. CRS results from the secretion of multiple proinflammatory cytokines, including IL-6, TNFα, and IFNγ, secondary to antigen-mediated activation of CAR T cells [3]. In severe cases, CRS may progress to macrophage activation syndrome, which presents with hemophagocytosis, hyperferritinemia, pancytopenia, liver insufficiency, coagulopathy, and neurologic symptoms [75]. Most cases of CRS can be managed with supportive measures. In cases of severe CRS, the administration of the anti-IL-6R monoclonal antibody tocilizumab results in the rapid resolution of the symptoms associated with CRS [75]. The administration of corticosteroids is usually employed after cytokine blockade in the management of severe CRS due to their lymphocytic activity [11]. CRS frequency and severity appear to correlate with tumor burden at the time of CAR T cell infusion, as shown in patients with ALL [3, 76]. This association suggests that effective debulking of patients prior to CAR T cell infusion or the treatment of patients with MRD might reduce significantly the risk of severe CRS.

Neurologic toxicity: Neurologic toxicity, including seizures, obtundation, delirium, and dysphasia, has been reported in up to 29% of patients in some studies treating patients with CD19-directed CAR T cells [3, 61, 63, 77]. The pathogenesis of this toxicity remains unclear as CAR T cells infiltrate the cerebrospinal fluid, but there is no evidence of CD19 expression in brain tissue. Neurologic symptoms are generally short-lived and usually reversible with supportive measures. Some patients with ALL have been reported as having self-limited encephalopathy after resolution of CRS, which frequently does not respond to tocilizumab [3]. Recently, Juno Therapeutics has stopped the development of JCAR015, a CD19-directed CAR T cell program being developed in adult patients with adult B cell ALL in the phase II Rocket trial. In that study, five patients died due to cerebral edema, which appeared to be treatment related. Fludarabine was removed from the lymphodepleting regimen after the first two deaths in an attempt to ameliorate therapy-related toxicity in the early post-infusion period. However, three more patients died of the same complication after lymphodepletion involving exclusively cyclophosphamide. Further, there is evidence indicating that the addition of fludarabine to the lymphodepletion regimen improves CAR T cell expansion, persistence, and long-term clinical outcomes [65, 78].

These severe toxic events emphasize the need for a careful selection and qualification of highly specialized centers to conduct adoptive T cell therapy studies and highlight the importance of close monitoring by medical personnel with expertise in cell therapies and the early institution of corticosteroid and/or IL-6 blocking therapy in patients experiencing CRS and/or neurologic

toxicity. While the toxicity profile observed in clinical trials of TCR T cell therapies is similar to that observed in CD19-directed CAR T cell therapy trials, the rates and severity of the most important adverse events (i.e., CRS and neurotoxicity) have been much lower. A recent analysis of 53 patients treated with NY-ESO-1^{c259} SPEAR® in Adaptimmune-sponsored studies in synovial sarcoma, melanoma, ovarian cancer, and multiple myeloma have shown that the most common adverse events were rash (49%), diarrhea (40%), fatigue and pyrexia (36% each), nausea (26%), anemia and lymphopenia (25% each), and neutropenia (23%) [71]. Most of these toxicities are likely related to the fludarabine/cyclophosphamide lymphodepleting regimen. Related serious (grades 3–4) adverse events were relatively infrequent, with CRS, neutropenia, and pyrexia occurring only in 8% of patients each, graft versus host disease in 6% (observed only in patients with multiple myeloma posttransplantation), and death related to bone marrow failure in one patient with synovial sarcoma. No grades 3–4 neurotoxicity events have been observed to date. Importantly, CRS rates are different depending on indication, with no cases of severe observed in patients receiving NY-ESO-1^{c259} SPEAR® T cells post-autologous SCT, despite high IL-6 levels [5].

Gene-engineered T cell therapies show impressive results in patients with cancers against which effective therapies are lacking. However, in order for these therapies to realize their full potential, physicians need to understand the risks associated with them and their management. Important inroads have been made in our understanding of the pathophysiology of CRS, which has allowed the development of efficacious treatment strategies. However, more work is needed to better understand the pathophysiology of the neurotoxic events arising in some patients after CAR T cell infusion, which seem to be distinct from CRS. Future work will assess the effectiveness of CRS prophylaxis with cytokine blockade prophylaxis and better patient selection as well as the role of molecular safety switches to ameliorate CRS-related toxicity of patients receiving CAR or TCR T cell therapy.

42.7 The Challenge to Maximize Safety and Efficacy for CAR T Cell and TCR T Cell Medicines

Given the potency of T cells, it is vital to control their activity as far as possible to prevent on-target, on-tumor off-tumor, and off-tumor toxicities, including those that could result from the endogenous TCR alone or mispaired with an introduced TCR and those that result from excessive activation and cytokine production.

As introduced previously, there are a number of technologies at the concept stage or in development that aim to improve the safety window of CAR T cell or TCR T cell therapies:

1. The immune receptor can be fine-tuned in terms of specificity and affinity for its target to differentiate between healthy and tumor tissues.
2. Activity or suicide switches or specific markers can be introduced into the therapeutic cell to enable activation or elimination of the therapeutic cell by a small molecule drug or monoclonal antibody.
3. The immune receptor can be introduced into the endogenous TCR locus, or the endogenous TCR can be shut off by RNA interference mechanisms to limit the T cell specificity only to that of the tumor-specific immune receptor.
4. The immune receptor can be provided in an inactivated state requiring activation by conditions (e.g., proteolytic activity) present in the tumor microenvironment.
5. Logic gated receptors could be developed that require the integration or multiple signals including negative signals to differentiate healthy from tumor tissue.
6. The cell might be engineered using novel notch-based technologies to precisely control the gene expression program following recognition of the target antigen. This concept enables therapeutic T cells sensing one tumor-associated antigen to induce expression of a receptor for a second tumor-associated antigen, thereby limiting expression of a CAR or TCR to only the therapeutic T cells that have entered the tumor microenvironment, and/or

to express only a precisely defined set of cytokines, chemokines, and other effectors.

It will also be vital to optimize the efficacy of CAR T cell and TCR T cell therapies such that they can realize their potential as single infusion therapies that can offer long-term disease control or even cure. While CAR T cell therapies have shown promise against hematological malignancies, response rates have been disappointing in solid tumors. Evidence from TCR T cell therapies and TILs demonstrate that high levels of efficacy are achievable, but the cell product will still need to overcome the highly suppressive tumor microenvironment, either through design or combination with suitable immunotherapies. Currently, a very large number of potential approaches to enhance the efficacy of CAR T cell and TCR T cell therapies are being tested in preclinical or clinical studies [79], as shown in Fig. 42.2. Such plethora of approaches reflects the novelty of the field and the numerous contexts where CAR T cell or TCR T cell therapies are being attempted. Nevertheless, it is anticipated that this research efforts will result in a much smaller number of optimal approaches which will then be widely adopted. Since the PD-1-PD-L1 axis has been clinically validated in several malignancies and tumors often upregulate PD-L1 in response to IFN-γ from T cells, it is likely that approaches to tackle this axis will prove useful for CAR T cell and TCR T cell therapies. In addition, there may also be immune suppressive pathways such as TGF-β for which no approved systemic inhibitory therapy exists, which would benefit from highly localized therapeutic cell-specific inhibition by gene engineering, due to the essential role of TGF-β in normal tissue homeostasis.

In summary, the technological progress that has brought so many different innovations to enhance the safety and efficacy of CAR T cell and TCR T cell therapies has not yet reached the level of maturity necessary to test in the short-term solutions to safely confer long-term disease control to a broad cancer patient population beyond CD19+ hematological malignancies. It is inevitable that the field will learn how to make the best use of the new technologies that have now become available and deliver increased benefit to a larger number of patients in the future.

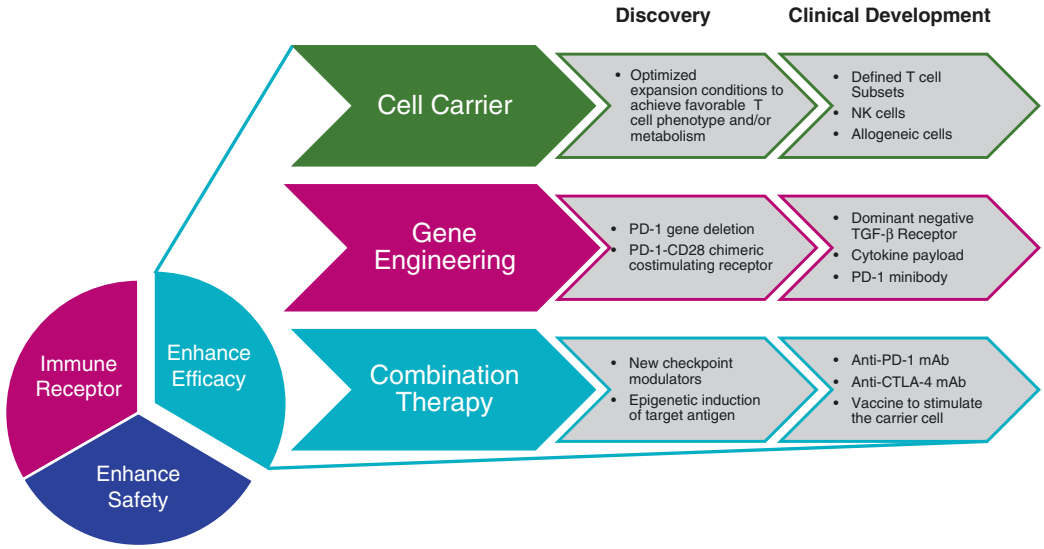

Fig. 42.2 Enhancing the efficacy of CAR T cell and TCR T cell therapies. Selected strategies to enhance the efficacy of CAR T cell and TCR Tcell therapies fall into three categories: enhancements to the quality or nature of the cell carrier, additional gene engineering steps to "arm" the therapeutic T cell to resist the tumor microenvironment, or the use of combination therapies

42.8 The Challenge of Achieving Maximum Access for CAR T Cell and TCR T Cell Medicines

While this book chapter focuses on the biology of engineered T cells, it is worth touching upon the critical aspects of virus and drug supply for autologous cell products. The high costs to supply engineered T cells to patients are one remaining key obstacle for broader patient access of such therapies. The two major cost drivers are those associated with the production of vectors to introduce the immune receptor into the T cells as well as the costs incurred during manufacturing of the engineered cells. The number of facilities that can manufacture GMP-grade vector and cell products is still limited, and the capacity of the existing facilities is so far not tailored to support supply of larger batches and markets. Most clinical trials to date utilize lentiviral vectors that are difficult to manufacture as they require packaging cell lines expressing multiple gene products leading to transduction-competent and replication-deficient viral vectors. A few specialized CMOs and pharmaceutical companies have now begun to develop more scalable processes overcoming current limitations that may allow much higher yields and manufacturing much larger scale that will lead to significant decrease of costs over the next 3–5 years. A couple of emerging virus-free gene transfer systems based on DNA transposon-based approaches [80, 81] might enable further reduction of manufacturing costs. However, it is still too early to define whether or not plasmid-based systems will replace lentiviral gene transfer.

The biggest cost driver is the cell transduction process which requires T cell activation, gene transduction, and subsequent expansion over a period of 7–12 days. The current state-of-the-art stipulates the need for one class B clean room with a class A laminar flow for the manufacturing of one product for a single patient. Multiple technology providers are now developing alternative solutions that allow introducing automation for some or all steps of the cell manufacturing process using closed bag systems. The first clinical trials in which patients will receive a product using a wholly automated tabletop-sized commercial cell processing device have recently been initiated. The biggest opportunity arises from the ability to place and run several of those "gene therapy in a box" devices into one ballroom suit that may have a lower clean room class. A recent manuscript from A. Kaiser described the advantageous of automation and introduces yet another concept that may ultimately lead to a further manufacturing cost reduction which lends support to the concept of pushing manufacturing away from central industrial facilities and closer to if not at the point of care [82].

Novel nuclease technologies that allow multigene editing of CAR and TCR T cells [83–85] or the use of homology-directed recombination (HDR) that can be used to couple delivery of a therapeutic gene cassette with targeted genomic modifications to generate engineered human T cells with therapeutic activity [86] may be a game-changer as it allows to generate off-the-shelf products from single donors that can be administered to multiple patients as they lack expression of potentially alloreactive endogenous TCRs. Multiple nuclease technologies exists such as zinc-finger nucleases, TALEN nucleases, meganucleases, or CRISPR/Cas9. Recently, universal CAR19 T cells were generated by lentiviral transduction of nonhuman leukocyte antigen-matched donor cells and simultaneous TALEN-mediated gene editing of T cell receptor α chain and CD52 gene loci and used to treat to children with ALL [87]. The gene-edited universal CAR T cells induced molecular remissions within about 4 weeks and persisted until conditioning prior to allogeneic stem cell transplantation. While this bridge-to-transplantation strategy demonstrates the therapeutic potential of gene-editing technology, longer-term data is required to understand the extent to which universal CAR T cells will be able to replace autologous cell products that will show a longer persistence in vivo.

An even more disruptive approach may be the future use of surface-engineered viral vectors that may very efficiently target a particular subset of resting T cells. Such an approach may lead to significant decrease of manufacturing costs due to shortened process times or even enable gene

modification of T cells directly in vivo [88]. At this stage, it is unclear when such in vivo approaches will become available for clinical testing in patients the future.

In summary, we believe the cost of goods for CAR and TCR T cell therapy will fall substantially in the coming period due to considerable investments into GMP-grade processes to scale viral vectors for gene delivery or their replacement by cheaper alternative gene delivery platforms and the advent of automation technology that will enable innovative supply chain solutions. Such investments are being made by multiple players in the field and will ultimately lead to stepwise reduction of the cost of goods for engineered cell products. It will probably take another 4–5 years until the fully fledged technology solutions will become more broadly available to enable clinical trials and subsequently to fuel future market supply for larger patient populations. Further in the future, the field may potentially switch to in vivo CAR/TCR gene delivery approaches for at least a fraction of the products, leading to yet another significant decrease in the cost of goods.

42.9 Conclusions and Future Perspective

We conclude that lymphocytes engineered to express CARs or TCRs bear an enormous clinical potential for patients suffering from cancer. We described three key obstacles that inhibit the full exploitation of the clinical and commercial potential of engineered cells and provide solutions. Novel enabling technologies have now become available that bear the potential to develop best medicines with increased safety and efficacy that can deliver transformational benefit to patients beyond CD19 as a target. Innovative supply chain solutions will become available that tackle the major cost drivers for autologous cell products and support enhanced access for these new medicines to larger populations with high unmet medical need. Once patient benefit has been maximized and costs have been minimized through development of key enabling technologies, the field will be able to transfer the added value to larger pipelines of CAR and TCR T cell medicines targeting multiple shared and unique personalized antigens expressed in tumors. Initial CAR T cell products using simple CAR constructs that were lacking additional co-stimulatory domains were assessed in patients between 1998 and 2008. These pioneering clinical trials did not deliver strong antitumor activity in patients. The picture radically changed when second-generation CAR constructs became available that led to unprecedented clinical activity in patients with ALL, DLBCL, and CLL. These results ignited a whole new industry around cell therapies, and the expectation is that the first CAR T cell products may receive marketing authorization in 2017. While B cell malignancies compose a relatively small market opportunity in oncology and while costs for supplying engineered lymphocytes are still very high, we expect continued technological progress to drive further success of cell therapies. It is difficult to predict what will happen in the future, but at the current speed of technology progress, it seems inevitable that the field will see more CAR T cell and TCR T cell therapies successfully reaching an increasing number of patients.

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Part VI

Concluding Remarks

Concluding Remarks

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Pedro Romero and Wolf H. Fridman

In the 1890s, a surgeon working at what today is known as the Memorial Sloan Kettering Cancer Center in New York had the momentous insight that the regression of a relapsing sarcoma observed in a patient undergoing erysipelas, as a complication of surgery, could have been a case of tumor rejection triggered by the body defenses that had been activated against the infection. W. Coley, as was his name, decided to test the validity of this idea by inoculating laboratory-grown bacteria, the state of the art in microbiology in those days, directly into the tumors and, to his amazement, observed complete tumor responses in some of the treated patients. These results ignited a new field of research known as tumor immunology. It took 120 years for this branch of immunology to, together with cancer biology and clinical oncology, fully come of age. The scientific and oncology communities have driven a renaissance of the field in the last 10 years. The major successes of immune checkpoint blockade during the last 5 years have reignited the field in a major way. To date, immunotherapy is becoming the fourth pillar of cancer treatment joining surgery and chemo- and radiotherapy. Monoclonal antibodies blocking the PD-1/PD-L1 axis have emerged as the backbone of immunotherapy in various tumor types including melanoma, lung, renal, bladder, and Hodgkin's lymphoma.

What have we learned? We now know that the immune system often recognizes tumors early during carcinogenesis up, in some cases, to the metastatic state. Strong supports for this statement are the favorable prognostic impact of Th1-oriented and cytotoxic T cell infiltration and the identification of T cells specific for mutated tumor antigens in the tumor microenvironment. The interactions between evolving and developing tumors and the immune system are dynamic and lead to a reciprocal and progressive sculpting. Tumor variants selected upon immune pressure evolve resistance to the main mechanisms of antitumor immunity, such as IFNγ or cytolytic lymphocytes. Conversely, various components of innate and adaptive immunity may be co-opted by tumors to provide niches favorable to their growth, migration, invasion, and seeding at distant sites. In clinically manifest tumors, a significant fraction of them are "T cell inflamed." The proportion of tumors that are infiltrated by T cells is variable from one tumor type to the other and within patients bearing cancers with similar histology. Many reasons may explain this variability. The intrinsic immunogenicity of tumors may play a significant role including tumors with a high load of somatic mutations, such as melanoma or lung cancer, which are likely to display a high density of neoantigens to T cells. However, an intact dendritic cell compartment, in particular

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cross-presenting BATF3+ dendritic cells, is required for neoantigens to be "visible" to the host's immune system. Moreover, in addition to the intrinsic tumor immunogenicity and the antigen processing and presentation arm, the access to tumors by migrating T cells is critical. The endothelial cells lining the neovessels are a barrier which primed T cells need to cross in order to infiltrate tumors. They go across the endothelial cell walls by a well-regulated set of mechano-cell biological processes involving sequential rolling along the vessel wall, tethering, stopping, and crawling across the endothelial cell junctions until reaching the tissue space beyond tumor blood vessels. Extravasated T cells then need to migrate into the tumor parenchyma. In short, variable and relatively high proportions of cancers are so-called immune deserts owing to the many steps in the immune cell infiltration process that may be disrupted in the advanced tumors.

The "T cell inflamed" tumors are likely to be sensitive to immunotherapy in general and to, more specifically, immune checkpoint blockade therapy. We have also learned that there are possibly countless mechanisms that impinge on the antitumor T cell activity in situ. Several categories of such immunosuppressive mechanisms have been established. These include the tumor-intrinsic loss of antigen in the immune escape variants and the selection of tumors able to secrete large amounts of immunosuppressive factors notably IL-10 and TGF-β. Moreover, tumor extrinsic mechanisms involve the recruitment of various immune/inflammatory cell types that may contribute to establish an immunosuppressive milieu.

A wealth of results has been streaming from the increasing number of large phase III clinical trials of immune checkpoint blockade. They confirm that around 30% in average of cancer patients may respond to these new agents, with the exception of Hodgkin's lymphomas with overall response rates equal or higher to 70%, although with low mutation rates. In the face of this still limited number of patients that may benefit from immune checkpoint blockade, there is a need for biomarkers allowing the precise identification of patients sensitive to immunotherapy. Some predictive biomarkers have gained credence. In particular, the expression of PD-L1 in the tumor as assessed by immunohistochemistry has proven useful at predicting response to the PD-1/PD-L1 blockade therapy. However, it is still far from a perfect biomarker as variable proportions of cancer patients with PD-L1+ tumors do not respond, while there can be patients responding to treatment whose tumors are negative for this marker. One of the complexities of such a biomarker is the fact that IFNγ is efficiently inducing its expression, an event likely to occur as patients respond to treatment. Thus, this is a dynamic marker whose expression depends for a good part on the status of the antitumor immune response. Systems biology approaches may in the future provide gene signatures or immune response phenotypic marker combinations with enough predictive power. So far, some leads such as polygene signatures or neoantigen load are showing some promise and may be examined in the future in appropriate clinical trials.

It is possible that the large and variable fractions of tumors that are poorly infiltrated by immune cells, particularly by T cells, may be rendered responsive to immune checkpoint blockade therapy by vaccination and/or adoptive T cell transfer. The goal of these two therapy modalities is to achieve significant tumor infiltration by tumor reactive T cells with powerful effector function and able to last for prolonged periods of time. However, current cancer vaccines have modest clinical efficacy, and adoptive T cell transfer therapy has shown promise in some hematological malignancies but remains difficult to apply to solid tumors. Basic research is needed to advance our understanding of the biology of antigen specific T cells and gain insights into efficient pharmacological means to modulate their differentiation and ability to remain functional in the immunosuppressive microenvironment. T cell memory induction by vaccination also needs much more mechanistic preclinical studies before being able to translate to adequate cancer vaccine formulations.

Concerning therapeutic vaccines, these may be suboptimal in various respects. The antigens

targeted by vaccination may not be strong tumor rejection antigens, and the T cell repertoire available to tumor-associated antigens is of low affinity and largely ineffective. The adjuvants available for vaccination may also lack potency, and progress in the understanding of the signals leading to innate immune activation should provide newer and more potent compounds to include in next-generation vaccines. Effective vaccines aiming at inducing T lymphocyte responses need to achieve not only high T cell numbers but also selectively favor the expansion of the highest affinity tumor antigen-specific T cells with robust polyfunctional differentiation and, at the same time, induce long-lived memory T cell responses. These constitute major challenges. Addressing them necessitates, on the one hand, progress in basic immunology research and, on the other hand, innovative early phase clinical trials with adaptive design that allow to rapidly test a relatively large number of variables coupled to accurate immunomonitoring of vaccine-specific immune cell responses.

A next set of challenges in the development of effective immunotherapies is the need to face the development of resistance to immune mechanisms. The first cases of adaptive resistance in melanoma have been recently reported. In addition to the now approved immune checkpoint blocking agents, anti-PD-1, and anti-CTLA-4, the tumor immunology community continues active efforts to mine the rich trove of well-defined immunosuppressive mechanisms operating in the tumor microenvironment. These include additional immune checkpoints such as LAG-3, VISTA, or TIGIT on effector T cells. Moreover, a new class of immune checkpoints may be represented by ectoenzymes degrading nucleotides/nucleosides to generate adenosine, an inhibitor of immune function in T cells. Notably, CD39 expressed on Tregs and especially CD73, the critical enzyme irreversibly converting AMP into adenosine, expressed in various immune cells, including T cells and also in tumors. Two CD73-blocking monoclonal antibodies are in early phase clinical trials testing dose and safety. A complementary immunotherapeutic strategy is based on the use of agonistic monoclonal antibodies engaging costimulatory receptors. While CD28 had to be excluded of the target selection early on after the realization of its serious toxicity (Tegenero) several years ago, other receptors showing some promise are CD137, OX40, GITR, and CD40. The early-phase clinical trials have already been performed, and their development proceeds with varying degrees of success.

An additional class of targets includes a handful of enzymes overexpressed by various cell types in the tumor microenvironment. Arginase and inducible nitric oxide synthase (iNOS) are major mediators of the suppressive capacity of immature myeloid cells infiltrating tumors; indoleamine oxidases (IDO) 1 and 2 are induced by IFNγ in antigen presenting cells and overexpressed in various tumors as can be cyclooxygenase-2 (COX2). In the case of IDO, its ability to deplete tryptophan is deleterious for effector functions of T cells. The kynurenines generated by the degradation of tryptophan are agonists of the AH receptor on Th17 cells leading to inflammatory cytokines that may promote tumor growth and dissemination. The use of specific inhibitors of these enzymes has shown significant antitumor effects in preclinical models, and several IDO inhibitors are well advanced in clinical development. It is likely that combinations of IDO inhibitors with immune checkpoint blockade will become approved new combination immunotherapies in the near future.

Finally, the reprogramming of tumor-associated macrophages and/or myeloid-derived suppressor cells is another approach to enhance antitumor immune responses. Indeed, it has been shown that monoclonal antibodies blocking the CSF-1R have antitumor effects in vivo associated with a shift in the functional profile of TAM from a protumoral activity (M2) to a tumor immune protective effect (M1) rather than their depletion as originally thought.

The field has reached a high degree of sophistication. As detailed throughout the book and above in these concluding remarks, we have now reached a deep understanding of the cross talk between the many cellular types, both inflammatory and stromal, in the tumor bed and their client

tumor cells. The molecular underpinnings of protumor and antitumor inflammatory and immune responses have yielded, and continue to provide, a rich palette of actionable targets which are now at various degrees of clinical development. To date, there is a growing consensus that a solid immunotherapy backbone is treatment with anti-PD-1/PD-L1 agents with future algorithms guiding the choice of combinations with other compounds targeting specific immune suppressive loops that would provide additive clinical efficacy. The field is now moving its focus from signaling pathways to two major promising horizons. One is the understanding of the metabolic states underlying tumor evolution and antitumor immune responses. This has already prompted a revision of the understanding of the Warburg effect, and inflammatory cells and tumor cells are viewed as avid competitors for nutrients, particularly glucose, that adapt their metabolic rates to the relative supply of oxygen and nutrients available in their extracellular neighborhoods. Systems biology has now embraced metabolomics approaches that provide large-scale profiles of the metabolic states at the multicellular level and efforts are underway to increase the resolution to ever fewer cells in a given spot within tumors. The other horizon is that of gene expression. In addition to the spectacular progress in single cell transcriptomics, there is now strong interest in understanding epigenetic programs of regulation of gene expression again at the systems level. Future advances will provide single cell level atlases of the tumor microenvironment so that we will reach unprecedented levels of understanding and will have the opportunity to integrate all this knowledge into dynamic mathematical models with the help of computational biology. Future translational scientists will more and more depend on accurate models to figure out appropriate immunotherapeutic algorithms as well as to develop more strongly predictive immune biomarkers to guide treatment decision trees. Complexity will grow and provide at the same time models that may allow much needed simplification in the day-to-day decisions in the clinic. Combinatorial immunotherapies as well as combinations of immunotherapies with standard therapies will grow at a steady pace in the years to come.