Magdalena Klink *Editor*

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Preface

The tumor microenvironment is a dynamic network that consists of tumor cells, immune cells, fibroblasts, endothelial cells, extracellular matrix, cytokines, chemokines, and receptors. Every element of this network promotes neoplastic transformation and supports tumor growth and invasion, as well as protecting the tumor from host immunity. A full understanding of the mechanisms underlying tumor growth and progression requires the thorough study of the above-mentioned factors and cells within the tumor microenvironment.

The main purpose of this book is to summarize the knowledge concerning the interactions of various types of cells in the solid tumor microenvironment, as the main factors involved in tumor progression and metastasis. Infiltrating immune cells are a normal constituent of tumors. Immune cells such as lymphocytes, dendritic cells, macrophages, neutrophils, natural killer cells, and mast cells are the most important cells involved in a crosstalk with the tumor cells. They either suppress or promote tumor growth and metastasis, and are the key players in the complex microenvironment network. Another important element taking part in tumor development is stromal tissue, which consists of fibroblasts, myofibroblasts, endothelial cells, immune cells, and extracellular matrix. The stromal cells, mainly fibroblasts, secrete various factors that affect tumor cells and result in a more aggressive cancer phenotype. Currently, tumor stroma is regarded as an essential contributor to tumor progression and metastasis. Myeloid-derived suppressor cells belong to the heterogeneous family of cells that accumulate in blood, lymph nodes, and bone marrow, and at tumor sites. They inhibit both innate and adaptive anti-cancer immunity, as well as promoting tumor progression. The immune system not only protects the host from cancer development and/or eliminates cancer cells, but it also promotes tumor growth. The current concept of tumor immunoediting consists of three phases: elimination, equilibrium, and escape. The elimination phase (formerly known as tumor immune surveillance) occurs when innate and adoptive immune systems successfully eliminate developing tumor cells. The equilibrium phase occurs when the phase of elimination is incomplete, and tumor dormancy appears as a result of developing an equilibrium state between tumor and immune cells. This is a period of latency between the end of the elimination phase

and the beginning of the escape phase. However, the existence of this period is hypothetical and needs proving. The final, escape phase is when the host suppression and tumor-eliminating mechanisms fail and the tumor develops gradually.

The second aim of this book is the focus on immunotherapy as an attractive approach to cancer therapy. This book gathers information on the three main strategies: vaccines, monoclonal antibodies, and adaptive immunotherapy, with emphasis on recent human clinical trials. Current concepts on the development and use of tumor-infiltrating lymphocytes or genetically-engineered T-cells, monoclonal antibodies against cytotoxic T lymphocyte-associated antigen 4, as well as vaccination via dendritic cells, are included in this book.

The presented collective work features a comprehensive summary of the interaction between various types of cells in the solid tumor microenvironment. I hope this book will describe, in sufficient detail, why tumor cells can survive and spread in the host organism, despite anti-tumor activity of immune cells.

Finally, I would like to take the opportunity to express my gratitude to all the authors who have contributed to this volume. Their vast knowledge and experience in the field of tumor microenvironment made the creation of this book possible.

Lodz, Poland Magdalena Klink
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Chapter 1
Immune Cells Within the Tumor Microenvironment

Daniela Spano and Massimo Zollo

Abstract A plethora of intrinsic and extrinsic factors, including communication between tumorigenic cells and infiltrating immune cells, fibroblasts, epithelial cells, vascular and lymphatic endothelial cells, cytokines and chemokines, constitute the tumor microenvironment. Although cancer cells can be immunogenic, tumor progression is associated with the evasion of immune surveillance, the promotion of tumor tolerance, and even the production of pro-tumorigenic factors by tumor-infiltrating immune cells. Here we will review the different types of immune cells within the tumors, with a focus on their fundamental role in tumor growth and immune escape, namely "cancer immunoediting." Unraveling their roles and the molecular mechanisms of action represents today an important issue for the development of new therapeutic approaches to fighting cancer.

List of Abbreviations

APC	Antigen-presenting cell
ARG1	Arginase 1
bFGF	Basic fibroblast growth factor
BST2	Stromal cell antigen 2
BTLA	B and T-cell lymphocyte attenuator
CD25	Interleukin(IL)-2 receptor α chain

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COX-2	Cyclooxygenase-2
CSF-1	Colony-stimulating factor 1
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CTLs	Cytotoxic T lymphocytes
DCs	Dendritic cells
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FGF2	Fibroblast growth factor-2
FOXP3	Transcription factor forkhead box P3
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
ILT7	Immunoglobulin-like transcript 7
iNOS	Inducible nitric oxide synthase
KLRG-1	Killer cell lectin-like receptor subfamily G, member 1
LAG	Lymphocyte-activation gene
M-CSF	Macrophage colony-stimulating factor
mDCs	Myeloid DCs
MDSCs	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
NGF	Nerve growth factor
NK	Natural killer cells
NKT	Natural killer T cells
NO	Nitric oxide
PD-1	Programmed cell death protein 1
pDCs	Plasmacytoid DCs
PDGF	Platelet-derived growth factor
PD-L1	Programmed cell death protein 1 ligand
PGE2	Prostaglandin E2
PyMT	Polyoma middle T
ROS	Reactive oxygen species
SCF	Stem cell factor
SDF1	Stromal-cell-derived factor 1
TAMs	Tumor-associated macrophages
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
Tim-3	T cell immunoglobulin and mucin-domain-containing molecule-3
TNF-α	Tumor-necrosis factor-α
Tregs	Regulatory T cells

TSLP Thymic stromal lymphopoietin
uPA Urokinase plasminogen activator
VEGF Vascular endothelial growth factor

Contents

1.1 Introduction

Over the past decade, solid tumors have increasingly been recognized as "organs" that show a complexity that approaches, and may even exceed, that of normal healthy tissue [1]. Solid tumors comprise not only the malignant cells, but also several other non-malignant cell types (including fibroblasts, resident epithelial cells, pericytes, myofibroblasts, vascular and lymphovascular endothelial cells, and infiltrating cells of the immune system), which together constitute the stromal tissue. Altogether, the non-malignant cells of stromal tissue produce a unique microenvironment that can modify the neoplastic properties of the tumor cells. In fact, it is now recognized that there is an extensive interplay between tumor cells and tumor microenvironment cells, in which the incipient neoplasias recruit and activate stromal cell types that assemble in an initial pre-neoplastic stroma. The latter, in turn, responds by enhancing the neoplastic phenotype of the nearby cancer cells, which again feed signaling back to the stroma, to continue its reprogramming [2, 3]. Thus the tumor microenvironment is a bidirectional, dynamic, and intricate network of interactions between the cells of the stromal tissue and the cancer cells. Therefore, a full understanding of both the tumor biology and the molecular mechanisms underlying tumor development and malignant progression requires the study of both of these individual specialized cell types within the tumor microenvironment.

An important role in tumor development and malignant progression is played by the tumor-infiltrating immune inflammatory cells. Once recruited into the tumor microenvironment, these cells can contribute to the malignant progression of the cancer-cell phenotype. Moreover, they establish a complex network of interplay that contributes to the promotion and maintenance of an immunosuppressive

microenvironment, which itself promotes immune escape, and as a consequence, enhances tumor progression.

This review focuses on the immune inflammatory cells of the tumor microenvironment. As such, we emphasize both their roles and functions in the promotion of the tumor malignant progression, and within the network of connections that they have with each other and with the cancer cells. An overview of the immune cell types in the tumor microenvironment is illustrated in Table 1.1. Figure 1.1 shows the network of communication of the immune cells with each other and with the cancer cells.

1.2 Roles of Immune System in Tumorigenesis

In the adult, hematopoiesis (that is, the process that generates the immune system cells from the hematopoietic stem and progenitor cells) occurs in bone marrow. The egress of hematopoietic stem and progenitor cells and the generated immune cells from the bone marrow to the blood circulation is necessary for surveillance, as part of host defense and repair mechanisms [4, 5]. There is a growing body of evidence that indicates that the immune system has both positive and negative effects on tumor development and progression. As a tumor-preventing system, the immune system can protect the host from virus-induced tumors by eliminating or suppressing viral infections. Moreover, the timely elimination of pathogens and prompt resolution of inflammation can prevent the establishment of an inflammatory environment conducive to tumorigenesis. Furthermore, the immune system can specifically identify cancerous and/or precancerous cells on the basis of their expression of tumor-specific antigens or molecules induced by cellular stress, and eliminate them before they can cause harm. This latter process is referred to as tumor immune surveillance. Despite tumor immune surveillance, tumors do develop in the presence of a functioning immune system. Therefore, a more complete explanation for the role of the immune system in tumor development has been formulated, thus leading to the concept of "cancer immunoediting." According to this theory, the immune system not only protects the host against tumor development but can also sculpt the immunogenic phenotype of a developing tumor, resulting in promoting or selecting tumor variants with reduced immunogenicity that are better suited to survive in an immunologically intact environment. Thereby, this process provides developing tumors with a mechanism to escape immunologic detection and elimination [6, 7]. The tumor immunoediting is divided into three phases, called elimination, equilibrium, and escape [8]. In the elimination phase of cancer immunoediting, corresponding to tumor immune surveillance, the immune system detects and eliminates tumor cells that have developed as a result of failed intrinsic tumor suppressor mechanisms. The elimination phase can be complete, when all tumor cells are cleared, or incomplete, when only a portion of tumor cells are eliminated. In the case of partial tumor elimination, the host immune system and any tumor cell variant that has survived the elimination process enter

Table 1.1 Immune cells infiltrating solid tumors

Cell type	Abbreviation	Surface molecular signature
Tumor-associated macrophages	TAMs	IL-10^{high} IL-12^{low} IL-1ra^{high} and IL-1 decoyR^{high}
Myeloid-derived suppressor cells	MDSCs	*Murine subpopulations:* granulocytic CD11b^{+} Gr1^{+} (Ly6G^{+}) monocytic CD11b^{+} Gr1^{+} (Ly6C^{+} Ly6G^{-}) *Human subpopulations:* granulocytic CD15^{+} CD33^{+} CD11b^{+} lin^{-} IL-4Rα^{+} CD80^{+} CD115^{+} VEGFR1^{+} CD62L^{low} CD16^{low} monocytic CD14^{+} HLA Dr$^{-/low}$ CD66b^{+} IL-4Rα^{+} CD80^{+} CD115^{+}
Dendritic cells	DCs	*Mouse myeloid DCs: CD11c^{+} subpopulations:* Bone marrow: Lin^{-} CD115^{+} Flt3^{+} CD117^{low} Circulation: CD11c^{+} MHCII^{-} SIRPα^{low} Spleen: CD11c^{high} MHCII^{+} CD8^{+} CD205^{+} SIRPα^{-} CD11b^{-}; CD11c^{high} MHCII^{+} CD8^{-} 33D1^{+} SIRPα^{+} CD11b^{+} Thymus: CD8^{+} CD205^{+} CD11b^{low}; CD8^{-} SIRPα^{+} CD11b^{high} Lung, liver, kidney: CD11c^{high} MHC^{+} CD103^{+} CD11b^{-}; CD11c^{high} MHC^{+} CD103^{-} CD11b^{high} Intestine: CD11c^{high} MHC^{+} CD103^{+} CD11b^{low} CX3CR1^{-}; CD11c^{high} MHC^{+} CD103^{-} CD11b^{high} CX3CR1^{+}; CD11c^{high} MHC^{+} CD103^{+} CD11b^{+} CX3CR1^{-} Lymph node (resident): CD11c^{high} MHCII^{+} CD8^{+} CD205^{+}; CD11c^{high} MHCII^{+} CD8^{-} CD11b^{+} Lymph node (migratory): CD11c^{+} MHCII^{high} langerin^{+} CD40^{high} Langerhans cells (dermis): CD103^{+} CD11b^{low} langerin^{+}; CD103^{-} CD11b^{high} langerin^{-} Langerhans cells (epidermis): CD11c^{high} CD205^{low} langerin^{+} EpCAM^{high} *Mouse plasmacytoid DCs:* B220^{+} CD45RB^{+} CD11c$^{-/low}$ CD11b^{-} *Human myeloid DCs:* CD11c^{+} CD123^{-} *Subpopulations:* CD16^{+}; CD1c^{+} (BDCA-1^{+}); CD141^{+} (BDCA-3^{+}) *Human plasmacytoid DCs:* CD11c^{-} MHC-II^{+} CD123^{+}
Tumor-infiltrating T cells	T cells	CD8^{+} *subpopulations* *T cells showing anergy:* NA *exhausted T cells*: B7-H1^{+} PD-1^{+} 2B4^{+} BTLA^{+} CTLA-4^{+} CD160^{+} LAG-3^{+} Tim-3^{+} *senescent T cells:* CD28^{-} Tim-3^{high} CD57^{high} KLRG-1^{high}
Regulatory T cells	Tregs	CD4^{+} CD25^{+} FOXP3^{+}
Mast cells		c-KIT^{+}

Cell type, abbreviation, and surface molecular signature are listed above. *NA* not available

Fig. 1.1 The tumor microenvironment. Overview of the network of connections within the tumor microenvironment, showing the tumor and immune cells that populate the tumor microenvironment and the complex interplay between these cells. *ECM* extracellular matrix, *TAM* tumor-associated macrophage, *MDSC* myeloid-derived suppressor cell, *Tregs* regulatory T cells, *DC* dendritic cell, *NK* natural killer cell

into a dynamic equilibrium. In the equilibrium phase, the immune system exerts a potent selection pressure on the tumor cells that is enough to contain, but not fully extinguish, a tumor containing many genetically unstable and rapidly mutating tumor cells. During this period of selection, many of the original escape variants of the tumor cell are destroyed, but new variants arise, carrying different mutations

that provide them with increased resistance to immune attack. This immune escape mechanism active at the level of the tumor can be ascribed to defects in components of the interferon (IFN)-γ receptor signaling pathway [9], in the expression of major histocompatibility complex (MHC) class I, in the processing of MHC class I restricted antigens, or in the presentation machinery of the antigens. These abnormalities can be the result of activation of oncogenes within tumor cells, with one example seen in Her2/neu, which enables tumors to take on a "stealth" phenotype and thus to hide from detection by cytotoxic T lymphocytes [10]. In conclusion, the failure of the immune system to completely eliminate the tumor results in the selection of tumor cell variants that are able to resist, avoid, or suppress the anti-tumor immune response, leading to the escape phase.

Another tumor immune escape mechanism arises from the ability of the progressing tumor to interfere with the host immune system. To this end, the tumor induces and/or recruits immunosuppressive cells which normally serve as safeguards against overwhelming inflammation or autoimmunity. By turning the host immune system against itself, tumors can gain an impressive arsenal of weapons to hamper the induction and progression of anti-tumor immune activity. Within the tumor microenvironment, the tumor-promoting inflammatory cells include macrophage subtypes (tumor-associated macrophages—also called TAMs), mast cells, and neutrophils, as well as T and B lymphocytes [1, 11–13]. These cells can secrete several signaling molecules that serve as effectors of their tumor-promoting actions. These include epidermal growth factor (EGF), the angiogenic growth factor vascular endothelial growth factor (VEGF), other proangiogenic factors, such as fibroblast growth factor-2 (FGF2), and several chemokines and cytokines that amplify the inflammatory state. In addition, these infiltrating immune cells can produce proangiogenic and/or proinvasive matrix-degrading enzymes, including matrix metalloproteinase (MMP) 9 and other MMPs, cysteine cathepsin proteases, and heparanase [13, 14]. As a consequence of the expression of those effectors, these cells induce and support tumor angiogenesis, which stimulates cancer-cell proliferation, facilitates tissue invasion, and sustains metastatic dissemination [2, 3]. Within the tumor mass, in addition to fully differentiated immune cells, a variety of partially differentiated myeloid progenitors have been identified [13]. These cells are intermediates between the circulating cells of bone-marrow origin and the fully differentiated immune cells in normal and inflamed tissues, and they show tumor-promoting activities. Of particular interest for their multiple functions, the myeloid-derived suppressor cells (MDSCs), are characterized by the expression of the macrophage marker CD11b and the neutrophil marker Gr1, and they show significant immunosuppressive activity [14, 15].

In conclusion, during the escape phase the immune system is no longer able to contain tumor growth, and a progressively growing tumor results.

1.3 Tumor-Promoting Immune Cell Types in the Microenvironment of Primary Tumor

1.3.1 Tumor-Associated Macrophages (IL-10^{high} IL-12^{low} IL-1ra^{high} and IL-1 decoyR^{high})

Macrophages originate from blood monocytes that are not fully differentiated cells, and they are profoundly susceptible to several environmental stimuli. When recruited into peripheral tissues from the circulation, monocytes can differentiate rapidly into distinct, mature macrophages, which have specific immunological functions. TAMs are mature M2-polarized macrophages which originate from blood monocytes that are recruited at a tumor site by molecules that are produced by neoplastic and stromal cells. TAMs express IL-10^{high}, IL-12^{low}, IL-1ra^{high}, and IL-1 decoyR^{high}, and they have a pivotal role in tumor growth and progression. In the tumor milieu, TAMs carry on their pro-neoplastic role by producing molecules that affect neoplastic cell growth directly (e.g., EGF), enhancing neoangiogenesis, tuning inflammatory responses and adaptive immunity, and catalyzing structural and substantial changes in the extracellular matrix (ECM) compartment [16–18]. TAMs perform these actions by secreting several chemokines, including CCL2, CCL5, CCL7, CXCL8, and CXCL12, and cytokines such as VEGF, platelet-derived growth factor (PDGF), and the growth factor macrophage colony-stimulating factor (M-CSF; also known as CSF-1) [19, 20]. In particular, CCL2 is the main molecule that is involved in monocyte recruitment. Furthermore, TAMs can themselves produce CCL2, which suggests that they can act as an amplification loop. Within the tumor mass, the monocytes are surrounded by several signals that can induce their differentiation towards mature M2-polarized macrophages. The M2 polarization factors are IL-4, IL-6, IL-10, and IL-13, M-CSF, glucocorticoids, transforming growth factor-β (TGF-β), and prostaglandin E2 (PGE2), and these can be produced within the tumor mass by neoplastic cells and fibroblasts (e.g., IL-10, TGF-β), and by Th2 lymphocytes (e.g., IL-4, IL-13) [20].

TAMs stimulate the angiogenesis by secreting the growth factors VEGF, PDGF, TGF-β, and members of the FGF family [21], the angiogenic factor thymidine phosphorylase, which promotes endothelial cell migration in vitro [22], and angiogenesis-modulating enzymes, such as MMP2, MMP7, MMP9, MMP12, and cyclooxygenase-2 (COX-2) [16, 18, 23], and several chemokines including CXCL12, CCL2, CXCL8, CXCL1, CXCL13, and CCL5 [19]. Moreover, TAMs secrete lymphatic endothelial growth factors that strongly promote peritumoral lymphangiogenesis [24].

Within the primary tumor microenvironment, at least two mechanisms have been proposed by which TAMs facilitate tumor metastasis. The first relates to the secretion of proteases within the tumor microenvironment, such as urokinase plasminogen activator (uPA), cathepsins B and D [25], MMP2, and MMP9 [26], which can digest the tumor basement membrane, thus facilitating tumor-cell

escape. A second mechanism is through direct enhancement of an early stage of the metastatic cascade [27]. An in-vivo invasion assay showed that TAMs promote carcinoma-cell motility and invasion through a paracrine signaling loop between the tumor cells and the TAMs. Within this loop, the macrophages express EGF, which promotes formation of elongated protrusions and cell invasion by the carcinoma cells. In addition, EGF promotes the expression of CSF-1 by the carcinoma cells. This CSF-1 promotes the expression of EGF by macrophages, thereby generating a positive-feedback loop. Disruption of this loop by blockade of the signaling of either the epidermal growth factor receptor (EGFR) or the CSF-1 receptor is sufficient to inhibit both macrophage and tumor-cell migration and invasion [28]. Furthermore, in-vitro evidence has demonstrated that the production and secretion of the Wnt-ligand Wnt5a by macrophages can stimulate the planar-cell-polarity noncanonical Wnt signaling pathway in carcinoma cells, with the consequent promotion of cell invasion [29]. Indeed, the gene expression profile of invasion-promoting TAMs isolated from MMTV-Polyoma middle T (PyMT) mice, as compared to that of control isolated TAMs, showed that these macrophages are specifically enriched in molecules involved in Wnt signaling. There is thus extensive evidence that the Wnt signaling pathway is involved in this TAM-mediated tumor-cell motility [30].

Finally, within the tumor microenvironment, TAMs have strong immunosuppressive activity, not only through their production of IL-10, but also by their secretion of chemokines (e.g., CCL17 and CCL22), which preferentially attract T-cell subsets that are devoid of cytotoxic functions, such as regulatory T cells (Tregs) and Th2 lymphocytes [19]. In addition, TAMs secrete CCL18, which recruits naïve (that is, antigen inexperienced) T cells, whereby their recruitment into this microenvironment characterized by M2 cells and immature dendritic cells (DCs) is likely to induce T-cell anergy. This is the mechanism that is responsible for the inability of an immune T cell to mount a complete response against its target, thus promoting an immunosuppressive tumor microenvironment [31].

1.3.2 Myeloid-Derived Suppressor Cells ($CD11b^+$ $Gr1^+$)

An additional class of immune cells within the tumor microenvironment is the MDSCs. These cells comprise a phenotypically heterogeneous population of immature myeloid cells at different stages of differentiation. They derive from bone-marrow progenitors that have not completed their maturation into granulocytes, monocytes, or DCs [32]. In mice, these cells express the membrane antigens Gr1 and CD11b, and based on the expression of different epitopes of Gr1, they can be further subdivided into the granulocytic, such as $CD11b^+$ $Gr1^+$ ($Ly6G^+$), and monocytic, such as $CD11b^+$ $Gr1^+$ ($Ly6C^+$ $Ly6G^-$) subclasses [33]. In humans, MDSCs appear to be more like a "family of MDSCs," which includes different cell populations with varying phenotypes and biological diversities (Table 1.1).

The major function of MDSCs is to orchestrate other cells of the immune response, to promote an immunosuppressive and anti-inflammatory phenotype, which results in tumor immune escape [34]. For this function in tumor-bearing hosts, MDSCs need to expand in the lymphoid organs and to subsequently be recruited to the primary tumor site [15, 32], processes being directed by tumor-associated inflammation and by angiogenic and chemoattractant factors.

Pro-inflammatory cytokines (e.g., IL-1β, IL-6) and bioactive lipids (e.g., PGE2), the major contributors to the inflammatory milieu of a tumor, can induce MDSCs in tumor-bearing hosts [35–37]. S100A8 and S100A9, pro-inflammatory proteins released by neutrophils, also induce MDSCs. In particular, S100A9 blocks differentiation of myeloid precursors into functional DCs or macrophages, through a STAT3-dependent pathway [38]. S100A8 and S100A9 are also highly expressed at tumor sites, thus leading to the strong recruitment of MDSCs [39]. Furthermore, MDSCs produce and secrete these proteins, which can lead to an autocrine loop of engagement.

Within the tumor microenvironment, the main factors responsible for expansion of MDSCs are VEGF [40] and MMP9, which play a role also in the maintenance of MDSCs [41]. Within the ECM, tumor-derived stem-cell factor (SCF) leads to myelopoiesis and the expansion of MDSCs through the inhibition of differentiation of myeloid precursors to functional DCs [42]. The recruitment of MDSCs to tumor sites is also driven by chemoattractant molecules, such as CCL2/CCR2 [43], stromal-cell-derived factor 1 (SDF1)/CXCR4, CXCL5/CXCR2 [44], and uPA [45].

The suppressive function of MDSCs is turned on by several factors, including IL-4, IL-13, interferon (IFN)-γ, IL-1β, and TGF-β. Activated MDSCs suppress the anti-tumor immune response in the tumor microenvironment directly through the expression of inducible nitric oxide synthase (iNOS) and ARG1. MDSCs that express ARG1 deplete L-arginine from the microenvironment, and thus limit its availability to T cells. Consequently, T cells are deficient in the CD3ζ chain of the T-cell receptor (TCR), and they are arrested in the G_0–G_1 phase of the cell cycle. This results in inhibition of both their function and their proliferation [46]. The high expression of iNOS in MDSCs increases the production of nitric oxide (NO) and reactive oxygen species (ROS). ROS inhibit MDSC maturation [47], induce DNA damage in immune cells in the tumor microenvironment, inhibit the differentiation of MDSCs into functional DCs, and recruit MDSCs to tumor sites [32]. Moreover, extracellular ROS catalyze the nitration of TCR, which consequently inhibits the T cell-peptide-MHC interaction, which results in T-cell suppression [48]. MDSCs also impair T-cell activation by abrogation of the expression of L-selectin on both $CD4^+$ and $CD8^+$ T cells, which thus suppresses the homing of these cells to tumor sites, where they would then be activated [45]. Another mechanism by which MDSCs interfere with T-cell activation is their ability to expand the Tregs in the tumor microenvironment [46, 49]. As discussed above, Tregs inhibit anti-tumor immune response [50]. Furthermore, MDSCs inhibit natural killer cells (NK) and B cells [34], and induce M2 polarization of TAMs through secretion of high levels of IL-10 [37]. As a consequence, the balance between immunosuppressive cells and anti-tumor immune cells is further tilted towards a dominant immunosuppressive

microenvironment. Finally, similarly to TAMs, MDSCs promote angiogenesis [41], thus facilitating metastatic growth through improved delivery of nutrients and oxygen within the tumor microenvironment.

1.3.3 Dendritic Cells

DCs are bone-marrow-derived cells that are seeded in all tissues [51]. They are professional antigen-presenting cells (APCs) found in peripheral tissues and in immunological organs such as the thymus, bone marrow, spleen, and lymph nodes [51]. DCs represent a critical link between innate and adaptive immunity, and are essential for the development of antigen-specific immune responses. In fact, their role is to scan peripheral tissues where they recognize, take up, and process pathogens, and present pathogen-derived antigenic peptides in the context of MHC molecules to naïve T lymphocytes at lymphoid tissues [51].

Both mice and humans have two major subsets of DCs: myeloid DCs (mDCs; also known as conventional DCs and classical DCs) and plasmacytoid DCs (pDCs). In mouse, mDCs show high level of CD11c expression. In the steady state physiological condition, several mDC subpopulations which colonize lymphoid organs and other tissues have been described in the mouse model. These mDC subpopulations are characterized by a specific surface signature listed in Table 1.1. The mouse pDCs are characterized by the expression of B220, CD45RB, low or null levels of CD11c, and absence of CD11b. The human mDC express CD11c and do not express CD123, and have been further divided into three subgroups characterized by the expression of CD16, CD1c (BDCA-1), and CD141 (BDCA-3) [51]. The human pDCs do not express CD11c, and express MHC-II and CD123.

On interaction with DCs, naïve $CD4^+$ T cells and $CD8^+$ T cells can differentiate into antigen-specific effector T cells with different functions. $CD4^+$ T cells can become T helper 1 (TH1) cells, TH2 cells, TH17 cells or T follicular helper (TFH) cells that help B cells to differentiate into antibody-secreting cells, as well as Tregs that downregulate the functions of other lymphocytes. Naïve $CD8^+$ T cells can give rise to effector cytotoxic T lymphocytes (CTLs) [51, 52]. Moreover, DCs also have an important role in controlling humoral immunity. They do so both directly by interacting with B cells, and indirectly by inducing the expansion and differentiation of $CD4^+$ helper T cells [53, 54]. DCs are conspicuous members of the microenvironment of several types of cancer [55, 56]. Tumors have the capability to attract and reprogram the biology of DCs, inducing them to exert immunosuppressive or angiogenic functions [57]. Tumor-associated cytokines such as VEGF, IL-10, and PGE2 can profoundly affect the nature of DCs [58]. Liu et al. showed that tumor-cell-derived TGF-β and PGE2 drive DCs to differentiate into regulatory DCs, with a $CD11c^{low}$ $CD11b^{high}$ Ia^{low} phenotype and high expression of IL-10,

NO, VEGF, and arginase 1 (ARG1), which inhibited $CD4^+$ T cell proliferation both in vitro and in vivo [58]. Therefore, these data demonstrate that tumors can educate DCs to differentiate into a regulatory DC subset, which contributes to constitution of the immunosuppressive tumor microenvironment and promotes tumor immune escape. Tumor-associated DCs can induce Tregs expansion and contribute to angiogenesis by producing angiogenic molecules such as MMP, VEGF, angiogenin, heparanase, and basic fibroblast growth factor (bFGF) [59, 60]. Tumors can prevent the DC-induced priming of tumor-specific T cells by switching differentiation of monocytes to macrophages, which is mediated by the interplay of IL-6 and M-CSF, rather than DCs [61]. The tumor glycoproteins carcinoembryonic antigen (CEA) and mucin 1 (MUC1), which are endocytosed by DC, can be confined to early endosomes, which thus prevents efficient processing and presentation to T cells [62]. Tumors also interfere with DC maturation. First, they can inhibit DC maturation through the secretion of IL-10 [63], which leads to antigen-specific anergy. Second, tumor-derived factors can alter the maturation of mDCs, and so yield cells that indirectly promote tumor growth ("pro-tumor" DCs). An example is tumor-derived thymic stromal lymphopoietin (TSLP) which induces DCs to express OX40 ligand, which directs the generation of TH2 cells. These skewed $CD4^+$ T cells accelerate breast tumor development through the secretion of IL-4 and IL-13 [64]. These cytokines prevent tumor cell apoptosis and indirectly promote the proliferation of tumor cells by stimulating TAMs to secrete EGF. pDCs also play a role in tumor progression. These cells express on their surface an orphan receptor, immunoglobulin-like transcript 7 (ILT7). Cao et al. demonstrated that tumor cells express bone marrow stromal cell antigen 2 (BST2), which is a ligand of ILT7. The interaction of ILT7 on pDC with BST2 on tumor cells results in inhibition of IFN-α and pro-inflammatory cytokine production by pDCs [65]. These pDCs induce naïve $CD4^+$ T cells to differentiate into IL-10-producing T cells with immunosuppressive functions. Finally, DCs can have direct pro-tumor effects: mDCs directly promote the survival and clonogenicity of multiple myeloma tumor cells [66, 67].

1.3.4 Tumor-Infiltrating T Cells

Tumors are also infiltrated by tumor-associated antigen-specific $CD8^+$ T cells. Although T cells have the potential to kill tumor cells, frequently they have low avidity and unable to control tumor growth [68]. Although the molecular and cellular mechanisms that contribute to the failure of T cells to eradicate the tumor are not well-defined, early evidence indicates that in the tumor microenvironment the T-cell dysfunction is due to T-cell anergy, exhaustion, and senescence.

T-cell activation relies on the TCR recognizing its cognate antigen in the context of MHC molecules from APC or an APC-like cell (tumor cell). Interaction between

co-stimulatory molecules CD80 (B7-1), CD86 (B7-2), and CD28 is crucial for appropriate T-cell activation. T-cell anergy is generally described as the induced hyporesponsive state with low IL-2 production or incomplete activation, to which naïve T cells fall upon low co-stimulatory and/or high co-inhibitory stimulation. Human tumors and tumor-associated APCs often express high levels of B7-H1 (CD274 or programmed cell death protein 1 ligand (PD-L1)), B7-H2 (CD275 or ICOS-L), B7-H3 (CD276), B7-H4 (B7S1 or B7x), and B7-DC (CD273 or PD-L2) co-inhibitory molecules with low-to-absent expression of B7-1 (CD80) and B7-2 (CD86) co-stimulatory molecules [69–72]. This indicates that the tumor microenvironment is poor co-stimulatory and high co-inhibitory, thus promoting T-cell anergy.

Exhausted T cells are described as effector T cells with decreased cytokine expression and effector function, and being resistant to reactivation [73]. T-cell exhaustion occurs when T cells are chronically activated at sites of chronic inflammation, such as cancer, autoimmunity, and chronic infection. Within the tumors, the exhausted T cells show a significant decrease in IL-2, IFN-γ, and TNFα expression as well as cell cycle arrest; this effect defines T-cell exhaustion. In tumors, exhausted T cells express high levels of multiple inhibitory surface molecules, which effectively prevent T-cell activation. These inhibitory receptors include B7-H1, programmed cell death protein 1 (PD-1), 2B4 (CD244), B and T-cell lymphocyte attenuator (BTLA), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), CD160, lymphocyte-activation gene (LAG)-3, and T cell immunoglobulin and mucin-domain-containing molecule-3 (Tim-3) [74–79]. Although the detailed molecular mechanism of T-cell exhaustion is incompletely defined, it is suggested that the B7-H1/PD-1 signaling pathway mediates $CD8^+$ T cells functional exhaustion, inhibiting PI3K/AKT signaling activation and downstream signals of the TCR [80].

Senescent T cells are described as unresponsive and terminally differentiated T cells characterized by telomere shortenings, cell cycle arrest, and phenotypic change such as the loss of CD28 expression, high expression of Tim-3, CD57, and killer cell lectin-like receptor subfamily G, member 1 (KLRG-1) [76, 81–84]. These cells manifest defective killing abilities and the development of negative regulatory functions [85].

In conclusion, the peripheral T-cell tolerance mechanisms observed in tumorigenesis process include T-cell anergy, exhaustion, senescence, and regulatory T cells (Tregs) (described below) that impair ongoing T-cell function and enable tumor escape.

1.3.5 Regulatory T Cells ($CD4^+$ $CD25^+$ $FOXP3^+$)

Another mechanism by which tumors can evade immune response is the recruitment of Tregs into the tumor microenvironment [86]. These cells actively suppress pathological and physiological immune responses, thereby contributing to the

maintenance of immunological self-tolerance and immune homeostasis. Furthermore, they are involved in suppressive control of a broad spectrum of immune responses, including those against autologous tumor cells, allergens, pathogenic or commensal microbes, allogeneic organ transplants, and the fetus during pregnancy [87, 88]. Tregs represent a family of specialized T cells that are subdivided into two major groups: natural Tregs and induced or adaptive Tregs [89]. Natural Tregs express CD4 and the IL-2 receptor α chain (CD25). These cells are physiologically produced by the normal thymus as a functionally mature and distinct population, and their development and function depend on the expression of the transcription factor forkhead box P3 (FOXP3) [87, 89]. Adaptive Tregs are induced from naïve T cells by specific modes of antigenic stimulation, especially in a particular cytokine milieu [87, 89].

They include IL-10-secreting T regulatory 1 (Tr1) cells, TGF-β-secreting T helper (Th) 3 cells, certain γ/δ TCR-expressing $CD4^-$ $CD8^-$ T cells, and $CD8^+$ $CD28^-$ T cells. $CD4^+$ $CD25^+$ $FOXP3^+$ natural Tregs suppress the activation and/or expansion of multiple types of immunocompetent cells. In fact, they suppress the activation and expansion of $CD4^+$ T cells, the function of effector T cells, the activation and/or proliferation and cytokine formation of $CD4^+$ and $CD8^+$ T cells, the B-cell proliferation and immunoglobulin production and class switch, the cytotoxic functions of NK and natural killer T cells (NKT), the function and maturation of DCs [90]. Several molecular and cellular mechanisms have been described to explain how Tregs can suppress immune responses [90]. One possible mechanism of Tregs-mediated suppression is the so-called "cell-to-cell contact-dependent suppression." There is evidence that CTLA-4 and LAG3 molecules, expressed by Tregs, and CD80 and CD86 co-stimulatory molecules, expressed by APCs, have key roles in this Tregs-mediated suppression. It has been postulated that CTLA-4 on Tregs might interact with the CD80 and CD86 molecules on APCs and transduce a co-stimulatory signal to Tregs, thus resulting in the activation of Tregs to exert suppression. Another possible role of CTLA-4 is that it might directly mediate suppression. CTLA-4 expressed on Tregs triggers induction of the enzyme indoleamine 2,3-dioxygenase (IDO) in DCs by interacting with their CD80 and CD86 [91]. IDO catalyzes the conversion of tryptophan into kynurenine and other metabolites, which have potent immunosuppressive effects in the local environment of DCs by means of cytotoxicity, or possibly by inducing de-novo generation of Tregs from naïve $CD25^-$ $CD4^+$ T cells [91]. Tregs might also downregulate DC expression of CD80 and CD86 via CTLA-4, hampering activation of other T cells by DCs [92]. Alternatively, CTLA-4 on Tregs might ligate CD80 and CD86 expressed by activated responder T cells, and directly transduce a negative signal to the responder T cells [93]. Moreover, CTLA-4 might augment the physical interaction between Tregs and APCs, thus enhancing the activation of Tregs or their suppressive interaction with other T cells or APCs [94]. The secretion of immunosuppressive cytokines by Tregs represents another mechanism by which Tregs mediate suppression or condition a suppressive milieu. Tregs secrete IL-10 and TGF-β. IL-10 could induce the immunosuppressive co-stimulatory molecule B7-H4 on DCs [95], while TGF-β is required for the maintenance of natural Tregs

and for the induction of Tregs from naïve T cells [96]. Another mechanism of Tregs-mediated suppression could be the killing of effector cells. In fact, Tregs release perforin and granzyme A which might induce death of T cells, monocytes, and DCs [97]. Interestingly, Tregs possess a more robust thioredoxin system compared to the other immune cells [98], which results in a reduced sensitivity toward ROS-mediated impairments, thus presenting them with a major survival advantage within the tumor microenvironment [99].

1.3.6 Mast Cells (c-KIT$^+$)

Mast cells derive from bone-marrow hematopoietic progenitors. While still immature, mast cells migrate from vascular to peripheral tissues, where their maturation depends on the microenvironmental conditions. From a physiological perspective, mast cells participate in tissue remodeling, wound healing, and angiogenesis [100]. These cells have pathological effects in acute disorders, in chronic allergic disorders [101] and in autoimmune diseases [102].

There is a lot of evidence that implicates mast cells in cancer proliferation and metastasis. Mast-cell infiltration and activation in tumors is mainly mediated by tumor-derived SCF and the receptor c-KIT, which is mainly found on these cells [103]. Once activated, mast cells can release several mediators that are involved in the processes of tumor microenvironment remodeling, thus facilitating tumor metastasis. Indeed, mast cells also produce proteases, such as tryptase, chymases, MMP9, and cathepsin, which together promote inflammation, modulate immune responses by hydrolyzing chemokines and cytokines, and degrade the ECM [104]. Mast-cell-derived leukotrienes promote the recruitment of neutrophils [105], induce vascular permeability, trigger chemotaxis in various cells, and increase mucus production [106]. Other released mediators include vasoactive factors (e.g., histamine, IL-8, VEGF, PGED, and substance P), which lower endothelial barriers [107], and proangiogenic factors (e.g., VEGF, PDGF, MMP9, and PGE2), which together induce angiogenesis. In addition, mast cells contribute to the establishment of inflammatory and immunosuppressive conditions in the tumor microenvironment by secretion of pro-inflammatory and immunosuppressive cytokines, and of chemokines. The pro-inflammatory cytokines (e.g., IL-1β, IL-6, IL-8, and TNF-α) increase the interstitial fluid volume by plasma effusion, and extend the distance that oxygen needs to be delivered to the oxygen-hungry cells, thus leading to hypoxia-induced metastasis [108]. The immunosuppressive cytokines (e.g., TGF-β and IL-10 [109]) favor immune suppression. The chemokines (e.g. CCL5 and CXCL8 [110]) act as chemoattractants for additional effector immune cells, thus remodeling the immune and inflammatory microenvironment of the tumor. Finally, mast cells secrete growth factors [e.g., EGF, insulin-like growth factor (IGF), nerve growth factor (NGF) and bFGF] that favor tumor-cell survival and proliferation, thus again facilitating metastasis [111].

There is a complex interplay between mast cells and the other immune cells within the tumor microenvironment. The factors secreted by mast cells lead to CCL2 production and IL-17 up-regulation in MDSCs. CCL2 signaling recruits more MDSCs, leading to more IL-17 production, which further exacerbates the inflammatory tumor microenvironment. IL-17 leads to the up-regulation of IL-9, IL-10, IL-13, CCL17, CCL22, CD39, and CD73. This results in various actions: CCL17 and CCL22, in turn, are chemoattractants that bring more Tregs to tumor sites; CD39 and CD73 enhance the suppressor function of Tregs; IL-9 produced by Tregs helps to maintain the survival of mast cells; and IL-10 and IL-13 induce ARG1 expression by MDSCs. Mast-cell modulation of the recruitment and the suppressor function of MDSCs and Tregs represents another mechanism by which they promote the tumor immune escape.

1.4 Conclusions and Future Remarks

The therapeutic strategies to cancer treatment have been evolved from relatively nonspecific cytotoxic agents to selective, mechanism-based therapeutics. These targeted approaches aim to inhibit molecular pathways that are crucial for tumor growth and maintenance. The growing body of evidence of the roles played by immune cells of tumor microenvironment in promoting tumor progression indicate that it is conceivable that these cells can serve as novel therapeutic targets in the treatment of cancers. Several immuno-therapeutic approaches have been developed to target the immune cells that infiltrate the tumor. The discussion of these strategies are far away from the purpose of this review, but are extensively reviewed by [112–119]. These immuno-therapeutic approaches endeavor to stimulate a host immune response that effectuates long-lived tumor destruction. These findings support the rationale for developing combinatorial strategies for targeting both tumor and immune cells. Moreover, targeted therapies and cytotoxic agents also modulate immune response. Some chemotherapies such as cyclophosphamide are directly toxic to immunosuppressive Tregs [118]; gemcitabine and 5-fluorouracil (5-FU) both selectively kill MDSCs [118]; dipyridamole [120] and bindarit [121] decrease the infiltration of TAMs and MDSCs in breast cancer primary tumors. All together, these data raise the possibility that these treatment strategies might be effectively combined with immunotherapy to improve clinical outcomes.

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Chapter 2
Tumor–Stroma Interaction and Cancer Progression

Neill Y. Li, Paul C. Kuo, and Philip Y. Wai

Abstract The understanding of how normal cells transform into tumor cells and progress to invasive cancer and metastases continues to evolve. The tumor mass is comprised of a heterogeneous population of cells that include recruited host immune cells, stromal cells, matrix components, and endothelial cells. This tumor microenvironment plays a fundamental role in the acquisition of hallmark traits, and has been the intense focus of current research. A key regulatory mechanism triggered by these tumor–stroma interactions includes processes that resemble epithelial–mesenchymal transition, a physiologic program that allows a polarized epithelial cell to undergo biochemical and cellular changes and adopt mesenchymal cell characteristics. These cellular adaptations facilitate enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components. Indeed, it has been postulated that cancer cells undergo epithelial–mesenchymal transition to invade and metastasize.

In the following discussion, the physiology of chronic inflammation, wound healing, fibrosis, and tumor invasion will be explored. The key regulatory cytokines transforming growth factor-β and osteopontin and their roles in cancer metastasis will be highlighted.

Keywords Cancer • Tumor microenvironment • Immunoediting

List of Abbreviations

bFGF	Basic fibroblast growth factor
BM	Basement membrane
BSP	Bone sialoprotein
CSC	Cancer stem cell

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CSF-1	Colony-stimulating factor
DMP1	Dentin matrix protein 1
DSPP	Dentin sialoprotein
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial–mesenchymal transition
GAG	Glycosaminoglycan
GMCSF	Granulocyte–macrophage colony-stimulating factor
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
Hh	Hedgehog
HSC	Hepatic stellate cells
IFN	Interferon
IL	Interleukin
LEF	Lymphoid enhancer factor
LLC	Large latency complex
LOX	Lysyl oxidase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDCK	Madin–Derby canine kidney
MDSC	Myeloid-derived suppressor cell
MEPE	Matrix extracellular phoshoglycoprotein
MET	Mesenchymal–epithelial transition
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
miR	microRNA
MSC	Mesenchymal stem cell
NK	Natural killer
OPN	Osteopontin
PDGF	Platelet-derived growth factor
PMA	Phorbol 12-myristate 13-acetate
SIBLING	Small integrin-binding ligand N-linked glycoprotein
TAM	Tumor-associated macrophages
TGF-β	Transforming growth factor
TME	Tumor microenvironment
TNF	Tumor necrosis factor
UUO	Unilateral ureteral obstruction
VEGF	Vascular endothelial growth factor

Contents

2.1 Introduction

The understanding of how normal cells transform into tumor cells and progress to invasive cancer and metastases continues to evolve. This expanding knowledge has inspired revision of the "hallmarks of cancer" that were established as the modern foundation for describing tumor progression [1]. In addition to acquired mutations, genomic instability, and epigenetic changes that characterize tumor cell transformation, there is the concept that within the heterogeneous complex of cells that is termed "tumor mass" resides a repertoire of recruited host immune and stromal cells. These cells, rather than attenuating tumor progression, seem to enable tumor growth, invasion, and metastasis. This recruited "tumor microenvironment" (TME) plays a fundamental role in the acquisition of hallmark traits, and has been the intense focus of current research. Cumulative evidence has shown that the components of the microenvironment, including the extracellular matrix (ECM), fibroblasts, myofibroblasts, leukocytes, endothelial cells, pericytes, and smooth muscle cells, dendritic cells, macrophages, lymphocytes, mesenchymal cells, and cancer-associated fibroblasts interact through a complex network of cytokines, mitogens, and growth factors to activate tumor growth. As such, the current generation of cancer hallmarks include the (1) sustainment of proliferative signals, (2) evasion of growth suppressors, (3) resistance of cell death, (4) establishment of replicative immortality, (5) induction of angiogenesis, (6) activation of invasion and metastasis, (7) reprogramming of energy metabolism, and (8) evasion of immune destruction [2].

Recently, epithelial–mesenchymal transition (EMT) has been shown to be a critical process that occurs in the TME and drives certain cancer hallmark traits. EMT is normally a physiologic process that allows a polarized epithelial cell, which normally interacts with basement membrane, to undergo biochemical and cellular changes that enable it to assume a mesenchymal cell phenotype. These cellular adaptations facilitate enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components [3, 4]. During the final stage of EMT, the basement membrane is degraded, and the enhanced mesenchymal characteristics facilitate cellular migration away from the epithelial

layer. Elaborate molecular cascades coordinating transcription factor activation, expression of specific cell-surface proteins, reorganization and expression of cytoskeletal proteins, production of ECM-degrading enzymes, and changes in the expression of specific microRNAs are required to complete EMT. Cancer cells undergo EMT to invade and metastasize. Importantly, cancer cells may adopt mesenchymal characteristics to differing extents, with some cells retaining some epithelial traits while others become fully mesenchymal. The specific mechanisms that induce EMT in carcinoma cells remain incompletely understood.

In this chapter, we discuss the interactions within the TME that enable tumor growth and invasion. Specifically, we will review the current concepts concerning (1) the components of the TME, (2) the similarities between the cellular processes of chronic inflammation, fibrosis, wound healing, and tumor progression, (3) EMT in tumor progression and the role of transforming growth factor (TGF)-β, and (4) the role of osteopontin (OPN) in cancer-EMT.

2.2 TME: The Non-immune Components

2.2.1 Epithelial Cells

Although carcinoma cells can arise from a variety of cells, the majority of solid tumors arise from epithelial cell types. Epithelial cells reside in the linings of organs, cavities, and glands. Cell shape and type vary according to function: cuboidal and columnar cells are commonly secretory in nature and form glands; squamous or stratified squamous cells are protective and provide support in the lining and protection of viscera and skin; transitional epithelial cells have the capacity to expand, allowing them to function in organs such as bladder which require dynamic kinetics. Epithelial cells derive their functional utility by forming stable sheets of cells through homodimeric E-cadherin and desmosome associations. As these cells often reside at the interface between the body's organs and the external environment and/or function in a location where rapid cell-cycle turn-over is required, there is a common predisposition to exposure to injurious toxins, infectious agents, growth factors, or hormones (Table 2.1; [5]). Injury and cellular turnover can lead to the accumulation of genetic alterations required for cancer cell development [6]. The myriad molecular mutations and epigenetic changes that occur in carcinogenesis are beyond the scope of this discussion, but we wish to highlight that the source of these changes often derives from the epithelial cell type that characterizes the organ of interest (Table 2.1).

2.2.2 Basement Membrane and Extracellular Matrix

As cancerous epithelial cells develop, they are initially confined within a fortified layer of stromal tissue called the basement membrane (BM). Normally, during

Table 2.1 Types and incidence of epithelial cancers in the United States [5]

Epithelial cancer	New cases in US (2012)	Deaths in US (2012)
Anal	6,230	780
Bladder	73,510	14,880
Breast	226,870 (female), 2,190 (male)	39,510 (female), 410 (male)
Cervix	12,170	4,220
Colorectal	103,170 (colon), 40,290 (rectal)	51,090 (colon and rectal)
Endometrial	47,130	8,010
Esophageal	17,460	15,070
Gallbladder	9,810	3,200

organogenesis and tissue remodeling, epithelial cells secrete several types of collagen and protein to produce the BM. The BM acts as a scaffold for epithelial tissue growth and regeneration [7], and is primarily composed of the basal lamina (type IV collagen) and lamina reticularis (type III collagen). Type VII collagen, anchoring fibrils, microfibrils (fibrillin), and perlecan, a proteoglycan that acts as a reservoir of water and growth factors, provide further strength to the BM. The rigidity and strength of the BM support its function as a barrier between the epithelial cells and the underlying ECM. Epithelial cells are strongly anchored to the BM through integrins and hemidesmosomes [8]. In consequence, tumor progression requires molecular strategies to detach transformed epithelial cells from the BM and to penetrate the BM and allow for tumor escape into distant sites.

The ECM is composed of a variety of non-cellular components including water, proteins, and polysaccharides that fill interstitial spaces to provide scaffolding and cushioning against external forces and protection of interstitial cells [9]. Proteoglycans and hyaluronic acid make up the majority of the polysaccharides in the matrix. Proteoglycans are proteins surrounded by carbohydrate polymers, glycosaminoglycans (GAGs), creating a net negative charge that attract Na^+ ions and water. Hyaluronic acid is composed of non-sulfated GAGs that have increased efficiency for water retention. In addition to the cushioning properties, the creation of this hydrated matrix allows for the sequestration of growth factors. During cancer progression, proteoglycans are digested by enzymes and heparanases. The enzymatic digestion serves to promote tumor growth and metastasis [10]. Hyaluronic acid also contributes to tumor growth by binding to CD44 receptors located on malignant cells, promoting cell differentiation and migration [11, 12]. Together, these data support the theory that the presence of malignant cells within the interstitial matrix leads to remodeling cascades that rearrange the polysaccharide-matrix into components that promote growth, differentiation, and cancer-cell invasion.

The ECM is also rich in fibrillar proteins such as fibronectin, collagen, and elastins, which provide matrix structural integrity and the anchors for cell motility. Fibronectins are glycoproteins that connect cell-surface integrins with collagen and elastin fibers. Collagen is the most abundant protein in the ECM, which provides tensile strength, cell adhesion, and chemotaxis. Collagen and elastin cross-linking is mediated by lysyl oxidase (LOX) which forms highly reactive aldehydes from lysines to create stiff collagen and elastin fibers [13]. Engagement between

Table 2.2 Key growth factors found within the TME [19]

Growth factor	Function	Sources
Fibroblast growth factor—FGF	Endothelial cell proliferation, fibroblast proliferation, stimulate proliferation, migration, differentiation of epithelial cells	Fibroblasts
Epidermal growth factor—EGF	Cellular proliferation, differentiation, survival	Platelets, macrophages
Hepatocyte growth factor—HGF or scatter factor (SF)	Cell growth, motility, morphogenesis, matrix invasion by binding to the c-Met receptor	Mesenchymal cells
Insulin growth factor—IGF	High sequence similarity to insulin, cell proliferation, inhibition of cell death	Hepatocytes, endothelial cells, pericytes
Platelet-derived growth factor—PDGF	Angiogenesis, fibroblast differentiation	Platelets, pericytes, endothelial cells
Transforming growth factor-a—TGF-a	Epithelial development, can bind EGF receptor by close homology	Macrophages, keratinocytes
Transforming growth factor-B—TGF-B	Epithelial–mesenchymal transition, epithelial motility, cellular survival, anti-proliferative factor in epithelial cells at early stages of oncogenesis	Mesenchymal stem cells, macrophages
Tumor necrosis factor-a—TNF-a	Inflammation, immune cell regulation	Macrophages
Vascular endothelial growth factor—VEGF	Angiogenesis, vasculogenesis, endothelial cell differentiation	Endothelial cells, tumor cells, pericytes

integrins, collagen, and elastin fibers enable cells to move through the ECM. The significance of LOX is demonstrated through breast cancer studies, where LOX loss-of-function decreases the cell motility of highly invasive MDA-MB-231 breast cancer cells. Conversely, gain-of-function addition of LOX to poorly invasive MCF-7 breast cancer cells demonstrated increased motility and migration [14, 15].

Tumor cells secrete growth factors and enzymes to remodel and stiffen the ECM. The fibrillar proteins are primarily affected, enhancing survival and invasiveness of these cells. All the constituent cells of the TME contribute to growth factor release and heterotypic signaling [16]. Under normal conditions, growth factor release is limited in order to repress unwanted growth and proliferation. Such regulation of growth factors serves to regulate senescence and maintain cellular turnover through apoptosis [17]. In the TME, increased growth factor release enhances heterotypic signaling between stromal cells and malignant cells or amongst the malignant cells themselves. For example, mitogens that stimulate cell division are overproduced in cancer cells to produce an autocrine proliferative signal pattern [18]. Cancer cells can also enhance their sensitivity to growth factors by up-regulating growth-factor receptors so that available ligands transmit a greater and more efficient response [16]. Important growth factors that drive tumor progression within the TME are listed in Table 2.2 [19].

2.3 TME: The Immune Components, Chronic Inflammation, Wound Healing, and Tumor Progression

The complementary oncogenic events that transform tumor cells, and the inflammatory processes derived from the enabling cells in the TME, have been defined as the "intrinsic" and "extrinsic" pathways respectively [20]. The intrinsic pathway encompasses the mutational events and genomic changes that activate oncogenes and inhibit tumor suppressors, driving transformation within targeted cells. Tumor cells generated in this fashion subsequently produce cytokines that recruit and populate the inflammatory TME. Alternatively, the extrinsic pathway are environmental stimuli amplified into inflammatory or infectious processes that serve to amplify the cancer risk (e.g., inflammatory bowel disease, hepatitis, *Helicobacter pylori*). These two mutually dependent pathways eventually converge, appropriating necessary components and signals from the other while also supplying reciprocally useful building blocks to fuel transformation and metastasis in a cooperated fashion. It is no coincidence that inflammation and wound-healing physiology parallels the tissue remodeling processes that occur in cancer progression. Dvorak [21] recognized that the composition of the tumor stroma strongly resembles the granulation tissue of healing skin wounds. These cascades promote important, essential inflammatory processes such as cell proliferation, migration, invasion through the extracellular matrix, angiogenesis, and ultimately provide the necessary components for host tissue repair and survival. In many types of cancer, these attributes brought on by an inflammatory milieu can be subverted by nascent tumor cells as tools for cancer progression and metastasis.

During tissue repair and wound healing, the restorative steps of the inflammatory cascade are well-characterized. Tissue injury created by toxins, infection, or a chronic inflammatory stimulus results in a host response focused on recruiting cells that initiate healing (Fig. 2.1). Key cellular components that are enlisted into this milieu include neutrophils, monocytes, macrophages, mast cells, dendritic cells, fibroblasts, and endothelial cells. The wound-healing process often involves partially overlapping phases: blood clotting, inflammation, new tissue formation, and tissue remodeling [22]. Different cell types arrive into this niche during specific phases in a highly coordinated fashion. Important pro-inflammatory signals produced during this process include interleukin (IL)-1β, IL-6, IL-23, tumor necrosis factor (TNF)-α, and TGF-β1. Activation of the selectin family of adhesion molecules (L-, P-, and E-selectin) facilitates leukocyte "rolling" along the injured vascular endothelium, activating integrin binding and immobilization (α4β1 and α4β7 binding to VCAM-1 and MadCAM-1), and ultimately transmigration through the endothelium into the site of injury [22]. Release of cytokines, chemokines, and prostaglandins to recruit additional inflammatory cells, the production of reactive oxygen species (ROS) to destroy infectious vectors, the generation of pro-angiogenic factors, and modulation of apoptosis represent other essential, activated functions.

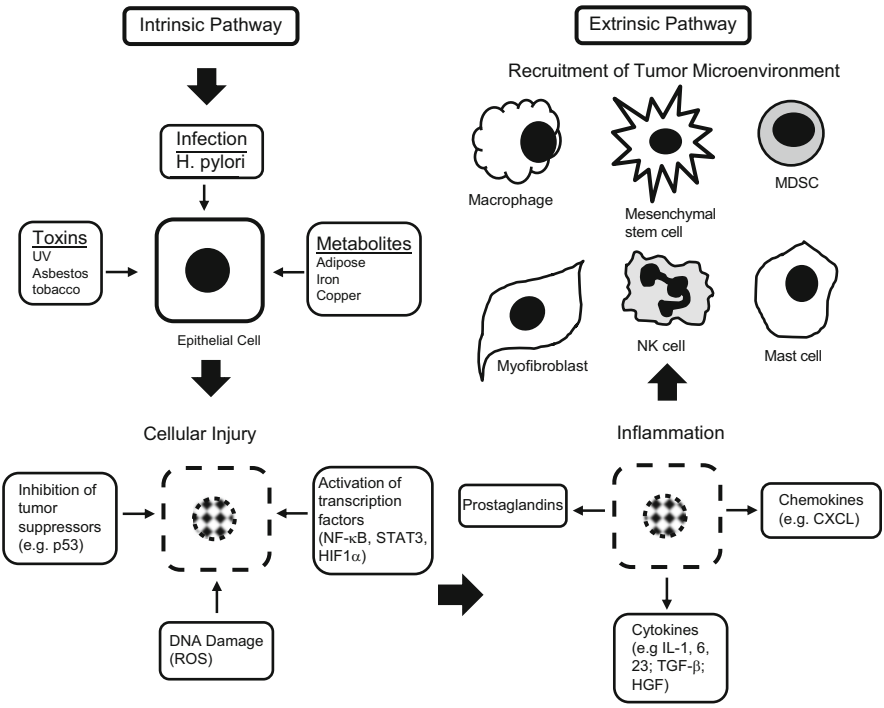

Fig. 2.1 The intrinsic and extrinsic pathways combine to create a local microenvironment around the injured and transformed hepatocyte to augment tumor promoting mechanisms (*ROS* reactive oxygen species, *HIF1α* hypoxia inducible factor 1 alpha, *NK cell* natural killer cell, *MDSC* myeloid-derived suppressor cell, *IL* interleukin, *TGF-β* transforming growth factor Beta, *HGF* hepatocyte growth factor, *NF-κB* nuclear factor kappa-light-chain-enhancer of activated B cells, *CXCL* chemokine ligand, *STAT3* signal transducer and activator of transcription 3)

Physiological inflammation is often self-limiting through downstream release of anti-inflammatory regulators (IL-10, IL-11, IL-13) which temper the pro-inflammatory cascade. However, cancer-associated inflammation is often directed by intercellular signals to persist, or be driven without regulation, to elicit pathologically persistent signals for cellular proliferation, migration, basement membrane invasion, and angiogenesis. In this context, tumors have been comparatively described as "wounds that do not heal" [21]. For example, in chronic disease states of the liver, an environment is often created that enable tumor growth. When the liver is exposed to injury and fibrosis ensues, this begins at first as a reversible wound-healing response. This primary injury event is characterized by inflammation, accumulation of ECM, and ultimately scarring, as described above. If the injury is self-limiting, the inflammatory changes are transient and the liver tissue is restored to its normal configuration as the event resolves. However, when the injury or the resultant inflammatory response is persistent, the liver architecture is irreversibly transformed, leading to progressive fibrosis and then cirrhosis. Agents that

injure the liver in such a way include toxins (CCL4, alcohol, or bile from biliary stasis), chronic infections (hepatitis B, hepatitis C), or remodeling processes (metabolite deposition from iron or copper, adipose tissue in non-alcoholic fatty liver disease). Chemical toxins, viral antigens, and metabolites damage hepatocytes, and these injuries recruit reparative cells. Immune cells remove or repair damaged cells, establish defense against further infection or injury, and regeneration or repair tissue. Conversely, chronic inflammation due to repetitive injury (toxin) or inability to remove the offending agent (viral infection) results in a deranged, decompensated response (Fig. 2.1).

The key immune-cells residing in the TME that enable tumor growth are the same components that facilitate wound healing and inflammation as described above. However, the tumor-associated cells recruited often display altered functions that lend themselves to cancer development. This alteration in function derives from the up-regulated expression of pro-tumor cytokines. For example, dendritic cells in neoplastic infiltrates are regulated by tumor-derived granulocyte–macrophage colony-stimulating factor (GMCSF) and IL-4 and are frequently immature, less effective at capturing antigens, and defective in T-cell stimulatory capacity [23]. IL-10 released into the TME is a potent inhibitor of dendritic cell activation and differentiation, allowing evasion of host adaptive immunity [20]. Increased serum levels of IL-10 is associated with poor prognosis and reduced survival in patients with various types of cancer [24–26]. IL-10 exerts immunosuppressive effects in a variety of ways [27], including inhibition of dendritic cell maturation and differentiation, down-regulation of co-stimulatory molecules and major histocompatibility complex (MHC) class I and II, inhibition of antigen priming of naïve T-cells [28–31], induction of tolerance and promotion of regulatory T-cells [32], and reduction of tumor recognition by cytotoxic lymphocytes [33, 34]. Experimental studies have shown that IL-10 administration before anti-cancer vaccination results in tumor progression [35–37]. Recently, hepatocellular carcinoma (HCC) progression has been demonstrated to be associated with IL-10 mediated elimination of memory B-lymphocytes in the development of hepatomas in hepatitis B [38]. Glycyrrhizae polysaccharide treatment of HCC in H22 hepatoma-bearing mice decreased tumor burden through down-regulation of regulatory T-cells, decreased lymph node IL-10 mRNA expression, and decreased serum IL-10 [39]. In patients with hepatitis C virus (HCV)-related HCC, an increase in the percentage of regulatory $CD4^{+}CD57^{+}$ T cells correlated with increasing tumor stage, with increased IL-10 levels and decreased anti-tumor interferon (IFN)-γ-producing capability in peripheral blood lymphocytes [40]. In analyzing cells isolated from human HCC specimens, Kuang et al. demonstrated that IL-10 released from activated monocytes stimulated monocyte expression of PD-L1. In turn, the PD-L1($^{+}$) monocytes effectively suppressed tumor-specific T cell immunity, and contributed to the growth of HCC in vivo [41].

Macrophages represent key mediators in the TME that function as first-responders and are unique in their ability to orchestrate both the innate and adaptive immune responses. Macrophages can be generally classified into M1 or M2

subtypes. M1 macrophages are associated with the acute inflammatory response, capable of killing pathogens and priming anti-tumor immune responses, while M2 macrophages are induced in vitro by IL-4, and IL-13, and consequently down-regulate MHC class II and IL-12 expression while increasing IL-10, scavenger receptor A, and arginase, amongst other cytokines. M2 polarization is associated with a tumor-permissive environment producing tumor-associated macrophages (TAM) [42, 43]. TAMs produce a number of potent angiogenic and lymphangiogenic growth factors, cytokines, and proteases that mediate neoplastic progression. TAMs have been shown to express vascular endothelial growth factor (VEGF)-C, VEGF-D, and VEGF receptor-3 to promote angiogenesis in human cervical carcinogenesis [44]. In a murine mammary cancer metastasis model, colony-stimulating factor (CSF)-1 regulates tumor growth by supporting and cultivating the TME. In CSF-1−/− mice, advanced mammary tumors and pulmonary metastases fail to develop due to decreased TAM recruitment into the neoplastic tissue [45]. CSF-1 has been shown to promote progression of mammary tumors to malignancy as replacement of transgenic CSF-1 into mammary epithelium restores macrophage recruitment, primary tumor development, and metastatic potential [45]. In addition to these mechanisms, the inhibition of tumor-suppressor pathways represent yet another strategy for promoting tumor growth. Macrophage migration inhibitory factor (MIF) released from TAMs is a potent cytokine that suppresses *p53* transcriptional activity. MIF released into the TME creates a niche with a deficient response to DNA damage [46]. TAMs will be diverted into the M2 phenotype in human tumors so that macrophage functions will be focused on promoting tumor growth, remodeling tissues, promoting angiogenesis, and suppressing adaptive immunity [43, 47] (Fig. 2.2).

A powerful stimulus for tumor progression within the TME includes the ROS derived from infiltrating leukocytes. In the presence of chronic inflammation and repetitive injury, leukocytes and other phagocytic cells induce DNA damage in proliferating cells through the generation of reactive oxygen and nitrogen species such as peroxynitrite. Irreversible DNA mutations generated by these reactive species can provide the critical trigger for neoplastic transformation.

Another class of cells that are recruited to the TME include the myeloid-derived suppressor cells (MDSCs). These cells are abundant in tumors and strongly inhibit anti-tumor immunity [22]. MDSCs represent an immature population of myeloid cells that inhibit both innate and adaptive immunity, and are present in cancer patients and in experimental animals with sizable tumor burden [48]. Although no definitive molecular characterization exists, many investigators have found human MDSCs to express CD33, CD11b, and CD15 cell surface markers [48]. MDSC inhibition of anti-tumor immunity is mediated by suppression of $CD4^+$ T-cells [49], inducing T regulatory cells [50], by down-regulating macrophage production of the type 1 cytokine, IL-12 [51], and potentially suppressing natural killer (NK) cell cytotoxicity [52]. In tumor models, trafficking and accumulation of MDSCs appears to be gp130-dependent ,and down-regulation of NK cell cytokine production to be NKp30-dependent [53]. Recent studies have also focused on the

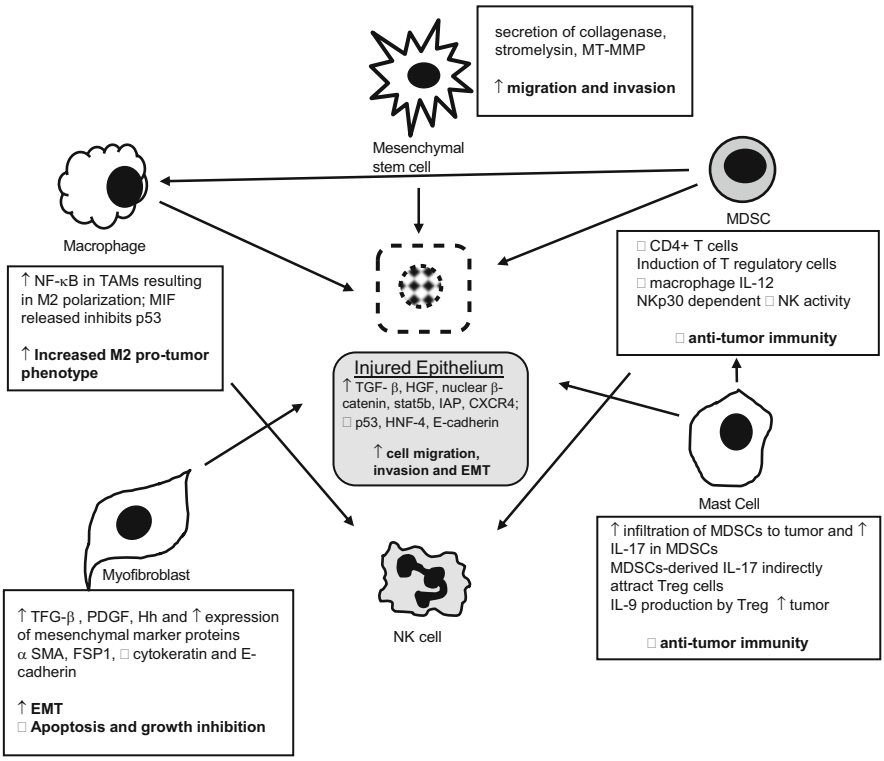

Fig. 2.2 The complex cellular network in the tumor microenvironment mediated by chemokines, cytokines, and cellular transcription factors (*NK cell* natural killer cell, *MDSC* myeloid-derived suppressor cell, *IL* interleukin, *TGF-β* transforming growth factor Beta, *αSMA* alpha smooth actin, *FSP-1* fibroblast specific protein, *PDGF* platelet-derived growth factor, *Hh* hedgehog, *NF-κB* nuclear factor kappa-light-chain-enhancer of activated B cells, *CXCL* chemokine ligand, *STAT3* signal transducer and activator of transcription 3, *Treg* T regulatory cells, *MT-MMP* membrane type matrix metalloproteinase, *HNF-4* hepatocyte nuclear factor-4, *EMT* epithelial–mesenchymal transition)

myofibroblast as another cell type that is commonly found in wounds and in the TME and has been implicated in tumor progression.

The presence of large numbers of fibroblasts and myofibroblasts is a hallmark of cancer with many tumors producing a desmoplastic response [22]. Although tumor fibroblasts can be derived from the stroma surrounding tumors, there is evidence to suggest that cells recruited from the bone marrow also "home in" on the TME [54]. Myofibroblasts are modulated fibroblasts that express α-smooth muscle actin and integrate with the actin–myosin contractile system, providing the necessary tension for wound closure [55]. These cells secrete collagen I and III, fibronectin, and proteoglycans that coalesce into a desmoplastic or "reactive" stroma. Desmoplasia is defined as "hard" or dense ECM created by excessive collagen and scaffolding protein deposition [56]. In normal physiologic wound healing,

recruited myofibroblasts form desmoplastic stroma and exist in the wound for a duration lasting days. However, in the TME this stroma can be maintained for months to years, as high levels of TGF-β in the tumor microenvironment differentiate recruited fibroblasts into myofibroblasts. This deranged desmoplastic response is regulated by cytokines such as TNF-α, microvascular injury, or platelet-derived growth factor (PDGF) secretion by tumor cells [57]. Auto- and paracrine PDGF- and TGF-β-dependent signaling centered on the myofibroblast is considered fundamental to the development of EMT, generation of cancer stem cells (CSCs), and ultimately to tumor progression. CSCs exhibit a $CD44^{high}/CD24^{low}$ antigenic phenotype, demonstrate upregulation of the mesenchymal markers and the transcription factors, N-cadherin, fibronectin, vimentin, FOXC2, SIP1, Hedgehog (Hh), Snail, and Twist, and possess self-renewal capability enabling CSCs to exit tissue reservoirs, enter and survive in the circulation, and exit into secondary tissue sites ("stemness") [58]. Cancer-associated fibroblasts are important contributors to the TME [59], and their precise origin continues to be unclear, with a variety of cells able to generate stem-cell characteristics including hepatocytes, oval cells/hepatic progenitor cells, and bone-marrow-derived cells [60].

2.4 EMT and TGF-β

EMT is a regulatory program used in normal embryogenesis, development, tissue regeneration, and fibrosis. As described above, EMT has now been implicated as a paradigm by which transformed epithelial cells subvert the molecular machinery native to inflammation and acquire the properties for invasion, inhibition of apoptosis, and dissemination [61–64]. As with many physiologic processes, execution of the EMT process can occur along a spectrum of partial to complete transition, and also in a transient or stable fashion during tumor progression and invasion [4]. During normal embryogenesis and development, induction of key regulatory transcriptional factors including Snail, Slug, Twist, and zinc finger E-box binding homeobox 1 Zeb1/2, Goosecoid, and FOXC2 [65–68] arise from signals emanating from the stroma. In the case of cancer and the TME, signals such as HGF, epidermal growth factor (EGF), PDGF, and TGF-β appear to be responsible for the elaboration of these EMT-inducing transcription factors. Various combinations of these factors function in a pleotropic fashion in a number of malignant tumor types, and they have been shown in experimental models of carcinoma to regulate invasion [69–71]. The downstream cellular processes activated by these transcription factors include the loss of adherens junctions, conversion from a epithelial to a spindle-cell or fibroblast morphology, expression of matrix-degrading enzymes, increased motility, and increased resistance to apoptosis. E-cadherin biology significantly governs the adhesiveness of cells derived from epithelial origin, and many of the activated molecular cascades directly inhibit E-cadherin gene expression and promote "cellular detachment" or escape from the anchoring niche of the basement membrane during EMT [72]. Coordinating mechanisms between these

transcription factors remain incompletely understood, with specific programs reflecting unique combination of transcription factor expression, and reciprocal effects on related signaling cascades. An additional layer of programming complexity derives from the heterogeneous nature of cancer cells. For example, cells at the invasive margins of carcinomas can be seen to have undergone an EMT, while cells residing in the core of the tumor may be shielded from these signals, interactions, or stimuli [73]. Understanding the molecular cascades that regulate cancer-EMT becomes important, as the modulation of this process can potentially reverse the cancer-activating programs. The promise of this reverse process, termed mesenchymal–epithelial transition (MET), remains elusive, as conclusive evidence supporting this therapeutic possibility remains to be convincingly demonstrated. However, as described above, the EMT program has been shown to be a spectrum of phenotypes where some cancer cells may enter into an EMT program only partially or incompletely, retaining and co-expressing both epithelial and mesenchymal genes and traits. In effect, this partial or incomplete programming reflects a true dichotomous state, which may lend itself to plasticity and reversion to a nascent epithelial state. Moreover, the tumor cells seen at the invasive front of solid tumors are considered to be the cells that eventually undergo EMT and exhibit properties such intravasation, transport through the circulation, extravasation, and formation of micrometastases [4, 74–76]. Paradoxically, cancer cells established at distant secondary sites often resemble the primary tumor from which they were derived, prior to EMT. These observations suggest the metastasizing cancer cells must be capable of reversing their mesenchymal phenotype via MET during the course of secondary tumor formation [77].

2.4.1 TGF-β Signaling

Although the molecular regulation of EMT involves a variety of signals that are beyond the scope of this chapter, we focus on *TGF-β*, a critical signal, in the following discussion. TGF-β is secreted by a variety of cell types, and exists as three isoforms (TGF-β1, TGF-β2, and TGF-β3) in mammals. The homo- or heterodimers are secreted into the ECM as part of a complex known as the large latency complex (LLC) [78]. TGF-β is activated when it disengages from this complex. The TGF-β receptors are membrane-bound receptors with serine threonine kinase activity. TGF-β binds as a ligand to the type II receptor, TGFβ-RII, in conjunction with the type III receptor, TGFβ-RIII. The heterotetrameric complex phosphorylates the type I receptor, TGF-βRI, which functions through the downstream family of proteins in the Smads family (primarily promoting binding to Smad2 and Smad3). Receptor-regulated Smads (R-Smads) form a complex with Smad4 and function in transcriptional regulation. Cooperative interaction occurs with the transcriptional enhancers p300/CBP, Forkhead, homeobox, zinc-finger, AP1, Ets, and basic helix–loop–helix families of transcription factors [79]. Ubiquitination by E3 ligases and Smurf family proteins contribute to degradation

of TGF-β pathway constituents. In this context, Smurf 1 and 2 often interact with Smad7 to regulate ubiquitin-mediated degradation [80]. The functions of TGF-β are diverse and often seemingly contradictory. TGF-β can function as a tumor suppressor by arresting cell cycle progression. However, non-canonical TGF-β signaling can promote a cellular program that enables tumor growth. Indeed, cumulative evidence has shown that TGF-β enables tumor progression and metastasis [81–83] as well as inducing cancer-EMT [4, 84]. The heterogeneity of ligands and downstream effectors that participate with TGF-β signaling, the variety of transcription factors and complexes at play, and the enormous amount of crosstalk between the TGF-β signaling network and other canonical signaling pathways result in a wide variety of effects of TGF-β on cancer growth and metastasis [85].

TGF-β provides a vital role in activating pro-EMT signals [86, 87]. The downstream transcriptional activation of Snail, Slug, Zeb1, Twist, and BHLH [88–90] results in the dismantling of cell–cell tight junctions and rearrangement of the actin cytoskeleton [88]. Recently, a novel Smad4 mutation was found to increase homodimerization of Smad4 with the receptor Smads and promote nuclear localization; this resulted in reduction in E-cadherin, increase in N-cadherin, increased fibroblastic phenotype, and ability to grow in anchorage-independent conditions of papillary thyroid cancer cells [78, 91]. TGF-β has been implicated as a mechanistic mediator of cancer cell resistance to chemotherapy and radiation. Radiation treatment has been shown to lead to increased TGF-β levels and increased circulating tumor cells and lung metastases [92], and ionizing radiation was found to promote TGF-β related EMT and associated increases in invasiveness and migration in six different cancer cell types [93]. TGF-β functions in hepatocellular cancer progression, and many liver cell types, including hepatic stellate cells (HSCs), hepatocytes, and liver sinusoidal endothelial cells are regulated by TGF-β [94]. Often, the dual role of TGF-β is regulated through modulation of receptor expression. For example, loss-of-function of TGF-β type II receptor results in enhanced susceptibility to tumorigenesis, providing evidence again that TGF-β normally retains tumor-suppressor functions [95]. Alternatively, transgenic mice with up-regulated Smad7 expression restricted to hepatocytes demonstrate significantly diminished liver damage and fibrosis, suggesting that TGF-β signaling in hepatocytes is required for fibrogenesis progression [96]. The significance of the dual nature of these effects is unclear, but they suggest that the effectors of TGF-β may be time- and context-dependent. For example, inactivation of type II TGF-β receptor in an animal model of breast carcinoma increases CXCL5- and CXCL12-mediated recruitment of MDSCs, which are potent suppressors of the adaptive immune response to tumors [20]. Smad7 activation or RNA interference against Smad4 decreases TGF-β signaling and attenuates the expression of pro-fibrotic genes [96, 97]. However, hepatocytes isolated from livers exposed to high TGF-β in vivo demonstrate elongated, fibroblastoid hepatocytes expressing vimentin and collagen I in comparison to healthy mouse livers [98]. Cumulative evidence from the Fabregat group demonstrates that TGF-β signaling regulates seemingly contradictory processes in normal liver cells and in HCC. TGF-β-mediated growth inhibition and apoptosis (tumor suppressor characteristics) occur in non-transformed human

fetal hepatocytes, while trans-differentiation into a mesenchymal-stem cell-like phenotype with increased expression of Snail, decreased E-cadherin expression, increased vimentin and N-cadherin expression (pro-tumor) is also TGF-β-mediated [99]. Indeed, parallel experiments using siRNA-mediated downregulation of Snail showed that hepatocytes became sensitized to TGF-β-mediated apoptosis, and that Snail and induction of the EMT phenotype impairs TGF-β apoptosis in cancer cells [100].

In other signaling pathways, β-catenin and lymphoid enhancer factor (LEF) also cooperate with Smads in inducing EMT [4, 101–103]. These studies demonstrate that the TGF-β/Smad/LEF/PDGF axis is an important inducer of an EMT phenotype in cancer. Evidence indicates that p38 mitogen-activated protein kinase (MAPK) and RhoA can mediate an autocrine TGF-β-induced EMT in NMuMG mouse mammary epithelial cells in an integrin-mediated fashion [104]. Fibulin-5, an ECM molecule, augments TGF-β-induced EMT in a MAPK-dependent mechanism [105]. Other MAPK-related mechanisms included TGF-β induction of an EMT in Ras transformed hepatocytes, mammary epithelial cells (via MAPK), and Madin–Derby canine kidney (MDCK) cells [106–108]. Interestingly, in mouse models of skin carcinoma and human colon cancer, the absence of TGF-β receptor expression confers improved prognosis [109, 110]. Loss of E-cadherin expression by cancer cells and passage through an EMT has also been shown to be TGF-β dependent [111, 112]. Cytosolic β-catenin sequestration maintains epithelial features of cancer cells, and acquisition of the mesenchymal phenotype correlates with β-catenin translocation into the nucleus, where it complexes with Tcf/LEF [103, 113]. β-catenin accumulation in the nucleus is often associated with loss of E-cadherin expression [74, 114]. Noncoding microRNAs including microRNA 200 (miR200) and miR205 inhibit the repressors of E-cadherin expression, ZEB1, and ZEB2, and maintain epithelial cell characteristics [115, 116].

2.5 Osteopontin and EMT

Osteopontin (OPN) was initially discovered as an inducible tumor promoter, is over-expressed in tumors, is the major phosphoprotein secreted by malignant cells in advanced metastatic cancer, is a key mediator of tumor cell migration and metastasis, is a lead marker of HCC progression and metastasis, and induces EMT [117–119]. OPN was initially characterized in 1979 as a phosphoprotein secreted by transformed, malignant epithelial cells [120]. Investigators have since independently detected this molecule as secreted phosphoprotein I (Spp1), 2ar, uropontin and early T-lymphocyte activation-1 (Eta-1) [121]. OPN is a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family of proteins which include bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), dentin sialoprotein (DSPP), and matrix extracellular phosphoglycoprotein (MEPE) [122]. Elevated OPN expression has been implicated as an important mediator of tumor metastasis, and has been investigated for use as a biomarker for advanced

disease and as a potential therapeutic target in the regulation of cancer metastasis. The molecular structure of OPN is rich in aspartate and sialic-acid residues, and contains unique functional domains [123]. These structural motifs mediate critical cell–matrix and cell–cell signaling through the $\alpha_v\beta$ integrin and CD44 receptors in a variety of normal and pathologic processes. Interestingly, the role of OPN appears to be maintained across species, with similar expression and functions detected in humans and rodents [121]. Cell types which express OPN include osteoclasts, osteoblasts, kidney, breast and skin epithelial cells, nerve cells, vascular smooth muscle cells and endothelial cells. Activated immune cells such as T-cells, NK cells, macrophages and Kupffer cells also express OPN. The secreted OPN protein is widely distributed in plasma, urine, milk, and bile [124–126]. The induced expression of OPN has been detected in T lymphocytes, epidermal cells, bone cells, macrophages, and tumor cells in remodeling processes such as inflammation, ischemia-reperfusion, bone resorption, and tumor progression. An important area of investigation involves the transcriptional regulation of OPN expression during tumorigenesis and metastasis, and the identification of *trans*-elements that could potentially affect the metastatic phenotype. A variety of stimuli including phorbol 12-myristate 13-acetate (PMA), 1,25-dihydroxyvitamin D, basic fibroblast growth factor (bFGF), TNF-α, IL-1, IFN-γ, and lipopolysaccharide (LPS) upregulate OPN expression [121].

In the context of tissue repair and fibrosis, upregulated expression of OPN has been demonstrated during the inflammatory phase of wound healing. OPN provides important regulation during significant steps in this process. The dependency on the duration of expression is critical to balancing the normal effects of OPN versus the pathologic stimulation that is associated with persistent expression. Excessive expression of OPN leads to fibrosis and scar formation, functioning in a dose- and time-dependent fashion. Using animal models, OPN has been implicated in the progression of both renal interstitial fibrosis and glomerular fibrosis. Investigators have demonstrated that upregulation of kidney OPN mRNA and protein correlates with progression to glomerular fibrosis [127]. Using the animal model for unilateral ureteral obstruction (UUO), OPN null mice demonstrated less interstitial fibrosis in comparison with wild type mice [128]. The primary function of OPN is the recruitment, regulation, and differentiation of fibroblasts and myofibroblasts [129]. Acting as a chemo-attractant for fibroblasts, OPN functions in ECM deposition and collagen matrix formation. OPN-null mice exhibited healing wounds with reduced organization in matrix architecture, reduced numbers of collagen fibers, and decreased fibril diameter [130]. The wound beds were characterized by an ECM with increased porosity. In addition, these OPN-null mice showed reduced expression of collagen type I mRNA, matrix metalloproteinase 9, fibronectin, and TGF-β mRNA [131]. Although the fibroblasts in OPN-null mice showed no response to stimulation by TGF-β1, transformation into myofibroblasts expressing α-SMA was still detected, suggesting a redundant alternative regulatory pathway. Interestingly, more efficient re-epithelialization and wound closure was also demonstrated in OPN-deficient conditions [131]. In comparison, investigators using a corneal injury model showed that wound closure was delayed in conditions

where OPN function was lost [132]. These contrasting results suggest that the role of OPN is tissue-dependent, may be altered with context, and may serve dual functions based on regulating stimuli.

Recently, our laboratory sought to determine whether OPN represents a target for altering EMT induction mediated by TGF-β. Using a co-cultured model for breast cancer, we analyzed the interaction between cancer cells and mesenchymal stem cells (MSCs). In MDA-MB231, which expressed high levels of OPN, we found that the OPN-stimulated MSCs subsequently expressed high levels of TGF-β (unpublished results). TGF-β then acts in a paracrine fashion to initiate EMT in the breast cancer cells, as measured by expression of increased levels of vimentin, tenascin-C, FSP-1, and SMA. MCF7 breast cancer cells that do not express OPN were co-cultured with MSCs as a control, and resulted in no observed increase in TGF-β expression and an absence of EMT. These data corroborate findings by other researchers. Using various cancer models, investigators have implicated OPN as an important regulator of metastatic behavior [117]. Medico et al. [133] used cDNA microarrays to identify OPN as a major target for the transcription factor, hepatocyte growth factor, and demonstrated that OPN mediated cell adhesion in MLP-29 murine cancer cells. In human HCC samples, Ye et al. [117] used microarray gene expression profiling to examine changes associated with HCC metastasis. These authors found that OPN correlated with the metastatic potential of primary HCC. Additional in-vitro studies showed that OPN neutralizing antibody significantly blocked invasion of SK-Hep-1 cells. Using archived HCC resection specimens, OPN mRNA expression correlated closely with intrahepatic metastasis, early recurrence, and late-stage/higher grades of HCC [119]. Additional immunohistochemistry studies demonstrated that OPN is expressed primarily on cancerous cells, especially in HCC with capsular invasion and in areas adjacent to stromal cells. Zhao et al. [134] used polyethylenimine nanoparticles to deliver a short-hairpin RNA for depletion of OPN expression in HCC cells. This resulted in the inhibition of HCC cell growth, anchorage-independent growth, adhesion with fibronectin, and invasion through extracellular matrix in vitro, and suppressed tumorigenicity and lung metastasis in nude mice. In an alternative approach, Sun et al. [135] used lentiviral delivery of micro-RNA against OPN, and suppressed in-vitro proliferation and in-vivo tumor growth of HCCLM3.

Recent studies from our laboratory and that of other investigators have examined the relationship between OPN and EMT in tumor progression. Saika et al. determined that OPN expression is up-regulated in the injured mouse lens before initiation of EMT [136]. Using OPN-null mice, these authors found that absence of OPN was associated with inhibition of EMT as measured by SMA, transforming growth factor-β, and collagen type 1. In non-small-cell lung cancers, OPN expression was associated with increased expression of the EMT markers, matrix metalloproteinase-2, Snail-1, Snail-2, transforming growth factor-β1-R, matrix metalloproteinase-9, N-cadherin, vimentin, SOX-8, and SOX-9 [137]. Based on our studies, OPN expression in HCC was also associated with integrin-dependent expression of EMT markers and enhanced in-vitro measures of growth and metastasis [119]. Using an animal model, OPN and EMT markers were

significantly increased in the metastatic cohort. OPN-aptamer inhibition decreased tumor adhesion, migration/invasion, and decreased EMT protein markers, SMA, vimentin, and tenascin-c. In-vivo treatment with OPN-aptamer inhibition decreased HCC growth by more than tenfold [119].

2.6 Summary

Tumor progression, invasion, and metastasis is dependent not only on mutational events arising in the transformed cell, but also on key interactions between the cancer cell and the recruited stromal cells and tissues surrounding it. The EMT–MET properties of cells has transformed our understanding of how tumors can simultaneously adopt invasive properties while also house themselves at distant metastatic sites. OPN is an interesting key mediator of the metastatic phenotype in various cancers, and we have recently explored its function in EMT. These results may offer therapeutic modulation of the invasive tumor phenotype.

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Chapter 3
The Role of Tumor-Associated Macrophages (TAMs) in Tumor Progression

Astrid Schmieder and Kai Schledzewski

Abstract Tumors are organ-like structures composed of neoplastic as well as non-malignant stroma cells. One of the most prominent cellular components of the tumor stroma are tumor-associated macrophages (TAMs). Pre-clinical as well as clinical studies have shown an inversed correlation between macrophage infiltration and patients' prognosis, indicating a macrophage supporting role for tumor progression. Macrophages are a heterogeneous cell population with many different functions for the organism. They have been broadly classified into pro-inflammatory, classically activated macrophages (M1; stimulated by IFN-γ or LPS) and anti-inflammatory, alternatively activated macrophages (M2; stimulated by either IL-4/IL-13, IL-1β/LPS in combination with immune complexes or by IL-10/β/glucocorticoids). TAMs have been shown to possess a M1-like phenotype in tumor initiation, while during tumor progression they acquire characteristics of M2-like macrophages. The latter TAMs support tumor growth by their pro-angiogenic, anti-inflammatory and matrix-remodeling abilities. They do so by secreting diverse growth factors like VEGF, PDGF, or EGF and chemokines and cytokines such as IL-10, TGF-β, CCL2, and CXCL12. In addition, they support tumor invasion by secreting matrix remodeling molecules such as matrix metalloproteinases (MMP) and cathepsins. These abilities qualify TAMs as important novel adjuvant therapeutic targets for human cancer. First approaches directed against tumor-promoting TAM populations as well as their functions with, for example, bisphosphonates have already shown some promising results.

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List of Abbreviations

AID	Activation-induced cytidine deaminase
ANG2	Angiopoietin-2
bFGF	Basic fibroblast growth factor
CCL	Chemokine (C–C motif) ligand
CCR2	CC-chemokine receptor 2
COX-2	Cyclooxygenase-2
CSF-1R	Colony-stimulating factor receptor 1
CX3CR1	CX3C-chemokine receptor 1
CXCL	CXC chemokine ligand
EGF	Epidermal growth factor
EMT	Epithelial–mesenchymal transition
GM-CSF	Granulocyte–macrophage colony-stimulating factor
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
HPV	Human papilloma virus
IDO	Indoleamine dioxigenase
IFN-γ	Interferon γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase.
LPS	Lipopolysaccharide
M1	Classically activated macrophage
M2	Alternatively activated macrophage
M-CSF/CSF-1	Macrophage colony-stimulating factor 1
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MIF	Migration inhibitory factor
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
MPS	Mononuclear phagocytic system
MRC1	Mannose receptor c1
NF-κB	Nuclear factor κB
PDGF	Platelet-derived growth factor
PlGF	Placenta growth factor
PyVT	Polyoma virus middle T antigen
ROS	Reactive oxygen species
STAT3	Signal transducer and activator of transcription 3
TAM	Tumor-associated macrophage
TEM	Tie2-expressing macrophage
TGF-β	Transforming growth factor β
Th1	T helper cell type 1
Th2	T helper cell type 2
TLR	Toll-like receptor

TNFR I	Tumor necrosis factor receptor 1
TNF-α	Tumor necrosis factor α
VEGF	Vascular endothelial growth factor

Contents

3.1 Introduction

In recent years it became evident that tumors can not just be seen as a bunch of malignant cells, but are more like *organ-like structures* composed of vessels, hematopoietic cells, and mesenchymal cells. As discussed in the previous chapter, each tumor has its own nourishing microenvironment responsible for tumor development, progression, and metastasis in which multidirectional interactions between neoplastic cells, the non-malignant cellular as well as the extracellular compartment, contribute to the enormous heterogeneity of neoplastic tissue [1]. In the process of tumor development under attack of the host immune system, several tumor stromal cells seem to contribute to tumor survival; among them are cells of myeloid origin such as macrophages.

Macrophages are a heterogeneous population of resident tissue cells, which originate mostly from blood monocytes and are part of the *mononuclear phagocytic system* (MPS). The MPS is an ontologic definition, which includes bone-marrow-derived precursor cells, blood monocytes, macrophages and some dendritic cells originating from myeloid precursors [2]. Cells of the myeloid lineage express the colony-stimulating factor receptor 1 (CSF-1R) on their surface. Its stimulation by macrophage colony-stimulating factor 1 (M-CSF/CSF-1) is essential for the development of macrophages from blood monocytes [3].

After birth, macrophages are found in nearly all tissues e.g. as Kupffer cells in the liver or microglia cells in the brain, where they fulfill a plethora of different functions. They are “all-rounder cells” required not only as front fighters in infections but more importantly as guardians of homeostasis in the body. They phagocytose death cellular debris and secrete growth factors important for tissue

regeneration and angiogenesis, thereby contributing to a permanent imperceptible regeneration of the body. This diversity of functions has called for an attempt to classify macrophages.

3.2 Macrophages Classification

Macrophages were first described by Elie Metchnikoff as phagocytic cells present in vertebrates and invertebrates with homeostatic and bactericidal functions important for maintaining the integrity of an organism [4]. Findings of molecular mRNA and protein expression profiles in the last decades of the twentieth century allowed researchers to describe and sub-classify cells in more and more detail. In terms of macrophages, a simple but relevant was introduced by separating two functionally diverse macrophage populations. *M1 macrophages* were defined as macrophages found in pro-inflammatory environments induced by interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), and granulocyte macrophage colony-stimulating factor (GM-CSF), and are important for killing intracellular bacteria and viruses. This is achieved by their high antigen presentation rate with their MHC class II complexes and the secretion of pro-inflammatory cytokines such as interleukin (IL)-12 and IL-23. *M2 macrophages*, on the other hand, are involved in homeostatic processes such as angiogenesis, tissue remodeling, wound healing, and anti-inflammation, and express high amounts of the anti-inflammatory cytokines IL-10 and low amounts of IL-12. They are mainly induced by typical TH2 cytokines such as IL-4, IL-13, glucocorticoids, and M-CSF [5–8]. In addition, they express typical M2 markers such as the scavenger mannose receptor [8], Stabilin-1 [9], chemokine (C–C motif) ligand (CCL)18 [10] in humans, and arginase-1 in mice [11]. This in-vitro classification has soon proven to be an oversimplification of the in-vivo situation, as macrophages not only show many different shades in between the two opposite ends of the macrophage activation spectrum, but they also have the capability to interconvert in between these two main forms [5]. Other classification attempts were made by sub-classifying the M2 macrophage population into M2a, M2b, and M2c, where M2a was induced by IL-4 and IL-13, M2b by immune complexes together with Toll-like receptor (TLR) agonist, and M2c by glucocorticoids and IL-10 [7]. The most flexible classification was proposed by Moser et al., who depicted macrophage activation states on a color wheel, with primary colors resembling the three main macrophage functions: immune regulation, wound healing, and host defense [12]. This illustration makes it possible to depict macrophages as a part of a continuum with overlapping functions according to the microenvironment which surrounds them in vivo. Although oversimplifying the heterogeneity of macrophages, the original classification into M1 versus M2 macrophages by Stein et al. still represents a valid classification model, as it communicates with clarity the basic points of macrophage activation. This

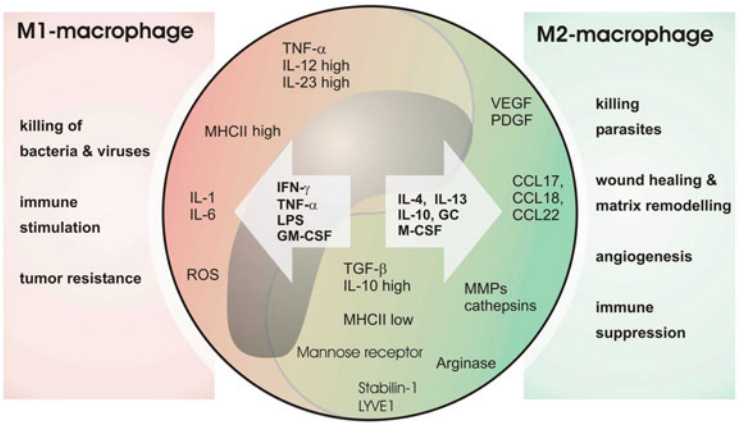

Fig. 3.1 The M1/M2 model of macrophage activation. In the presence of IFN-γ, LPS, GM-CSF, and high concentrations of TNF-α, macrophages acquire a pro-inflammatory phenotype involved in the killing of bacteria, viruses, and tumor cells. These cells secrete high amounts of IL-12, IL-23 and ROS. In addition, they show a high antigen presentation capability supported by their surface expression of MHC class II molecules. On the other hand, the M2 macrophage phenotype is induced by IL-4, IL-13, IL-10, glucocorticoids, and M-CSF. These macrophages are engaged in killing of encapsulated parasites and in processes important for the preservation of tissue homeostasis. They secrete high amounts of IL-10, TGF-β, several growth factors, and extracellular matrix degrading enzymes

classification will therefore be used in this chapter despite its limitations for the sake of clarity (Fig. 3.1).

3.3 The Link Between Cancer and Inflammation

Established tumors have been described as chaotically structured organs that need to interact and communicate with its host. This is obvious in terms of nutrient supply and removal of toxic waste products, a necessity managed by the vascular system. Without connection to the host vessel bed, tumors would reduce in size and therefore concepts of targeting angiogenesis were developed [13]. After several years in clinical use, it became obvious that adjuvant anti-angiogenic therapy is not a sufficient cancer treatment and consequently further development of established therapeutic strategies—such as classical radiotherapy, anti-mitotic drugs, tumor-cell-directed target therapies (antibody–drug conjugates)—but also novel concepts were required.

The link between cancer and inflammation was already, made by Virchow in the nineteenth century, who suggested chronic irritation by inflammation as a possible trigger for cancer development [14]. Since then, several epidemiological studies have underlined this connection, as several chronic infections such as *Helicobacter pylori* or human papilloma virus (HPV) are associated with a higher risk to develop cancer or lymphomas. Accordingly, anti-inflammatory drugs such as non steroidal

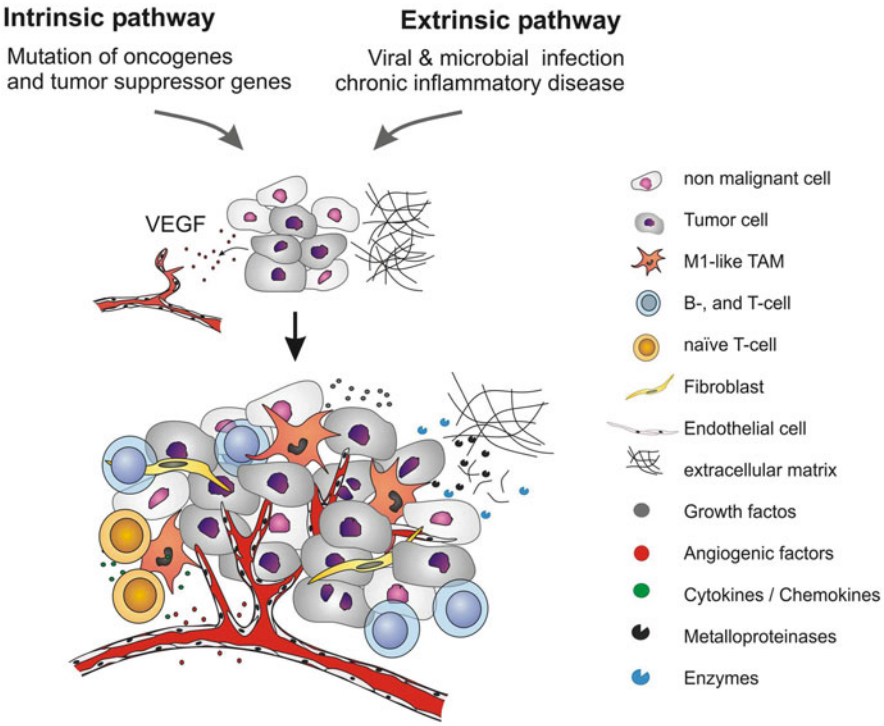

Fig. 3.2 The link between tumor inflammation and cancer. Inflammation and cancer are linked together by the intrinsic and extrinsic pathway. In the intrinsic pathway, mutations lead to the permanent activation of oncogenes tumor suppressor genes; this results in the production of inflammatory mediators, which attract leukocytes important for tumor development. In the extrinsic pathway, a chronic inflammatory environment caused by chronic bacterial infections or chronic inflammatory diseases increases the risk of tumor development. In vivo, both pathways contribute to tumor initiation

anti-inflammatory agents reduce the cancer incidence [15]. A helpful model to study the link between inflammation and cancer is the subdivision in an intrinsic and extrinsic pathway. The *intrinsic pathway* requires a genetic event in neoplastic cells that activates a transcriptional program reminiscent of the one found in inflammatory conditions, e.g., the activation of the BRAF-MAPK signaling pathway in melanoma increases the secretion of soluble factors such as IL-10, vascular endothelial growth factor (VEGF), and IL-6, which contributes to the establishment of an immunosuppressive and angiogenic tumor environment [16]. Similarly, inactivating mutation of the type II transforming growth factor β (TGF-β) receptor in a breast carcinoma model unleashes production of CXC chemokine ligand (CXCL)5 and CXCL12, leading to the attraction of immune cells with immunosuppressive skills such as myeloid-derived suppressor cells (MDSC) [17]. Such data provide evidence that initial mutations in certain oncogenes might already pave the way for the development of a tumor-nourishing environment providing the perfect conditions for tumor establishment (Fig. 3.2).

In contrast, the *extrinsic pathway* is defined as an inflammatory environment caused by infections and chronic inflammatory diseases present during or even before tumor development. Such local conditions contribute to the activation of pro-inflammatory signaling pathways and transcription factors such as nuclear factor κB (NF-κB) or signal transducer and activator of transcription (STAT) 3 in potential tumor cells, which enhances neoplastic transformation and resistance to apoptosis. In addition, the plethora of secreted cytokines and chemokines present in such environments attracts more inflammatory cells such as macrophages and T-cells important for the production of angiogenic, immunosuppressive, and mitogenic factors [17, 18].

In vivo, however, *both pathways* contribute simultaneously to tumor initiation. This was recently confirmed by using a murine genetically modified tumor model of pancreatic carcinoma with an inducible expression of the *K-ras* oncogene in adult mice. The expression of the mutated alone did not suffice for the development of pancreatic ductal adenocarcinoma, but needed the combination with chronic pancreatitis (extrinsic pathway) induced by caerulein [19].

3.4 The Role of Tumor-Associated Macrophages in Tumor Initiation

In both the intrinsic and extrinsic pathways, macrophages are major components of the cancer related inflammatory environment. In the phase of *tumor initiation*, the balance between anti-tumor immunity and pro-tumor inflammation is dependent on cytokines, chemokines, and growth factors, in addition to the activation state of diverse immune and stroma cells present in the tumor environment [20, 21]. Malignant transformation of cells in the body is a frequent process, and is kept under control by a regular immune response. Therefore, at early time points during tumor development malignant cells are attacked by M1 macrophages and other leukocytes. However, a clear association of M1 macrophage differentiation with successful tumor immunosurveillance is not possible, as most of the confirmed tumor-inducing cytokines are listed within the typical "M1 cytokines." Accordingly, reactive oxygen species (ROS) secreted by M1 macrophages in inflammatory environments have been accused of inducing DNA damage and genomic instability [22], although it is still unclear whether ROS are able to penetrate the nucleus of adjacent cells. Alternatively, TNF-α secreted by macrophages or other immune cells might lead to ROS accumulation in pre-cancerous cells, causing mutations by oxidative damage in diverse oncogenes and tumor-suppressor genes such as, for example, p53 [23]. Also, a direct inactivation of mismatch repair enzymes was attributed to ROS [24, 25], which promotes accumulations of inflammation-induced mutations. Convincing evidence links also cytokines and growth factors secreted by macrophage with tumor initiation. Activation of the NF-κB transcription factors by IL-1 and TNF-α contributes to proliferation and survival of cancer cells [26]. In addition NF-κB activation induces the transcription of activation-induced cytidine deaminase (AID), found to induce genomic instability in critical cancer genes such

as Bcl-6, Trp53, and c-Myc [24], thereby contributing to the formation of gastrointestinal cancers and lymphomas [27, 28]. Another important transcription factor implicated in the process of tumorigenesis is STAT3, which is found constitutively activated in both immune cells and in tumor cells [18]. The activation of STAT3 is achieved by diverse macrophage-derived cytokines and growth factors such as epidermal growth factor (EGF) or IL-6. This transcription factor has been described to be involved in inducing a stem-cell-like phenotype or to stimulate stem-cell expansion, enlarging the cell pool that can be affected by environmental mutations [29]. In addition, STAT3 protects tumor cells from apoptosis [30].

Although the phenotypical characteristics of macrophages within early, initiative steps of neoplastic transformation remain still a matter of discussion, the primary immune reaction against malignant cells will create a milieu characteristic for a Th1 immune response. Under such pro-inflammatory conditions, infiltrating macrophage precursors will most probably differentiate into M1 macrophages. In developed solid tumors that obviously evade immune surveillance, TAMs have been analyzed in humans as well as in murine experimental models in more detail. Here, TAMs share characteristics of M2 macrophages [31].

The change from the M1 to the M2 macrophage phenotype has also been verified using a murine hepatocellular carcinoma model, in which TAMs express high levels of MHC-class II molecules, IL-1β, IL-6, IL-12, and inducible nitric oxide synthase (iNOS) in early tumor stages, while in late tumor stages mostly major histocompatibility complex (MHC) class II low TAMs with typical M2-macrophage markers as mannose receptor c1 (MRC1), arginase, and IL-10 prevail [32]. The mechanisms behind the switch from the M1 to the M2 phenotype during cancer progression are still poorly understood. However, there is emerging evidence that the profound changes in oxygen tension, glucose levels, and pH occurring during tumor growth are partly to blame for this phenomenon [33].

During this switch, TAMs acquire a defective NF-κB response to inflammatory stimuli such as lipopolysaccharide (LPS), with impaired secretion of pro-inflammatory cytokines as IL-12, IL-1, and TNF-α [34]. The NF-κB family of transcription factors is composed of five members who are able to form homo- or heterodimers: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel [35]. P50 homodimer formation has been described in TAMs [36] as a possible negative regulatory mechanism of the inflammatory response in TAMs. In contrast to the other NF-κB family members, p50 and p52 lack a transcription activation domain.

3.5 The Role of Tumor-Associated Macrophages in Tumor Progression

Several epidemiological as well as clinical studies exist, which link a high macrophage infiltration in tumors with a poor clinical outcome [37]. A high count of TAMs in lung adenocarcinoma was, for example, associated with accelerated lymphangiogenesis and lymph node metastasis [38]; a high TAM count was also an independent negative prognostic factor for clear renal cell carcinoma [39],

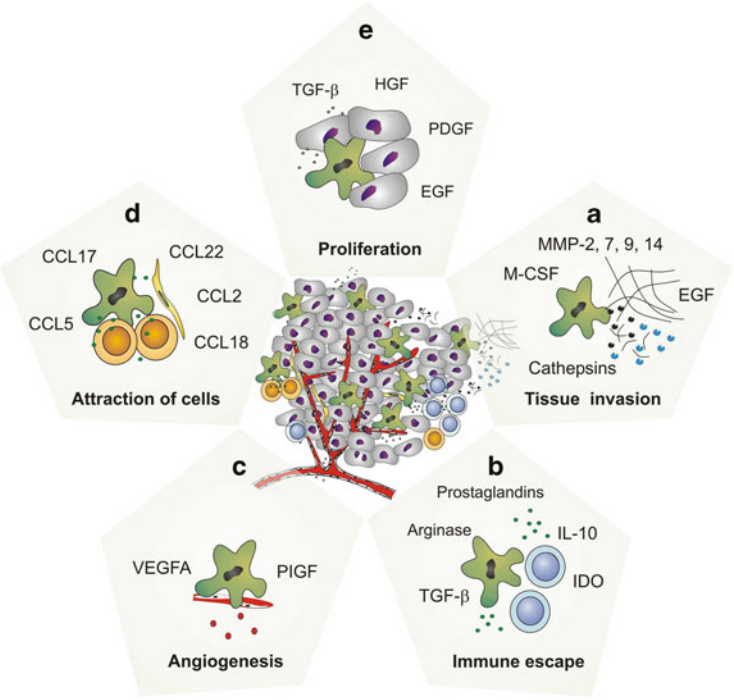

Fig. 3.3 Tumor-associated macrophages support tumor progression. **a** Tumor-associated macrophages support tumor cell invasion by the secretion of diverse matrix-degrading enzymes and tumor cell attracting growth factors such as EGF. **b** Immunosuppression by macrophages is achieved by the secretion of immunosuppressive cytokines such as IL-10, TGF-β, and by environmental deprivation of essential amino acids for T-cells. **c** The process of angiogenesis is supported by the release of angiogenic growth factors such as VEGF and PIGF. **d** In order to create a tumor environment in favor of tumor progression, stromal cells and immune cells with tumor-supporting abilities need to be attracted. Macrophages sustain this process by secreting many different cytokines and chemokines. **e** Macrophages support proliferation of tumor cells by the secretion of different growth factors such as EGF, PDGF, HGF, and bFGF

Ewing sarcoma [40], and Hodgkin lymphoma [41, 42]. This is not surprising, as many skills of M2-macrophages can support the multistep process in cancer development and progression proposed in 2000 by Hanahan and Weinberg, encompassing six traits required for malignant growth [43]. These *six hallmarks of cancer* encompass self-sufficiency from external growth factors, resistance to growth suppressors and cell death signals, a limitless replicative potential, and the ability to induce angiogenesis and to invade tissue and metastasize. Macrophages provide support for tumor growth by secreting growth factors and cytokines that activate anti-apoptotic pathways in tumor cells, and stimulate angiogenesis. In addition, macrophage-derived metalloproteinases pave the way for tissue invasion and metastasis of tumor cells. Therefore, tumor cells recruit M2-like macrophages with trophic functions and immunosuppressive abilities as innocent partners in crime which support the establishment of a proper microenvironment in favor of the tumor (Fig. 3.3).

Mirroring the six hallmarks of cancer, Condeelis and Pollard in 2006 proposed the *six extrinsic traits conferred by macrophages* that contribute to tumor incidence, progression, and metastasis, which include chronic inflammation, matrix remodeling, tumor cell invasion, intravasation, angiogenesis, and seeding at distant sites [44]. As chronic inflammation has already been discussed before, the following sections will focus on the role of TAMs for tumor angiogenesis, malignant invasion, and immunosuppression.

3.5.1 Angiogenesis

Similarly to organs, tumors also contain a complex structure of endothelial cells forming a vascular bed. Tumor vessels supply nutrients and oxygen, and remove waste products and carbon dioxide [45]. Without such connection to the host blood stream, tumors would not exceed a size of more than 1 mm^3 and remain asymptomatic [46]. In contrast to organ development in embryogenesis, where the vessels are formed in a process termed vasculogenesis, tumor vessels develop by sprouting endothelial cells out of the locally established vessel system. This form of vessel formation is called angiogenesis, and in adults, apart from the tumor environment, is limited to the female reproduction circle, to acute or chronic inflammation, and to wound healing. In order to proliferate, resting endothelial cells have to differentiate into an angiogenic form. This angiogenic switch is turned on under dominant stimulation with pro-angiogenic growth factors such as VEGFs or placenta growth factor (PlGF) [47, 48]. Such stimulated endothelial cells weaken their intercellular contacts and partially degrade the continuous basement membrane by releasing proteases such as matrix metalloproteinase (MMP)-9. Subsequent proliferation and matrix invasion of these endothelial cells results in the formation of a new vessel from an existing one. Whereas in normal angiogenetic processes, such as wound healing, sprouting ends with the formation of mature capillaries, in the tumor, endothelial cell layers are non-continuous, with an impaired cell-to-cell attachment and a missing continuous basement membrane. This leads to vessels that are irregularly shaped, tortuous and dilated, and in consequence leak into the tumor microenvironment and remain partially insufficient in oxygen supply. During tumor progression, hypoxia triggers the expression of VEGFs by tumor cells as well as by other cellular components of tumor stroma. A positive correlation between TAMs and angiogenesis has been described in human breast carcinoma [49, 50] and many other tumors such as colorectal cancer [51] or squamous cell carcinoma [52]. The link between infiltrating TAMs and angiogenesis has been further confirmed by introducing animal models. Mice deficient for expression of M-CSF in a MMTV-PyMT mouse model of breast carcinoma showed a reduction of tumor vessel density to only 50 % in response to an impaired TAM infiltration. Herewith, the authors showed that the angiogenetic switch is dependent on the presence of TAMs in the tumor environment [53]. The positive impact of macrophages on angiogenesis in vivo has also been demonstrated in a skinfold chamber model where small

nodules of breast carcinoma cells (spheroids) were grown with or without human macrophages [54]. As depletion of macrophages either by anti-M-CSF antibodies [55] or chlodronate treatment [56] also confirmed the relevance of macrophages for tumor vessel growth, the question regarding the underlying mechanism of TAM driven angiogenesis arose. Within developed tumors of the MMTV-PyMT mouse model of mammary carcinoma, macrophages localize to hypoxic regions or in close association to developing tumor neo-vasculature [57]. Hypoxia in macrophages triggers the overexpression of the α-subunits of hypoxia-inducible factors (HIF)-1 and HIF-2 which form active dimers with the constitutively expressed β-subunit of HIF. This complex binds as a potent transcription factor to hypoxic response elements in the promoters of oxygen-sensitive target genes [58]. Such genes include VEGF-A, migration inhibitory factor (MIF), angiopoietin-2 (ANG2), and CXCL8, known to be potent stimulators of angiogenesis [59, 60]. In addition, HIF-2α has been shown to induce the expression of arginase 1, which indicates the role of HIFs in M1/M2 macrophage differentiation [61]. NF-κB is another transcription factor induced in the initial phase of hypoxia [62], which in turn elevates HIF-1α levels in macrophages. However, upon prolonged hypoxic stimulation, hypoxia-associated genes like VEGF-A were expressed independent of NF-κB pathway. This suggests a role of NF-κB pathway only in the early response of macrophage to hypoxia present in the tumor [60].

In addition, macrophages are producers of potent pleiotrophic factors known to stimulate other cells present in the tumor stroma to contribute to the pro-angiogenic milieu. Such potent pleiotrophic factors expressed by TAMs are IL-1β [63], TGF-β, and TNF-α [64]. TGF-β, for example, has been shown to induce the expression of VEGF in cultured fibroblasts as well as adenocarcinoma cells [65]. Another important feature of TAMs is linked to secretion of proteases such as MMP-7 [59], MMP-9 [66], or MMP-12 [67]. By their proteolytic activity, these enzymes are potent modulators of extracellular matrix and support migration and invasion of activated endothelial cells in the sprouting process. Lysis of extracellular matrix via MMPs also converts matrix-bound VEGF-A into its bioactive form via intramolecular processing [68]. This is in line with the experimental finding that vascular remodeling in a murine gliobastoma model strictly depends on MMP-9 provided by myeloid cells [69]. Another class of proteases active in extracellular matrix turnover and tumor angiogenesis generated by TAMs are cathepsins [70]. In a model of pancreatic tumor growth, cathepsin B and cathepsin S expressed by TAMs have been identified as critical components for promoting tumor angiogenesis and invasion in vivo [71].

The observation of close interactions of angiogenic endothelial cells with macrophages resulted in the question whether cells of the haematopoietic lineage like macrophages were able to contribute to vessel formation by differentiation processes. Recently, the application of pleiotrophin, an angiogenic factor produced by several types of human cancer cells together with M-CSF derived from malignant plasma cells, was able to transdifferentiate peripheral blood monocytes into endothelial cells [72]. Although such transdifferentiated cells successfully integrate into tumor vessels in a model of human multiple myeloma in SCID mice, the biological

relevance of such processes for the development of tumor vasculature in humans unresolved so far. Another macrophage subtype sharing some traits with endothelial cells has been identified as Tie2 expressing macrophages in a transgenic mouse model [73–75]. Here, GFP was expressed under control of the endothelial-specific Tie-2 promoter. FACS analysis of cells grown in a murine mammary carcinoma subclassified two Tie2 GFP-positive fractions. The main fraction was characterized as $Tie2^{+}CD31^{+}CD45^{-}$, and therefore represent $CD31^{+}$ endothelial cells, whereas 5 % of Tie2 GFP-positive cells were identified as $Tie2^{+}CD11b^{+}CD45^{+}$, indicating a lineage of Tie2-expressing macrophages (TEM) [73]. Tie2-expressing macrophages (TEMs) do not dominate the TAM population, but have been mostly detected in close proximity to growing vessels. Depletion of TEMs in an experimental tumor model shrank tumor size, supporting a functional role of TEMs for tumor angiogenesis [76]. A molecular analysis of TEMs identified molecules partially known to be expressed by M2 macrophages such as MRC1 and hemoglobin scavenger receptor CD163 [77]. In addition, TEMs express Stabilin-1 as well as LYVE-1 [77], a protein originally identified as marker-specific to lymphatic vessels [78]. The pro-angiogenic potential of TEMs has been related to elevated expression levels of factors like VEGF-A, MMP-9, Cyclooxygenase (COX)-2, and cathepsin B [79]. Therefore, TEMs may promote sprouting vessels in the tumor [130].

3.5.2 Matrix Remodeling and Malignant Invasion

In biological systems, migration is an important ability inherent to many cell types at different time points, especially during embryogenesis and wound healing. The process of cell migration is commonly understood as a *single cell movement* consisting of five chronological steps: localized polymerization of actin to filaments, pseudopod formation, loosening of cell–cell contacts, adhesion to extracellular matrix proteins with β1 and β2 integrins, activation of contractile forces by engagement of actin-binding proteins such as myosin, finally leading to movement of the entire cell towards the adhesion side. In malignant cells, the cellular protrusions used for single cell movement are called invadopodia, which are able to penetrate vertically into the basement membranes using proteolytic extracellular matrix degrading enzymes such as MMP-14, -2, and -9 [80]. But, especially in the context of cancer-cell invasion, other forms of cell movements are important. Malignant cells seem to prefer a movement in cell groups, sheets, or strands—a process called *collective cell migration*. In this form of migration, coherent neoplastic cells held together by cell adhesion molecules such as integrins and cadherins develop cytoskeletal dynamics only in cells at the leading edge; the other cells in the cell group are then moved by traction. This form of movement requires proteolytic capacities, underlined by results showing that collective cell migration can be abrogated with protease inhibitors [81].

It has been proposed that malignant cells can also use the amoeboid form of movement. This form dispenses with focal proteolysis, but rather adapts the cell shape to preformed matrix structures, squeezing through by extending lateral food

holds. Such an amoeboid migration is mainly used by leukocytes such as macrophages and, with some exceptions, has been recently questioned as a relevant in-vivo form of movement in cancer cells [82].

The role that *TAMs* play in tumor invasion and metastasis is a hot topic in the field of oncology. It has long been known that co-culture of macrophages with malignant cells promotes the invasive abilities of cancer cells [83]. Using a transgenic MMTV-PyMT mouse model, Lin et al. were able to prove this concept in an in-vivo situation. They crossed MMTV-PyMT mice, a mammary cancer-susceptible strain, with mice containing a recessive null mutation in the M-CSF gene, which resulted in the delayed development of breast carcinoma metastasis to the lungs [84].

It has been known for a long time that a high activity of diverse proteolytic enzymes such as the zinc-dependent *metalloproteinases* in the tumor environment is associated with an increase in distant metastasis and with a shorter disease-free and overall survival in cancer patients [85]. Identification of TAMs as a major source of MMPs was achieved elegantly with co-culture experiments. Co-culture of malignant cells with macrophages increased the expression of MMP-2, -9, -3, and -7, accompanied by an increased MMP-2 activity. The increase in MMP secretion was, however, not observed when macrophages were co-cultured with a benign mammary epithelial cell line. In addition, it was possible to significantly diminish the increase in MMP secretion in this co-culture experiment when a blocking TNF-α antibody was used, which points to a major role of the *TNF-α pathway* for MMP expression. Further data coming from diverse murine tumor models support the notion that TNF-α enhances the metastatic behavior of cancer cells [86]. In addition, an autoregulatory loop between TNF-α and MMPs exists, as MMP inhibition leads to a reduction in TNF-α secretion, probably by the inhibition of TNF-α shedding by MMP-7 [87, 88]. TNF-α can bind to two receptors, of which the tumor necrosis factor receptor (TNFR) I governs the TNF-α response in the tumor environment [89]. While high TNF-α concentrations lead to the induction of cell death, lower levels seem to stimulate an M2-like macrophage phenotype and to enhance the activation of pro-invasive pathways involving JNK and NF-κB [87, 90].

In malignant cells TNF-α as well as TGF-β are able to induce *epithelial–mesenchymal transition* (EMT) features. EMT is characterized by the loss of epithelial markers such as cadherins, and the up-regulation of mesenchymal ones such as snail, twist, and vimentin [91]. The EMT confers a high motility on malignant cells by loosening cell–cell contacts, which predisposes to faster single cell movements [92]. Other factors derived from TAMs such as *cathepsins*, a family of lysosomal cysteine proteases, participate in the induction of an EMT phenotype in tumor cells by direct cleavage of E-cadherin on the surface of tumor cells.

Evidence that macrophages in tumors are not only responsible for extracellular matrix degradation and phenotypical changes in tumor cells, but actively instigate tumor cells to a tissue-invasive behavior came from a chemotaxis-based in-vivo assay combined with intravital imaging. Microneedles filled with Matrigel and

gradients of either M-CSF or EGF attracted TAMs as well as breast cancer cells in a ratio of 1:4, although cancer cells expressed only the EGF receptor while macrophages only the CSF-1R in this tumor model. This provided evidence for a macrophage co-migration with cancer cells and vice versa [93]. In addition, using the same intravital multiphoton imaging, this research group demonstrated that cancer cells with an invasive behavior predominantly reside in macrophage-rich sites at the tumor margin [94]. As TAMs are the major source of EGF, an *EGF-M-CSF paracrine loop* responsible for co-migration of macrophages and tumor cells has been postulated. In addition, stimulation of the EGF receptor pathway has been shown to induce the formation of invadopodia in cancer cells, thereby stimulating single cell migration [80]. M-CSF, on the other hand, can induce proliferation in tumor cells expressing CSF-1R. An autocrine loop which increases invasiveness of malignant cells has been detected in MDA-MB 231 cells, which express CSF-1R and secrete M-CSF [95].

That macrophages not only promote local invasion of tumor cells, but are also involved in the establishment of distant metastasis, has been underscored by the cancer cell–leukocyte fusion theory. Leukocyte–cancer hybrids have been documented in animal tumors as well as in human neoplasias, which bear the genetic and functional traits of both cell types. As myeloid cells are highly mobile cells with low tissue stringency, a macrophage–tumor cell heterotypic fusion could facilitate immune escape and distant metastasis [96].

Taken together, macrophages are actively involved in tumor cell invasion and metastasis by promoting extracellular matrix degradation, instigation of cancer cell motility, and immune escape.

3.5.3 Immunosuppression, Tumor Growth, and Immune Cell Attraction

In developed tumors, immune reactions against tumor cells are mostly missing. The tumor immune escape is mainly achieved by three different mechanisms: (1) elimination of tumor cells with a high immunogenicity, (2) secretion of immunosuppressive cytokines, (3) attraction of immunosuppressive stroma cells. As tumors have an inherent genetic instability, it is likely that immune-sculpting by tumor cells is a process present at all times during tumor progression [97]. One of the most efficient ways to maintain an immunosuppressive environment is the secretion of tumor-derived soluble factors that supports the development of an M2-like TAM phenotype in the tumor stroma. Such factors include M-CSF and IL-10. M2-like TAMs have a decreased ability to present tumor antigens, which leads to a reduced tumor specific T-cell response [31]. In addition, TAMs show a defective LPS/IFN-γ response with a reduced expression of IL-12 [34]. IL-12 is essential for the differentiation of naïve T-cells to $CD4^+$ Th1 cells. As $CD4^+$ Th1 cells are a major source of IFN-γ, a defect in IL-12 production of TAMs leads to an impaired

cell-mediated immunity in the tumor environment. However, not only the IL-12 production is impaired, but also other proinflammatory cytokines such as TNF-α, IL-6, CCL3, and IL-1β show a defective induction by LPS [36]. In contrast, secreted mediators with immunosuppressive ability as IL-10, TGF-β, prostaglandins, and indoleamine dioxigenase (IDO) have been found to be over-expressed in TAMs [31, 36]. IL-10 has been shown to inhibit the production of pro-inflammatory cytokines and antigen presentation in an autocrine manner [98]. IDO is a tryptophan catabolic enzyme actively involved in T-cell suppression by inducing a local critical tryptophan shortage [99]. This shortage leads to the translation of LIP, the inhibitory isoform of the immune regulatory transcription factor NF-IL6, which alters the expression of central immune mediators important for tumor progression as IL-6, TGF-β, and IL-10 [100]. Immunosuppression by amino acid deprivation is however not only restricted to tryptophan and its catabolic enzyme IDO, but has also been described for arginine and cysteine. Cysteine is an essential amino acid for T-cells as they lack a functional cysteine transporter [101]. Their need for cysteine is supplied by antigen-presenting cells, which are in the possession of a functional cystathionase and x_c-transporter and secrete cysteine in the extracellular space, which than can be efficiently imported by T-cells via their alanine–serine–cysteine transporter [102]. The mechanism of T-cell inactivation by cysteine deprivation is mainly mediated by MDSC, immature precursors of TAMs [103]. An important mechanism of T-cell inactivation used by TAMs, however, is the deprivation of extracellular L-arginine. Arginase and inducible iNOS are two competing enzymes involved in the metabolization of L-arginine. While iNOS in macrophages is up-regulated by Th1-type cytokines as IFN-γ, IL-2 and TNF-α, the induction of arginase is best achieved by Th2 cytokines such as IL-4, IL-13, and IL-10. In late stage tumors, mostly M2-like TAMs with a high activity of arginase are found. Arginase produces urea and ornithine, the latter being important for the generation of diverse polyamines such as putrescine. The production of these polyamines supports tumor cell proliferation and angiogenesis [104]. In addition, a high arginase activity depletes L-arginine from the extracellular space, which is required for a proper T-cell activity. Interestingly, not only T-cells need L-arginine for a proper function. A recent study provided evidence that also the proliferation of some tumor cells depends on the presence of extracellular L-arginine (L-arginine auxotrophic tumors). This opens the door for a new therapeutic approach in L-arginine auxotrophic tumors [105].

In addition to their immunosuppressive activities, TAMs promote tumor development also by the secretion of diverse growth factors and numerous cytokines and chemokines. That TAM infiltration correlates with a higher proliferation rate of neoplastic cells has already been demonstrated for several tumor entities. Apart from the above-mentioned growth factors VEGF and EGF, important for tumor angiogenesis and tumor cell migration, several other growth factors such as platelet-derived growth factor (PDGF), TGF-β, hepatocyte growth factor (HGF), and basic fibroblast growth factor (bFGF) have been described as originating from TAMs [106]. Chemokines derived from TAMs include CCL13, CCL17, CCL18, and CCL22. CCL17 and CCL22 attract mainly Th2-cells and regulatory T-cells

[107], while CCL18 recruits naïve T-cells [108]. By secreting those chemokines, TAMs indirectly contribute to the generation of an immunosuppressive environment. The repertoire of cytokines and chemokines is however immense, and involves also CCL2, CCL5, CXCL9, CXCL10, and CXCL16; some of them contribute not only to cell recruitment, but also to angiogenic processes in the tumor [36]. All these data point to TAMs as key components in the tumor stroma, able to promote nearly every aspect necessary for tumor progression.

3.6 TAM Heterogeneity

Developed tumors can be subdivided into an invasive front, central necrotic and hypoxic areas with lack of vessels, and regions with high as well as low rates of tumor cell proliferation. This results in many distinct tumor microenvironments. As macrophages are highly plastic cells able to change their phenotype in response to different environmental stimuli, it is obvious that these microenvironments influence the development of many dissimilar distinct TAM phenotypes. TAMs have been found to accumulate around vessels, where they secrete chemotactic mediators in order to stimulate intravasation of tumor cells. In hypoxic areas, TAMs express a broad array of pro-angiogenic factors, which stimulate neo-angiogenesis and tumor cell proliferation, while in the periphery of tumors TAMs are involved in matrix degradation supporting tumor cell invasion. Although heterogeneity seem to be the stable trait of TAM differentiation, first attempts to classify TAMs systematically used the concept of M1/M2 polarization, and defined TAMs in developed tumors as rather M2- than M1-differentiated [31, 87]. Later on, the anti-inflammatory M2-like TAM phenotype was further confirmed by gene profiling experiments in murine tumors [36, 77, 109]. In a systematic analysis of mouse mammary carcinoma and lung adenocarcinoma, several subsets of TAMs isolated by CD11b were sub-classified by their differential expression of LY6C, MHC class II molecules, CX3C-chemokine receptor 1 (CX3CR1), CC-chemokine receptor 2 (CCR2), and CD62L. This resulted in distinct subsets that seem to contribute in different ways to promote tumor growth [110].

As no single marker specific to TAMs exists so far, defining clear TAM populations should be based on the use of two or even a set of three surface antigens. Here, the clear description of the Tie2-expressing CD11b positive macrophage subset (TEM) discussed in the above sections with pro-angiogenic functions seems to be the first description of such a TAM sub-entity common to many different tumors. Although many other TAM subpopulations such as stabilin-1^{+}Lyve^{+}CD11b^{+} tumor-infiltrating macrophages in murine melanoma [111] or Ms4a8a^{+}/CD68^{+} TAMs in murine adenocarcinoma [112] have been described so far, TAM classification in the field of macrophage heterogeneity remains almost at an initiative stage [113].

3.7 TAMs as Promising Therapeutic Targets in Cancer Therapy

Therapeutic approaches for cancer have rapidly evolved in the past few years. While originally only standard surgery, chemotherapy, and radiotherapy were available for tumor patients, new targets in cancer therapy include pathologically activated signaling pathways, anti-angiogenetic therapies, and lately immunotherapeutics. The myeloid cell lineage of innate immune systems bears the potential to work as a rational novel therapeutic target, as these cells with their pro-angiogenetic, immune-suppressive and matrix-degrading abilities work as central supporters of tumor progression. Attractive strategies for targeting myeloid cells are: (1) the sequestration of cytokines or chemokines, which either activate the central pathologically regulated signaling pathways in TAMs or are involved in the attraction of immunosuppressive stroma cells, (2) TAM re-education into macrophages with anti-tumor activities, and (3) adoptive transfer of macrophages with anti-cancer properties as drug delivery system for chemotherapeutics [114, 115] (Fig. 3.4).

Sequestration of Cytokines and Chemokines and Inhibition of TAM Recruitment. High serum concentrations of *IL-6* correlate with chemoresistance and reduced survival of ovarian cancer patients [116]. IL-6 induces the Jak/STAT3 pathway in TAMs, thereby fostering their tumor-supporting abilities. Evidence that targeting IL-6 might act on modulation of TAMs in the tumor environment comes from a phase II trial: a good therapeutic response to a chimeric antibody targeting IL-6 correlated with a reduction of CCL2 and CXCL12, two chemokines important for TAM and MDSC recruitment [117]. In addition, a direct blockade of the main TAM attractor in tumors, *CCL2*, was shown to inhibit metastatic seeding of mammary cancer cells [118]. Accordingly, inhibition of CSF-1R signaling by a blocking antibody reduced TAM infiltration [119]. Interestingly, no effect of macrophage recruitment to non-cancer inflammatory environments has been noted with this therapeutic approach, indicating that maturation of TAMs necessitates *M-CSF* signaling, while monocytes with inflammatory functions do not. Results of M-CSF signaling inhibition in humans as a novel therapeutic approach in cancer are expected in the near future. Also, depletion of TAM by using *liposomal chlodronate* prior to radiotherapy in a murine model has proven effective in inhibiting tumor relapse [120]. Un-encapsulated bisphosphonate is a validated therapy of osteoporosis and bone metastasis of diverse tumors in humans, as it inhibits osteoclast functions. Recently, though, it seems that the therapeutic effect of intravenous bisphosphonates in cancer patients goes beyond the inhibition of bone resorption, as in oestrogen-responsive early breast cancer patients the bisphosphonate zolendronic acid improved the disease-free survival [121]. Whether this therapeutic success can be attributed to its effect on TAM modulation has to be evaluated.

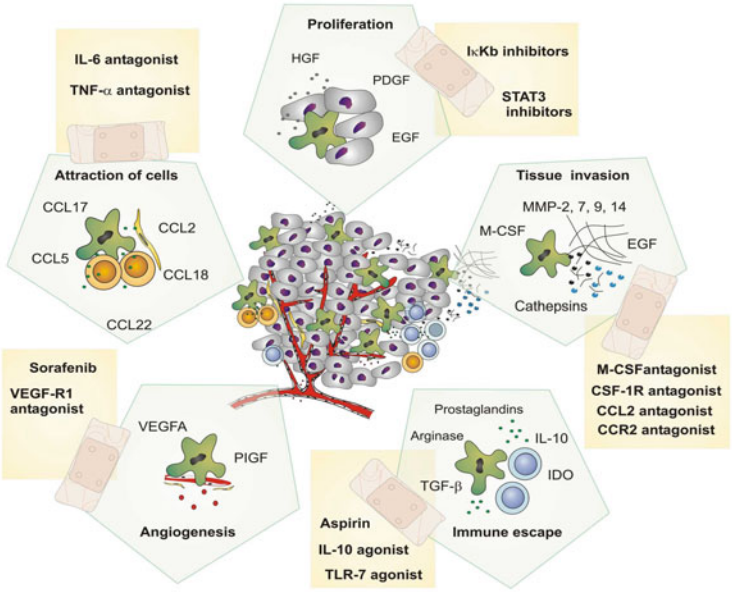

Fig. 3.4 Tumor-associated macrophages as novel targets in cancer therapy. Tissue invasion supported by TAMs can be antagonized by blocking the effect of the M-CSF signaling pathway and by targeting CCL2/CCR2. Immune escape mediated by essential amino acid depletion, by prostaglandins, and by immunosuppressive cytokines such as IL-10 and TGF-β can be attenuated by aspirin, IL-10 antagonists, and a TLR7 agonist, the latter working as an unspecific activator of the innate immune system. Sorafenib and VEGF-R1 inhibit also the angiogenetic abilities of macrophages. Attraction of tumor-supporting immune cells can be inhibited by the application of IL-6 and TNF-α antagonists. STAT3 and NF-κB inhibitors are able to attenuate the secretion of different growth factors by TAMs important for tumor growth

TAM Re-education. As during tumor progression TAMs are hijacked by tumors to develop their M2-like macrophage characteristics, a proposed model to target TAMs is their re-education towards an M1-like phenotype [122]. A dysregulation of the IKKβ/NF-κB pathway is held responsible for the M1/M2 phenotype switch during cancer progression. Accordingly, IKKβ deletion or inhibition has already been proven effective in a colitis-associated tumor model and in a chemically induced hepatocellular cancer model in mice [123]. Such an inhibition of the NF-κB pathway in bone marrow stromal cells has become reality with the introduction of bortezumib, a proteasome inhibitor that among other effects blocks the degradation of the NF-κB-inhibitory protein IkBa [124]. Recently, a therapy targeting CD40 in pancreatic ductal adenocarcinoma and the raf/MEK/ERK signaling inhibitor sorafenib has been shown to generate tumoricidal macrophages able to subvert the immunosuppressive tumor environment [125, 126]. Many other therapeutic agents such as rituximab or imiquimod are in clinical practice, and already showed the ability to induce macrophages with tumoricidal abilities and

improved clinical outcomes of tumor patients [127, 128]. This highlights the validity of the TAM re-education approach.

Adoptive Transfer. As macrophages with tumoricidal abilities have been shown to be effective weapons against some tumor entities, adoptive transfer of such M1 macrophages to the tumor environment is not an insensate approach. An attempt was made in 1974 in mice with metastatic B16 melanoma. Adoptive transfer of thioglycolate-stimulated peritoneal macrophages reduced the incidence of pulmonary metastases. In humans, intraperitoneally delivered macrophages were able to reduce the volume of malignant ascites in patients suffering from ovarian cancer, but unfortunately no effect was seen on the tumor mass [129].

Nevertheless, the idea behind the adoptive transfer of tumor fighting macrophages should be followed, especially as irrespective of their tumoricidal abilities, macrophages could be also used as vectors for anti-cancer cell directed drugs or as a delivery systems for gene therapy [115].

3.8 Conclusion

In conclusion, cancer cells attract macrophages by simulating a wound-healing environment, and use their angiogenetic, tissue remodeling, and immunosuppressive abilities for their own advantage. As macrophages are a large cellular component of tumors, they need to be taken into account when developing immunotherapeutic strategies to combat cancer. This necessitates a profound knowledge of the M1/M2 pathophysiology of these cells. Here, specific care needs to be taken to identify macrophage differences between humans and mice, as many M1- or M2 macrophage markers identified on mouse macrophages are not present on the human counterpart. A lot of research work needs still to be done in this regard; it will, however, be exciting to follow the future developments in this immunological field.

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Chapter 4
Cancer Immunotherapy via Dendritic Cells

Karolina Palucka and Jacques Banchereau

Abstract Owing to their properties, dendritic cells (DCs) are often called "nature's adjuvants," and thus have become the natural targets for antigen delivery. DCs provide an essential link between the innate and the adaptive arms of the immune system. DCs control both tolerance and immunity. Therefore, DCs are key targets for both preventive and therapeutic vaccination. Herein, we will discuss the physiology of DCs as it applies to immunotherapy of cancer.

Contents

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4.1 Introduction

The immune system has the potential to eliminate neoplastic cells. Perhaps the most compelling evidence of tumor immunosurveillance in humans is provided by paraneoplastic diseases which link neurological disorders to an anti-tumor response [1]. Onconeural antigens, normally expressed on neurons, can also be expressed in breast cancer cells [2]. Some patients develop a strong antigen-specific $CD8^+$ T cell response which controls cancer but concomitantly results in autoimmune cerebellar degeneration, causing a severe neurologic disease [3, 4].

The adoptive transfer of cancer antigen-specific effector T cells in patients can result in rejection of established tumors, thereby illustrating the potential of tumor immunotherapy [5]. Ideally, one would like to directly induce efficient tumor-specific T cells through vaccination, including effector T cells able to reject tumors, and memory T cells able to prevent tumor relapse. DCs are essential in generation of immune responses, and as such represent targets and vectors for vaccination. Furthermore, murine models demonstrate that the generation of protective anti-tumor immunity depends on the presentation of tumor antigens (Ags) by DCs [6, 7]. There, DCs can capture tumor antigens released from tumor cells, either alive or dying, and cross-present these antigens to T cells in tumor,draining lymph nodes. This antigen presentation results in the generation of tumor-specific effector T cells that contribute to tumor rejection [6, 7]. Thus, DCs represent important targets for therapeutic interventions in cancer.

Preventive vaccines are designed to initiate protective humoral immune responses. Today, more than 70 preventive vaccines have been licensed for use against approximately 30 microbes, sparing countless lives [8]. However, effective vaccines remain elusive for diseases such as human immunodeficiency virus (HIV)-induced acquired immune deficiency syndrome, plasmodium-induced malaria, virus-induced hepatitis C, and *Mycobacterium*-induced tuberculosis, to cite a few [8]. These require therapeutic vaccines able to elicit strong cellular immunity, in particular in cytotoxic T cells, which is necessary to eliminate existing disease, i.e., the cells that are infected with the causative agent and/or malignant cells (Fig. 4.1). Here, we will discuss the biology of human DC subsets in the context of vaccination.

4.2 The Biology of DC Subsets

4.2.1 Basics of DC Biology

DCs are a rare cell type that was discovered by Ralph Steinman in 1973. After four decades of research, it is now clear that DCs are at the center of the immune system through their ability to control both tolerance and immunity [9, 10]. DCs are bone-marrow-derived cells that seed all tissues (reviewed in [10]). They are poised to

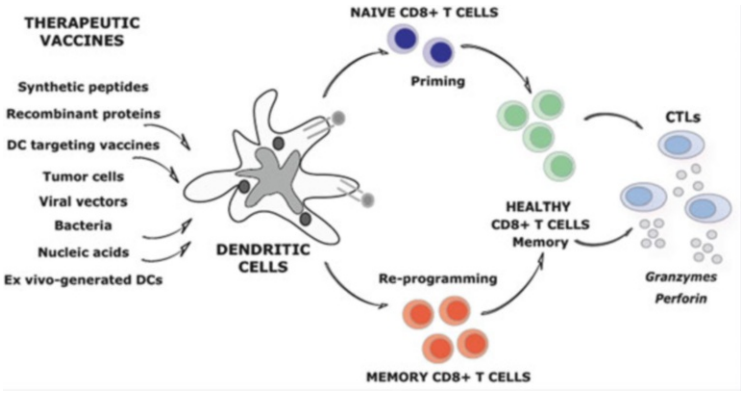

Fig. 4.1 Therapeutic vaccines. Therapeutic vaccines are designed to elicit cellular immunity, that is to say, prime new T cells, as well as induce a transition from chronically activated non-protective $CD8^+$ T cells to healthy $CD8^+$ T cells. The features of healthy $CD8^+$ T cells include their ability to generate cytotoxic T lymphocytes (CTLs) that reject cancer and to provide long-lived memory $CD8^+$ T cells able to rapidly generate new effector T cells secreting cytotoxic molecules thereby preventing relapse. Numerous approaches to therapeutic vaccines are being pursued. Their common denominator is the action via DCs

sample the environment and transmit the gathered information to cells of the adaptive immune system, i.e., T cells and B cells [9, 10]. In peripheral tissues, DCs capture Ags through several complementary mechanisms. DCs launch the immune response by presenting the captured Ag in the form of peptide-MHC complexes to naïve, i.e., antigen-inexperienced T cells in lymphoid tissues. Upon interaction with DCs, naïve $CD4^+$ and $CD8^+$ T cells differentiate into antigen-specific memory T cells with different functions. $CD4^+$ T cells can become Th1, Th2, Th17, and T follicular helper (Tfh) cells that help B cells differentiate into antibody-secreting cells, as well as regulatory T cells (Tregs) that downregulate the functions of other lymphocytes. Naïve $CD8^+$ T cells can give rise to cytotoxic effector lymphocytes (CTLs). The plasticity of DCs in response to extrinsic signals, and the existence of distinct DC subsets with distinct functions, contribute to the mounting of highly diverse immune responses. DCs are essential in pathogen resistance including different viruses, bacteria, and parasites, as demonstrated using DC-depleted mice [11].

In the steady state, non-activated (immature) DCs present self-antigens to T cells, thereby leading to tolerance through either T-cell deletion or differentiation of regulatory/suppressor T cells. These immature DCs have special characteristics, including: (1) the ability to efficiently capture antigens, (2) accumulation of MHC class II molecules in the late endosome–lysosomal compartment, (3) low levels of costimulatory molecule expression, (4) a unique set of chemokine receptors that allow their migration to lymphoid tissues (e.g., CCR7), and (5) a limited capacity to secrete cytokines [12]. In contrast, mature antigen-loaded DCs can launch the differentiation of antigen-specific T cells into effector cells with unique functions

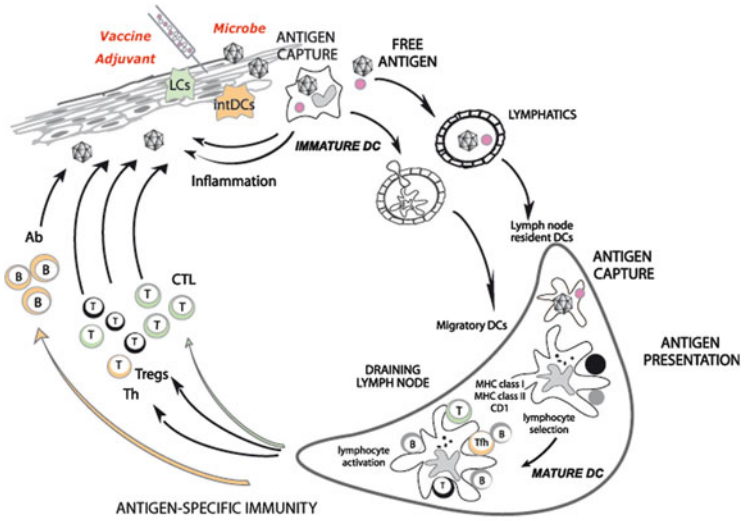

Fig. 4.2 Launching of the immune response. Antigen(s) can reach lymph nodes via two pathways: one pathway is via lymphatics where the antigen is captured by lymph-node resident DCs. The other pathway is mediated by tissue resident DCs. There, immature DCs capture antigens; tissue- and/or pathogen-derived signals trigger DC activation, their migration towards secondary lymphoid organs and simultaneous maturation. DCs display antigens in the context of classical MHC class I and class II or non-classical CD1 molecules, which allow selection of rare antigen-specific T lymphocytes. Activated T cells drive DCs towards their terminal maturation, which induces further expansion and differentiation of lymphocytes into effector cells. If DCs do not receive maturation signals, they will remain immature, and antigen presentation will lead to immune regulation and/or suppression

and cytokine profiles (Fig. 4.2). Indeed, immature DCs promptly respond to environmental signals, and differentiate into mature DCs. DC maturation is associated with (1) the down-regulation of antigen-capture activity, (2) the increased expression of surface MHC class II molecules and costimulatory molecules, (3) the ability to secrete cytokines [12], and (4) the acquisition of CCR7, which allows migration of the DC into the draining lymph node [12]. However, DC maturation does not result in a unique phenotype. Rather, in response to different signals that are provided by different microbes either directly or through the surrounding cells, DCs acquire distinct phenotypes that eventually contribute to diverse immune responses. In addition to cytokines or direct microbial signals, the ligation of CD40 represents an essential signal for the differentiation into fully mature DCs able to launch adaptive T cell immunity [13].

4.2.2 Basics of DC Subsets

Human blood DC subsets can be distinguished by the differential expression of three surface molecules: CD303 (BDCA-2), CD1c (BDCA-1), and CD141 (BDCA3). $CD303^+$ pDCs represent a front line of anti-viral immunity through their ability to secrete large amounts of type I interferon (IFN) in response to virus encounter [14]. Their pre-synthesized stores of MHC class I may permit a rapid initial $CD8^+$ T cell response to viral infections [15]. pDCs-derived type I IFN may promote the immunogenic maturation of other DC populations [16], thereby helping in the activation of novel T-cell clones. In their resting state, pDCs are considered as playing an important role in tolerance, including oral tolerance [16].

Human $CD141^+CD1c^-$ DCs uniquely express Toll-like receptor (TLR) 3, produce interleukin (IL) 12, and efficiently cross-prime $CD8^+$ T cells when activated with poly I:C [17–23]. However, other human DCs such as epidermal Langerhans cells (LCs) [24, 25] and $CD1c^+$ DCs also cross-present antigens to $CD8^+$ T cells [19, 21, 22].

The human skin hosts epidermal LCs and dermal interstitial DCs (dermal DCs). The dermal DCs can be further subdivided into $CD1a^+$ DCs and $CD14^+$ DCs. Earlier studies of human cutaneous DCs demonstrated their phenotypic and functional heterogeneity with regard to cellular immunity and priming of highly efficient CTLs [26]. Our studies concluded that human $CD14^+$ DCs can directly help activated B cells as well as induce naïve T cells to differentiate into cells with properties of T follicular helper cells (Tfh) [24]. They may thus be specialized for the development of humoral responses [24]. On the contrary, LCs are more efficient in cross-presenting peptides from protein antigens to $CD8^+$ T cells and prime $CD8^+$ T cells into potent CTLs.

The DC subsets that sit in tissues under the steady state are dependent upon fms-related tyrosine kinase 3 (FLT3) and macrophage colony-stimulating factor receptor (MCSF-R). However, inflammatory processes such as those initiated by microbial invasion, or vaccine adjuvant, substantially alter the DC compartments. The origin of DCs that are recruited to inflammation sites is still under investigation, though it is clear that monocytes give rise to inflammatory DCs in vivo [27]. DCs express numerous non-clonal recognition receptors, including lectins, TLRs, NOD-like receptors (NLRs), and helicases through which they can sense microbes and microbial products such as, for example, nucleic acids, thereby allowing the launching of protective type I interferon production [28, 29]. Indeed the experimental adjuvants CpG and imiquimod bind to TLR9 and TLR7/8 respectively [29]. Most recently biochemical approaches revealed novel sensors of nucleic acid function from the DExD/H-box helicase family molecules in DCs [30, 31].

4.2.3 Human DC Subsets and Humoral Immune Responses

T helper (Th) subsets, specialized for promoting particular types of immune responses and eventually inflammations, function through their secretion of cytokines, enabling unique immune responses (reviewed in [32]). Among those, T follicular helper (Tfh) cells help B cells to differentiate into antibody-secreting cells and govern the germinal center reaction, the main site of immunoglobulin somatic mutation and isotype switching [33, 34]. Human blood $CXCR5^+CD4^+$ T cells represent circulating memory Tfh cells. Blood $CXCR5^+CD4^+$ T cells comprise three subsets: Tfh1, Tfh2, and Tfh17 cells. Tfh2 and Tfh17 cells efficiently induced naive B cells to produce immunoglobulins via IL-21 [35]. In contrast, Tfh1 cells have been found to lack the capacity to help naïve B cells [35]. In-vitro studies demonstrated that Tfh development is regulated by a specific DC subset, interstitial $CD14^+$ DCs [24]. This requires IL-12 both in vitro [36] and in vivo, as IL-12Rβ1 deficient humans have displayed substantially less circulating memory Tfh and memory B cells than control subjects [37]. Importantly in the context of vaccination, expansion of Tfh1 cells at day 7 correlates with protective antibody titers at day 28 after influenza vaccination in healthy adults and children [38]. Whether the induction of Tfh differentiation depends on the same mechanisms in mice remains to be established.

4.2.4 Human DC Subsets and Cellular Immune Responses

$CD8^+$ T cells recognize peptide-MHC (pMHC) class I molecules expressed by DC, and develop into CTLs able to kill cells presenting a specific pMHC complex [10]. The ideal properties of vaccine-elicited $CD8^+$ T cells include: (1) high avidity for pMHC on tumor cells, (2) high levels of granzyme and perforin, molecules essential for cytotoxic activity against cancer/infected cells, (3) expression of surface molecules allowing trafficking into the tumor; and (4) resistance to regulatory mechanisms present in the tumor [24, 39]. At least four components of the immune response are necessary for that ideal response to happen: (1) the presence of antigen presenting DCs, (2) the quality of induced $CD4^+$ helper T cells [40], (3) the elimination of Tregs which can inhibit CTLs via the secretion of various cytokines including transforming growth factor β (TGF-β), and can compete with $CD8^+$ T cells for IL-2 via constitutive expression of CD25 [41], and (4) the breakdown of the immunosuppressive tumor microenvironment. As discussed earlier, our studies with human Langerhans cells and interstitial DCs showed their specialization in priming $CD8^+$ T cell immunity and humoral immunity respectively. Skin LC efficiency in priming naïve $CD8^+$ T can be at least partially explained by their surface expression of IL-15 [42, 43] and/or upregulation of CD70 upon viral exposure [44]. Furthermore, interstitial DCs play a major role in generation of suppressor $CD8^+$ T cells [45]. Recent studies have further analyzed lymph-node-resident and skin-migratory DC subsets in the human [23, 46]. Both $CD1c^+$ and

CLEC9A-expressing CD141$^+$ DCs isolated from human lymph nodes were able to cross-present long peptides (requiring processing) of melanoma-tissue-derived antigen (MART-1) to T cell lines [46], whereas blood DCs can cross-present when activated via Toll-like receptor ligands [18, 19].

Antigen-specific CTLs must traffic into the tumor bed, an area that is not clearly understood [39]. A dysregulation of chemokine homeostasis might prevent the CD8$^+$ T cells to enter the tumor bed. Tumors might actively repulse CD8$^+$ T cell [47]. Finally, the tumor-infiltrating myeloid-derived suppressor cells [48, 49] might inhibit effector CD8$^+$ T cell functions. The negative cues of the tumor environment can be counteracted by a series of therapeutic modalities. These include antibodies neutralizing cytokines such as IL-10, IL-13, and TGF-β [50]. Antibodies such as anti-CTLA-4 and anti-programmed cell death protein-1 ligand 1 (PD-L1) which block the immune-inhibitory signals in lymphocytes can also be used in combinations with cancer vaccines to off-set regulatory mechanisms [40]. The roles of DCs in the regulation of tumor microenvironment are discussed later.

4.3 Cancer Immunotherapy via DCs

4.3.1 Vaccination via DCs

DCs can be engaged in the action of therapeutic vaccines in a number of ways, including indirect involvement as, for example, with GVAX [51] or listeria-based vaccines [52] to name a few (Fig. 4.3). DC can also be used directly following their generation ex vivo and injection to patients (reviewed in [53]). These studies concluded that DC-based vaccines are safe and can induce the expansion of circulating CD4$^+$ and CD8$^+$ T cells that are specific for tumor Ags [53–56]. Objective clinical responses have been observed in some patients. A recent study focused on optimizing vaccine immunogenicity, and demonstrated in phase I/II clinical trials that provision of MHC class II epitopes from defined melanoma tumor antigens results in improved immunogenicity [57]. Furthermore, novel approaches are being developed, including the pre-operative vaccination of patients with her2$^+$ breast cancer [58] as well as combination therapies in ovarian cancer utilizing autologous DC vaccines and adoptive T-cell transfer to enhance vaccine efficacy [59]. More recent studies have utilized pDCs as cell-based vaccines [60]. Patients with metastatic melanoma received intranodal injections of activated and tumor antigen-associated peptide loaded-pDCs. Several patients mounted vaccine antigen-specific CD4$^+$ and CD8$^+$ T-cell responses. Despite the limited number of administered pDCs, an IFN signature was observed after each vaccination [60]. Whereas the clinical efficacy of elicited immunity will need to be determined in larger cohorts and long-term follow-up, type IFN response is highly desirable in melanoma [7, 61].

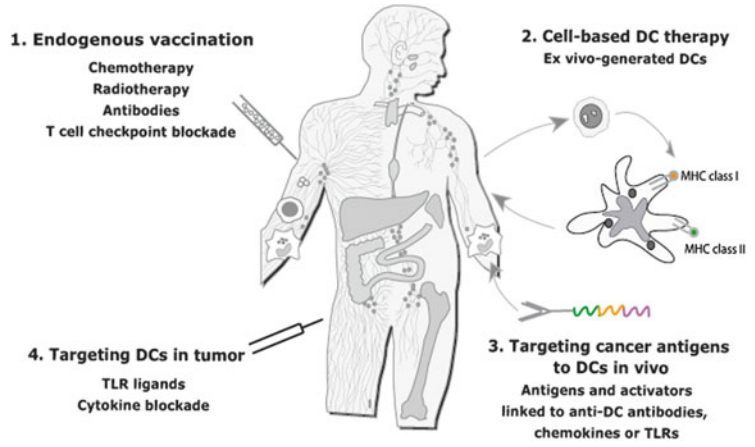

Fig. 4.3 DCs and cancer immunotherapy. DCs can be exploited for cancer immunotherapy in numerous ways including: (1) their random targeting in "endogenous" vaccination resulting from in-vivo antigen release in a process of immunogenic cell death in response to chemotherapy, radiotherapy as well as immune modulation approaches targeted at T cells, (2) vaccines based on ex-vivo generated tumor antigen-loaded DCs that are injected back into patients, (3) vaccines based on specific in-vivo DC targeting with anti-DC antibodies fused with antigens and with DC activators, and (4) targeting DCs in the tumor environment to reprogram pro-tumor inflammation towards tumor rejection

4.3.2 Vaccination via DCs: In Vivo DC Targeting

Following the pioneering studies from Ralph Steinman and Michel Nussenzweig labs with anti-DEC 205 antibodies [62–64], numerous studies performed in mouse models and in human in vitro systems have demonstrated the efficacy of targeting DCs [10]. Most particularly, targeting antigens through the DC surface lectins DCIR [25, 65], DC-SIGN [66], Dectin [67], Clec9A [68], and Langerin [69] results in humoral and/or cellular $CD4^+$ and/or $CD8^+$ T-cell responses. In the absence of adjuvants, targeting $DEC205^+$ DCs in vivo can induce tolerance [62]. Provision of adjuvants such as TLR3 or TLR7/8 agonists or DC activation signal via CD40 enables the concomitant maturation of vaccine-engulfing DCs [70]. Furthermore, targeting different DC receptors generates quantitatively and qualitatively different immune responses [64, 71]. Injection of antigens coupled to antibodies against DC surface molecule Clec9A results in production of strong antibody responses, even without co-administration of adjuvants [72]. That happens via antigen presentation by DC on MHC class II and consequent Tfh expansion [73]. These results in the mouse are in line with prior studies showing the essential role of DCs in the generation of antibody responses, and show that these can be amplified by targeting antigen to DC surface receptors in vivo. Importantly, CLEC9a is also a receptor for necrotic cells, and has been shown to facilitate cross-presentation [74]. As opposed to antibody response, CLEC9A-dependent generation of $CD8^+$ T-cell responses

requires adjuvant. Generation of different responses by targeting distinct DC receptors is further exemplified by recent studies targeting DC-asialoglycoprotein receptor (DC-ASGPR), a lectin-like receptor, which is a known scavenger receptor. Targeting antigens to human DCs via DC-ASGPR in vitro but not lectin-like oxidized-low density lipoprotein (LDL) receptor, Dectin-1, or DC-specific ICAM-3-grabbing non-integrin favored the generation of antigen-specific suppressive $CD4^+$ T cells that produce IL-10 [75]. Furthermore, comparing the cross-presentation of identical antigens conjugated with antibodies against different DC receptors that are targeted to early or late endosomes at distinct efficiencies revealed remarkable differences [76]. Thus, in human $BDCA1^+$ and monocyte-derived DCs, CD40 and mannose receptor targeted antibody conjugates to early endosomes, whereas DEC205 targeted antigen primarily to late compartments. Surprisingly, the receptor least efficient at internalization, CD40, was the most efficient at cross-presentation. This did not reflect DC activation by CD40, but rather its relatively poor uptake or intra-endosomal degradation compared with mannose receptor or DEC205 [76]. DC targeting-based vaccination studies in non-human primates demonstrated robust T-cell immunity in prime-boost design with HIV gag-DEC205 targeting vaccine [77]. Early clinical trials in the human analyzed the delivery of gonadotropin [hCG-b]) to antigen-presenting cells (APCs) by antibody-mediated targeting of a mannose receptor [78]. Delivery of this product with granulocyte–macrophage colony-stimulating factor (GM-CSF) and TLR3/TLR7/8 agonists induced consistent humoral and cellular immune responses to hCG-b [78]. Several studies are ongoing testing the immune efficacy of HIV antigens or NY-ESO1 cancer antigen targeted via DEC-205 in healthy individuals and in cancer patients (clinicaltrials.gov).

4.3.3 Modulating DCs in Tumor Environment

Another approach to immunotherapy via DCs is focused on exploiting DCs in the tumor microenvironment (Fig. 4.3). Indeed, DCs are found in most tumors in humans and mice. DCs can sample tumor antigens through the capture of dying tumor cells and through the nibbling of live tumor cells (reviewed in [79]). Tumors can prevent antigen presentation and the establishment of tumor-specific immunity through a variety of mechanisms. By converting immature DCs into macrophages, i.e., through IL-6 and M-CSF, tumors can prevent the priming of tumor-specific T cells [80, 81]. Alternatively, the tumor glycoproteins carcinoembryonic antigen (CEA) and MUC-1 that are endocytosed by DCs stay confined in the early endosomes, therefore preventing efficient processing and presentation to T cells [82].

Tumors can inhibit DC maturation, for example through the secretion of IL-10, leading to Ag-specific anergy [83]. Tumor-derived factors can alter DCs maturation so as to yield cells that indirectly help tumor growth (“pro-tumor” inflammation) [84]. As an example, we have shown that thymic stromal limphopoietin (TSLP) that

is produced by tumor cells, induces DCs to express OX40L that directs the generation of Th2 cells [85, 86]. These skewed $CD4^+$ T cells accelerate breast tumor development through the secretion of IL-4/IL-13 [85, 86]. These cytokines prevent tumor cell apoptosis and promote the proliferation of cancer cells indirectly by stimulating tumor-associated macrophages to secrete EGF [87]. A similar pathway operates in pancreatic cancer [88].

pDCs that are infiltrating breast carcinomas produce little type I interferon upon TLR ligation [89]. These pDCs induce naïve $CD4^+$T cells to differentiate into IL-10-producing T cells with suppressive functions. Such inhibition of type I interferon secretion might also impact the generation of effector T cells, as DCs require type I interferon signals to cross-present tumor antigens [6, 7]. Whether this mechanism explains why pDC are associated to poor prognosis [90] remains to be determined. Finally, DCs can have direct pro-tumor effects. In multiple myeloma mDCs directly promote the survival and clonogenicity of tumor cells [91]. In ovarian cancer, pDCs contribute to tumor angiogenesis by secreting pro-angiogenic cytokines [92]. Thus, understanding the functions of DCs in the tumor bed might represent a rich field of investigation. Ultimately, rewiring the "pro-tumor" DCs into "anti-tumor" DCs might represent a novel approach for cancer immunotherapy.

4.4 Final Remarks

Immunotherapy is moving to the forefront of cancer therapy, owing to recent progresses in the field. For example, an antibody that blocks the function of CTLA-4, a molecule providing negative regulatory feedback in T-cell activation, has been approved in 2011 by the Food and Drug Administration (FDA) for the treatment of melanoma [93]. The classical cancer therapies that are based on chemotherapy might in fact be effective through the engagement of the immune system. For example, chemotherapeutic agents such as anthracyclines and oxaliplatin induce cancer cells to undergo apoptosis, which is associated with cell surface exposure of calreticulin. Surface calreticulin might contribute to the capture of apoptotic bodies by DCs and the elicitation of tumor-specific $CD8^+$ T cell immunity. These T cells might contribute to the elimination of the tumor cells [94] that have not responded to the chemotherapy. There is now strong evidence that antibody therapy with agents such as anti-CD20 and anti-HER 2 involve the adaptive immune system beyond the elicitation of antibody-dependent cytotoxicity (ADCC). Indeed, antibodies against Her-2 can enhance cross-presentation of tumor antigens, most likely by DCs, leading to the break of tolerance against this antigen [95]. Accordingly, patients responding to trastuzumab (Herceptin) show enhanced $CD8^+$ T cell immunity to Her-2 [95].

Nearly 40 years after their discovery, the importance that DCs have achieved in physiology and medicine has been recognized by the award to Ralph Steinman of the Nobel Prize for Medicine and Physiology. The new knowledge represents a

fertile ground to work on to design better strategies for intervening in numerous clinical situations. The capacity of LCs and $CD14^+$ DCs to preferentially prime cellular immunity and humoral immunity respectively has significant implications, most particularly in the context of novel human vaccines. Thus, DCs are moving to the forefront of cancer immunotherapy.

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Chapter 5
Myeloid-Derived Suppressor Cells and Tumor Growth

Lizzia Raffaghello and Giovanna Bianchi

Abstract The tumour microenvironment is a heterogeneous and complex environment, characterized by the presence of malignant cells and non-neoplastic cellular elements, including immune and stromal cells, as well as blood vessels. Tumour progression is associated with profound alteration of myelopoiesis, which gives origin to myeloid-derived suppressor cells (MDSCs) from immature myeloid progenitors. MDSCs accumulate in the blood, secondary lymphoid organs, bone marrow, and at tumour sites, as has been observed in different cancer patients and experimental tumour models in response to pro-inflammatory cytokines and growth factors released by tumour. Upon recruitment, MDSCs exert various immunosuppressive effects to block innate and adaptive anti-tumour responses. This chapter reviews the origin and features of MDSCs, as well as the immunosuppressive mechanisms used by these cells in order to promote tumour progression.

List of Abbreviations

APC	Antigen-presenting cells
ARG1	Arginase 1
ATRA	All-*trans* retinoic acid
BCL-XL	B cell lymphoma XL
BM	Bone marrow
C/EBPβ	CCAAT/enhancer-binding protein-β
COX2	Cyclooxygenase 2
DCs	Dendritic cells
ERK	Extracellular-signal-regulated kinases
G-CSF	Granulocyte CSF

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GM-CSF	Granulocyte/macrophage colony-stimulating factor
G-MDSCs	Granulocyte MDSCs
iDCs	Immature dendritic cells
IFN-γ	Interferon-γ
IL	Interleukin
IMC	Immature myeloid cells
iNOS	Inducible nitric oxide synthase
JAK-1	Janus kinase-1
M-CSF	Macrophage CSF
MDSCs	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
M-MDSCs	Monocyte-MDSCs
MMP9	Metalloproteinase 9
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor-κB
NK	Natural killer
NKT	T natural killer
NO	Nitric oxide
PB	Peripheral blood
PDE5	Phosphodiesterase 5
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PMNs	Polymorphonuclear neutrophils
ROS	Reactive oxygen species
SCF	Stem cell factor
STAT 3	Transcriptional factor signal transducer and activator of transcription 3
TAMs	Tumour-associated macrophages
TDSF	Tumour-derived soluble factors
TGF-β	Transforming growth factor-β
TLRs	Toll-like receptors
Treg	Regulatory T cells
VEGF	Vascular endothelial growth factor

Contents

5.1 Introduction

Established tumours are heterogeneous and complex masses characterized by the presence of malignant proliferating cells and non-transformed cellular elements including stromal cells, blood vessels, and inflammatory cells [1–3]. There is now considerable evidence that non-neoplastic cells present in the tumour microenvironment functionally interact with tumour cells, and in this way promote tumour progression and metastasis [4]. Various cells belonging to the adaptive immune system such as T and B lymphocytes, and to the innate immune system including macrophages, dendritic cells (DCs), polymorphonuclear neutrophils (PMNs), natural killer (NK) cells, eosinophils, mast cells, and myeloid-derived suppressor cells (MDSCs), have been identified [5].

Unlike cells found in secondary lymphoid organs, T lymphocytes present in tumours are often disregulated and unable to mount specific responses against neoplastic cells [3, 6]. This latter effect is due to immunosuppressive molecules released, such as transforming growth factor-β (TGF-β) and interleukin-10 (IL-10), and proteins expressed (galectin-1 and indoleamine-2,3-dioxigenase: IDO) by the tumour itself [7–9]. In addition, malignant cells may inhibit the development of fully differentiated immune cells, and participate in the generation of immature non-functional immune cells such as immature dendritic cells (iDCs). With regard to different factors released by tumour cells such as vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), IL-10, and macrophage-colony stem factor (M-CSF), they activate the transcriptional factor signal transducer and activator of transcription (STAT) 3 and consequently inhibit maturation of DCs [10, 11]. Additional immunosuppressive immune cells found in tumours include regulatory T cells (Tregs) expressing CD4, CD25, and Foxp3 markers, T natural killer (NKT), and MDSCs.

Treg have been demonstrated to inhibit antitumor immune responses through different mechanisms, including: (1) secretion of TGF-β and IL-10, which inhibit antitumour effector responses promoted by $CD4^+$, $CD8^+$ and NK cells, (2) metabolic disruption through deprivation of cytokines such as IL-2, or generation of immunosuppressive adenosine by the ectoenzymes CD39 and CD73, (3) inhibition of DC maturation and functions, and (4) induction of cytolysis of $CD8^+$ lymphocytes by granzymes A or B and perforin [12]. Several studies of mice and human models revealed higher number of Treg both in the periphery and within tumours of different histology and demonstrated that depletion of Treg significantly improved antitumoural immunity [13–15].

The NKT represent a immune cell population involved in different diseases such as autoimmune disorders, infections, and cancer. NKT express an invariant α/β TCR $\alpha_{24}\beta_{11}$ which recognizes glycolipids associated to CD1d molecule. Upon

direct or indirect activation by DCs, the NKT secrete Th1 and Th2 cytokines including interferon-γ (IFN-γ), IL-3, and IL-14, and are involved in immunosuppressive cell recruitment [16]. An additional immunosuppressive effect mediated by tumour cells is represented by alterations of myeloid cell differentiation. As a result, normal pathways involved in the generation of DCs, granulocytes, and macrophages are blocked, and/or the development of monocyte MDSCs (M-MDSCs), granulocyte MDSCs (G-MDSCs), suppressive DCs and tumour-associated macrophages (TAMs) is promoted [10, 17].

The MDSCs represent a heterogeneous population of cells of myeloid origin that are expanded and activated in response to growth factors and cytokines released by tumours. Once MDSCs are activated, they accumulate in lymphoid organs and tumours, where they exert T-cell immunosuppression. In the following section we will discuss the origin, the functions, and the mechanisms of action of MDSCs, as well as the strategies to target these cells for the therapeutic benefit of cancer patients.

5.2 Origin and Features of MDSCs

The bone marrow (BM) represents the site in which myelopoiesis takes place under the control of different soluble factors such as cytokines, IL-3, and growth factors: granulocyte/macrophage colony-stimulating factor (GM-CSF), macrophage CSF (M-CSF), and stem cell factor (SCF). In particular, hematopoietic stem cells give origin to common myeloid progenitors from which immature myeloid cells (IMC) are generated [10]. In healthy individuals, IMCs migrate to peripheral organs and differentiate into mature granulocytes, DCs, and macrophages. In contrast, under pathological conditions such as cancer, infections, trauma, or sepsis, specific factors inhibit IMC differentiation into mature myeloid cells, and stimulate MDSC expansion and activation [10]. Tumour-bearing mice represent suitable models where it's possible to identify and isolate MDSCs that preferentially accumulate in the BM, spleen, and peripheral blood, and to a lesser extent in lymph nodes [18, 19]. In contrast, in cancer patients MDSCs have been preferentially found in peripheral blood (PB) and tumours [10].

The soluble factors involved in expansion and activation of MDSCs can be divided into two main groups. The first group includes molecules primarily produced by tumour cells that mediate MDSC expansion through stimulation of myelopoiesis. These factors include VEGF, SCF, GM-CSF, granulocyte CSF (G-CSF), M-CSF, gangliosides, prostaglandins, IL-6, IL-10, IL-12, metalloproteinase 9 (MMP9), and CCL2 [10, 20, 21]. Most of these factors converge on the activation of the STAT3 that has a crucial role in the following processes: (1) MDSC expansion, (2) contribution of MDSCs to angiogenesis, (3) MDSC accumulation in cancer patients, and (4) MDSC immune suppressive activity [22]. The second group of soluble factors, implicated in MDSC activation, is produced by tumour stromal cells and activated T cells. These factors, including IFN-γ, ligands for Toll-like receptors (TLRs), IL-4, IL-13, and TGF-β, are responsible for activation of different transcription factors such as STAT6, STAT1, and

nuclear factor-κB (NFκB) [10]. It is noteworthy that MDSCs acquire immunosuppressive activity only after their activation.

The MDSCs have been identified in the spleen of tumour-bearing mice on the basis of their expression of two specific markers, i.e., CD11b and Gr1, and also their ability to inhibit $CD8^+$ T lymphocyte activation through different mechanisms [23]. Murine MDSCs are also $F4/80^{int}$ $CD11c^{low}MHC\text{-}II^{low}$. In addition, some markers, including IL-4Rα, the receptor for M-CSF, and the co-stimulatory molecule CD80, have been used in order to identify an immunosuppressive MDSC fraction [24–26]. However, these latter molecules are strictly related to the tumour model used and cannot be used as general markers for MDSC identification.

More recently, different groups have shown that the antibody against Gr1 made it possible to distinguish two different MDSC fractions based on their intensity of Gr1 expression, i.e., $Gr1^{high}$ which express Gr1 at high intensity and prevalently constituted by granulocytes, and $Gr1^{low}$ with a low intensity mainly characterized by monocytes and other myeloid immature cells [24, 27]. In addition, the antibody against Gr1 molecule binds two different molecules belonging to the Ly6 superfamily, Ly6G and Ly6C, which reside on the surface on granulocytes and monocytes respectively [18, 28]. On these bases, two major classes of MDSCs, i.e., G-MDSCs, consisting of $CD11b^+$ $Ly6G^{high}$ $Ly6C^{low}$, and M-MDSCs, which are $CD11b^+$ $Ly6G^{low}$ $Ly6C^{high}$, have been identified in the spleen of tumour-bearing mice. In most tumour models, the G-MDSC subset is the predominant, representing almost 70–80 % of tumour-derived MDSCs [18, 28]. While G-MDSCs produce high levels of reactive oxygen species (ROS) and low levels of nitric oxide (NO), due to the increased activity of STAT3 and NADPH oxidase, M-MDSC subset has up-regulated expression of STAT1 and inducible nitric oxide synthase (iNOS) with consequent high levels of NO and low concentrations of ROS [18, 28].

More recent studies have described a novel immunomagnetic method which made it possible to separate MDSCs into three fractions, i.e., $Gr1^{low}$, $Gr1^{int}$, and $Gr1^{high}$ [29]. The fraction constituted by $Gr1^{high}$ characterized by granulocytes $Ly6G^{high}$ was shown to possess a weak immunosuppressive effect on the response mediated by allogeneic or antigen-specific T lymphocytes [29]. In contrast, the fractions $Gr1^{low}$ and $Gr1^{int}$, composed of monocytes and immature myeloid cells with a ring shape nucleus respectively, were highly immunosuppressive [29]. Similarly to the splenic M-MDSCs and G-MDSCs, analogous subsets have been also identified in tumour infiltrates. In two different tumour models, more than 90 % were M-MDSCs $CD11b^+Gr1^{low}F4/80^+IL4\text{-}R\alpha^+CCR2^+CX_3CR1^+$, and the remainder were G-MDSCs $Gr1^{high}F4/80^{low}$ [30].

5.3 Mechanisms of MDSC Immunosuppressive Activity

MDSCs suppress multiple effectors of adaptive and innate immunity (Fig. 5.1) [17]. In particular, MDSCs have been shown to:

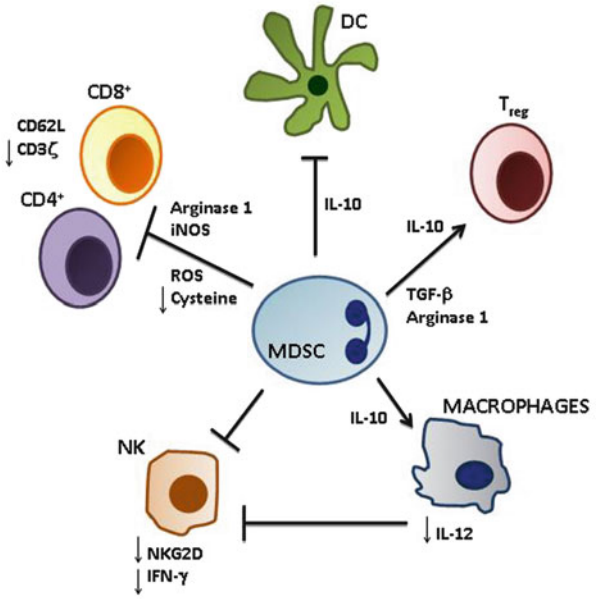

Fig. 5.1 Immunosuppressive mechanisms mediated by myeloid-derived suppressor cells. $CD4^+$ and $CD8^+$ T cell activation is inhibited by arginase 1, inducible nitric oxide synthase (iNOS), generation of reactive oxygen species (ROS) and cysteine deprivation, and induction of T regulatory cells (Tregs) is mediated by IL-10 and transforming growth factor-β (TGF-β). Innate immunity is suppressed by down-regulation of dendritic cell (DC), production of IL-12 by macrophages, and by inhibition of natural killer (NK) cell cytotoxicity. Myeloid-derived suppressor cells (MDSCs) produce a high amount of IL-10 that induces Treg and Th2 cells and inhibits IL-12 production

1. Inhibit $CD4^+$ and $CD8^+$ activation and proliferation in a major histocompatibility complex (MHC) restricted or unrestricted and antigen-specific manner [31, 32]
2. Indirectly affect T-cell activation by inducing Treg expansion thanks to the production of IL-10 and TGF-β or arginase 1 (ARG1) [33]
3. Stimulate the conversion of macrophages into M2 phenotype through the secretion of IL-10 and downregulation of M1 macrophage production of IL-12 [34]
4. Inhibit cytotoxicity of NK cells and their IFN-γ production [35]. However, the role of MDSCs on NK cell activity is controversial, since it has been reported that MDSCs expressing Rae-I, the ligand for NKG2D, can activate NK cells as well [36]
5. Interact with type II iNKT that facilitate tumour progression by producing IL-13, which induces the accumulation of MDSCs [27, 32, 37]

Multiple mechanisms by which MDSCs mediate immune evasion have been elucidated in the mouse models and will be discussed below.

5.3.1 T-Cell Deprivation of Essential Amino-acids

The MDSCs cause the depletion of two amino-acids required by lymphocytes for their growth and differentiation, i.e., L-arginine and L-cysteine. Extracellular L-arginine is depleted through its consumption by ARG1, which is induced in the cytosol of MDSCs by Th2 cytokines such as IL-4 and IL-13 and TGF-β in a STAT6-dependent and -independent manner [38]. MDSC activation of ARG1 reduces the extracellular levels of L-arginine, which leads to down-regulation of the CD3ζ chain of the T-cell receptor (TCR) and its signal transduction [39, 40]. In addition, the depletion of L-arginine can cause a G_0-G_1 cell cycle arrest through inhibition of the phosphoinositide 3-kinase (PI3K)/mammalian target of the rapamycin (mTOR) pathway [10, 41].

Cysteine is an essential amino-acid that serves as a fundamental substrate for generation of glutathione, a major intracellular molecule that protects cells from oxidative stress [42, 43]. Cysteine can be synthesized from intracellular cystine through the action of cystathionase, or alternatively can be imported as the oxidized form of cystine through ASC neutral amino-acid plasma membrane transporter. T cells lack cystathionase, and have a defective cystine transporter [42]. As a result, T cells must obtain cysteine from extracellular sources. DCs and macrophages normally have large amounts of cysteine that in part derives from the import of cystine, which is sequentially reduced, and in part is intracellularly synthesized through the enzyme cystathionase. During antigen presentation, DCs, which are in close proximity to lymphocytes, release the surplus cysteine that is readily taken up by T cells. In contrast to DCs, MDSCs, which do not express cystathionase and ASC transporter, generate cysteine from imported cystine. As a result, MDSCs deplete the environment of cystine, do not export cysteine and consequently prevent T-cell proliferation and activation [38].

5.3.2 Generation and Release of Oxidizing Molecules

The MDSCs express different enzymes involved in the production of ROS and NO, including NADPH oxidase (also known as NOX2) and iNOS respectively [10]. MDSCs also express the calcium-binding proteins S100A8 and S100A9, which together with gp91phox are part of the NOX complex, responsible for the increased production of ROS. ROS include superoxide anion (O_2^-) that is converted to hydrogen peroxide (H_2O_2) [23]. The latter molecule is then involved in the downregulation of the CD3ζ chain of TCR, thereby inhibiting T-cell activation through TCR [44]. iNOS is responsible for NO production, which interacts with O_2^- to form the highly reactive peroxynitrite anion ($ONOO^-$). NO is able to block the phosphorylation and subsequent activation of proteins associated to IL-2

receptor such as Janus kinase-1 (JAK-1), JAK-3, STAT5, extracellular-signal-regulated kinases (ERK), and AKT [45–47]. Moreover, NO can also decrease the stability of mRNA of IL-2 and the release of IL-2 [48]. It is worthy of note that in the presence of low cytosolic levels of L-arginine, iNOS activity can be modified and converted to induce the production of O_2^- together with NO [49, 50]. Peroxynitrite anion and NO cause the nitration or nitrosylation of TCR, CD8, and CD3 chains, thereby blocking T-cell activation. In particular, the first studies performed by Kusmartsev et al. [51] demonstrated that peroxynitrite may cause apoptosis of activated T lymphocytes through the inhibition of phosphorylation events in T cell signal transduction. Recently, it has been shown that peroxynitrite can also impair the TCR recognition of MHC-I-peptides due to nitrosylation of TCR tyrosines [31]. Furthermore, CCL2, a chemokine involved in inducing T-cell migration upon interaction with CCR2 expressed by T cells, can be modified by nitrosylation and inhibited in its function of T lymphocyte recruitment [52].

These observation prove that ARG1 and iNOS represent an important immunosuppressive mechanism mediated by MDSCs, and that the simultaneous inhibition of these enzymes may in part reconstitute T lymphocyte responsiveness [40].

5.3.3 *Induction of Development and Expansion of Treg*

In an experimental model of murine colon carcinoma, MDSCs have been shown to induce the expansion of Treg by IL-10, TGF-β, decrease of L-arginine and upregulation of CD40–CD40L interactions (essential for Treg activation) in an IFN-γ-dependent manner [25, 53]. In the model of ovarian carcinoma, MDSCs expressing high levels of CD80 cooperate with $CD152^+$ Treg in order to inhibit T-cell activation [26]. Additionally, MDSCs mediate Treg induction with a mechanism that requires ARG1 but is transforming growth factor-beta independent [33]. Interestingly, human $CD14^+HLA\text{-}DR^{low/-}$ MDSCs promote the transdifferentiation of TH17 cells into $FOXP3^-$-induced T_{reg} by producing TGF-β and retinoic acid [33].

5.3.4 *Interference with T-Cell Migration and Viability*

The expression of metalloproteinase ADAM17 by MDSCs induces the cleavage of CD62L, which is necessary for T-cell migration to draining lymph nodes [54]. Furthermore, MDSCs express galectin 9 which binds to T immunoglobulin and mucin domain-containing protein 3 (TIM3) on lymphocytes and induces T cell apoptosis [55]. As discussed above, CCL2 may be modified by MDSC-derived peroxynitrite, thereby impairing $CD8^+$ migration to the tumour core [52].

5.4 Molecular Mechanisms of MDSC Activation in Cancer

Expansion and activation of MDSCs are promoted by different soluble factors which can be classified into two groups. The first group includes tumour-derived soluble factors (TDSF) that induce MDSC expansion through stimulation of myelopoiesis and inhibition of differentiation of mature myeloid cells [11]. In contrast, the second group is characterized by factors released by activated T lymphocytes and tumour-derived stromal cells implicated in MDSC activation [11].

Typical TDSF are represented by cytokines (IL-3, IL-1β, IL-6, IL-10), growth factors (GM-CSF, VEGF, SCF, M-CSF, TGF-β), chemokines (CCL2), prostaglandins (PGE2), and proinflammatory proteins such as S100A8/S100A9. In general, none of these factors is sufficient to induce and activate MDSCs by itself [11].

IL-3 represents one of the first cytokines able to induce myelopoiesis in tumour-bearing mice and in particular to promote the expansion of immunosuppressive MDSCs [56]. GM-CSF, commonly released by different human tumours and murine tumour cell lines, has been shown to induce $CD11b^{+}Gr1^{+}$ without the participation of other cytokines. Accordingly, the administration of recombinant GM-CSF in mice affected by cancer promotes recruitment of MDSCs and their immunosuppressive function [56–59]. The presence of GM-CSF has been proposed as a negative prognostic factor in patients with head and neck squamous carcinoma. Thus patients with tumours releasing relevant amounts of GM-CSF showed higher incidence of relapse and metastases than those characterized by low GM-CSF production [60]. However, it is worth mentioning that GM-CSF is also used to transfect tumour cells administered as potent vaccines, since this cytokine is also able to recruit and expand professional antigen-presenting cells (APC) [61]. The contradictory effect mediated by GM-CSF seems to be dependent on the dosage used; only at high doses could GM-CSF mobilize myeloid precursors from the bone marrow, and induce immunosuppressive MDSC accumulation in the PB and lymphoid organs [62]. VEGF, which is secreted by various tumours and is often associated with poor prognosis, has been shown to inhibit in-vivo DC differentiation and promote MDSC $Gr1^{+}$ expansion [63–66]. The use of amino-biphosphonates, inhibitors of MMP-9 which regulates VEGF bioavailability, made it possible to reduce the accumulation of MDSCs [67].

Another factor able to induce MDSC recruitment is SCF, whose silencing or neutralization by specific antibodies is shown to reduce $CD11b^{+}Gr\text{-}1^{+}CD115^{+}$ in the BM of tumour-bearing mice, to decrease the tumour progression and angiogenesis, and to restore the proliferation of T lymphocytes [68].

PGE2, produced by cyclooxygenase 2 (COX2), represents a crucial factor in inducing MDSC expansion [69, 70]. PGE2 interacts with different PGE2 receptors (EP1, EP2, and EP4), among which EP4 seems to be involved in the MDSC induction of ARG1 [70]. Similarly, knock-out mice for EP2 showed decreased tumour growth and less accumulation of intra-tumour MDSCs [17].

Pro-inflammatory cytokines such as IL-1β and IL-6, which are present in the microenvironment of many tumours, have been shown to dramatically increase the

rate of immunosuppressive MDSC accumulation [71–73]. IL-1β also increases MDSC suppression of innate immunity by facilitating cross-talk between MDSCs and macrophages. Similarly, the pro-inflammatory S100A8/A9 proteins, highly expressed by tumour-infiltrating leucocytes, participate in an autocrine loop produced by MDSCs and involved in the recruitment of these latter cells [74–76]. In this manner, an immunosuppressive tumour microenvironment is maintained. Most of the factors listed above converge toward the activation of JAK proteins and STAT3 transcription factor, which represent the main regulatory factors of MDSC expansion [77–79]. In this connection, STAT3 has been demonstrated to upregulate different target proteins including:

- S100A8, S100A9, two calcium-binding proinflammatory proteins involved in MDSC accumulation [75, 76, 80]
- iNOS and NADPH oxidase (NOX2), related to NO and ROS production by MDSCs [81]
- Cyclin-D, MYC, BCL-XL, which promote MDSC survival [11]
- CCAAT/enhancer-binding protein-β (C/EBPβ), an important regulator of differentiation of myeloid progenitors to functional MDSCs [82]

Reduced expansion of MDSCs in $STAT3^{-/-}$ conditional knockout mice, as well as normal mice, treated with specific inhibitors of STAT3, supports the hypothesis that STAT3 has a crucial role in MDSC expansion and activation [78, 83]. Moreover, persistent activation of STAT3 in myeloid progenitors prevents differentiation into mature myeloid elements and promotes expansion of MDSCs [80].

The transcription factors STAT1 (mainly activated by IFN-γ) and STAT6 (activated by IL-4 and IL-13) are additional important regulators of MDSC activation through its effects on iNOS and ARG1 expression, whereas STAT5 (activated by GM-CSF) is involved in promoting MDSC expansion through the induction of cyclins, survivin, B cell lymphoma XL (BCL-XL), and MYC [11, 84–86]. Finally, NF-κB, upon activation by TLR ligands, has a relevant role in inducing the mobilization of MDSCs to sites of tumour growth, probaly through targeting ARG1 and iNOS and the pro-inflammatory mediators PGE2 and COX2, which enhance MDSC accumulation and suppressive activity [11, 70, 87]. Figure 5.2 summarizes the molecular mechanisms involved in tumour-mediated induction of MDSCs.

5.5 MDSCs in Cancer Patients

MDSCs have been identified in the peripheral blood and in tumour infiltrates of patients affected by different tumour types; however, the MDSC phenotype in humans is less definite in comparison to that found in mice. Similarly to MDSCs found in mice, in humans there have been two different MDSC subtypes identified so far, for the monocyte and the granulocyte. The prevalence of M-MDSCs compared to G-MDSCs depends on the tumour type; for example the PB of patients

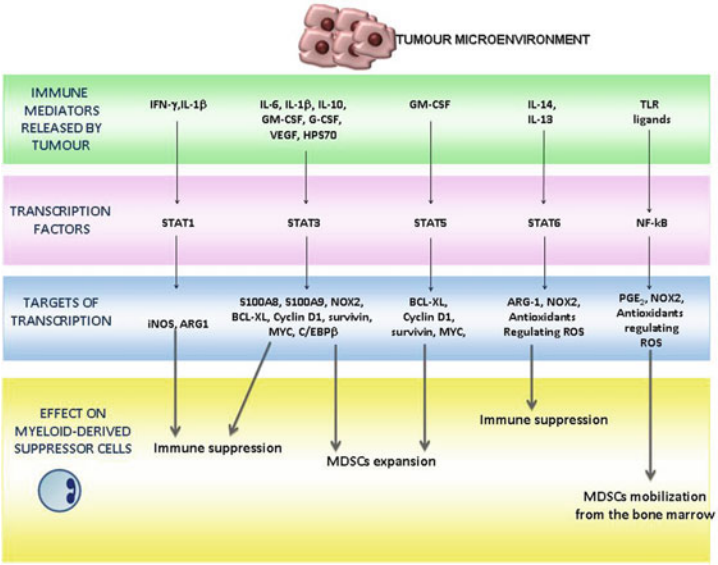

Fig. 5.2 Molecular mechanisms involved in tumour-mediated induction of myeloid-derived suppressor cells. Tumour cells release different pro-inflammatory cytokines and growth factors that are responsible for the induction of multiple transcription factors including signal transducer and activator of transcription (STAT)1, STAT3, STAT5, STAT6, nuclear factor-κB (NF-κB). Each of these factors regulate the expression of proteins involved in myeloid-derived suppressor cell (MDSC)-mediated functions such as inducible nitric oxide synthase (iNOS), NADPH oxidase (NOX2), arginase 1 (ARG1), S100A8, S100B, B-cell lymphoma XL (BCL-XL), cyclin D1, survivin, MYC, CCAAT/enhancer-binding protein-β (C/EBPβ), prostaglandin E2 (PGE2). *G-CSF* granulocyte colony-stimulating factor, *GM-CSF* granulocyte–macrophage colony-stimulating factor, *IFNγ* interferon-γ, *IL* interleukin, *ROS* reactive oxygen species, *TLR* Toll-like receptor, *VEGF* vascular endothelial growth factor

affected by head and neck carcinoma, non small lung cancer, prostate and breast carcinoma, and multiple myeloma is characterized by the presence of M-MDSCs that are able to inhibit allogeneic and antigen-specific T responses, and may differentiate into mature DCs in presence of GM-CSF e IL-4 [59, 88, 89]. More recently, an highly immunosuppressive MDSC population has been identified in patients affected by metastatic melanoma. These cells are $CD14^{+}HLA\text{-}DR^{-/low}$, and induce immunosuppression through a mechanism mediated by TGF-β independently from ARG1 and NOS [59]. What is more, $CD14^{+}HLA\text{-}DR^{-/low}$ have been also isolated from the PB of hepatocarcinoma patients, where they inhibit lymphocyte proliferation in an ARG1-dependent manner and induce T_{reg} [90].

The first evidence of the presence of G-MDSCs in cancer patients was found in the research that characterized cells $CD11b^{+}CD15^{+}CD14^{-}$ expressing ARG1 in patients affected by renal carcinoma [91]. Subsequently, the same group defined more precisely that G-MDSCs in renal carcinoma patients belonged to a subset of activated granulocytes $CD66b^{+}VEGFR1^{+}CD62L^{low}CD16^{low}$ able to secrete ARG1

after degranulation [92]. Additional studies revealed the presence of IL4Rα marker (typically expressed in MDSCs isolated from different experimental tumour models developed in mice) also in G- and M-MDSCs defined in melanoma and colon carcinoma patients [93]. However, the immunosuppressive activity was identified only in the granulocytic $CD14^+$ fraction [93].

Other important issues addressed in cancer patients are whether MDSC presence correlates with clinical cancer stage, and whether MDSC presence in PB can be modulated by chemotherapy. In this regard, an interesting paper by Diaz-Montero et al. demonstrated that MDSCs, defined as $Lin^{low/-}$, $HLADR^-$, $CD33^+$, $CD11b^+$, were present at higher percentages in the peripheral blood of cancer patients than in that of healthy donors [94]. Moreover, among stage IV patients, those with extensive metastatic burden had the highest number of MDSCs. Two chemotherapy regimens were evaluated, and in both cases MDSCs were increased after therapy [94].

5.6 Therapeutic Targeting of MDSCs

It has become increasingly clear that successful cancer immunotherapy relies on the elimination of the immunosuppressive barrier induced by different elements including MDSCs [10]. For this purpose, different therapeutic approaches are currently being explored in order to:

- Induce myeloid differentiation
- Inhibit MDSC expansion
- Eliminate MDSCs
- Inhibit MDSC functions

One of the most promising approaches is based on the promotion of myeloid differentiation. Vitamin A has been identified as a compound that mediates this effect. Specifically, vitamin A metabolites such as retinoic acid have been shown to promote the differentiation of myeloid progenitors into DCs and macrophages [95]. Furthermore, administration of therapeutic concentrations of all-*trans* retinoic acid (ATRA) in renal-cell cancer patients induced reduction of MDSC numbers and promoted MDSC differentiation into DCs and macrophages [95–97]. Similar effects have been obtained in tumour-bearing mice in which ATRA eliminated immature myeloid cells and improved the effect of vaccination [97]. The mechanism of ATRA effect on MDSCs occurred through upregulation of glutathione synthesis and reduction of ROS [98]. Vitamin D3 is another compound that has the potential to decrease MDSC numbers and promote myeloid differentiation in cancer patients [99].

Since MDSC expansion is regulated by soluble factors released by tumours, a promising approach is the use of agents able to neutralize the effect of these factors. In this regard, it has been shown that inhibitors of SCF signaling decreased MDSC expansion, an antibody against VEGF (Avastin) reduced the size of $CD11b^+VEGFR1^+$

population in PB of cancer patients, and an inhibitor of MMP9 diminished the number of MDSCs in the spleen and tumours of cancer-bearing mice [65, 67]. An additional approach is the use of agents that inhibit MDSC function, such as inhibitors of COX2 and ROS. COX2 is a well-known enzyme involved in the production of PGE2, which upregulates ARG1 expression by MDSCs [69, 70]. Accordingly, COX2 inhibitors were found to downregulate the expression of ARG1 by MDSCs and to improve the therapeutic efficacy of immunotherapy [91, 100]. Nitroaspirin, a non steroidal anti-inflammatory drug, and phosphodiesterase 5 (PDE5) inhibitors such as sildenafil have been found to limit the activity of ARG1 and iNOS in splenic MDSCs and to improve the function of tumour-antigen-specific T cells [89, 101].

Finally, MDSCs can be eliminated using some chemotherapeutic drugs. For example, gemcitabine reduced the number of MDSCs and improved T-cell responses in cancer-bearing mice [102]. MDSC recruitment can also be reduced by the use of STAT3 inhibitors such as sunitinib that decreased the number of MDSCs in patients with renal cancer [103].

The identification of different MDSC subpopulations with different immunosuppressive functions will permit the design of more specific therapeutic strategies able to inhibit only the most immunosuppressive population, thereby avoiding the induction of strong myeloablation.

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Chapter 6
Tumor-Infiltrating Lymphocytes and Their Role in Solid Tumor Progression

Theresa L. Whiteside

Abstract Tumor-infiltrating lymphocytes (TIL) are an important component of the tumor environment. Their role in tumor growth and progression has been debated for decades, with the current emphasis on survival benefits TIL appear to bestow on the host when present in situ as large aggregates of activated T and B cells. Gene signatures and protein profiling of TIL provide clues about their potential functions in the tumor, and correlations with clinicopathological tumor characteristics, clinical outcome, and patients' survival data indicate that TIL exert influence on the disease progression, especially in colorectal carcinomas and breast cancer. At the same time, the recognition that TIL signatures vary in composition and with time, and that TIL interactions with the tumor cells are complex, calls for a more careful assessment of their prognostic significance. The mechanisms tumors utilize to subvert the host immune system are well-known. The balance between pro- and anti-tumor responses of TIL might be orchestrated by the tumor serving as a measure of its aggressiveness and potentially providing a key to selecting therapeutic strategies and to prognosis.

Keywords Cancer • Tumor-infiltrating cells • Lymphocytes • Prognosis

List of Abbreviations

Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
CFC	Cytokine flow cytometry

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CRC	Colorectal cancer
CTL	Cytolytic T cell
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
DC	Dendritic cells
ER	Estrogen receptor
IGKC	IgG kappa chain
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
NK	Natural killer cells
NSCLC	Non-small cell lung cancer
PD-1	Programmed cell death protein-1
PGE2	Prostaglandin E2
PMN	Polymorphonuclear neutrophils
TA	Tumor-associated antigen
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
Th cell	T helper cell
TIL	Tumor-infiltrating lymphocytes
TMA	Tissue microarrays
Treg	Regulatory T cells
VEGF	Vascular endothelial growth factor

Contents

6.1 Introduction

The immune cells present in the tumor microenvironment belong to both the adaptive and innate arms of the immune system, and are found in virtually all human solid tumors. They may be present at various densities ranging from subtle infiltration to overt inflammation. As lymphocytes usually constitute the largest component of these immune infiltrates, they are commonly referred to as "tumor-infiltrating lymphocytes" or TIL. Attention given to TIL has progressively grown in the last decade, largely because of the perception that these cells might play a critical role in carcinogenesis and also might be therapeutically useful.

Technological advances have allowed for a better examination of tumor infiltrates and for the identification of immune-related gene signatures expressed in the tumor microenvironment. Phenotypic and functional characteristics of TIL, their interactions with the tumor cells or the tumor stroma, and their prognostic and predictive significance have become a subject of intense investigations worldwide. As a result, inflammatory infiltrates into tumors have achieved the status of one of the "Hallmarks of Cancer" by Hanahan and Weinberg [1], in recognition of the role they play in tumor progression and in tumor escape from the host immune system. It has also been recognized that cancer cells have a complex relationship with the immune system, and that subtle differences in immune cell infiltrates into the tumor can result in eradication of cancer cells or in enhancement of their growth. The dynamic relationship existing between TIL and the tumor has been difficult to discern in human specimens, and much of the available insights have come from animal, largely mouse, models of tumor growth [2].

In this brief review, I will summarize recent progress in the TIL field, focusing only on T and B lymphocytes and natural killer (NK) cells. While it is clear that other leukocytes, M1 and M2 macrophages, dendritic cells (DC), and neutrophils (PMN) are all important components of the tumor microenvironment, it is the TIL that have captured the highlights, because of the new insights which indicate that TIL have prognostic or predictive significance in cancer.

6.2 Anti-tumor Effects of TIL

Traditionally, T lymphocytes, and especially $CD8^+$ cytolytic T cells (CTL), have been considered the major anti-tumor immune effector cells. Their MHC class I-restriction and specificity for tumor-associated antigens (TA), combined with the ability to produce perforin and other cytotoxins upon activation, confers upon them functions which lead to death of tumor cells but sparing of normal cells. A subset of $CD4^+$ T helper (Th) cells is essential for providing cytokine-mediated support for CTL expansion and functions. NK cells, which are not MHC-restricted and do not require prior sensitization to antigens, can also recognize and eliminate tumor cells by mechanisms that involve a release of perforin, granzymes, and cytokines [3]. These lymphocytes are mediators of cellular anti-tumor immunity. B cells, which upon Ag-specific activation give rise to antibody (Ab)-producing plasma cells, mediate humoral anti-tumor immunity. It has been debated whether it is T or B cells that play a more important role in the control of tumor progression. Contributions of NK cells to anti-tumor immunity have been largely considered in the context of antibody-dependent cytotoxicity (ADCC) during cancer therapy with antibodies. Today, it is evident that cooperative interactions of these cells are critical for the development of effective anti-tumor responses. The presence of B cells among TIL has been recently recognized as a prognostically significant biomarker [4, 5], and the involvement of infiltrating NK cells in cooperative anti-tumor effects has been confirmed [6].

6.2.1 $CD8^+$ Cytolytic T Cells

Favorable associations of dense T-cell infiltrates with improved prognosis of many human cancers have been reported for decades [7]. While the presence and functions of T cells in the tumor were the major concern in many earlier studies, more recent data emphasize the diversity in cellular composition of immune tumor infiltrates in various tumor types, with B cells, NK cells, M1 and M2 macrophages, granulocytes, or mast cells contributing substantially to the "immune signature" that uniquely characterizes each solid tumor. More recently, tumor-associated T cells have been shown to be independently associated with improved survival in a variety of epithelial-type cancers [8, 9]. The most comprehensive studies correlating TIL with disease outcome have been performed in colorectal cancer [10–12]. The group of Freedman, Galon, and colleagues has used state-of-the-art systems-based approaches and an objective scoring method to examine hundreds of colorectal cancer samples and to correlate the type, density, and location of immune cells within tumors with clinical outcome [11–13]. They showed that a strong local immune reaction, including $CD3^+$, $CD8^+$, and memory $CD45RO^+$ T cells, correlates with a favorable prognosis regardless of the local extent of the tumor or the regional lymph node involvement [11–13]. At the same time, a number of reports from various laboratories on the nature and cellular composition of TIL in human tumors have given support to the role these cells play in estimating clinical outcome in cancer [14]. For example, Mahmoud et al. evaluated the prognostic significance of $CD8^+$ T cells in over 1,300 breast cancer patients diagnosed between 1987 and 1998 [15]. Tissue microarrays (TMA) obtained from these patients' tumors were stained with anti-CD8 Ab, and numbers of infiltrating T cells determined. The total number of $CD8^+$ T cells in the tumor correlated with a higher tumor grade, and was associated with better patient survival. In these and other studies, T-cell infiltrates emerged as a stronger independent prognostic factor than the current clinicopathological criteria such as tumor size, depth of infiltration, differentiation, or the nodal status [16]. A proposal has been made recently for introducing a routine evaluation of the TIL density, location, phenotype, and function in order to define "an immune score" for each tumor as a part of the standard pathologic examination [16]. However, it is unclear how readily the immune score will be accepted by the pathologists as a routine procedure, largely because of requirements for automated image analysis and for standardization of methods. Nevertheless, globally-collected data strongly support the merit of the "immune score," which emerges as the first immunologic marker of risk in cancer with the potential to be incorporated into prognostically-relevant immune classification of human cancer equal to or better than the conventional TNM classification of malignant tumor [16].

In addition to the TIL immune score, the availability of standardized single-cell assays able to detect tumor-antigen-specific T cells (ELISPOT), cytokine flow cytometry (CFC), and tetramer binding among TIL has greatly facilitated evaluations of their potential value as prognostic biomarkers in cancer [17]. However, it has been also observed that tumor epitope-specific $CD8^+$ T cells present in

situ or in the peripheral circulation of patients with cancer were often preferentially eliminated either directly via the Fas/FasL or Trail/TRAILR pathways [18] or indirectly through the release of tumor-derived exosomes carrying death receptor ligands [19]. The propensity of TIL isolated from human solid tumors to undergo spontaneous apoptosis was measured by annexin V binding in flow cytometry assays, and tumor-epitope reactive, activated $CD8^+$ T cells which expressed Fas were shown to be particularly sensitive to tumor-induced effects [20]. Specifically, $FasL^+$ tumor-derived exosomes isolated tumor cell supernatants or plasma of cancer patients have been recently linked to tumor progression, demonstrating that the presence of membrane-tethered FasL, and potentially of other molecules such as PDL-1 (programmed cell death protein-1) or transforming growth factor-β (TGF-β), could contribute to apoptosis of anti-tumor effector T cells among TIL and thus to tumor escape from the host immune system [21]. These studies suggest that the presence of death-inducing ligands on tumor cells or in tumor-derived exosomes might contribute to elimination of TIL responsible for anti-tumor effects in the tumor microenvironment [21]. In aggregate, anti-tumor effects of $CD8^+$ T cells accumulating in the tumor microenvironment that are potentially responsible for improved outcome have to be counterbalanced by immunosuppressive activities of the tumor which favor tumor progression. For this reason, the concept of the "immune score" as a biomarker of outcome might not be entirely valid, at least in aggressive tumors characterized by the strongly immunosuppressive phenotype.

Although $CD8^+$ T cells are present and are activated in many human tumors, these tumors do not undergo spontaneous regression. This is probably due to regulatory mechanisms which inhibit T-cell responses in the tumor microenvironment [22]. These mechanisms can operate at the level of tumor cells, inducing, for example, loss of tumor antigens or down-regulation of class I MHC molecules, rendering the tumor invisible to $CD8^+$ effector T cells [23]. Alternatively, T cells can and do up-regulate immune checkpoints or inhibitory pathways that are hard-wired into all T-cell responses to prevent excessive activation and tissue damage. For example, following T-cell receptor (TCR) engagement by an antigen, T cells up-regulate cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), an inhibitory molecule that counteracts the stimulatory receptor CD28 [24]. Tumor cells often express PD-L1, a ligand for another inhibitory receptor, PD-1. Activation of the PD-1/PD-L1 pathway in T cells decreases their proliferation, survival, and cytokine production [25]. Still another regulatory break is the presence in the tumor microenvironment of suppressor cells, such as regulatory T cells (Treg; see below) or myeloid-derived suppressor cells (MDSC). These regulatory cells produce inhibitory cytokines (e.g., interleukin 10, IL-10; TGF-β) or suppressive factors which dampen or abrogate anti-tumor immunity [26, 27].

6.2.2 $CD4^+$ Helper T Cells

This subset of T cells is present in solid tumors with a frequency that equals or exceeds that of $CD8^+$ T cells. Several subsets of helper T cells are recognized, including Th1, Th2, Th17, and regulatory T cells (Treg). The well known "Th1/Th2"

paradigm [28] refers to the balance that exists between the functionally distinct subsets of Th cells. Th1 cells produce cytokines, notably IL-2 and interferon γ (IFN-γ), which play a role in activating and enhancing expansion as well as effector functions of $CD8^+$ T cells and NK cells [29]. Th1 cells also influence the antigen-presenting capacity of DC, thus shaping CTL responses [29, 30]. In contrast, Th2 cells secrete cytokines that are important for B-cell maturation, clonal expansion, and class switching, thus promoting humoral immune responses. The Th1/Th2 ratio is altered in cancer and other diseases, with Th2 cells often outnumbering Th1 cells in the blood and tumor tissues of patients with cancer [31]. There are no surface markers distinguishing these two Th subsets, but cytokine production and gene expression profiles have been used to discriminate Th1 from Th2 responses [32]. In a study of 400 ER-negative breast tumors, the Th1 profile (IL-2, IL-12, IFN-γ) was inversely correlated with the Th2 profile (IL-13, TGF-β), and Th1 responses associated with a lower risk for distant metastases [33]. Th2 responses were associated with a higher risk. The combination of both pathways allowed for a better prediction of metastasis-free survival than either of the pathways alone [33]. This example emphasizes the potential importance of Th1 versus Th2 responses at tumor sites for disease outcome, and indicates that immune response developing in the microenvironment of tumors is an important prognostic factor.

A relatively recent addition of Th17 cells, characterized by the production of IL-17, to the T-cell repertoire has altered the Th1/Th2 paradigm. The Th17 cells play a major role in autoimmunity, and their involvement in cancer has been less well studied. A recent study of human breast tumors identified Th17 cells as a prominent component of infiltrates, and established a negative association between their presence and the disease stage or number of involved lymph nodes, suggesting that Th17 are involved in anti-tumor responses [34]. In a study of patients with ovarian carcinoma, Kryczek et al. reported that patients with higher numbers of Th17 cells had significantly improved overall survival, irrespective of the tumor stage. Further, the frequency of Th17 cells inversely correlated with that of tumor-infiltrating $FOXP3^+$ Treg [35]. However, experiments in mouse models of cancer indicate that Th17 may also be involved in pro-tumor functions by promoting angiogenesis [36]. IL-17 has been shown to induce expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), angiotensin, IL-8 and prostaglandin E2 (PGE2) in stromal, endothelial, and tumor cells [36]. The exact cellular mechanisms that determine pro- vs anti-tumor functions of $Th17^+$ TIL remain unclear and need further investigations. Nevertheless, given that angiogenesis remains a major feature of progressing tumors, the presence and quality of Th17 infiltrates are likely to be of considerable importance in cancer prognosis.

6.2.3 Regulatory T Cells (Treg)

This relatively minor subset of $CD4^+$ T cells (~5 %) is well-represented among TIL, and Treg play a major role in modulating immune responses in situ. Tumors appear to recruit Treg to the tumor microenvironment, where they accumulate,

representing a substantial component of TIL in multiple tumor types (reviewed in [26]). The presence and functional competence of Treg inversely correlates with outcome in many, but not all, human tumors [26, 37]. The existing conflicting reports with respect to the role of Treg in promoting tumor progression vs its regression have largely originated from the lack of a definite phenotypic profile for human Treg. While the $CD4^+CD25^{high}FOXP3^+$ natural (n) Treg normally responsible for maintaining peripheral tolerance control cancer-associated inflammation [38], another subset of Treg, inducible (i) Treg, which may or may not be $FOXP3^+$ but produce adenosine, IL-10 or TGF-β and arise by tumor-driven conversion of conventional $CD4^+$ T cells to highly suppressive therapy-resistant cells, appear to be responsible for down-regulating in situ anti-tumor responses [38]. The iTreg promote tumor growth, expand and accumulate in cancer, and their presence in TIL predicts poor outcome. In ovarian carcinoma, melanoma, breast cancer, and glioblastoma, the frequency of Treg among TIL correlated with tumor grade and reduced patient survival [37]. Because Treg are heterogeneous, consisting of many subsets of functionally distinct cells, and because no universal distinguishing marker for human Treg is currently available, their use as a biomarker of prognosis is limited and has to be taken with caution. Additional studies evaluating the role of Treg present in the tumor microenvironment as an independent predictor of prognosis in cancer are necessary.

6.2.4 B Cells

B cell originate in the bone marrow and then migrate to secondary lymphoid organs, e.g., lymph nodes, where they interact with antigens, differentiate into plasma cells, and produce the antigen-specific Abs. TIL populations in human solid tumors include variable proportions of infiltrating B cells. While a search for promising immune correlates of cancer diagnosis, prognosis, and survival has been largely limited to T-cell responses, newer reports indicate that B cells might be critically important for outcome. Two recent independent studies provide useful insights into the prognostic role of B cells in cancer. Schmidt and colleagues have reported data that validate the B-cell signature as the most robust prognostic factor in breast cancer and other human tumors [4, 39]. These investigators identified the immunoglobulin G kappa chain (IGKC) as an immunologic biomarker of prognosis and response to chemotherapy in hundreds of patients with breast cancer, non-small cell lung cancer (NSCLC), and colorectal cancer (CRC) [4, 40]. In this multi-institutional study, the IgG kappa chain (IGKC) was microscopically identified as a product of plasma cells present in the tumor stroma, and was validated as a prognostic biomarker by the RNA- and protein-based expression studies independently performed in thousands of formalin-fixed, paraffin-embedded specimens at 20 different centers [4]. Expression of the IGKC transcript was the strongest discriminator of patients with breast cancer with and without metastases among the 60 genes found in the B-cell metagene, while transcripts of the T-cell metagene had lesser prognostic

significance [4, 39]. Infiltrates of both T and B cells were found to be associated with better prognosis. However, the most important finding was that IGKC predicted responses to neoadjuvant therapy in breast cancer, which thus qualifies it as the first immune marker of response to cancer treatment. The finding of the B-cell signature as a validated biomarker of prognosis and response to therapy provides a strong support for the role of humoral immunity in controlling cancer [4].

In support of this key role of the B-cell signature, Nielsen and colleagues [5] reported that among TIL present in high-grade serous ovarian carcinomas, $CD20^+$ B cells co-localized with activated $CD8^+$ T cells and expressed markers of antigen presentation, including MHC class I and class II antigens, CD40, CD80, and CD86. These B cells were antigen-experienced. The presence among TIL of both $CD20^+$ B and $CD8^+$ T cells correlated with a better patient survival than that compared to $CD8^+$ T cells alone. Although these $CD20^+$ B cells had an atypical CD27(−) memory B-cell phenotype, together with $CD8^+$ T cells, they promoted favorable prognosis in ovarian cancer [5].

The emerging evidence for a significant role of the B cell signature as a biomarker of prognosis and possibly of metastasis in several human malignancies deserves careful attention, particularly in view of novel insights into functional heterogeneity of this lymphocyte subset, which appears to play a pivotal role in regulating T-cell responses [41]. Most recently, human B cells were found to express CD39 and CD73, the ectoenzymes hydrolyzing exogenous ATP to adenosine [42]. The ability of activated $CD19^+$ B cells to regulate T cells via the adenosine pathway and adenosine receptor signaling places these lymphoid cells in the category of regulatory elements potentially as effective as Treg [42].

6.2.5 Natural Killer Cells

Natural killer (NK) cells mediate innate immune responses and can mediate direct cellular cytotoxicity without a need for prior sensitization [6]. In contrast to T cells, NK cells are not HLA-restricted. They are regulated by a set of receptors, such as killer inhibitory receptors or KIRs, and of activating receptors, such as NKG2D and others [6], which calibrate anti-tumor functions of these cells. As a result, NK cells eliminate tumors that lack MHC class I expression or that overexpress ligands for NKG2D, including MICA and B. There is little evidence for an association of the NK-cell presence in the tumor microenvironment and clinical outcome in solid tumors. Nevertheless, there is evidence that NK cells, which express high levels of low-affinity Fc receptors (CD16) for IgG, are critical for antibody-mediated cellular cytotoxicity (ADCC). NK cells are also strong IFN-γ producers [37, 43, 44]. Unfortunately, NK cell functions are often found to be down-regulated in cancer, and in a recent study of highly aggressive NSCLC, NK cells were found to have an altered phenotype and were impaired in the ability to secrete IFN-γ [45]. Tumor- and peripheral blood-derived NK cells in patients with cancer are frequently compromised, and in some cases this impairment has been linked to the tumor progression and poor prognosis [46].

6.3 Summary and Conclusions

The immune response, which is mediated by subsets of lymphoid cells, can have a powerful influence on the survival of patients with cancer. In this respect, evidence is especially strong for colorectal and breast cancers [47, 48]. Patients with large infiltrates of T or B cells or increased expression of genes encoding T-cell or B-cell signatures tend to have better survival compared to those with few tumor-infiltrating immune cells [4, 5, 47, 48]. TIL can be divided into at least three distinct cell types: effector cells, regulatory cells, and inflammatory cells, all of which can influence each other's functions through production of cytokines and soluble factors. Tumor cells themselves also produce immunosuppressive cytokines and factors, which have direct effects on immune cells recruited to the microenvironment. Therefore, cellular composition of the tumor microenvironment and interactions of cells and cytokines established within it determine the outcome of an anti-tumor immune response. As neither the composition nor the cytokine milieu in the microenvironment are constant, because they undergo changes as tumors progress from pre-malignant to malignant and eventually metastatic phenotype, the impact TIL may have on outcome is variable. Current data suggest that it may be dependent on the balance existing between inflammatory and regulatory TIL. This balance may be a critical part of the underlying mechanism responsible for the influence TIL exert on patient outcome. Understanding of the cellular and molecular mechanisms involved in creating and maintaining this balance is, therefore, necessary for determining how TIL contribute to survival of patients with cancer.

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Chapter 7
Polymorphonuclear Neutrophils and Tumors: Friend or Foe?

Magdalena Klink and Zofia Sulowska

Abstract The tumor microenvironment is a dynamic network which apart from cancer cells includes also cells of the immune system such as polymorphonuclear neutrophils. Neutrophils are implicated in the interaction with cancer cells and, due to their ability of secreting the variety of active proteins and factors, play an important role in tumor progression and/or tumor destruction. In the tumor environment, neutrophils exist as anti-tumor phenotype (N1) and pro-tumoral phenotype (N2), analogous to the polarization of tumor-associated macrophages. The N1 phenotype of neutrophils is characterized by a cytotoxic and pro-inflammatory activity, while the N2 phenotype of cells has strong immunosuppressive properties. During cancer progression and metastasis, neutrophils facilitate and intensify extravasation of tumor cells as a result of the release of neutrophil metalloproteinases and elastase, which destroy the components of extracellular matrix, thus helping tumor cells to go through the endothelial barrier. On the other hand, tumor cells have a strong impact on the functional activity of neutrophils, and by causing immune system suppression in the host organism they promote the progression of the cancer. This review summarizes the pro- and anti-tumoral activity of neutrophils as the result of their direct contact with cancer cells and the release of different active mediators.

List of Abbreviations

BMs	Basement membranes
ECM	Extracellular matrix
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte–macrophage colony-stimulating factor

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huGCP-2	Human granulocyte chemotactic protein 2
ICAM-1	Intracellular adhesion molecule 1
IFN	Interferon
IL	Interleukin
IRAK 1	Interleukin-1 receptor-associated kinase 1
IRS-1	Insulin receptor substrate-1
MIP-1 α	Macrophage inflammatory protein-1 α
MMP	Metalloproteinase
NE	Neutrophil elastase
NK	Natural killer
NN	Naive neutrophils
PI3K	Phosphatidylinositol-3 kinase
ROS	Reactive oxygen species
TAMs	Tumor-associated macrophages
TANs	Tumor-associated neutrophils
TGF-β	Transforming growth factor β
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
TRAIL	TNF-related apoptosis-inducing ligand
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

Contents

7.1 Introduction

The response of the host to the appearance of tumor cells involves mechanisms of both the innate and adaptive immune systems. Polymorphonuclear neutrophils represent the first line of host defense against infection, and are potent effectors of non-infectious inflammation. The still-unanswered question is whether these

cells support tumor growth and metastasis, or whether they are involved in the destruction of tumor cells [1].

Tumor cell infiltration into blood vessels, resulting in metastasis and neutrophil penetration into the tumor microenvironment, requires the cells to pass through matrix proteins and endothelial barriers. In both processes, the presence of proteases such as neutrophil elastase and matrix metalloproteinases as well as various cytokines is strongly required [2, 3]. Here, we will focus upon the double role of neutrophils in the facilitation and prevention of tumor metastasis.

7.2 Tumor-Associated Neutrophils: N1–N2 Polarization

Neutrophils are the most common leukocytes found in the peripheral blood. They are short-lived cells that respond to chemotactic stimuli to enter sites of infection or inflammation. In contrast to the well-established role of neutrophils in host defense against infection, relatively little is known about their involvement in the development, growth, and progression of human cancer or the antitumor response and/or tumor destruction. Due to their response to and production of cytokines, neutrophils are involved in a complex cross-talk with not only immune and endothelial cells but also cancer/tumor cells. Numerous articles have described a double neutrophil impact on tumor biology and antitumor reactions.

The tumor environment is a dynamic network that includes cancer cells, immune cells, fibroblasts, endothelial cells, extracellular matrix, cytokines, and receptors [4–6]. Among infiltrating tumor leukocyte populations, neutrophils are detected in the milieu of various types of solid tumors, e.g., renal [6], gastric [7, 8], melanoma [9], and pancreatic [10]. To reach the tumor, neutrophils must leave the circulation (extravasation). This step requires coordinated interactions between neutrophils and endothelial cells. However, several potent pro-inflammatory mediators and chemotactic factors are secreted by tumor cells, and these factors stimulate neutrophil migration into the tumor microenvironment and their later intratumoral accumulation. The stimuli attracting/recruiting neutrophils include chemokines (interleukin 8, IL-8; macrophage inflammatory protein-1 α, MIP-1α; human granulocyte chemotactic protein 2, huGCP-2) and cytokines (tumor necrosis factor-α, TNF-α; granulocyte colony-stimulating factor, G-CSF; and granulocyte–macrophage colony-stimulating factor, GM-CSF), which are produced in the tumor microenvironment [11–13]. When neutrophils reach the tumor, they are termed tumor-associated neutrophils (TANs), and are defined by the surface marker CD11b. However, little is known about their phenotype and relationship to naive neutrophils (NNs). Fridlender et al. [14] used a transcriptomics approach in mice to compare the two types of cells. Their study has provided evidence that primary and secondary granule proteins, such as myeloperoxidase, proteinase-3, cathepsin-G, and lactoferrin, are down-regulated in TANs in comparison to NNs. Additionally, higher expression of receptors and proteins responsible for the recognition of foreign antigens, including Toll-like receptors (TLRs) 1, 2, and 4, interleukin-1 receptor-associated kinase 1 (IRAK-1), myeloid differentiation (Myd88) protein,

and MHC class II, was found in TANs compared to NNs. Similarly, most chemoattractants for T and B cells and macrophages (e.g., CCL-2, -3, -7, -17, CXCL-1, -2, -9, -10) were also upregulated in TANs.

TANs are fully capable of modifying tumor growth and invasiveness, and their presence may be indicative of a better or worse host anti-tumoral response. Several authors have reported that the presence of intratumoral neutrophils constitutes an independent factor suggesting the poor survival of patients with renal cell carcinoma [6], gastric carcinoma [8], melanoma [9], and head and neck cancer [15]. Regardless of the presence of intratumoral neutrophils, there is some evidence that an increased number of neutrophils in the peripheral blood is also associated with the poor survival of patients with metastatic melanoma [16, 17]. However, another report has indicated that a very high concentration of TANs reduces the mortality of patients with advanced gastric carcinoma [7]. Using animal models, other studies have also demonstrated the anti-tumoral activity of neutrophils. Neutrophils isolated from the peripheral blood of healthy rats have been shown to have a highly cytotoxic and anti-proliferative effect on Walker 256 carcinoma cells (W256). Such neutrophils administered at the site of tumor in rats bearing W256 tumors significantly prolong the survival of animals and increase tumor regression [18–20].

Fridlender at al. [21] have provided evidence for the existence of N1 (anti-tumoral) and N2 (protumoral) TAN phenotypes analogous to the polarization of tumor-associated macrophages (TAMs) toward a protumoral (M2) or anti-tumoral (M1) phenotype. According to these authors, neutrophil polarization is regulated by transforming growth factor β (TGF-β). TGF-β contributes to the accumulation of N2 TANs and prevents the generation of N1 neutrophils [12, 13, 21]. TGF-β plays an important role in tumor growth, acting as both its suppressor and promoter. The mechanism underlying this dual role is unclear. TGF-β also exerts an effect on immune cells present in the tumor microenvironment. It directly suppresses cytotoxic T cell activity and inhibits natural killer (NK) cell proliferation and function. However, it induces T-cell differentiation into $CD4^+CD25^+$ Treg and Th17 cells. It also induces the pro-tumorigenic phenotype of TANs (N2). TGF-β blockade (inhibition) increases the number of neutrophils in the tumor microenvironment and leads to a shift to N1 neutrophils with anti-tumor activity [13, 21, 22].

The alteration of neutrophil phenotypes is most likely connected to different degrees of neutrophil activation. Highly activated TANs with an N1 phenotype produce higher levels of pro-inflammatory and chemotactic cytokines activating NK and cytotoxic T cells (TNF-α; CCL-3; CCL-9; interleukin 12, IL-12), express Fas and ICAM-1 molecules on their surface, and demonstrate low levels of arginase and matrix metalloproteinase-9 (MMP-9). N1 TANs can also kill cancer cells and promote the recruitment and activation of $CD8^+$ T cells. In contrast, N2 TANs do not produce considerable levels of immune-activating cytokines, although they do produce a large amount of arginase, which is immunosuppressive for T cells and inactivates their effector function via the down-regulation of T cell receptor (TCR) expression. N2 TANs also highly express pro-angiogenic and pro-metastatic factors such as MMP-9 and vascular endothelial growth factor (VEGF) and acute inflammatory chemokines (CCL-2, CCL-5). In addition, the nuclei of N1 and N2

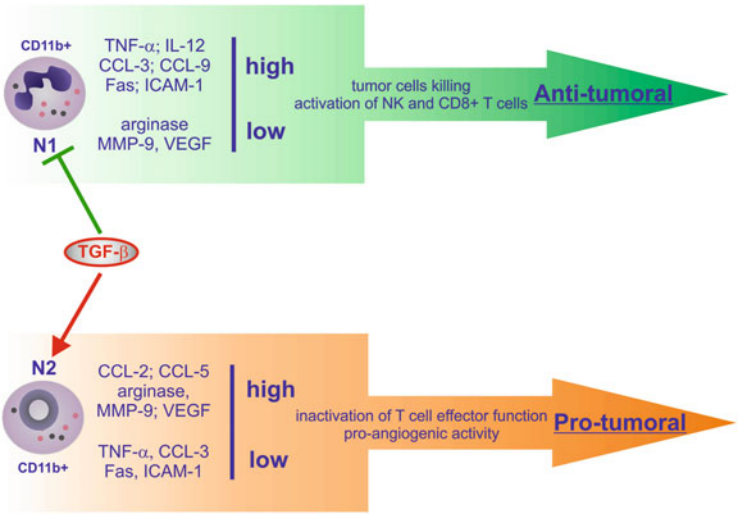

Fig. 7.1 Polarization of tumor-associated neutrophils (TANs). Tumor-associated neutrophils (TANs) undergo polarization into an anti-tumoral (N1) phenotype or a pro-tumoral (N2) phenotype. N1 TANs produce a high level of pro-inflammatory and chemotactic cytokines that activate cytotoxic natural killer (NK) and $CD8^+$ T cells. N1 TANs highly express molecules such as Fas and ICAM-1. They also produce low amounts of immunosuppressive arginase, pro-angiogenic factors, metalloproteinase 9 (MMP-9), and vascular endothelial growth factor (VEGF). By contrast, N2 TANs produce low amounts of pro-inflammatory and chemotactic cytokines, and have a low level of surface Fas and ICAM-1 expression. However, N2 TANs produce a large amount of acute inflammatory chemokines, arginase, MMP-9, and VEGF. N2 TANs inactivate the effector function of T cells and induce angiogenesis

neutrophils differ in shape; N1 TANs have a hypersegmented nucleus, whereas N2 TANs have circular nuclei [13, 21, 23, 24] (Fig. 7.1).

7.3 Anti-tumoral Effect of TANs

Neutrophils per se are not capable of recognizing tumor cells specifically. Tumor cells are also too large to be ingested by neutrophils. Recruited neutrophils produce several cytotoxic mediators, including reactive oxygen species (ROS), proteases, membrane-perforating agents, and soluble factors such as TNF-α and IL-1β, which are involved in the dysfunction of tumor cells and, finally, in tumor destruction.

7.3.1 ROS Production

Activated neutrophils produce and release a variety of powerful ROS. Radical species such as superoxide anion ($O_2^{-\bullet}$) and hydroxyl radical ($OH^\bullet$) as well as non-radical species such as hydrogen peroxide (H_2O_2) are produced by neutrophils

during the complex series of reactions named "respiratory burst." It is assumed that a plasma membrane-bound NADPH oxidase complex catalyzes a one-electron reduction of oxygen to $O_2^{-\bullet}$, which then is converted into H_2O_2 spontaneously or via the action of superoxide dismutase (SOD). In the reaction of H_2O_2 with Cl^- catalyzed by myeloperoxidase (MPO), highly toxic HOCl is formed. The oxygen metabolites are released either extracellularly or intracellularly into the phagosome [25–27].

ROS have a dual effect on cancer cells. On the one hand, ROS exert a genotoxic effect that initiates DNA damage, resulting in tumor establishment. On the other hand, their cytotoxic effect leads to tumor regression. The cytotoxic activity of ROS is related to various types of DNA damage such as oxidation, depurination, methylation, deamination, and single- and double-strand breaks. In addition to DNA, ROS also affect nuclear and cytoplasmic enzymes and various signaling proteins. Another action of ROS is lipid peroxidation [28, 29]. The involvement of ROS produced by neutrophils in the lysis of tumor cells has been proven by Zivkovic et al. [30] and Dallegri et al. [31]. They demonstrated that phorbol-12-myristate-13-acetate (PMA)-activated neutrophils induce tumor cell lysis (melanoma B16-F16 cells, B lymphoblasts) via ROS.

7.3.2 Fas/FasL System

The Fas/Apo-1 (CD95)/Fas ligand (FasL) system plays an important role in immune surveillance against cancer through inducing tumor cell apoptosis. The Fas molecule is a death receptor that belongs to the TNF super family of receptors. Its primary and best known function is the induction of apoptotic cell death after interaction with its physiological ligand FasL [32, 33]. Fas is expressed on N1 TANs [13]. Membrane-bound FasL (mFasL) has been documented on the membrane of colorectal [34], colon [35, 36], renal [35], liver [37], and breast cancer cells [38]. The Fas/FasL system has been shown to be involved in the cytotoxic activity of neutrophils against tumor cells [39, 40]. Additionally, tumor cells expressing FasL induce a massive infiltration and accumulation of neutrophils within tumors that leads to the induction of the antitumor response or even tumor rejection [39]. Other authors have reported that soluble FasL (sFasL), which is generated by cleaving mFasL, is a potent neutrophil chemoattractant but not a neutrophil activator [41, 42]. Reports have also shown that mFasL is protective against cancer, whereas sFasL is deleterious (promotes tumorigenesis), suggesting that sFasL and mFasL could have different functions [43, 44]. However, it should be noted that the interaction of FasL on tumor cells with Fas on neutrophils also can initiate neutrophil apoptosis, which is one form of tumor escape from immune surveillance [45].

7.3.3 TRAIL

TNF-related apoptosis-inducing ligand (TRAIL) is a membrane protein belonging to the TNF superfamily. This type II transmembrane protein is produced by and

expressed on the surface of several activated immune cells, including major players in anticancer immunity such as human NK cells and activated cytotoxic T cells. One of the characteristics of TRAIL is its ability to induce apoptosis via appropriate receptors only in transformed or cancer cells, not in normal cells [46, 47]. Membrane-bound TRAIL (mTRAIL) is cleaved by cysteine proteases and released from the cell surface as a soluble protein (sTRAIL) [48, 49]. Freshly isolated neutrophils have been shown to express TRAIL mRNA. Additionally, neutrophils can release sTRAIL, particularly after stimulation with interferon-γ (IFN-γ) or lipopolysaccharide (LPS) (reviewed in [50]). The potential antitumor significance of neutrophil-derived TRAIL has been previously examined and published. Koga et al. [51] have demonstrated that IFN-γ-stimulated neutrophils produce TRAIL and exert their cytotoxic effect on leukemic cells, at least partly, in this way. Tecchio et al. [52] reported that sTRAIL, present in supernatants harvested from interferon α (IFN-α)-activated neutrophils, exerted remarkable pro-apoptotic activity against TRAIL-sensitive cells (Jurkat J32 clone and MEG-01).

7.3.4 Matrix Metalloproteinase-8

MMP-8, also called collagenase-2, is the principal secreted neutral proteinase capable of initiating the degradation of native fibrillar collagens of types I, II, and III. It is primarily produced by neutrophils, monocytes, macrophages, T cells, and various cancer cells [53]. Neutrophil-produced MMP-8 has been shown to retard tumor growth. The unexpected finding that MMP-8 might have tumor-fighting functions was derived from a study by Balbin et al. [54]. They demonstrated that the absence of MMP-8 strongly increased the incidence of skin tumors in male *Mmp-8*$^{-/-}$ mice. Bone marrow transplantation studies provided additional evidence that neutrophil-derived MMP-8 was sufficient to restore the antitumor protection mediated by this metalloproteinase. Similar findings have also been reported by Gutiérrez-Fernández et al. [55]. Their studies revealed that MMP-8 reduced experimental lung metastasis in a mouse model. Additionally, high MMP-8 expression in breast tumors correlates with a lower incidence of lymph node metastasis, and confers a good prognosis to breast cancer patients. Therefore, the authors concluded and proposed that MMP-8 is an immunoprotective factor against tumors, which also has the ability to reduce the metastatic potential of malignant cells in both mice and humans.

7.4 Protumoral Effects of TANs

7.4.1 Neutrophil Elastase

Neutrophil elastase (NE) is a neutral serine protease produced by neutrophils that is stored in azurophilic granules. It was first identified as an enzyme with bactericidal activity. Currently, it is known that NE has various biological functions with the ability to destroy extracellular matrix (ECM) components. This serine protease has specificity against various components of the ECM such as elastin, fibronectin, proteoglycans, and type IV collagen. NE has been implicated in a variety of inflammatory diseases including chronic obstructive pulmonary disease, acute respiratory distress syndrome, ischemic-reperfusion injury, arthritis, and cancer [56, 57]. NE may promote the development, invasion, and metastasis of many cancers. The degradation of basement membranes (BMs) and the ECM is crucial for the invasion and metastasis of malignant cells. Moreover, NE promotes tumor growth and, at a modest concentration, directly induces tumor cell proliferation. TANs secrete NE near the tumor cell surface where it enters into tumor cells via clathrin-coated pits and gains entry into endosomes. In the endosomal compartment, among its various potential protein substrates, NE degrades insulin receptor substrate-1 (IRS-1). IRS proteins are capable of both regulating and activating phosphatidylinositol-3 kinase (PI3K), depending on the cell of origin. In the absence of IRS-1, the activity of PI3K is increased. The activation of PI3K leads to the phosphorylation of the serine/threonine kinase B (PKB) known also as AKT [3, 58–60]. Activated AKT phosphorylates a variety of substrates that are crucial in maintaining cell growth and survival, and it also regulates glucose metabolism [61]. Some reports indicate that patients with a higher concentration of NE in tumor extracts have a shorter survival time compared to individuals with a lower NE level. Higher amounts of NE are associated with stage III (FIGO classification) diseases [62, 63]. Houghton et al. [59] also demonstrated that NE directly induced tumor cell proliferation in both human and mouse lung adenocarcinomas via IRS-1 degradation. Another important role of NE in tumor growth was described by Gaida et al. [64]. They demonstrated that NE degraded E-cadherin, an adhesion molecule that mediates contact between tumor cells, resulting in significantly increased migratory capacity of pancreatic tumor cells.

In addition to the direct effect of NE on tumor cells, this serine protease can be involved in the modulation of the functional activity of various cells in the tumor microenvironment. NE appears to play a focused role in targeting the conversion of latent TGF-β and pro-MMP-9 into their biologically active forms [65]. NE has an important effect on cell–cell interaction and adhesion. It binds directly to CD11b/CD18 and regulates the integrin-mediated adhesion of neutrophils to fibrinogen [66]. NE also has the ability to cleave intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) from the cell surface (reviewed in [57]).

7.4.2 Matrix Metalloproteinase-9

Tumor invasion, metastasis, and angiogenesis require the controlled degradation of ECM, and the increased expression of MMPs is associated with the metastatic activity of tumor cells. MMPs are a family of peptides secreted by various types of cells, and they aid the degradation of ECM components to facilitate cell infiltration into tissue. MMPs have been divided by researchers into several groups based on their structure and substrate specificity. MMP-9 is a gelatinase that primarily hydrolyzes components of the basal lamina, including gelatin and collagen IV. MMP-9, also called gelatinase B, is involved in tumor growth, angiogenesis, and metastasis. Degradation of the ECM by MMPs can support tumor and endothelial cell migration [67, 68].

Within neutrophils, MMP-9 is stored inside secondary granules and released upon stimulation by IL-8, which is generally considered a major neutrophil chemotactic factor [69, 70]. Increasing evidence describes the pro-angiogenic function of neutrophil-derived MMP-9, which proteolytically releases VEGF sequestered within the ECM. Nozawa et al. [71], using a model of pancreatic islet carcinogenesis in mice, have shown that MMP-9 secreted by neutrophils mediates tumor growth and angiogenesis. Bakes et al. [72] then demonstrated that tumor formation by highly disseminating variants of human fibrosarcoma (HT-1080) and prostate carcinoma (PC-3) lines depended on the influx of inflammatory neutrophils that released elevated levels of angiogenesis-inducing MMP-9. Notably, various tumor cells release MMP-9 as a pro-enzyme (proMMP-9) in complex with the tissue inhibitor of metalloproteinase (TIMP), which negatively regulates its activation. However, in contrast to other cell types, human neutrophils uniquely release TIMP-1-free proMMP-9, poising it for activation and allowing it to rapidly exert its catalytic activity [73–76]. In an in-vitro study, Bausch et al. [77] have demonstrated that PMN-derived MMP-9 acts as a potent, direct, and VEGF-independent angiogenic factor for pancreatic ductal adenocarcinoma. To summarize, neutrophil-derived MMP-9 is essential for blood vessel formation, tumor cell intravasation, and spread.

7.4.3 Direct Involvement of Neutrophils in Tumor Cell Metastasis

Circulatory cancer cells must survive blood flow shear forces and immune system challenges, and after entering into capillaries, they must pass through the endothelial vessel wall (extravasation) into areas surrounding or distant from tumor tissues. This process plays a key role in tumor metastasis [78]. Several reports have indicated that neutrophils facilitate and enhance tumor cell extravasation. However, it remains unclear whether circulating neutrophils play an accidental or deliberate role in the formation of metastases. Wu et al. [79] have demonstrated that factors

present in a tumor-conditioned medium increase neutrophil attachment to cells of the human breast tumor cell line MDA-MB-231 and facilitate tumor cell transendothelial migration. Importantly, MDA-MB-231 cells alone did not transmigrate. Slattery and Dong [80] have reported that neutrophils enhance the migration of human melanoma cells (C8161) under flow conditions and improve C8161 cell adhesion to fibroblast L-cells. In later studies, Dong et al. [81] concluded that neutrophils facilitated melanoma cell tight adhesion on the endothelium and subsequent melanoma cell trans-endothelial migration. Recently, using in-vivo models of metastasis, Spicer et al. [82] showed that neutrophils promoted cancer cell adhesion within liver sinusoids, and intravital microscopy showed that neutrophils might act as a bridge to facilitate interactions between cancer cells and the liver parenchyma.

Neutrophil-mediated tumor cell extravasation has been extensively studied with melanoma cells. One hypothesis assumes that transmigration involves (1) neutrophil tethering on the endothelium and (2) tumor cell attachment to the tethered neutrophils. In this manner, their maintenance close to the endothelium facilitates extravasation. An alternative hypothesis assumes that tumor cells first interact with neutrophils to form "heterotypic aggregates" and next bind to the endothelium through neutrophils [83–85] (Fig. 7.2). Neutrophil tethering depends on L-selectin expressed on neutrophils and P- and E-selectins presented on the surface of endothelial cells [86]. Selectins bind to the carbohydrate sialyl Lewis^x (sLe^x) antigens present on both tumor and endothelial cells. This binding has low affinity that allows neutrophil tethering along the endothelium [79, 87]. Interaction between neutrophils/tumor cells/endothelial cells involves β-integrins (CD11a/CD18, LFA-1; CD11b/CD18; Mac-1) present on the neutrophil surface and E-selectin and ICAM-1 expressed on endothelial cells and ICAM-1 on tumor cells respectively [13, 79, 85]. Although several cytokines and chemokines have been implicated in influencing the adhesive activity of neutrophils and tumor cells, IL-8, alternatively known as CXCL8, is particularly interesting. IL-8 is overexpressed in various human cancers including skin, breast, stomach, prostate, melanoma, lung, ovarian, and colon cancer (review in [69]). Tumor cells, endothelial cells, macrophages, and neutrophils express the receptors for IL-8 (CXCR1 and CXCR2). As previously mentioned, IL-8 acts as a chemotactic factor for leukocytes and mediates neutrophil migration to the site of inflammation, and enhances their adhesive properties via the upregulation of Mac-1 and LFA-1 expression. In addition, IL-8 activates endothelial cells and promotes angiogenesis [81, 88]. The important roles of this chemokine in neutrophil and tumor cell interactions as well as in tumor metastasis have been proven by Huh et al. [89]. They demonstrated that the reduction of IL-8 expression in melanoma cells using small interfering RNA (siRNA) decreased the interaction between melanoma cells (WM35 melanoma cell line) and neutrophils, and diminished melanoma cell tethering to the endothelium. Additionally, decreasing IL-8 secretion from melanoma cells reduced neutrophil-mediated melanoma cell transit across the endothelial cell layer. However, melanoma cells are able to induce IL-8 expression in neutrophils, which can amplify the adhesive properties of neutrophils via an autocrine mechanism [90].

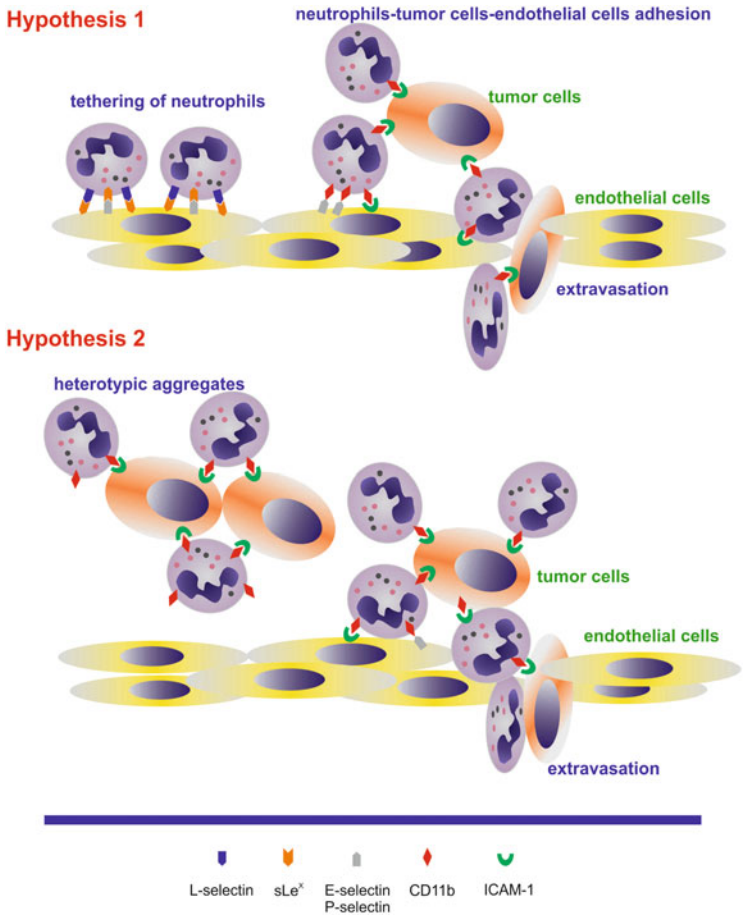

Fig. 7.2 Involvement of neutrophils in tumor cell extravasation. One hypothesis assumes that neutrophils first tether to the endothelium and then catch tumor cells and maintain them close to the endothelium, finally facilitating tumor cell extravasation. Neutrophil tethering depends on L-selectin expressed on neutrophils and P- and E-selectins presented on the surface on endothelial cells [86]. Selectins bind to the carbohydrate sialyl Lewis^x (sLe^x) antigen. Interactions between neutrophils/tumor cells/endothelial cells involve β-integrins (CD11b) present on the neutrophil surface and E-selectin and ICAM-1 expressed on endothelial and ICAM-1 on tumor cells. The second hypothesis assumes that tumor cells first interact with neutrophils and form "heterotypical aggregates" and next bind to the endothelium through neutrophil. In the presence of neutrophils, extravasation is facilitated. The formation of aggregates depends on CD11b-ICAM-1 interaction

However, it should be stressed that metastasis involves more mechanisms than those mentioned above. Glinsky et al. [91] have reported that metastatic breast and prostate carcinoma cells form multicellular homotypic aggregates at the site of their primary attachment to the endothelium. The process of metastasis most likely also

includes simply catching all cancer cells or passage through a leaky vasculature or metastasis through the lymphatic system [92–94].

7.5 Impact of Cancer Cells on Neutrophils

Tumor cells actively modulate the functions of neutrophils. One effect of tumor cells is the intensification of neutrophil inflammatory activity. Trellakis et al. [15] have reported that head and neck squamous carcinoma cells (HNSCCs) release factors that modulate the cellular functions of neutrophils, enhancing the survival, migration, and chemotaxis of peripheral blood neutrophils. Cancer cells induce neutrophil production of a high level of mediators such as lactoferrin and MMP-9, which upregulate the inflammatory activity of neutrophils. The authors concluded that interaction between HNSCCs and neutrophils may promote tumor progression. Hor et al. [95] have shown that co-culture with human glioma cell lines (U-373MG or U-118MG) increases neutrophil survival as a result of the augmented production of IL-8, IL-6, and TNF-α by neutrophils and tumor cells. The induced improvement of neutrophil survival by glioma cells requires cell-to-cell contact and the engagement of Fas/FasL cross-talk.

Our studies have also clearly demonstrated that cancer cells enhance the inflammatory properties of neutrophils. We have shown that ovarian cancer cells isolated from ovarian tumors pre-activate autologous blood neutrophils to enhance ROS production in response to stimulation with PMA and fMLP, as well as upregulate CD11b/CD18 expression on neutrophils [1]. The impact of ovarian cancer cells on neutrophils is caused by direct cell-to-cell contact. In our most recent study, we demonstrated that the activation of neutrophils isolated from ovarian cancer patients by ovarian cancer cells was dependent on the interaction of inducible heat shock protein HspA1A, which was produced by the ovarian cancer cells, with Toll-like receptor (TLR) 2 and 4, which are expressed on the neutrophil cell surface [96] (Fig. 7.3). HspA1A (formerly known as Hsp70) is secreted from dead and living cells, and exerts a powerful effect on the immune system [97, 98]. HspA1A interacts with target cells via numerous cell surface receptors, e.g., CD91, CD36, CD40, TLRs 2 and 4, and other c-type lectin receptors [99, 100]. Our data may have a practical implication for targeted anticancer therapies based, among other factors, on the inhibition of HspA1A expression in cancer cells. Preventing the enhancement of neutrophil pro-inflammatory activity by ovarian cancer cells (via expressed HspA1A) may protect against tumor progression and metastasis.

Another form of tumor cells action on the immune system is their ability to phagocytose other cancer cells. This phenomenon is termed cell cannibalism, and it has been documented primarily by light and electron microscopy of cytological preparations. As documented by others, tumor cannibalism is not only directed against sibling tumor cells but also against other cells present in the tumor microenvironment, including tumoricidal immune cells, e.g., neutrophils and

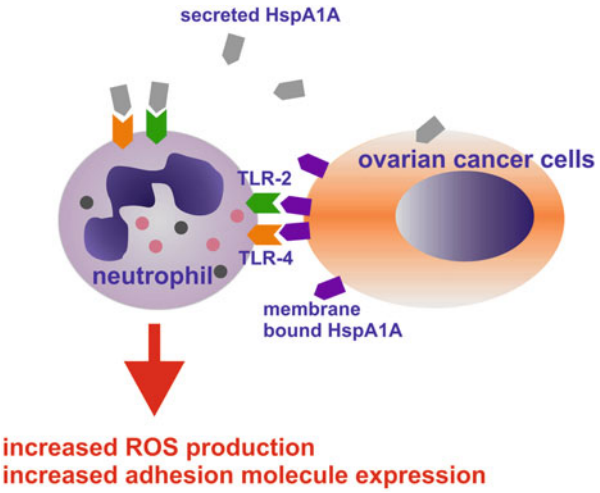

Fig. 7.3 The impact of ovarian cancer cells on neutrophils. Interaction of neutrophils with ovarian cancer cells results in an increase in neutrophil activity. This activation depends on the interaction of inducible heat shock protein HspA1A expressed on the surface of cancer cells, or its release as a soluble form with Toll-like receptor (TLR) 2 and 4 expressed on neutrophils

lymphocytes [101, 102]. This phagocytic activity of tumor cells is relatively rare, and is typically observed in high-grade/poorly differentiated malignancies. Cannibalism of neutrophils by tumor cells has previously been reported in certain carcinomas (lung, gall bladder, and pancreas), including lymphoma and melanoma. The cannibalistic activity of tumor cells significantly increases their survival and metastatic properties [103–105]. It is possible to believe that cannibalism by tumor cells is one method of survival, and is used as a special tumor immune escape mechanism by which tumor cells are able to phagocytose neutrophils and anti-tumor lymphocytes.

Tumor cells can affect endothelial cells in a specific manner to prevent subsequent neutrophil adhesion. Blaheta et al. [106] have shown that physical contact between tumor cells and the endothelium induces a distinct down-regulation of CD44 adhesion receptors on endothelial cells, leading to subsequent suppression of CD44-triggered neutrophil attachment and diminished neutrophil-HUVEC interactions. This may be a mechanism by which neutrophils do not facilitate cancer invasion, and instead, it may serve as an escape route for tumor cells from effective neutrophil attack.

7.6 In Summary

A large number of studies support the duality of neutrophil function in relation to tumors. Through mutual interactions with tumor/cancer cells, polymorphonuclear neutrophils appear to play both positive and negative roles. Some data show that neutrophils, similarly to other cells of immune system, increase the metastatic efficiency of tumor cells primarily through the degradation of the extracellular matrix and enhancement of angiogenesis (protumoral functions of neutrophils).

This role of neutrophils appears to predominate under conditions of chronic inflammation. Conversely, neutrophils activated as a result of physical contact with tumor cells destroy and eliminate the tumor cells (antitumor properties of neutrophils). However, the involvement of neutrophils in immune surveillance in the anticancer response of the host appears to be unsung or disregarded. The antitumor properties of neutrophils should not be ignored, particularly under conditions of acute inflammation. Targeting the antitumor properties of neutrophils may improve therapy for some cancers by preventing further infiltration and expansion into normal tissues.

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Chapter 8
Cancer Immunoediting: Elimination, Equilibrium, and Immune Escape in Solid Tumors

Jacek R. Wilczynski and Marek Nowak

Abstract Emphasizing the dynamic processes between cancer and host immune system, the initially discovered concept of cancer immunosurveillance has been replaced by the current concept of *cancer immunoediting* consisting of three phases: elimination, equilibrium, and escape. Solid tumors composed of both cancer and host stromal cells are an example of how the three phases of cancer immunoediting functionally evolve, and how a tumor shaped by the host immune system gets a finally resistant phenotype. Elimination, equilibrium, and escape are described in this chapter in detail, including the role of immune surveillance, cancer dormancy, disruption of the antigen-presenting machinery, tumor-infiltrating immune cells, and resistance to apoptosis, as well as the function of tumor stroma, microvesicles, exosomes and inflammation.

List of Abbreviations

AFP	Alpha-fetoprotein
APCs	Antigen-presenting cells
APM	Antigen-processing machinery
CAFs	Cancer-associated fibroblasts
CCR7	C–C chemokine receptor type 7
CEA	Carcinoembryonic antigen

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COX	Cyclooxygenase
CSCs	Cancer stem cells
CSF-1	Colony-stimulating factor-1
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CTLs	Cytotoxic T lymphocytes
CXCR1	Interleukin 8 receptor, alpha
DCs	Dendritic cells
DTCs	Disseminated solitary tumor cells
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial–mesenchymal transition or transformation
EOC	Epithelial ovarian cancer
FasL	Fas ligand
FGF	Fibroblast growth factor
GITR	Glucocorticoid-induced tumor necrosis factor receptor
GLI	Glioma-associated oncogene homolog
GM-CSF	Granulocyte–macrophage colony-stimulating factor
Hh	Hedgehog signaling
HIF-1α	Hypoxia-inducible factor-1α
HLA	Human leukocyte antigen
HPV	Human papilloma virus
Hsp	Heat-shock protein
IAPs	Inhibitor of apoptosis proteins
ICAM-1	Intercellular adhesion molecule 1
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
ILT	Immunoglobulin-like transcript
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
MAPK	Mitogen-activated protein kinases
M-CSF	Macrophage colony-stimulating factor
MDSCs	Myeloid-derived suppressor cells
MMPs	Metalloproteinases
MVD	Microvessel density
NF-κB	Nuclear factor-κB
NK	Natural killer cells
NKG2D	Activating receptor of NK cells
NKT	Natural killer T cells
NO	nitric oxide
PBLs	Peripheral blood lymphocytes
PD-1	Programmed death-1 and its ligand PD-L1 (also called B7-H1)
PDGF	Platelet-derived growth factor
PGE_2	Prostaglandin E_2

RANTES	Regulated on activation, normal T-cell expressed and secreted (CCL5)
RCAS1	Receptor-binding cancer antigen expressed on SiSo cells
RNS	Reactive nitrogen species
ROI	Reactive oxygen intermediates
STAT	Signal transducer and activator of transcription
TAA	Tumor-associated antigen
TAMs	Tumor-associated macrophages
TANs	Tumor-associated neutrophils
TAP	Antigen peptide transporter
TCR	T cell receptor
TEMs	Tie-2-expressing monocytes/macrophages
TGF-β	Transforming growth factor-β
TILs	Tumor-infiltrating lymphocytes
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
Tr1 cells	Type 1 regulatory T cells
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	T regulatory cells
TSA	Tumor specific antigen
uPAR	Urokinase plasminogen activator receptor
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

Contents

8.1 Introduction

The idea of *cancer immunosurveillance* has been built on the hypothesis that cancer cells are recognized as non-self and induce the host response. In fact, cancer cells differ from normal human cells. Neoplastic cells express on their surface antigens, which can be the targets for humoral or cellular response.

Initially, tumor antigens were divided into tumor-specific antigens (TSA), present only on cancer cells, and tumor-associated antigens (TAA), found also on non-cancer cells. However, during subsequent investigations, antigens initially thought of as TSA have been found also on normal human cells. Currently, the classification of tumor antigens is based on their molecular structure and origin. Thus, there are differentiation antigens (e.g., tyrosinase or gp-100 in melanoma), overexpression/amplification antigens (e.g., HER-2/neu in ovarian and breast cancer), mutational antigens (e.g., p53, Ras in various cancers), cancer testis antigens (e.g., NY-ESO-1 in ovarian cancer), glycolipid antigens (e.g., MUC-16 in ovarian cancer), oncofetal antigens (e.g., AFP in germ cell tumors, CEA in colorectal cancer), and viral antigens (e.g., HPV in cervical cancer) (reviewed in [1]). To date, more than 1,000 human tumor antigens have been described (Cancer Immunome Database). Conceptually, TAAs may be divided to three groups: self-antigens or embryonic antigens overexpressed or respectively aberrantly expressed on cancer cells, self-antigens modified by post-translational tumor-specific disturbances, and neo-antigens originating from mutations, chromosomal aberrations, and viral transformation [2].

Thus, the intact immune system may recognize TAAs and prevent the development of cancer, in a process initially termed immunological surveillance [3]. The host response involves both innate and adoptive immune system, which closely cooperate. Generally, the innate immunity is mainly responsible for early detection and elimination of malignant cells, while the adaptive immune system rather controls the tumor progression. However, cancer cells have developed variety of strategies to evade the host immune system. They shed surface antigens and down-regulate the expression of molecules necessary for interaction with immune cells. They also produce and release factors (cytokines, enzymes) that exert a modifying effect on the host-adaptive immune response or induce the apoptosis of immune cells [4, 5]. These host–tumor interactions may or may not result in cancer elimination. When the host-mediated antitumor immunity is stronger, tumor cells are eliminated; otherwise, cancer cells undergo immune escape and grow rapidly [1, 6].

Emphasizing the dynamic processes between cancer and host immune system, the concept of cancer immunosurveillance [3] has been replaced by the current concept of *cancer immunoediting* [7] consisting of three phases: elimination, equilibrium, and escape. In the process of *elimination*, nascent transformed cells are recognized and eradicated by innate and adaptive immune system—if all neoplastic cells are eliminated, cancer immunoediting is finished and consistent with cancer immunosurveillance. If all transformed cells are not eliminated at the beginning, immunological pressure leads to the selection of clones with decreased immunogenicity, which successively become resistant to the immune system in the *equilibrium* phase—tumors are usually still not detectable clinically. A developing tumor creates a proinflammatory and immunosuppressive microenvironment leading to the impairment of the host immune function and *escape* from immunosurveillance, resulting in tumor growth and metastases.

8.2 Immunosurveillance of the Host Against Cancer-Elimination

The main effectors of cancer immunosurveillance are natural killer (NK) cells, natural killer T cells (NKT) cells, γδ T cells, cytotoxic T lymphocytes (CTLs), interferon (IFN) γ, perforins, and system Fas/FasL. Their role in cancer immunosurveillance was firstly confirmed and described in immunologically manipulated mice (reviewed in [8, 9]). Subsequently, clinical findings have supported the conclusions driven from animal studies. The presence of high-density tumor infiltration by NK cells and tumor-infiltrating lymphocytes (TILs) was found in many cancers, and correlated with better prognosis and survival in patients with ovarian cancer, breast cancer, lung cancer, oral, oesophageal, gastric and colorectal cancer, and malignant melanoma [10–19]. Moreover, the presence of both tumor-specific cellular (T cells) and humoral (antibodies) response was connected with better prognosis in cancer patients [20].

The elimination process is initiated when developing tumor cells (for their progression) and also macrophages, stromal cells of the cancer site, release inflammatory cytokines which recruit and activate other innate effector cells such as NK, NKT, and γδ T cells. They recognize and destroy neoplastic cells by means of perforins, Fas/FasL, TRAIL, and IFN-γ [21–23]. Secreted IFN-γ exerts cytotoxic effects and induces apoptosis of the cancer cells [24, 25]. Necrotic tumor cells release tumor antigens which evolve an adaptive response. NK cells promote maturation of dendritic cells (DCs) and their migration to the regional lymph nodes. DCs ingest destroyed tumor cells and their tumor antigens, and after maturation and migration to the regional lymph nodes present the antigens to naïve $CD4^+$ T cells. This presentation generates clonal expansion of tumor-specific $CD4^+$ and $CD8^+$ T cells (CTLs). Tumor-specific CTLs infiltrate the tumor site and eliminate the rest of the cancer cells expressing tumor antigens [8]. When all cancer cells are destroyed, the elimination is completed. However, the end may be not so successful.

Dying transformed cells (and also normal human cells) release danger signals such as uric acid, heat shock proteins, and extracellular matrix derivates which may induce a proinflammatory response activating innate immune system [26, 27]. Limited inflammatory reaction usually helps eradication of tumor cells, but intense inflammation may promote tumor progression, among others by stimulation of release of immunosuppressive cytokines such as interleukin (IL) 10 and transforming growth factor β (TGF-β) as a feedback loop [28]. Moreover, genetic instability of cancer cells under host immunologic pressure creates less immunogenic types of cells [20]. Taken together, this weakening of the immune response and decreasing immunogenicity of transformed cells may lead to the next steps of cancer immunoediting—equilibrium and/or escape.

8.3 Cancer Dormancy and Cancer-Immune Equilibrium

Cancer dormancy defined as a clinical phenomenon is described by cancer systemic or local recurrence after a long time in a patient who has been considered as completely cured and free of the disease. Such situation has been observed in several tumors, including breast, prostate, renal, thyroid cancer, and melanoma [29]. The relapse of breast cancer after 10–20 years after the primary treatment has been noticed in a relatively steady population of 1.5 % of patients [30]. It has also been shown that circulating tumor cells were present in 36 % of breast cancer patients after mastectomy as long as 7–22 years after the surgery [31].

Clinical dormancy is probably connected to the existence of cancer stem cells (CSCs), which reside in bone marrow or survive in metastatic niches, are in a cell-cycle arrest, and are resistant to both chemo- and radiotherapy [32, 33]. Highly tumorigenic $CD133b^{+}$ melanoma CSCs and $CD44^{+}CD24$-$cytokeratin^{+}$ breast cancer stem-like cells have been observed in the bone marrow of patients [34, 35]. The stem cell phenotype depends on the cell reprogramming through a group of transcription factors including Sox2, c-Myc, Klf4, Oct4, Nanog and Lin28. The overexpression of these transcription factors has been demonstrated in various tumors, such as gastrointestinal, lung, and colon cancers (reviewed in [36]). Nanog is a common regulatory molecule for several pathways: c-Met receptor tyrosine kinase, hedgehog, and TGF-β signaling [37–39]. Moreover, transcription factors that regulate epithelial–mesenchymal transition or transformation (EMT) and down-expression of p53 were able to induce stem properties in cancer cells (reviewed in [36]). Microenvironmental signals could influence the development of cancer stem cells (CSCs). Hypoxia through hypoxia-induced factor 1α (HIF-1α), inflammation through signal transducer and activator of transcription (STAT) 3, nuclear factor (NF)-κB- and Hh-signaling, epidermal growth factor (EGF), TGF-β, and IL-6 are all inductors of CSC development. Finally some epigenetic events such as DNA demethylation and histone acetylation/methylation could activate the CSC phenotype (reviewed in [36]).

There have been many hypotheses to explain the behavior of dormant cancer cells and clinical dormancy, including insufficient angiogenesis, the presence of micrometastases, restoration of host immunosurveillance after successful treatment, reaction of disseminated all over the body cancer cells to hormonal or environmental factors, or regulation of a dormant cancer cell pool by organ size-controlling genes (reviewed in [29]). All of them were based on experimental and clinical proofs; therefore, it is a reasonable assumption that tumor dormancy is a complex phenomenon dependent on various mechanisms. Insufficient angiogenesis is one of the suspected reasons for dormancy of a small tumor mass, which may be both an initial growing cancer or a micrometastatic tumor (angiogenic dormancy) (reviewed in [40]). It can slowly proliferate, but is avascular either because of the lack of expression of angiogenic factors or because of the up-regulation of angiogenesis inhibitors. The rate of proliferation and apoptosis balance each other, and the tumor has stable dimensions [41]. Behavior of melanoma metastases

could confirm such a hypothesis. While micrometastases indicate low rate of proliferation and microvessel density, macrometastases show significantly higher both proliferation potential and vascularity [42]. Another reason for small tumor dormancy is an immunosurveillance reached by cytotoxic effectors-dependent apoptosis balanced by tumor proliferation rate (immune dormancy) [43].

Another problem is dormancy of solitary cells, which could originate either from small growing tumors producing early single-cell dissemination into distant organs, or from disseminated solitary tumor cells (DTCs) which have left after primary therapy. The small clinically "silent" tumors have been found in the breasts of 39 % of women aged 40–50, and in the prostates of 46 % of men aged 60–70, subjected to autopsies after death caused by car accidents, but we know that only 1–1.5 % of populations at this age have clinically recognizable tumors [44–46]. The possibility of very early dissemination was raised by the studies, suggesting that mammary ductal carcinoma in situ (DCIS) produced disseminated cells [47]. These early DTCs produced by pre-malignant lesions are not able to initiate a metastatic growth in target organs due to their insufficient genetic alterations and suppressive signals from the environment, and thus enter a state which prevents apoptosis but maintains dormancy [48]. Similarly, it has been found that about 30 % of patients diagnosed as having breast cancer already had micrometastatic disease in bone marrow; however, only 50 % of them presented with clinically evident bone metastases in the course of the disease [31]. Most of the dormant solitary cells have been isolated from bone marrow of various cancer patients and showed proliferative quiescence, based mostly on G_0/G_1 arrest with over-expression of p21 and p27 (cellular dormancy) [49]. Down-regulation of the urokinase receptor (uPAR), β1-integrins, focal adhesion kinase (FAK), and EGF receptor (EGFR) reduces proliferative signals from ECM. Prolonged uPAR suppression activates long-lasting dormancy, as was shown by inhibiting of uPAR in squamous cancer cell line. The possible mechanism that triggers dormancy is an uPAR-mediated establishing of an imbalance in the cancer cells that favors p38/stress-activated protein kinase (SAPK) over extracellular signal-regulated kinase/mitogen activated protein kinase (ERK/MAPK) signaling, which activates endoplasmic reticulum stress-like reaction and G_0/G_1 cell arrest (reviewed in [50]). The interactions between fibronectin and α5β1 integrin were also uPAR-dependent and modulated the ECM functions (reviewed in [51]). This means that inappropriate interactions with ECM may trigger mechanisms leading to cell dormancy (reviewed in [52, 53]). Impaired signaling through integrins and adhesion signal transducers has been noticed in DTCs of squamous and breast cancers [54–56]. Disturbed interactions with ECM may also trigger autophagy, a self-digestion process during which a cancer cell, through the regulated breakdown of cytosolic components, acquires a pro-survival phenotype. The presence of both autophagy and dormancy has been confirmed in ovarian cancer cells under stress conditions [57]. Another regulator of DTCs quiescence, transcription factor HES-1, which induced dormancy but prevented cell senescence and terminal differentiation, has been identified in melanoma cells [49].

An important observation made in the nineteenth century by Paget contributes to the contemporary understanding of dormancy. According to it, the metastatic cancer cell potential to survive depends not only on the inherent cell properties, but also on the existence of a hostile or hospitable environment in the target organ ("seed and soil" theory) [40]. Modern studies support that notion, as cancer cells could be turned into a quiescent state by the signals derived from the microenvironment and dependent on the therapy [58]. It has been shown that breast cancer patients with cells disseminated to the bone marrow had longer disease-free intervals than patients who displayed cell dissemination into other organs [59]. Squamous carcinoma cells have been shown to disseminate into multiple organs including lungs, liver, bone marrow, spleen, and lymph nodes; however, only inside lungs and lymph nodes they developed clinical metastases [60]. Moreover, murine models indicated that cancer cells disseminated to the bone marrow failed to expand unless they were transplanted into irradiated recipients [61]. The connection between environment and behavior of DTCs is further supported by the observation that genes responsible for DTCs quiescence, including MKK4, MKK6, KISS1, and some others, are exclusively activated in the target organs, but not in the primary tumor [62]. Some of these genes activate p38α/β and inhibit ERK 1/2 signaling, leading to an epigenetic regulation of cell quiescence (reviewed in [48]). TGF-β could be another regulator of cell dormancy, but its function depends on the type of the target organ, other signals, and the ability of cancer cells to activate alternative cellular pathways to benefit of the proliferative TGF-β activity [48]. The primary tumor could also influence the dormancy of DTCs, as its gene signatures may determine the behavior of the DTCs. This has been confirmed in breast cancer, where some genes predicted either a long or alternatively a short metastasis-free interval [48]. Regulation of DTCs may also occur via mechanisms of DTCs self-seeding into the primary tumor, which usually increases its aggressiveness, and via tumor instigation of distant micrometastases by endocrine factors (reviewed in [48]). The latter mechanism is interesting, as osteopontin secreted into the circulation by instigating tumor activates bone-marrow-derived cells, which migrate into the dormant tumor and stimulate cancer-associated fibroblasts (CAFs) to switch dormant cells into a proliferative malignant phenotype (reviewed in [63]).

The status of cancer-immune equilibrium is closely connected to the problem of tumor immune dormancy, when small tumors could be controlled by host effector cells. The murine studies showed that sarcomas transplanted into T cell-, IFN-γ-, and IL-12-deficient mice rose vigorously, but were eliminated when retransplanted into immunocompetent wild-type mice. Depletion of innate NK cells or neutralization of the activating receptor of NK cells (NKG2D) and TRAIL pathways had no effect (reviewed in [64]). Similarly, long-term survivals were demonstrated in mice subjected to adoptive immunotherapy which, however, did not eliminate completely transplanted prostate cancers, but instead controlled them in the phase of a small tumor [65]. The equilibrium between T $CD8^+$ cells and small skin tumors was also observed in another murine study [66]. These findings strictly indicate that adaptive T effectors, IFN-γ, and IL-12 play an important role in controlling tumor growth (reviewed in [64]). Tumors in cancer-immune equilibrium have been shown

to be slowly proliferating tumors with increased ratio of dying cells and the presence of host immune effectors (reviewed in [64]). Human clinical observations seem to support the cancer-immune equilibrium phase of tumor growth. It has been shown that late lung cancer remissions occurred mostly in immuno-defective persons, as well as small non-detected tumors transplanted unintentionally with the organs of immunocompetent donors became clinically evident in immunosuppressed recipients [67, 68].

8.4 Cancer Escape Mechanisms

8.4.1 Disruption of the Antigen-Presenting Machinery, HLA-G and Costimulatory Molecules

Tumor-associated antigens originate from self-antigens or embryonic antigens overexpressed or respectively aberrantly expressed on cancer cells, self-antigens modified by post-translational tumor-specific disturbances, and neo-antigens originating from mutations, chromosomal aberrations, and viral transformation (reviewed in [2]). As most solid tumors express self- or modified self-antigens, T effectors are unable to recognize them properly due to central and peripheral tolerance. Peripheral tolerance could be overcome by a process of cross-priming during which DCs, in order to effectively stimulate T effectors, need to encounter antigens associated with "danger signals" (pathogenic-associated molecular patterns—PAMPs) via Toll-like receptors (TLR). Usually the "danger signals" are derived from microorganisms; however, in cancer, necrotic cells could deliver damage-associated molecular pattern (DAMPs) signals including calreticulin and high-mobility group box-1 protein (HMGB1) [69, 70]. Low tumor-induced expression of TLR9 receptor on plasmacytoid DCs has been observed in head and neck squamous cancer [71]. In colon cancer patients loss, of functional TLR4 resulted in short progression-free survival [69]. DCs which have not been activated by "danger signals" are able to present tumor antigens in the context of MHC molecules; however, this process causes T-cell anergy and apoptosis in a mechanism of cross-tolerance [72]. Observations in cancer patients revealed the presence of soluble forms of HLA (sHLA). The data concerning the concentration of sHLA in cancer is not consistent, and depends on the tumor type and HLA allotypes. Increased, unchanged or decreased sHLA levels have been described in pancreatic, melanoma, and gastric cancer respectively. sHLA may down-regulate activity of CTL and NK cells (reviewed in [73]). The mechanism of tumor recognition by T effectors is also disturbed by abnormalities in antigen presentation machinery, including loss or down-regulation of HLA class I antigens due to gene mutations, loss of heterozygosity, and disturbed transcriptional regulation (reviewed in [2]). The presence of such mechanisms has been confirmed in oesophageal, prostate, and lung cancer. Tumors are capable of losing TAAs together with HLA antigens not

only spontaneously, but in the response to adoptive T $CD8^+$ therapy. Initially effective MART-1/Melan A-targeted adoptive T-cell therapy of HLA-A2 positive melanoma was found to be ineffective in metastases and recurrent tumors, due to the loss of expression of MART-1 and HLA-A2 molecules [74–76]. In melanoma and colon cancer, the mutation of β2-microglobulin has been observed [77]. Tumors are also characterized by acquired deficits in antigen peptide transporter (TAP) and low-molecular mass polypeptide (LMP)2 and LMP7 immunoproteasome subunits [78]. In melanoma and renal cancer, decreased expression of HLA class I antigen was shown to be caused by methylation of TAP-1 and -2 [79]. Interferon is capable of up-regulation of HLA molecules, but defects in IFN-γ signaling such as mutations of Janus kinases (JAK) 1 and 2 may also decrease their expression [80]. In head and neck squamous cancers, down-regulation of HLA class I antigen and defective function of members of antigen processing machinery (APM) were correlated with low T $CD8^+$ infiltration, metastases to regional lymph nodes and poor prognosis (reviewed in [81]).

Despite these mechanisms, activated NK cells should be able to recognize and kill HLA-negative tumor cells. However, to avoid both CTL and NK cell-dependent attack, tumor cells express an immunomodulatory non-classical HLA class I antigen HLA-G on their surface ([82], reviewed in [73]). Epigenetic changes such as demethylation or histone acetylation may be responsible for ectopic HLA-G expression on cancer cells [83]. Unfortunately, it seems that host immunosurveillance against tumor accounts for initiating HLA-G, as IFN-producing immune effector cells up-regulate HLA-G expression. Moreover, tumor-infiltrating immune cells also acquire the HLA-G positive phenotype, producing strongly immunosuppressive environment inside the tumor. Effector cells, by contact with HLA-G both on cancer and on regulatory cells, and via trogocytosis of membrane fragments containing HLA-G from DCs, become inhibited and turned into tolerogenic status (reviewed in [84]). Several receptors for HLA-G functioning as killing inhibitory receptors (KIRs) have been identified, including KIR2DL4/p49, immunoglobulin-like transcript (ILT)-2, and ILT-4, which were found to be expressed on NK cells, T and B lymphocytes, macrophages, and DCs. Therefore, HLA-G is capable not only of inhibiting NK cytotoxicity, but of modulating the activity of DCs, followed by inhibition of proliferative T-cell responses (reviewed in [84–87]). Through inhibitory ILT-2 receptor, HLA-G disturbs T-cell activation, and decreases CD3ζ phosphorylation and IL-2 secretion [88]. Additionally to the expression of membrane-bound HLA-G, tumors are capable of secreting its soluble form (sHLA-G), having strong systemic immunoregulatory properties. sHLA-G induces Fas-dependent apoptosis of activated T $CD8^+$ CTLs, and decreases T $CD4^+$ helper activity. Both membrane-bound and sHLA-G forms induce production of Th2 cytokines, including IL-10, which in this way creates an auto-enhancing regulatory loop. HLA-G could be also present in exosomes disseminated into the circulation from the tumor [84]. Inside established tumors, there are several factors that trigger and support HLA-G expression, including hypoxia (via HIF-1α), chronic inflammation (via NF-κB), and immunosuppressive IL-10 (reviewed in [89]). Activators of NF-κB transcription factor also stimulate the sHLA-G shedding from cancer cells

[90, 91]. The presence of HLA-G molecules has been confirmed in many cancers, especially these associated with inflammation [84, 92]. Concentration of sHLA-G correlates with tumor size [93]. In addition to HLA-G, some other non-classic HLA molecules like HLA-E and HLA-F have been described in tumors, including lung cancers, and their expression indicates a bad prognosis [94]. HLA-E exerts additional suppressive signals to lymphocytes through CD94/NKG2A KIR, and HLA-G has a stabilizing effect on this molecule [85].

The NKG2D (natural killer group 2, member D) receptor is expressed on the surface of NK and some T cells, including activated T $CD8^+$ and some T $CD4^+$, γ/δ T, and NKT cells respectively. Human NKG2D ligands comprise MHC class I-related chain (MICA and MICB) and UL16 binding protein family (ULBP) members. Ligands for NKG2D are induced on tissues upon inflammation, stress stimuli, and DNA damage during cancer transformation (reviewed in [73]). Tumors are capable of disturbing the recognition of surface ligands by NKG2D receptors through several mechanisms [95–97]. Firstly, constant over-expression of NKG2D ligands results in down-regulation of NKG2D expression. Moreover, by TGF-β production, cancer can directly down-regulate NKG2D expression [98, 99]. Soluble MIC molecules released from cancer cells could further disturb CTL and NK cell cytotoxicity by down-regulation of activating NKG2D receptor, natural cytotoxicity receptor NKp44, and chemokine receptors CCR7 and CXCR1 [100]. A model of prostate cancer studied on NKG2D-deficient mice indicated the growth of more aggressive tumors with high expression of NKG2D ligands compared to tumors in wild-type animals [101]. Expression of NKG2D ligands has been observed in human colorectal tumors; however, this varied between different tumor types and became less frequent in more advanced tumors. High expression of these ligands correlated with improved survival of patients and NK cell infiltration [102].

Costimulatory molecules which transfer positive or negative signals necessary to initiate T-cell responses belong either to the classic B7 family (CD80, CD86) or to the family of B7 homologues containing B7-H1, B7-H2, B7-H3, B7-H4, and some other members. Absence of classic co-stimulatory molecules CD80 and CD86 on the surface of tumor cells induces anergy in T $CD4^+$ lymphocytes recognizing HLA class II antigens [103]. Recently, the B7-H4 homologue, transferring a negative signal for T cell activation, has deserved greater attention, due to its abundance both in the tumor and immune cells of cancer patients (reviewed in [104]). The B7-H4 molecule, by arresting the cell cycle, inhibits the activation, proliferation, and clonal expansion of T $CD4^+$ and T $CD8^+$ cells, as well as secretion of stimulatory IL-2 and IFN-γ cytokines [105]. A soluble form of B7-H1 molecule has been observed in aggressive renal cancer [106]. To date, expression of B7-H4 has been confirmed in a variety of solid tumors including colon, prostate, lung, gastric, ovarian, pancreatic, and uterine cancer, and melanoma (reviewed in [104]). Regulatory T cells (Tregs) were reported to induce B7-H4 molecules on the surface of DCs and tumor-associated macrophages (TAMs), where it functioned as an inhibitor of T-cell activation and cytotoxicity ([107], reviewed in [108]). Moreover, B7-H4 mediated inhibitory effects on the growth of neutrophils [109]. In addition to regulatory effects on the function of the immune system, B7-H4 influenced the

tumorigenesis by enhancing the proliferation, migration, and invasiveness, and protecting cancer cells from apoptosis, as was shown in an ovarian cancer murine model [110, 111]. In ovarian cancer, the expression of B7-H4 and the level of soluble B7-H4 correlated with tumor stage, pathological type, and patients' poor prognosis (reviewed in [104]). Similarly, in breast cancer the over-expression of B7-H4 was connected with negative receptor status and HER-2/neu positivity [112].

8.4.2 Tumor-Infiltrating Lymphocytes and Immune Escape

Tumor-infiltrating lymphocytes (TILs) are the heterogenous population of immune cells, which upon existence of immunoregulatory conditions in tumor environment acquire in most circumstances an immunosuppressive or regulatory phenotype, and lose at least partially an anti-tumor effector activity. The composition and activation status of TILs depends on the expression of chemokines and cytokines originating from both cancer and immune cells in tumor environment.

Effectory T CD8$^+$ cells in the TIL population have been considered to be a good prognostic sign in ovarian cancer [113, 114]; however, there are suggestions that the T CD8$^+$/Tregs ratio could be a better indicator of good prognosis [15]. The presence of T CD8$^+$ effectors capable of recognition of tumor-associated antigens has been confirmed in several tumors. In melanoma patients, T CD8$^+$ effectors responsive against melanA/MART-1 cancer antigen were present in peripheral blood and regional lymph nodes, and most of them belonged to population of naïve CD28$^+$CD45RA^{high} T cells. The rest of melanA/MART-1-reactive T CD8$^+$ effectors belonged to memory T cells, and were abundant especially inside the tumor [115]. Similar observations were done for colorectal cancer [116]. However, the anti-tumor T CD8$^+$-mediated reactivity was not consistently found in peripheral blood of breast cancer patients, and was different compared to T cells isolated from the bone marrow of the same patients (reviewed in [117]). It seems that irrespective of possessing an effector phenotype, T cells might be unresponsive against some tumor antigens in vivo, which could result from both suppressive environment and antigen heterogenic immunogenicity [118]. Moreover, distinct regulatory mechanisms are probably engaged in control of the TIL function in different intratumor localizations. In ovarian cancer, increased intra-epithelial T CD8$^+$ lymphocyte density was correlated with better prognosis, while the intensity of stromal T CD8$^+$ infiltrate did not indicate such a correlation [119]. It has been shown in several tumors including ovarian cancer that many regulatory cytokines present in the tumor and ascites, including IL-10, TGF-β, tumor necrosis factor α (TNF-α), and vascular endothelial growth factor (VEGF) indicate immunosuppressive actions against effector TILs [120, 506]. Inside the tumor, effector TILs are functionally impaired, as was indicated by down-regulation of CD3ζ chain, decreased proliferation and expression of activation antigens (CD25, CD69, HLA-DR), and low secretion of stimulatory cytokines, such as IL-2, IL-4, and IFN-γ ([121–125], reviewed in [126]).

The mechanisms of effector TIL inhibition include also tolerance-inducing plasmacytoid DCs, B7-H4$^+$ macrophages, TAMs, and myeloid-derived suppressor cells (MDSCs) [107, 127–129]. Expression of galectins by tumor cells is another mechanism of effector TIL inhibition. Galectins are proteins possessing the same recognition domain as β-galactosides, and involved in cell proliferation, adhesion, migration, apoptosis, and angiogenesis. In human melanoma, the expression of galectin-3, although not consistently observed in every tumor, has been shown to be correlated with apoptosis of TILs [130]. Expression of galectin-1 (Gal-1) in the tumor cells and in its stroma has been correlated with malignancy and poor patient outcome. Expression of galectin-1 in stroma surrounding the cancer cells and in endothelium in tumor-penetrating vessels protects the tumor from host immune reaction. Expression of Gal-1 in head and neck squamous cancer correlated negatively with T effector infiltration, while blockade of Gal-1 activity in melanoma resulted in reduced tumor mass and more abundant T cell infiltrate (reviewed in [131]). Another immunoregulatory molecule influencing negatively effector function is indoleamine 2,3-dioxygenase (IDO), the expression of which has been noticed in a variety of cancers. Overexpression of IDO in colorectal, ovarian, and endometrial cancers affected the infiltration of tumor with T CD3$^+$, T CD8$^+$ and CD57$^+$ NK cells. In most cases of solid tumors, over-expression of IDO correlated with the abundance of Tregs infiltrate, metastases to regional lymph nodes and to distant sites, and short progression-free and overall survival, and was present especially in advanced tumors (reviewed in [132]). However, in different conditions and in certain tumor types the infiltration of effector TILs may be more vigorous than in most cancers. Tumors showing over-expression of chemokines CCL2, CCL5, CXCL9, and CCL22, activatory cytokines IL-2 and IFN-γ, and parallel low concentration of VEGF, were infiltrated with significantly increased T-cell numbers [120, 133]. The state of TIL effector anergy is not permanent, as cells tested outside the tumor hostile environment presented in in-vitro conditions expression of activation marker (HLA-DR) and co-stimulatory molecules (CD28, CD80, CD86), and indicated cytotoxicity against cultured ovarian cancer cells [124, 125, 134–137]. Not only TILs but also peripheral blood lymphocytes (PBLs) may be functionally impaired in cancer patients. The functional impairment and down-regulation of JAK3, STAT3, and CD3-zeta signaling molecules in PBLs of ovarian cancer patients have been noted [138, 139].

CD4$^+$CD25$^+$Foxp3$^+$ T regulatory cells (*Tregs*) are one of the most important cells promoting tumor escape and indicating an unfavorable prognosis for cancer patients. An increase in the number of Tregs in peripheral blood, lymph nodes, and spleen of cancer patients has been repeatedly noted (reviewed in [140]). Consistent with these observations, patients with gastric and esophageal cancers have shown increased numbers of circulating peripheral blood natural Tregs. A population of Tregs infiltrating tumors was also present inside tumors themselves, and was more abundant in advanced tumors compared to early-stage disease, being a poor outcome predictor in certain tumors [113, 114, 141]. Accumulation of Tregs has been observed in a variety of solid tumors including lung, pancreatic, breast, liver, ovarian, gastrointestinal, and head and neck cancers (reviewed in [2]). It seems, that expansion of Tregs includes both population of naturally circulating and locally

induced Tregs (reviewed in [142]). Tumor-derived TGF-β correlated with the intensity of Tregs infiltrate in gastric cancer, and was the inducer of a local population of Tregs from naïve T $CD4^+CD25^-$ cells [143]. In a variety of tumors including breast or gastric cancer and melanoma, the Tregs recruitment to the tumor site is regulated by the CCR4-dependent attraction induced by CCL22 or CCL17 secreted by the cancer cells, macrophages, and DCs (reviewed in [142, 144]). The method of attraction may influence the activation status of Tregs. One of the most important factors of Tregs promotion is expression of IDO by both cancer cells and myeloid DCs. The expression of IDO is associated with poor clinical outcome in ovarian cancer [145–149]. Similarly, tumors secreting increased levels of TGF-β have been characterized by increased Tregs infiltrate and disturbed T $CD8^+$ and T $CD4^+25^-$ effector activity, evidenced by a low secretion of IL-2, IFN-γ, and TNF-α [113, 114, 150]. A potent source of TGF-β are also intra-tumoral immature DCs. TGF-β induces in T cells an intra-cellular Smad-2 and -3 signaling pathway and signal transducer and activator (STAT) 3 and 5 activation, which result in a switch into the Tregs phenotype. Other regulators of Tregs expansion are mechanisms engaging interactions of T cell cytotoxic T-lymphocyte antigen 4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor (GITR) with corresponding ligands on DCs, as well as interactions between programmed death-1 (PD-1) on T cells with B7-H1 expressed on DCs and TAMs (reviewed in [142]). Immunoregulatory Tregs could effectively inhibit host defense against cancer based on cytotoxic effectors such as $CD8^+$ lymphocytes, NK, NKT cells, and antigen-specific T $CD4^+CD25^-$ lymphocytes, as well as could reversely block maturation of DCs [151, 152]. In-vitro studies on cultured human cells revealed that by blocking NKG2D receptor on NK cells with membrane bound TGF-β, Tregs were capable of blocking NK cells activity and IFN-γ secretion. Both low number of circulating NK cells and down-regulation of NKG2D expression on NK cells were poor prognostic factors in colon cancer patients [100, 153, 154]. It has also been presented that $CCR4^+$ Tregs utilized galectin-1 to inactivate NK cells in metastasizing breast cancer [155]. Tregs could also up-regulate expression of B7-H3 and B7-H4 immunosuppressive molecules on DCs, which contributed to DC-mediated inhibition of T effectors activity (reviewed in [142]). Murine studies indicated that Tregs were capable of impairing the expression of costimulatory CD80, CD86 and CD40 molecules on DCs and secretion of proinflammatory IL-12 and TNF-α molecules. Tregs-mediated suppression of antigen-presenting function of DCs is dependent on TGF-β and IL-10 secretion [156]. Tregs closely cooperate with MDSCs to promote tumor growth; however, they may have different roles. Tregs could protect tumors in early stages of proliferation and metastases when host anti-tumor defense is still effective, while MDSCs augment tumor progression and induce systemic suppression (reviewed in [157]). The glucocorticoid-induced TNF-related protein (GITR) has been discovered due to its role in reversing immunosuppressive effects of Tregs in mice. Expression of GITR in humans was confirmed on Tregs, and at low levels on T $CD4^+$ and T $CD8^+$ cells, and its action is mediated by combining with the GITR-ligand (GITR-L). It has been shown that gastrointestinal tumor cell lines indicate the expression of GITR-L.

The GITR/GITR-L signaling down-regulated the CD40, CD54, and EpCAM molecules, as well as induced TGF-β secretion by tumor cells. Constitutive expression of GITR-L by cancer cells diminished anti-tumor NK cell activity [158]. Independently from their detrimental effects on tumor host immunity, Tregs exert in some circumstances positive functions. Tregs triggered and stimulated by recognition of gut bacteria could reduce risk of gastro-intestinal tumors through down-regulation of inflammation [159]. In familial ovarian cancer, the observation that high Tregs density correlated with better prognosis was consistent with the clinical observation that patients with familial ovarian cancer and carriers of BRCA mutations have a better outcome, although their tumors are usually more aggressive [160].

Tr1 T lymphocytes represent another group of regulatory IL-10-producing cells generated upon immature DCs stimulation [161]. The detailed profile of secreted cytokines specific for Tr1 cells includes IL-10, TGF-β, and trace amounts of IFN-γ [162]. The possible role of Tr1 cells for human pathology and unfavorable outcome was confirmed in studies of different types of tumors [163, 164]. It has been shown that Tr1 cells primed by cyclooxygenase 2 (COX-2) were associated with inhibition of DCs maturation and contributed to increased growth of head and neck squamous cancer [165]. Moreover, a murine model revealed that IL-10-knockout or Tr1-depleted mice showed improved anti-tumor immunity [166]. The population of regulatory T cells with a profile similar to that of Tr1 cells of secreted cytokines are Th3 cells. In addition to TGFβ and IL-10, they are able to produce IL-4 [167]. The importance of Tr1/Th3 infiltrate for progression of B16 melanoma has been documented in murine studies, where inoculation of melanoma cells into mice resulted in expansion of Tr1/Th3 cells, inhibiting cytotoxic reactions from T $CD8^+$ and NK cells [168].

T $CD4^+$ Th17 cells, which upon stimulation by IL-23 produce IL-17 [169–171] are the next population of lymphocytes engaged in immuno- regulatory mechanisms existing inside the tumor. In a murine model, Th17 cells promoted growth of transplanted cervical cancers into the nude mice. An increased number of Th17 lymphocytes was noted in several solid tumors, including melanoma, breast, colon, and hepatocellular carcinoma, being in some of them a bad prognostic factor. Similarly, an increased number of peripheral blood Th17 lymphocytes was observed in gastric cancer patients. In most advanced cases, the Th17 cells were seen abundantly in tumor-draining lymph nodes (reviewed in [144]). High numbers of Th17 cells have been identified among ovarian tumor TILs, and IL-17 was consistently detectable in both serum and ascites of epithelial ovarian cancer EOC (patients) [172–174]. Tumor cells, cancer-associated fibroblasts, TAMs, T cells and antigen-presenting cells (APCs) produce pro-inflammatory cytokines (IL-1β, IL-6, IL-23, TNF-α) that facilitate the expansion of Th17 cells in a tumor environment [173, 174]. The Th17 up-regulation in the mouse model of ovarian cancer depended on the secretion of TNF-α by cancer cells. Consistent with this observation, treatment with anti-TNF-α antibody reduced serum IL-17 levels in EOC patients [174]. Chemoattraction of Th17 cells by both tumor- and CAFs-derived chemokines MCP-1 (CCL2) and RANTES (CCL5) has been demonstrated.

TAMs could participate in Th17 expansion by production of pro-inflammatory cytokines. The role of Th17 cells for enhancement of tumor growth is probably based on their vasculogenic abilities ([175], reviewed in [144]). However, the results of studies concerning the role of Th17 cells and IL-17 have been inconclusive, as they have indicated its functional ambiguity both for promotion and rejection of tumors [175–178]. It has been shown that Th17 cells secreting IFN-γ and IL-17 were able to up-regulate CXCL9 and CXCL10 chemokines, thus leading to chemoattraction of NK and T cytotoxic cells [179]. The protective role of Th17 cells against tumor progression was observed in ovarian and prostate cancers, and the number of Th17 cells increased in patients treated because of breast cancer and metastatic melanoma with monoclonal antibodies (reviewed in [144]).

Natural killer T lymphocytes (*NKT cells*) express both T-cell receptor and receptors characteristic for NK cells. Two subpopulations of NKT cells dependent on the presence (NKT I) or absence (NKT II) of the invariant Vα14Jα18 T cell receptor (TCR) Vβ chain have been recognized, and it was found that while NKT I cells mediate tumor rejection, the NKT II cells allow for its growth (reviewed in [180]). Both the number of NKT I cells and their responsiveness to α-galactosylceramide (α-GalCer—specific activator of NKT cells) stimulation were decreased in solid cancers, as well as their proliferative activity and capability of IFN-γ production (reviewed in [180]). Low circulating number of NKT I cells in head and neck squamous cancer were an independent predictor of poor survival, while high $V\alpha24^+$ NKT I cell infiltration in colorectal cancers was correlated with favorable prognosis of progression-free and overall survival [181, 182]. The role of NKT II cells for tumor promotion was confirmed in murine studies of renal cell cancer and fibrosarcoma models; however, studies indicated that the extent of suppression revealed by NKT II cells may vary between different tumors [183, 184]. The NKT cells inside tumors are engaged in a couple of regulatory networks. One of them counteracts the functions of NKT I and NKT II cells, probably by direct cell–cell interactions or through intermediary anergic plasmacytoid DCs [185]. In another network presented in a murine model, Tregs seemed to reduce the number, proliferative response, and cytokine secretion of NKT I cells [186]. Activated NKT I cells were shown to produce IFN-γ and IL-2, which together with IL-12 secreted by APCs activated NK cells [187]. They also induced maturation of DCs by up-regulation of costimulatory molecules, expression of class II MHC, and IL-12 secretion [188]. On the other hand, mDCs in the peripheral blood of melanoma and renal cancer induced NKT I cell reversible dysfunction mediated by TGF-β and IL-10 [189]. The suppressive NKT II cell activity is based on a function of IL-13, which promotes the expansion of M2-type macrophages and stimulates IL-13 receptor positive $Gr\text{-}1^+CD11b^+$ MDSC cells to inhibit T $CD8^+$ effectors by secretion of TGF-β [190, 191].

Lymphocytes B are a heterogenous population of cells which, according to recent studies, possess the protumoral regulatory activity. They could mediate suppression of immune reactions, as the loss or inactivation of B lymphocytes reduced the number of Tregs and MDSCs (reviewed in [157]). Production of immunoglobulins by B cells initiates creation of immune complexes, which could initiate FcR- and complement-dependent chronic inflammation, promoting cancer [192, 193].

Tumor-infiltrating B cells produce lymphotoxin α/β which through activation of STAT3 in prostate cancer cells sustain their growth. Moreover, immunoglobulins could function as carriers for immunosuppressive TGF-β [194, 195]. Lymphocytes B stimulate also M2-type polarization of macrophages by IL-10, and induce T-cell anergy, especially in the case of advanced tumors. They can also influence the Th1/Th2 balance (reviewed in [157]). B cell deficient mice were shown to be resistant to syngenic tumors including colon carcinoma and some types of melanoma, whereas partial B cell depletion was correlated with reduced tumor growth in a mouse model of colorectal cancer (reviewed in [196]). However, it seems that the precise role of B cells depends on the B cell subpopulation studied, the tumor type, and the particular immune situation inside, as syngenic mouse melanoma model depletion of B cells enhanced tumor growth and metastases [197]. Some populations of B lymphocytes possessing immunoregulatory properties and called Bregs have been described. The possible role for Bregs in cancer is suggested by the studies on breast cancer producing lung metastases. Bregs engaged in this pathology are characterized by a phenotype similar to immature B2 cells with high CD25, CD81, and B7-H1 expression. Their suppressive activity is based not on IL-10 secretion, but instead on generation of TGF-β-producing Tregs. Breg-like cells have been generated in vitro from B cells treated with conditioned media from breast, ovarian, and colon cancer cell cultures [198].

8.4.3 Immunoregulatory Function of Tumor-Associated Myeloid Cells

Tumor-associated myeloid cells (TAMCs) constitute the heterogenic population of cells of common myeloid lineage, and include at least four cell subpopulations: myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), and the angiogenic monocytes/macrophages expressing endothelial kinase-2 (Tie-2) called Tie-2-expressing monocytes/macrophages (TEMs) (reviewed in [199]).

Myeloid-derived suppressor cells (*MDSCs*), characterized in mice by the $CD11b^+/Gr\text{-}1^+$ phenotype (monocytic $Ly6C^+$ or granulocytic $Ly6G^+$), are a multifunctional population of marrow-derived cells involved in the immunosuppression of host immune responses against cancer, which function links the mechanisms of chronic inflammation and tumor progression [200]. In humans, MDSCs are characterized as $CD14^-CD11b^+$ cells, or alternatively $CD33^+$ cells lacking the expression of mature myeloid or lymphoid markers [201, 202]. It seems, however, that in humans the precise phenotype of the MDSCs depends on the tumor type (reviewed in [199]). Similarly to mice, human MDSCs could also belong to either the monocytic or granulocytic line. Monocytic MDSCs (M-MDSCs) are able to differentiate into macrophages and mature DCs, and exert their regulatory effects via nitric oxide (NO), suppressory cytokines, and arginase 1 activity. Granulocytic MDSCs (G-MDSCs) suppress immune responses via direct cell-to-cell contact and

reactive oxygen intermediates (ROI)/reactive nitrogen species (RNS) production (reviewed in [199]). MDSC cells are scarcely represented in spleen and almost absent in the lymphatic nodes; however, in the presence of tumor they expand and start to be abundant in spleen, lymph nodes, tumor sites, and malignant ascites [201, 202]. Receptor CCR2, C5a component of the complement, and pro-inflammatory S-100 proteins are responsible for chemoattraction of MDSCs into tumor (reviewed in [199]). This unique cell population possesses the common feature of suppressing, in both an antigen-specific and non-specific manner, host anti-tumor responses mediated by T $CD8^+$ CTLs, NK cells, and NKT cells, and of blocking DCs maturation [191, 201, 203, 204]. The pleiotropic effects of MDSCs are mediated through production of arginase 1 and ROI/RNS [201, 205, 206], inhibition of T $CD8^+$ CTLs, induction of T $CD4^+CD25^+Foxp3^+$ Tregs, and promotion of Th2-biased environment by secretion of IL-10 and blocking macrophage-derived IL-12 production [207, 208]. The tumor cells could participate in differentiation of MDSCs by secretion of granulocyte–macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), IL-6, VEGF and prostaglandin E_2 (PGE_2) [209]. Cytokines IL-1β, IL-6 and PGE_2 increase accumulation and suppressive activity of MDSCs [203, 210–212]. In the tumor site, the main activity of MDSCs is based on non-specific inhibition of immune effectors mediated by NO and arginase production. NO inhibits T effectors by interfering with intracellular JAK3 and STAT5 pathways, induction of T cell apoptosis, and down-regulation of MHC class II expression. Arginase 1 activity depletes arginine and causes the translational blockade of CD3 ζ chain. In the peripheral lymphoid organs, MDSCs inhibit T cell by production of ROI/RNS during the direct cell-to-cell contact [202]. Action of MDSCs against T $CD8^+$ CTLs is probably based on modification of TCR-binding activity caused by peroxinitrite activity [213]. A correlation between high peroxinitrite concentration and immunosuppression has been demonstrated in various cancers including pancreatic, head and neck, breast cancers, mesothelioma, and melanoma [202]. MDSC-inhibited T $CD8^+$ cells are unable to secrete IFN-γ and IL-2, and to kill the target cells [214]. It was also found that MDSCs inhibited T cells by depletion of cysteine, which is essential for T-cell activation. Moreover, they were capable of down-regulating CD62L selectin expression on T cells, thus reducing their migration into regional lymph nodes (reviewed in [215]). Myeloid-derived suppressor cells are also capable of inducing tumor mutations, and thus augmenting the tumor metastatic potential [200]. By production of IL-10, MDSCs could also skew the function of TAMs into pro-tumoral M2-type activity [208, 212]. They promote the formation of new blood vessels by expressing metalloproteinases and increasing the bioavailability of VEGF [216, 217]. Circulating MDSCs may differentiate in a hypoxic tumor environment into Gr1-F4/80^+ macrophages [218]. The expansion and functional activation of MDSCs is regulated by NF-κB, as IL-1β signaling crucial for recruitment of MDSCs into gastric cancer has been found to be NF-κB-dependent [219]. The STAT system also regulates MDSC function. STAT1 is responsible for MDSC interferon-dependent activation, and STAT5 is engaged in MDSC survival (reviewed in [199]).

Macrophages constitute one of the major immune cell population responsible for both tumor rejection and promotion [220–222], but their function is determined by the way they are activated. The presence of IFN-γ, GM-CSF, TNF-α, lipopolysaccharide (LPS), or other Toll-like receptors ligands shifts their activity into the so called M1 profile, while stimulation by IL-4, IL-10, IL-13, or TGF-β results in the M2 profile [223]. Tumor MDSCs in murine breast cancer model were shown to contribute to M2 switch of TAMs, similarly to cancer-associated fibroblasts (reviewed in [199]). It has been demonstrated that T $CD4^+$ lymphocytes by secretion of IL-4 and IL-13 potentiated the metastasis capabilities of adenocarcinoma by stimulation of M2-type TAMs [224]. Lymphocytes B also participate in skewing activity of TAMs into the M2 phenotype by stimulating Fcγ receptors on resident myeloid cells [225]. Several additional signals switching the differentiation of macrophages into M2-type have been identified, including hormones, growth factors, and bacterial products (reviewed in [199]). However, it seems that polarization into M1 and M2 phenotypes is somehow artificial and represents the extremal differentiation status, while many cells indicate a function being a mixture of M1/M2 phenotypes with the balance slightly pushed towards M1- or M2-type [226]. Different signals present in tumor environment could be the source of heterogenous activation contributing to different patterns of gene activation in macrophages. Macrophages with mixture of both M1 and M2 phenotypes have been identified in tumors (reviewed in [199]). Macrophages of M1-type could effectively destroy tumor cells through production of Th1 cytokines and stimulation of T $CD8^+$ CTLs [220]. Conversely, macrophages of M2-type produce mainly IL-6, IL-10, TGF-β, and VEGF, and have poor APC abilities. M2-type macrophages regulate inflammation into chronic phase, stimulate tissue healing and remodeling as well as angiogenesis. This cell subset constitutes the vast majority of tumor-associated macrophages (TAMs), which play a discreditable role in tumor progression [220, 221]. Mouse studies confirmed the importance of M2-type TAMs in tumor progression. Src homology-2-containing inositol-5′-phosphatase-1 (SHIP1)-deficient mice, which show spontaneous generation of M2-shifted macrophages, demonstrate increased growth of transplanted tumors [227]. And in contrast, p50 NF-κB-deficient mice, which are unable to mount M2 polarization, show resistance to transplantable tumors [228]. It has been shown that most aggressively growing tumors were infiltrated by large numbers of TAMs [229]. Recruitment of macrophages into tumors is regulated by Th2 cytokines, chemokines [221, 230, 231], urokinase plasminogen activator (uPa), microbial defensins, and hypoxia [232]. Some of the attractants are universal for many tumors, while some are exclusively secreted by certain tumor types, for instance uPa and defensins in prostate and gastric cancer respectively (reviewed in [199]). Colony-stimulating factor-1 (CSF-1) and TGFβ are major cytokines that are believed to play an important role in recruitment of macrophages into the tumors. Both of them are expressed constitutively on the surface of solid tumors [233, 234], correlate with intensity of TAMs infiltrate [233, 235], and indicate a poor prognosis for the patients [233, 236]. The chemokines CCL2 (monocyte chemotactic protein-1—MCP-1) and CCL5 (regulated on activation, normal T-cell expressed and

secreted—RANTES) were found to be expressed predominantly by the solid tumors [133, 237–242]. Their over-expression correlated with intra-tumor TAMs content as well as with bad survival ratio [133, 238–240]. They were also shown to regulate migration of peripheral blood monocytes into the tumor. Upon the tumor-derived M-CSF monocytes are able to differentiate to macrophages. High M-CSF production correlates with poor outcome in ovarian, breast, and endometrial cancers (reviewed in [243]). The chronic inflammation recognized as an important component of carcinogenesis is regulated by TAMs which, triggered by tumor-derived inflammatory cytokines (TNF-α) and components of necrotic cancer tissues, secrete in turn inflammatory chemokines (CCL2, CXCL1, CXCL8, CXCL12), IL-6, and TNF-α, generating a self-enhancing loop. IL-6 secreted by TAMs plays an important role in stimulation of both cancer and stromal cells. It activates STAT3 pathway in tumor cells, making them more proliferative and apoptosis-resistant (reviewed in [243]). The number of TAMs correlates with advancement of the tumor. High-grade ovarian tumors were characterized by more abundant $CD68^+$ and $CD163^+$ TAMs populations, and a correlation between $CD68^+$ macrophages and Tregs was noted, suggesting the cooperation between both populations existing on the regulatory level [160]. TAMs are also the most abundant mononuclear cell population in the ascites of ovarian cancer patients, where they contribute to suppression of T effector cells by secretion of IL-10 and TGF-β [244, 245]. A hypoxic environment inside solid tumors is another attractant for macrophages. Anaerobic conditions increase expression of endothelin-2 (ET-2) and VEGF, as well as chemokine CXCL12 and receptor CXCR4, which become a stimulus for macrophage recruitment into hypoxic areas of the tumor [246, 247]. Adaptation of TAMs to a hypoxic environment depends on function of HIF-1α, which not only helps TAMs to function in anaerobic environment, but also contributes to pro-angiogenic and pro-metastatic TAMs activity [248]. Clinical studies seem to confirm that there is an enhancement of invasiveness and peritoneal metastatic activity in ovarian cancer under hypoxic conditions [249, 250]. Tumor-associated macrophages secrete Th2 cytokines, enhance intra-tumor angiogenesis (by VEGF, TGF-β, and fibroblast growth factor—FGF) and augment extracellular matrix remodeling by metalloproteinases (MMPs), thus promoting tumor growth and intravasation of cancer cells into blood vessels, resulting in increased tumor metastatic potential ([220, 221], reviewed in [9]). TAMs secrete also some specific molecules such as semaphorin 4D (Sema4D) and growth-arrest specific-6 (Gas6) which promote cancer neo-angiogenesis and proliferation [251, 252]. Subsets of TAMs not completely biased towards M2-type activity may secrete some amounts of Th1 cytokines, for instance TNF-α. Although TNF-α is considered to be an anti-tumor cytokine, it has also some pro-tumor activities. It might contribute to DNA damage, induce angiogenic factors, and act as a growth factor for cancer cells [253]. Investigations performed on ovarian cancer indicated that TAMs were also able to inhibit host T effectors by expression of B7-H4 co-stimulatory molecule, which was identified as a negative regulator of T-cell activation [107]. Tumor-associated macrophages could also exert immunoregulatory effects by secretion of NO and ROI. Investigations have confirmed, that tumors compared to normal

tissues are characterized by both higher expression of inducible nitric oxide synthase (iNOS) and production of ROI, and that their activity is related to TAMs [254–257]. Defective M1-type functions showed by TAMs are probably caused by disturbed activation of NF-κB in response to pro-inflammatory stimuli present in advanced tumors, including TNFα [258, 259]. Factor NF-κB is responsible for regulation of transcription of many genes, including those for cytokines, chemokines, and anti-apoptotic molecules [220]. The STAT signaling molecules also play an important role for TAMs function. STAT3 and STAT6 are activated in M2-type TAMs, whereas STAT1 in M1-type TAMs respectively (reviewed in [199]).

Tumor-associated neutrophils (*TANs*) are a population of $CD11b^{+}Ly6G^{+}$ cells which have a longer life-span than typical neutrophils, due to hypoxia and IL-1 present in tumor environment, and are able to mediate chronic inflammation and angiogenesis. Despite the phenotypic similarity and partly overlapping markers, TANs and granulocytic MDSCs seem to be the distinct cell populations. The recruitment of TANs depends on the CXCL8 (IL-8) and TGF-β activity (reviewed in [199]). The presence of TANs has been verified and confirmed in several tumors, including kidney, breast, colon, and lung cancers, and consistently correlated to poor prognosis in renal, breast, and lung cancer (reviewed in [199]). TANs contribute to tumor growth by promoting angiogenesis, proliferation, and metastases, and on the contrary their depletion inhibits tumor growth. It seems that two subpopulations of TANs exist in the tumor environment: N1-type TANs capable of tumor rejection by TGF-β and ROI function, and N2-type TANs which are TGF-β-negative and promote tumorigenesis. It has been suggested that N1-type TANs are fully activated neutrophils, whereas N2-type TANs are immature ones ([260], reviewed in [199]). TANs were able to secrete hepatocyte growth factor (HGF) and oncostatin, which augmented invasiveness of cancer cells and up-regulated expression of CXCR4 (reviewed in [261]). Upon activation, neutrophils secrete fibers composed from proteins and chromatin, called neutrophil extracellular trap (NET), and used for entrapment and killing of microbes and activation of DCs and T cells. The presence of NET was observed in TANs infiltrating Ewing sarcoma, in patients with early relapse of the disease. The tumor-promoting role of NET could be an activation of tolerogenic DCs or degradation of extracellular matrix to augment metastases [262]. The peripheral blood neutrophils could also participate in tumor growth promotion, as IL-8 secreted by neutrophils together with up-regulation of CD11b/CD18 on their surface facilitated melanoma cell arrest on endothelium and tumor cell extravasation [263]. Moreover, in-vitro studies have shown that ovarian cancer cells could participate in potentiation of peripheral blood neutrophil inflammatory responses (enhancement of ROS formation) by direct cell-to-cell contact [264]. The activation of ovarian cancer patients' neutrophils by ovarian cancer cells was dependent on the interaction of HspA1A originating from ovarian cancer cells, with TLR2 and TLR4 expressed on the surface of neutrophils [138, 139].

Tie-2-expressing monocytes/macrophages (*TEMs*) are a population of $CD11b^{+}/Gr1^{low/-}/Tie\text{-}2^{+}$ cells which express endothelial kinase-2 (Tie-2) receptor for

angiopoietin [265]. They originate from peripheral blood Tie-2^+ monocytes which have been recruited to the tumor by hypoxia-triggered chemokine CXCL12 and Ang-2. Moreover, it seems that CXCR4 may be engaged in this recruitment, as CXCR4 blockade was connected with significant reduction of TEMs infiltrate in breast tumors [266]. Engagement of Ang-2 is not restricted to chemotactic attraction of TEMs, but also regulates tumor promotion by increase of IL-10 secretion by TEMs, stimulation of Tregs, and inhibition of M1-type TAM function (reviewed in [199]). TEMs are related to M2-type TAMs but have, however, a more M2-skewed functional signature, with pronounced expression of arginase I, scavenger receptors, and lowered expression of IL-1β, (COX2), IL-12, TNF-α, and iNOS. They also express pro-angiogenic molecules, such as VEGF and MMPs (reviewed in [199]). TEMs play a crucial role in tumor angiogenesis. They are seen mainly in the hypoxic areas of the tumor in proximity to the vessels. Mouse studies confirmed that ablation of Tie-2^+ macrophages inside the breast tumors and gliomas resulted in reduction of tumor vasculature and mass, whereas injection of tumor cells together with TEMs significantly augmented tumor vascularization [265].

8.4.4 Dendritic Cells as Tumor Growth Enhancers

Dendritic cells (DCs) are professional antigen-presenting cells of myeloid or plasmacytoid origin [267, 268]. Myeloid DCs (mDCs) are characterized by $CD11c^+CD33^+CD45RA^-CD123^-$, whereas plasmacytoid DCs (pDCs) by the $CD11c^-CD4^+CD45RA^+CD123^+$ phenotype. pDCs show exclusively expression of TLR7 and TLR9, as well as IFN secretion upon viral stimulation. On the other hand, mDCs indicate the expression of a broad spectrum of TLRs, excluding TLR7 and -9, and are not capable of secreting IFN on viral challenge. Dependent on the environmental factors and signals of activation, DCs are able to stimulate either Th2 or Th1 responses. Inside the tumor environment, DCs acquire regulatory properties (reviewed in [108, 269]). Presence of competent mature DCs is very rare in the tumors, as has been confirmed in ovarian, prostate, breast, and renal cancers (reviewed in [269]). If present, they occupy the peri-tumoral tissues. On the other hand, progressive tumors usually contain DCs having an immature $CD4^-CD8^-$ phenotype. As opposed to mature DCs, these cells indicate pro-tolerogenic functions, and are unable to effectively stimulate cytotoxic responses [270–272]. Moreover, they are able to inhibit tumor-specific T $CD8^+$ cytotoxic responses even in chemotherapy pre-treated mice, by capturing $CD8^+$ CTLs into DCs rich areas of the tumor [273]. There are tumor-derived immunoregulatory factors that are responsible for defective maturation and differentiation of DCs. Lack of immunostimulatory IL-12 and IFN-γ in tumors creates an environment which blocks DC maturation (reviewed in [269]). Tumor environment contains also many other cytokines and immunoregulatory factors that modulate DC function, and among them are cytokines such as VEGF, IL-10, IL-6, TGF-β, PGE_2, factors such as IDO and ROI, and finally tumor antigens and metabolites (reviewed in

[200]). The meaning of VEGF for DC function has been shown in murine studies, where use of VEGF-neutralizing antibody stimulated DC differentiation and raised the number of mDCs, while in the presence of VEGF the DCs showed disturbed antigen-presentation capacity [274, 275]. Murine studies found the presence of functionally immature $CD11c^+$ DCs expressing low levels of co-stimulatory CD86 and CD40 molecules in tumor and tumor-draining lymph nodes. Depletion of these DCs in tumor-bearing mice significantly retarded tumor progression [276]. Studies in human gastric and non-small lung cancer confirmed that differentiation of DCs was negatively affected by VEGF [277, 278]. Murine studies demonstrated that a population of immature mDCs acquired upon VEGF stimulation a proangiogenic $CD11c^+DEC205^+$VE-cadherin$^+$ phenotype, migrated to perivascular areas of the tumor, and maintained its vasculogenesis [279, 280]. -Interleukin-10 is responsible for down-regulation of costimulatory molecules on DCs, and thus cooperates with VEGF in worsening of APC function of DCs. It also blocks DC differentiation. The source of IL-10 is the tumor itself and TAMs. Similar effects showed exposition of DCs to TGF-β function (reviewed in [269]). Renal cancer cell-lines were shown to produce IL-6 and granulocyte–macrophage colony-stimulating factor (GM-CSF), which inhibited DCs differentiation. The blocking effect of IL-6 was also observed in myeloma [281]. Retention of DCs inside tumors and down-regulation of their migratory potential is probably mediated by CXCL8 (IL-8) produced by tumors, including hepatocellular, pancreatic, and colon cancers, which acts through CXC receptor (CXCR)-1 and -2 on DCs (reviewed in [269]). Expression of IDO on DCs deprives tryptophan to the T cells and promotes T-cell apoptosis or anergy. The presence of IDO-positive DCs has been confirmed in tumor-draining lymph nodes in the cases of melanoma, breast, colon, lung, and pancreatic cancers, and the intensity of such infiltrate was correlated with poor prognosis [282]. The population of cells which mediated entirely all IDO-dependent suppression in lymph nodes was the population of $CD19^+B220^+$ plasmacytoid DCs [283]. Expression of IDO on DCs is probably up-regulated by PGE_2 present in tumor environment [284]. IDO^+DCs are capable of inducing $CD4^+CD25^+Foxp3^+$ Tregs. Immature DCs exert also other activating T $CD4^+CD25^+Foxp3^+$ Tregs effects, mediated through TGFβ and IL-10, thus promoting tumor growth ([153, 154, 285], reviewed in [108]). Interactions between DCs and Tregs mediated through cytotoxic T-lymphocyte antigen-4 (CTLA-4) could compromise anti-tumor immunity in an IDO-dependent way [286]. DCs can also modulate the trafficking of Tregs into tumor site and lymph nodes, thanks to CCR4/CXCL22 interactions (reviewed in [108]). Tregs were shown to direct back regulatory signals towards DCs, mainly by down-regulation of costimulatory molecules on DCs, inhibition of their maturation, and impairment of APC functions by TGF-β and IL-10. Tregs have also been reported to induce immunosuppressive molecules B7-H3 and B7-H4 on the surface of DCs (reviewed in [108]). Accumulation of ROI in tumor localization creates a constant stress which has profound impact on DC functions and vulnerability to apoptosis, through modulation of NF-κB and c-Jun N-terminal kinase (JNK) pathways (reviewed in [269]). Molecule CD200 is a membrane protein belonging to co-stimulatory molecules, which exerts

suppressive effects through binding to CD200 receptor (CD200R). Both CD200 and CD200R are present on the surface of myeloid DCs. It was shown that stimulation of CD200R on DCs created tumor-supporting reactions mediated by Th2 cytokines and increased Tregs activity, while blocking CD200/CD200R interactions with monoclonal anti-CD200 antibodies resulted in a shift towards Th1 activity. Moreover, tumors themselves (including ovarian cancer) are capable of expressing CD200 molecules, thus influencing DC function [287, 288]. Myeloid DCs isolated from ovarian tumors also exhibited the expression of programmed cell death-1 ligand 1 (PD-L1, B7-H1). Accumulation of PD-$1^{+}B7^{-}H1^{+}$ DCs in the tumor was associated with suppression of $CD4^{+}$ T helper cells, $CD3^{+}CD8^{+}$ cytotoxic/regulatory T cell activity, decreased infiltration of T cells, and expansion of Tregs ([289], reviewed in [108]). In ovarian cancer, plasmacytoid DCs accumulate in tumor environment, preferentially in ascites, where they are attracted by CXCL12 [113, 114, 272]. Similarly to mDCs, ascitic pDCs have an immature phenotype. Plasmacytoid DCs promote the generation of immuno-regulatory IL-10^{+} T $CD8^{+}$ suppressors, which independently from T $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs down-regulate IFN-γ secretion mediated by T effectors and prevent them from proliferation [272, 290]. They also secrete TNF-α and IL-8, thus being capable of promoting angiogenesis [113, 114]. Tumor-associated pDCs were found to have a different phenotype compared to ascitic pDCs, and expressed a semi-mature phenotype with higher level of CD86 and CD40 expression, thus being capable of partial activation in tumor localization. Function of tumor-associated pDCs was modulated by tumor-derived TNF-α and TGF-β [291]. The intercellular machinery of DCs exposed to tumor-derived regulatory molecules inhibits their differentiation to a mature phenotype via induction of STAT3 signaling. Moreover, activation of STAT3 in tumors blocks secretion of pro-inflammatory factors and enhances DC immaturity (reviewed in [108]).

8.4.5 Inflammation and Cancer Escape

Chronic inflammation may account for about 15 % of cancers, due to the fact that inflammation mediators like TNF-α are able to initiate tumor growth by stimulation of NO synthase and ROI production, both being capable of DNA damage [292–294]. During progressive tumor growth, chronic inflammation caused by tumor-infiltrating immune cells contributes to cancer progression [231, 295]. Oxidative stress seems to play a pivotal role in this process by stimulating an inflammatory network based on COX2, iNOS, cytokines, chemokines, and transcription factors. Reactive oxygen intermediates participate in regulation of resistance to apoptosis, angiogenesis, proliferation potential, and metastasis formation of tumor cells (reviewed in [296]). Moreover, stromal cells could also contribute to chronic inflammation and initiate or promote tumor growth. Upon senescence, fibroblasts acquire a “senescence-associated secretory phenotype” (SASP) characterized by activation and production of pro-inflammatory cytokines (IL-6, IL-1β), chemokines

(IL-8, MCP-1, GRO-1/α), MMPs, adhesion molecules, and integrins [297, 298]. Senescent stromal fibroblasts have been detected in specimens of ovarian tumors in areas adjacent to malignant epithelium [299]. Chronic inflammation and oxidative stress also promote the generation of heat-shock proteins (Hsps), which prevent cells from apoptosis and enhance their survival. Over-expression of Hsp90 was found on several tumors, and correlated to metastatic potential and poor survival. Similarly, the presence of Hsp70 was noticed on colon, lung, breast, and pancreatic cancer metastases, and correlated with resistance of cancer cells against apoptosis (reviewed in [300]).

Toll-like receptor polymorphisms in genes encoding TLR6 and TLR10 increased risk of development of some cancers [301]. Activation of TLRs both on macrophages and on the cancer cells enhanced tumor growth by various mechanisms such as stimulation of growth-promoting cytokines or protection against apoptosis [302–304]. In ovarian cancer, stem-like slow-growing cell populations initiate tumor re-growth after surgery or chemotherapy by activation of the TLR4 pathway, which regulates the pro-inflammatory phenotype of these cells characterized by high NF-κB, IL-6, IL-8, MCP-1, and GRO-1/α activity [305]. Therefore, the $TLR4^+$ phenotype of ovarian cancer cells has been correlated with chemoresistance. Similarly, the expression of TLR9 has been connected to high metastatic potential of ovarian tumors [306].

Tumor necrosis factor-α is one of the pro-inflammatory cytokines stimulated by TLRs, which promotes tumor survival by stimulation of NF-κB-dependent pathways regulating anti-apoptotic molecules, tumor proliferation, neoangiogenesis, and metastatic properties [307, 308]. Polymorphisms leading to over-production of TNF-α have been connected with greater risk of cancer, including breast and gastric tumors [309]. Increased TNF-α concentrations have been observed in ovarian cancer patients in serum and cyst fluid, as well as in cancer tissues and ascites [310, 311]. Cancer patients have also been characterized by over-expression of receptor TNF-R2, which was further correlated with tumor stage and patient prognosis [312]. TNF-α expressed on tumor cells orchestrates the paracrine "TNF network", and together with IL-6 and CXCL12 regulates tumor growth [313]. Interactions between tumor-derived IL-6 and TAMs-derived TNF-α enhanced incidence of prostate cancer metastases both to the bones and regional lymph nodes [314]. Moreover, prostate tumors have been characterized by increased TNF-α, TNFR1, and TNFR2 levels, which correlated with poor prognosis [315].

Interleukin-6 is another pro-inflammatory cytokine which through activation of intracellular STAT3-pathway regulates cell proliferation, induces epithelial–mesenchymal transition and appearance of the cell migratory phenotype, and up-regulates resistance to apoptosis and chemoresistance [316–320]. Polymorphisms of the *IL6* gene promoter region could influence the risk of certain tumors [321]. In-vitro investigations in ovarian cancer showed that *p53* overexpression could regulate IL-6 secretion [322]. Interleukin-6 is produced either by tumor cells themselves or by M2-shifted tumor-associated macrophages, and together with IL-1, TNF-α, VEGF, and chemokines produces a cooperative network for promotion of tumor growth

[313, 323]. IL-6 was able to induce the suppressive Th2 phenotype in tumor-infiltrating T cells, and M2-type activity in TAMs [324, 325]. In-vitro studies showed that IL-6 augmented growth of colon carcinoma, which was confirmed in vivo by the observation that IL-6 serum levels correlated with the dimensions of the tumor [326, 327]. Increased IL-6 expression was related to an advanced stage of disease and decreased survival in colon cancer patients. These effects were mediated through IL-6-mediated promotion of tumor cell proliferation and inhibition of apoptosis through gp130 activation on tumor cells with subsequent signaling through JAKs and STAT3 [328]. Women with advanced ovarian cancer had significantly higher IL-6 levels both in the serum and ascites [329–332]. In these patients, IL-6 was engaged in neo-angiogenesis, spread of peritoneal metastases, and ascites production [333]. In several prostate cancer cell lines, IL-6 inhibited apoptosis and enhanced survival by activation of phosphatidylinositol-3-kinase signaling [334].

Transforming growth factor-β, despite its anti-tumor activity in early tumors, might also enhance tumor escape and contribute to tumor-associated inflammation in later stages. Mutations of the TGF-β-receptor, Smad signal transduction pathway genes, and TGF-β-inducible gene-h3 were associated with reduced p53 expression, ovarian cancer risk, and paclitaxel resistance respectively [335–337]. On the contrary, some polymorphisms of *TGF* gene make individuals less prone to development of lung cancer (reviewed in [338]). The source of TGF-β could be both tumor cells and M2-type TAMs [220]. Lung cancers overexpress TGF-β, and are characterized by several mutations of TGF-β receptors, which prevents cancer cells from negative autocrine regulation of growth by this cytokine. As a result, high TGF-β concentration produces a suppressory environment inside the tumor (reviewed in [338]). In advanced tumors, TGF-β is engaged in Th17 cell differentiation, inhibition of DCs maturation, stimulation of VEGF production, generating the $CD4^+CD25^+Foxp3^+$ Tregs, and decreasing activity of NKT, T $CD8^+$, and NK cytotoxic cells. It supports angiogenesis, metastasizing, and epithelial–mesenchymal transition [339–342]. In breast cancer, chemotherapy-induced TGF-β signaling enhances tumor recurrence through IL-8-dependent expansion of cancer stem cells (CSCs), while TGF-β pathway inhibitors prevent the development of drug-resistant CSCs [343]. TGF-β signaling induces a mammalian target on rapamycin (mTOR) complex 2 in cancer cells and regulates epithelial–mesenchymal transition [344].

Interleukin-10, similarly to TGF-β, exerts both anti-tumor and pro-tumor activity, which seems to be dependent on the tumor type and advance of the disease. IL-10 has been shown to be secreted directly by tumor cells, as well as by immunoregulatory Tr1/Th3, $CD4^+CD25^+Foxp3^+$ Tregs, TAMs, and MDSCs. In established tumors, IL-10 enhances the intra-tumor and peripheral blood immunosuppressive phenotype by stimulation of M2-type TAMs and Th2-type lymphocytes [168, 339, 340, 345, 346]. Autocrine activation of the STAT3-pathway by IL-10 in tumor cells up-regulates expression of Bcl-2 and HLA-G, thus protecting cancer cells from host effectors and apoptosis [84, 347]. In ovarian cancer patients, IL-10 concentrations were increased in peritoneal fluid and serum compared to benign ovarian disease [330, 331, 348]. Moreover, the expression

of IL-10 was found to correlate with tumor aggressiveness, the presence of metastases, and shorter progression-free survival [349, 350]. High levels of IL-10 in TAMs significantly correlated with stage, tumor size, lymph node metastasis, lymphovascular invasion, or histologic poor differentiation in non-small cell lung cancer [351]. In melanoma patients, IL-10 mRNA expression increased progressively from preinvasive, through primary invasive, to metastatic tumors, and correlated with vertical growth phase as well as metastatic competence [352].

Cyclooxygenase-prostaglandin E_2 (*COX-2-PGE_2*) inflammatory pathway is important for tumor development, as revealed studies showing anti-tumor effects of selective COX-2 inhibitors in colorectal cancer [353, 354]. Activity of the *cox2* gene has been proved to participate in ovarian carcinogenesis both in sporadic and in BRCA 1/2-conditioned cancer [355, 356]. Up-regulation of COX2-PGE_2 in tumor cells and TAMs results from hypoxia and HIF-1α, and influences several regulatory and signaling pathways including Ras/MAPK, PI3K/Akt, and NF-κB-mediated pathway [353, 354]. COX-2 over-expression stimulates VEGF and neo-angiogenesis, and its raised levels predict poor survival in some cancers [357–359]. In a murine model, COX-inhibitors administered together with taxol decreased the expression of VEGF and reduced microvessel density (MVD) of transplanted ovarian tumors [360]. Over-expression of COX-2 in ovarian cancer correlated also with resistance to platinum-based chemotherapy [361]. COX-2, microsomal prostaglandin E synthase-1 (mPGES-1), and prostaglandin receptor EP1 were positive not only in tumor epithelial cells, but also in the tumor stroma, indicating that CAFs participate in COX/PGE_2 signaling [355]. Lung cancers also over-express COX2 and produce several prostanoids and leukotrienes. The presence of COX2 over-expression seems to be the key factor in promotion of lung cancer growth, as the pharmacologic inhibition of COX2 reduced tumor growth in a lung cancer murine model. COX2 was capable to modulate MDSC activity through PGE_2-mediated ARG-1 expression, and to enhance expansion of Tregs also by PGE_2 (reviewed in [215]). Prostaglandin PGE_2 inhibits DCs maturation and migration towards regional lymph nodes, up-regulates IL-4 and IL-10 cytokines, and finally increases tumor migratory and metastatic potential [200, 353, 354]. Squamous, adenocarcinoma, and small-cell lung cancers are able to produce prostaglandin E_2, and express a variety of prostaglandin receptors. PGE_2 functions as a stimulator of lung cancer growth by augmenting angiogenesis and proliferation, and simultaneously inhibits T and NK effector cells (reviewed in [338]). Peroxisome proliferator-activated receptor-γ (PPARγ) is an inhibitor of COX-dependent inflammatory reaction, and in mouse studies produced a decrease of PGE_2 levels, reduction of MVD, enhanced tumor apoptosis and improved mice survival [362].

Pro-inflammatory cytokine IL-23 also documents the relationship between the cancer and inflammation. In ovarian cancer particularly, a high level of expression of genes regulating pro-inflammatory pathway including IL-23 was detected [174]. Moreover, *IL-23* receptor gene polymorphism was shown to correlate with advancement of tumors [319, 320]. Upon stimulation by tumor-derived IL-23, Th17 cells release IL-17 and other inflammatory mediators such as IL-1, IL-8, TNF-α,

and PGE_2 which produce a pro-tumor inflammatory environment. The increased expression of both IL-23 and IL-17 has been observed in many malignant tumors, and correlated with angiogenesis, expression of MMPs, and decrease of cytotoxic anti-tumor immune response [20, 363].

Interleukin-18 is a pro-inflammatory cytokine which activates immune CTL and NK cells and induces IFN-γ, and thus is capable of exerting anti-tumor effects. However, IL-18 has also been found to potentiate tumor growth (reviewed in [364]). The expression of IL-18 has been demonstrated on melanoma, squamous skin, breast, and gastric cancer, and was connected with the presence of distant metastases in breast and gastric cancers [365, 366]. In-vitro studies showed that transfection of poorly metastatic lung cancer cells with IL-18 construct enhanced their invasion ability and down-regulated E-cadherin, thus increasing metastasis potential [367]. In a murine melanoma model, the pro-metastatic IL-18 action was mediated by up-regulation of vascular cell adhesion molecule 1 (VCAM-1) [368]. In addition, the pro-angiogenic properties of IL-18 have been noticed in gastric cancer, where IL-18-dependent stimulation of thrombospondin-1 was discovered [249, 250]. Moreover, IL-18 induced Fas ligand expression on melanoma cells and made them less susceptible for effector destruction [508].

Interleukin-8 (IL-8, CXCL8) is a chemokine secreted by macrophages, neutrophils, and endothelial and tumor cells, mediating its biological effects through binding to CXCR1 and CXCR2 receptors present on both tumor and endothelial cells [369–371]. Hypoxia and oxidative stress are strong inducers of IL-8 expression on cells of several malignancies, including ovarian cancer, via *Ras* gene over-expression and activation of PI3K/Akt and p38 MAPK signaling [372]. Some *IL-8* gene polymorphisms are correlated with the overall risk of developing the intestinal type of gastric cancer [373]. Increased IL-8 was found in ascites and serum of ovarian cancer patients, while IL-8 over-expression was observed on tumor cells, both correlated with advancement, vascularity of tumors, and short patient survival [374, 375]. IL-8 is engaged in blocking of TRAIL-induced cancer cell apoptosis and in recruiting certain immune cells into the peritoneum, where they contribute to tumor spread and formation of ascites [376, 377]. It has been shown that chemoresistant ovarian cancers were characterized by increased expression of IL-8 [378]. IL-8 and CXCR1 have been found to be over-expressed in pancreatic cancer, and in-vivo studies showed that tumors from patients who had higher IL-8 levels grew faster [379]. In-vitro studies of gastric cancer revealed that IL-8 increased NF-κB and Akt signaling and adhesion molecules intercellular adhesion molecule 1 (ICAM-1) and VCAM-1 expression in cancer cells, thus increasing their migration, adhesion, and invasion [380]. Similarly, IL-8-transfected colon cancer cell lines demonstrated increased migration and proliferation in vitro, whereas in-vivo xenografted IL-8-expressing colon tumors indicated faster growth and enhanced microvessel density [381]. Over-expression of CXCR2 receptor inhibited cancer apoptosis, up-regulated VEGF on tumor cells, and was an indicator of poor prognosis [382].

The *Hedgehog* (*Hh*) *signaling* pathway plays an important role in human development. The expression of Hh ligands and the intensity of Hh signaling is

up-regulated by hypoxia and inflammation [383, 384]. The classical activation method requires binding of one of the Hh ligands (sonic—SHH, Indian—IHH or desert—DHH) to the membrane-bound receptor Patched (PTCH). The Hh-PTCH complex influences the Smoothed (SMO) factor which activates the glioma-associated oncogene homolog (GLI) transcription factors that up-regulate target genes (reviewed in [385]). During embryonic development, Hh signaling promotes cell proliferation, angiogenesis, EMT, and stem cell re-growth, all under hypoxic conditions; thus, the situation according to the Hh function resembles in some circumstances that found inside solid tumors. Inhibition of Hh signaling has been found to decrease the proliferation of cancer cells [386]. The Hh-GLI-mediated increase of proliferation has been observed in melanoma cells [387]. Target genes responding to Hh-GLI regulation include proliferation activators including cyclins, IGF-BP6, and osteopontin. Moreover, the Hh-GLI pathway up-regulates the expression of Bcl-2 anti-apoptotic molecule (in brain, gastric, and pancreatic cancers) and regulates stability of p53 (in breast cancer) [388–392]. In ovarian and endometrial cancer, Hh signaling down-regulates the p21 and p27 inhibitors of cell cycle progression, and correlates with advancement of the tumors [393, 394]. The Hh-GLI pathway is also engaged in angiogenesis via up-regulation of VEGF, and enhances invasiveness and migration in several tumors including skin, breast, ovarian, pancreatic, and prostate cancers, and melanoma (reviewed in [385]). It also represses E-cadherin expression, enhances MMPs, and activates stromal fibroblasts, thus inducing EMT [395–398]. One of the most important functions of Hh signaling is the maintenance of the cancer stem cells (CSCs), a slow-proliferating, self-renewing population of cells which are the reservoir for tumor re-growth [395, 396]. The stimulatory effect of Hh on viability of CSCs has been observed in a variety of tumors including breast, brain, ovarian, and colon cancers (reviewed in [385]).

8.4.6 Resistance to Apoptosis and Tumor "Counter Attack"

Apoptosis describes the highly selective process, occurring both in physiological and pathological circumstances, by which cells upon receiving certain activating stimuli enter the course toward a programmed death [399]. Resistance to apoptosis or its reduced efficacy have been repeatedly reported as one of the escape mechanisms observed in cancer development. The background for these phenomena could originate from disturbances of nearly all steps of apoptotic pathway inside tumors, including disrupted pro- and anti-apoptotic signaling, impaired caspase activity and defective death receptor function (reviewed in [400]). Some reports suggest that polymorphic variations in genes regulating apoptosis could interfere with the risk of cancer. An association with several cancer types and *TNFalpha* gene or *FAS* promoter region polymorphisms has been found [253, 401, 402]. On the contrary, presence of certain *DR4* and *CASP8* polymorphisms could have a protective effect against bladder and breast cancer respectively [403, 404].

Down-regulation of apoptosis mechanisms observed in tumor cells could augment tumorigenesis by influencing proliferative capabilities and drug resistance of the cancer. The next problems are resistance of tumors to T-cell dependent cytotoxicity and apoptosis, and a tumor cell "counter attack" against host immune effector cells using the apoptotic pathway.

Apoptosis-regulatory proteins that have been extensively studied in solid cancers belong to the Bcl-2 family of proteins. The Bcl-2 family of proteins is engaged in the intrinsic pathway of apoptosis, and acts in a mitochondria-dependent way [405]. The mutations of pro-apoptotic proteins and overexpression of anti-apoptotic proteins were observed in the cases of solid tumors. In transgenic mice having an enforced expression of Bcl-2 protein, an increased risk for cancer incidence occurred, however, it was rather low (about 10 %), and tumors developed in advanced age [406]. Hence, although Bcl-2 mutation is causally connected with the origin of cancer, it does not seem to be the only sufficient condition for malignant transformation. Bcl-2 rather promotes neoplastic transformation, and by prolonging the lifespan of the cells, allows them to accumulate additional oncogenic mutations [407]. The observation that double transgenic mice, overexpressing products of both *bcl-2* and *c-myc* genes, show accelerated appearance of breast cancer seems to confirm that notion [408]. Overexpression of Bcl-2 protein has been shown in prostate and breast cancers, and led to inhibition of TRAIL-mediated apoptosis [409, 410]. Bcl-2 is also highly expressed in small-cell lung cancer, and to a lesser extent in squamous lung cancers (reviewed in [338]). Some other members of the Bcl-2 family could also participate in tumorigenesis. Bcl-w protein was overexpressed in both colorectal and gastric adenocarcinomas, and it was shown to suppress cell death by blocking JNK activation pathway [411, 412]. Colorectal cancers characterized by microsatellite instability demonstrated the presence of mutations in the *bax* gene, resulting in impaired function of the pro-apoptotic Bax protein [413]. The stable tumor cell lines overexpressing the Bcl-xL protein were found to be apoptosis- and drug-resistant [414].

Inhibitor of apoptosis proteins (IAPs) are endogenous inhibitors of caspases. Amplification of chromosomal regions which encompass the IAPs-coding sequences has been observed in various tumors including esophageal squamous cell carcinoma [407]. The up-regulation of IAP family members expression has been documented in various cancers, including pancreatic cancer and glioma, and was responsible for chemoresistance [122, 123, 415]. Overexpression of survivin, another extensively studied member of the IAP family, has been demonstrated in non-small cell lung carcinoma [416]. In neuroblastoma, expression of survivin has been correlated with more aggressive and unfavorable disease [417].

Another example of apoptosis-regulatory protein that has been studied is the p53 suppressor protein, due to its multidirectional function frequently called the "guardian of the genome" [400, 418]. The p53 protein, found to be down-regulated in numerous cancers, functions as a regulator of some target genes involved in apoptosis-resistance and increased proliferation activity of melanoma [419]. It has also been shown that silencing of p53 mutants in cancer cell lines resulted in reduced cellular growth due to increased apoptosis [420]. Point mutations of p53

occurring frequently in lung cancers caused up-regulation of Bcl-2, with concomitant Bax hypo-expression (reviewed in [338]).

Reduced caspase activity is another mechanism of cancer apoptosis resistance. Caspases form the system of cytoplasmic enzymes engaged in inflammatory cytokine processing and apoptosis. Mutations of the *caspase-8* gene, including modification of the stop codon, missense mutation at codon 96, and the deletion of the leucine 62, have been found in head and neck cancer, neuroblastoma, and vulvar squamous cancer respectively [54, 55, 421, 422]. All of them prevented the proper activation of the caspase cascade. Similarly, silencing mutations in caspase-9 gene were associated with development of neuroblastoma and small-cell lung cancer ([423], reviewed in [338]). Loss of caspase-1 mRNA has been observed in gastric cancer and metastatic melanoma, and in both tumors correlated with clinical stage and bad prognosis [424, 425]. Both down-regulation of caspase activity and their decreased concentrations were described in various tumors, including colorectal, ovarian, breast, and cervical cancers, the fact that was correlated with poor clinical outcome [426, 427]. A deficiency of caspase-8 was described in small-cell lung cancer and neuroblastoma [428, 429]. And conversely, high levels of caspase-3 inside the tumor cells correlated with low malignancy and good outcome in pancreatic and lung cancers [430, 431]. However, dysregulation of apoptosis observed in some studies seems to be much more complex, and does not allow for simple conclusions. Expression of caspase-3 and -7 did not correlate with clinico-pathological features of breast cancer [432], and active caspase-6 concentrations were increased in progressive melanoma and its metastases compared to non-malignant naevi [433]. Therefore, despite the fact that disturbances of apoptosis regulation in various tumors are obvious, there is still no certainty regarding the problem whether these disturbances are primary or secondary events in cancer [407].

The death receptors Fas (CD95) and TRAILR1 and -R2 are the members of the TNF receptor superfamily characterized by the presence of intracellular death domain (DD), and together with their ligands, FasL and TRAIL play an important role in the regulation of the extrinsic apoptosis pathway. Tumors are able to inhibit the death receptor signaling at several steps. The spectrum of possible disturbances covers the down-regulation or impairment of receptor function and the reduced level of the death signals [400]. Loss of Fas has been attributed to mutations in *ras* and *TP53* genes [434, 435]. Tumor-associated mutations could also deregulate the function of Fas and TRAIL receptors. Missense mutations and loss of Fas gene were identified in myeloma and melanoma (reviewed in [436]). Deletions and mutations of TRAILR1 and -R2 receptors have been detected in many tumors, including non-small cell lung cancer (reviewed in [437]). Lack of cytoplasmic signaling domains of Fas and TRAILR1 and -R2 has been found in many tumors, including myeloma, gastric, and breast cancers (reviewed in [2]). Inactivating mutations of downstream Fas signaling molecules like FADD and caspase-10 have been found in non-small cell lung cancer [438]. The low expression of Fas and both FasL and TRAIL has been documented in neuroblastoma and pre-cancerous cervical lesions respectively [439, 440]. High levels of anti-apoptotic regulator FLICE inhibitory

protein (c-FLIP) have been demonstrated to correlate with TRAIL-mediated apoptosis in melanoma cells [441]. Overexpression of c-FLIP has been confirmed in several tumors in mice and humans, and in some of them was correlated to bad prognosis (reviewed in [2, 437]).

Activation of T cells during immune response is a self-limiting phenomenon, as activated T cells up-regulate Fas death receptor and enter activation-induced cell death (AICD). Some tumors, like melanoma, lung, pancreatic, gastric, colon, and breast cancers might accelerate AICD and escape from immune recognition and destruction, by overexpression of FasL and elimination of T effectors in FasL-dependent pathway (reviewed in [2, 442]). Expression of FasL on their surface is either constitutive or induced by chemotherapy (reviewed in [437]). A significant reduction in TILs and apoptosis of Fas-positive TILs has been observed in esophageal cancer and metastatic gastric carcinoma respectively. A similar correlation has been found in head and neck tumors and ovarian cancer. The expression of FasL and TILs apoptosis was more evident in metastatic colon cancer and in breast cancer lymph node metastases. High FasL/Fas ratio was a bad prognostic sign among patients with ovarian and hepatocellular cancers (reviewed in [442]). The meaning of FasL for tumor escape is sustained by the observation that down-regulation of FasL expression in colon cancer cells significantly reduced tumor growth in syngenic mice, and stimulated T cell anti-tumor response [443]. Moreover, soluble FasL (sFasL) which is produced by cleavage of membrane FasL by tumor metalloproteinases, as well as microvesicles containing FasL produced and released by melanoma, could kill effector immune cells and cause systemic immunosuppression ([444], reviewed in [442]). A significantly increased number of $CD3^+Fas^+$ apoptotic T cells were found in blood of patients with metastatic melanoma and head and neck cancers. Furthermore, T $CD8^+$ cells more frequently entered apoptosis than T $CD4^+$ cells, suggesting that T $CD8^+$ cells are more sensitive to apoptosis [445, 446]. This mechanism was called FasL "counter attack" [447]. It is directed against tumor-infiltrating and by-standing T lymphocytes, as upon tumor recognition T cells express substantial levels of FasL which induces "suicidal" and "fratricidal" T cell death (reviewed in [436, 448]). Moreover, human metastatic melanoma cells are capable of engulfing and ingesting T lymphocytes in a process called "tumor cannibalism" [449]. However, the function of FasL can also accelerate the rejection of tumor by induction of proinflammatory and anti-tumor effects mediated in vivo by activated neutrophils [450]. In addition, screening of the melanoma cell lines by RT-PCR and functional assays did not reveal expression of functional FasL [451]. To summarize these conflicting results, it was hypothesized that the local levels of FasL may determine the course of the events, with high FasL levels provoking neutrophil infiltration, and lower levels being capable of anti-tumor T response elimination. Activation of neutrophils might depend on the form of FasL (only membrane-bound FasL is an activator) and/or on the macrophages and DCs which upon FasL stimulation produce IL-1β and other pro-inflammatory proteins and chemoattractants [437]. The extension of FasL/Fas signaling could be

genetically determined, as different tumors are characterized by either frequent or rare Fas mutations, and p53 mutation, abundantly met in various tumors, can down-regulate Fas expression (reviewed in [442]). The effects of FasL/Fas signaling might also depend on the local environment, which through the action of some immunoregulatory molecules may create appropriate conditions for tumor escape. Up-regulation of FasL on tumor cells resulted from pro-inflammatory cytokines TGF-β, IL-10, prostaglandins, and reactive oxygen species (reviewed in [442, 448]).

Other molecules including RANTES and RCAS1 could augment FasL "counter attack" by inducing cycle arrest and apoptosis of anti-tumor activated T cells (reviewed in [436, 448]). Tumor cells also showed the ability to use a transmembrane or soluble decoy receptors with non-functional or absent death domain to avoid T-cell-mediated apoptosis. Decoy receptors such as soluble Fas (sFas) or various TRAIL receptors (-R3, -R4) have been described in tumors (reviewed in [2]). Increased serum level of sFas was detected in various tumors, and correlated with poor outcome in melanoma patients (reviewed in [437]). T cells can also eliminate target cells by the perforin/granzyme pathway. It has been demonstrated that tumors are resistant to perforin/granzyme-dependent killing by cytotoxic T cells, caused by the expression of granzyme B inhibiting serine protease inhibitor PI-9/SPI-6 present on the cells of melanoma, cervical, and breast cancers, and correlated with a poor patient outcome [452, 453]. Another immunological mechanism that contributes to cancer "counter attack" against cytotoxic T cells involves the interactions between PD-1 and its ligand PD-L1, also called B7-H1. Different tumors including ovarian, colon, lung, and breast cancers indicate the expression of PD-L1, similarly to tumor-infiltrating myeloid cells in non-small cell lung cancer [338]. Binding of PD-1 on T cells to its ligand on cancer cells resulted in inhibition of T cell activation via induction of FasL and IL-10. Moreover, blocking of PD-L1 reduced T-cell apoptosis in tumor models [448, 454, 455]. Over-expression of PD-L1 on ovarian cancer epithelial cells is a mechanism of possible importance for intra-epithelial T $CD8^+$ depletion and deactivation [119]. Lung tumors possessing a high expression of PD-L1 showed fewer TILs compared to B7-H1-negative tumors (reviewed in [338]). The precise mechanism of PD-1/PD-L1 interactions is probably based on up-regulated expression of the activator protein-1 (AP-1) subunit c-Fos in TILs. Immunosuppressive effect of c-Fos was mediated through induced expression of PD-1 via connection of c-Fos to the AP-1-binding site in PD-1 encoding gene. Knocking-out mutation of this binding site abrogated PD-1 induction and augmented T effector immunity [456]. Tumor cells subjected to apoptosis generate apoptotic bodies, a structures distinct from microvesicles and exosomes, which are formed from randomly blobbing cellular membrane vesicles having a couple of micrometers in diameter. They contain fragmented nuclei and organelles, and are able to transfer oncogenes into target cells and to suppress cytotoxic anti-tumor T $CD8^+$ lymphocytes (reviewed in [457]).

8.4.7 The Role of Tumor Stroma in Immune Escape

Solid tumors are composed not only from neoplastic cells, but also stroma containing fibroblasts, extracellular matrix, endothelial cells, and tumor-infiltrating immune cells. One of the most important populations of cells which are residents in tumor stroma are CAFs. These cells have met with growing interest, due to their capability of initiating and promoting tumor growth (reviewed in [458]). The population of CAFs gathers distinct subpopulations of fibroblasts; however, their precise functions and differences between them still await investigation. Another interesting question is the origin of CAFs. Most of them are modified local fibroblasts, but some additional sources of CAFs have been identified, which vary according to the tumor type. Some cells originate from mesenchymal stem cells, and some are a result of the mechanism called epithelial-to-mesenchymal transition (reviewed in [459]). The meaning of CAFs for tumor development is highlighted by the observation that for effective carcinogenesis the presence of cancer cells is not enough, and without cooperation with surrounding tissues, cancer cells cannot form an aggressive tumor. The interaction between the fibroblasts and ECM in cancer recalls the processes of tissue repair, however, disturbed during carcinogenesis (reviewed in [459]). CAFs produce growth factors exerting tumor-promoting activity, such as EGF, FGF, TGF-β, platelet-derived growth factor (PDGF), or insulin-like growth factor (IGF) [460, 461]. The population of CAFs has also shown expression of chemokines CCL5, CXCL12, and CXCL14, which are responsible for tumor metastatic potential [462], increased angiogenesis, and influx of macrophages into the tumor [463]. Previous studies have shown that CAFs are an alternative source of VEGF-A capable of compensating for the lack of tumor-derived VEGF-A (reviewed in [464, 465]). These factors act in a paracrine manner together with signaling from ECM components and integrins. CAFs-derived TGF-β modulates the growth and the oncogenic potential of adjacent epithelial cells, and promotes their resistance to apoptosis by up-regulation of NF-κB transcription factor (reviewed in [459]). Elevated TGF-β in tumor stroma activates CXCR4 expression in epithelial cells, making them unresponsive to growth-inhibitory signals. Expression of CXCR4 in prostate cancer is a bad prognostic sign [466]. IGF-1 expressed by prostate tumor stroma stimulates proliferation of epithelial cells by up-regulation of MAPK, Akt, and cyclin D1. In a murine model, overexpression of IGF-1 by CAFs promotes malignant transformation of epithelial cells, and increases metastatic potential which could be abrogated by blockade of IGF-1 receptor or MAPK. Activation of IGF-1 interferes with the TGF-β intercellular Smad pathway, and blocks apoptosis of epithelial cancer cells [467]. The cooperation between endothelial cells and CAFs could influence carcinogenesis in prostate cancer. Genetic instability of stromal fibroblasts reported in the patients contributes to malignant transformation of epithelial cells [468, 469]. Similarly, murine studies of breast cancer have indicated that implantation of tumor cells together with fibroblasts non-responding to TGF-β into laboratory animals augmented growth and metastases of implanted cancer [470]. The presence of

fibroblasts was not an indispensable condition for tumor growth stimulation in vitro, as supernatants from fibroblast culture were also activators of cancer progression, due to the presence of chemokines CXCL12 and CXCL14. Alternations of expression of many genes regulating fibroblast function were noted in breast cancer (reviewed in [459]). Pancreatic adenocarcinoma, which is one of the most lethal human malignancies, is characterized by intense stromal reaction. CAFs in pancreatic cancer produce ECM proteins, growth factors, and pro-inflammatory cytokines [471].

During some physiologic processes such as embryonic development and wound repair, there is a temporary need for epithelial cells to escape from the rules governing the tissue structure and adopt a mesenchymal phenotype which enables them to migrate. This is called epithelial-to-mesenchymal transition, and occurs also in pathological conditions during cancer development and progression. EMT is an active process during which epithelial cells lose inter-cellular connections and acquire migratory capacities [472]. Cell adhesion molecule epithelial E-cadherin belongs to the key negative regulators of EMT, being responsible for adherens junctions and epithelial integrity. Repression of E-cadherin is regulated by transcription factors called snail, twist, zeb, and slug. Loss of E-cadherin functions is a typical phenomenon met in human cancers, thus leading to EMT, decreased adhesion, and increased metastasizing capacity (reviewed in [215, 472]). Disturbed function of E-cadherin could depend on genetic mutations in its gene; however, most reasons cause inactivation of E-cadherin by promoter methylation and transcriptional repression [473, 474]. The initiating signal for EMT is delivered by both tumor- and stroma-derived TGF-β, which cooperates with the activated Ras pathway [475, 476]. EMT accelerates significantly upon TNF-α costimulation with TGF-β [472]. Following the changes of E-cadherin functions, alterations in expression of integrin αvβ6 receptor for fibronectin and tenascin occur. The inflammation and tissue repair mechanisms are both stimulators of this change (reviewed in [472, 507]). Up-regulation of αvβ6 integrin enhances the capability of colon cancer epithelial cells to migrate into the extracellular matrix and to metastasize into liver, and reversely stimulates TGF-β secretion, thus providing a self-perpetuating loop [477, 478]. As a result of EMT, single cancer cells migrate in the absence of any inter-cellular contact, and their survival depends on autocrine VEGF/Flt1 interactions [479]. Snail transcription factor expression has been confirmed in non-small cell lung cancer and melanoma, and correlated with shorter survival and predisposition to metastases respectively [480, 481]. Murine studies indicated that snail expression affects the function of MDSCs, as snail-knockout mice were characterized by reduced number and argininase activity (reviewed in [215]).

8.4.8 *Microvesicles and Exosomes: Mediators of Tumor Escape*

Microvesicles are small membrane-enclosed structures shed from a variety of cells, including cancer, which are present in both physiological and pathological conditions in body fluids such as blood, urine, or ascites. Tumor-derived microvesicles (alternatively called oncosomes or ectosomes) are uniquely generated by tumor cells. Microvesicles are a unique population of structures which are distinct from exosomes. Microvesicles originate from an outward budding and fission of the cellular membrane, and may have irregular shape and dimensions ranging from 200 nm to 1 μm [482]. Shedding of microvesicles is not just a passive process, as it occurs in specific places of the cell surface, needs exposure of phosphatidylserine, and requires energy input, RNA synthesis, and protein translation [482, 483]. However, compared to normal cells, tumor cells could shed microvesicles from the entire surface, especially from the invading cellular edges [484]. The function and contents of microvesicles depends on the cell type from which they originate [485]. Tumor-shed microvesicles contain cytokines, miRNA, mRNA, FasL, chemokine receptors, tissue factor, EGFR, Her-2, metalloproteinases, or other molecules (reviewed in [482]). Cellular proteins are selectively incorporated into microvesicles in ARF6-regulated endosome recycling, which activation has been linked to acquisition of invasive potential by the tumor (reviewed in [457]). The interaction with the cells occurs via microvesicle fusion with the target cell or their endocytosis. Microvesicles are released into the body fluids or extracellular milieu, where they play a regulatory role for ECM degradation and invasion, angiogenesis, metastases, and immune escape of the tumor [486]. It has been demonstrated in a mouse model that microvesicles shed from highly metastatic melanoma cells were able to change the phenotype of weakly metastatic melanoma cell-line into an aggressive phenotype capable of metastasizing [487]. Similarly, the oncogenic receptor EGFRvIII found on the aggressive gliomas was transferred to a non-aggressive populations of tumors [488]. Moreover, the number of microvesicles were shown to correlate with invasiveness of tumor in vitro and in vivo [489]. Similarly, the early stages of ovarian cancer were characterized by a lower number of microvesicles in malignant ascites compared to advanced disease [490]. Microvesicles containing mRNA, miRNA, or fragments of genomic DNA could influence the transcriptome of the target cells and augment tumor invasiveness (reviewed in [457]). Tumor-derived microvesicles stimulate endothelial cells and stromal fibroblasts to promote neo-angiogenesis and invasion. Cancer cell-lines were able to produce microvesicles containing VEGF, MMPs, and miRNA, which stimulated motility, invasiveness, and tubule formation by endothelial cells. Upon stimulation, the endothelial cells produced their own microvesicles with encapsulated MMPs, VEGF, and sfingomielin, which in autocrine manner further promoted endothelial invasion to the stroma. Those processes were stimulated by hypoxic conditions (reviewed in [482]). Microvesicles released by prostate cancer and lung cancer

cell-lines were shown to chemoattract and activate stromal fibroblasts, and by MMPs increased their motility and resistance to apoptosis. In turn, stimulated fibroblasts were capable of shedding microvesicles, facilitating tumor invasiveness and migration [491, 492]. Fusion of microvesicles produced by human melanoma and colon cancer cells with monocytes inhibited their differentiation, and switched them to immunosuppressive activity. On contact with tumor vesicles, monocytes acquired the $CD14^{+}HLA^{-}DR^{-}$ phenotype, indicated lack of co-stimulatory molecule up-regulation, and started to secrete TGF-β [493]. Fas-containing cancer-derived microvesicles induced apoptosis of T cells and abrogated their killing abilities [492]. Tumor cells can escape effector immune cell-mediated apoptosis by preventing the intracellular accumulation of caspase-3, and abrogation of microvesicle production has been shown to increase caspase-3 and apoptosis of tumor cells (reviewed in [484]). Presence of MMPs and other proteases inside tumor-derived microvesicles was correlated both in vivo and in vitro with acquisition of invasive capacity in ovarian and breast cancer respectively. Activity of proteases within vesicles was augmented in a hypoxic environment, and played a probable role in up-regulation of tumor-metastasizing capacity (reviewed in Chari et al. 2007). Association between the presence of tissue factor (TF)-containing microvesicles shed from the tumor and increased risk of thromboembolism suggests their role in the hypercoagulative state observed in cancer patients [494]. And finally, microvesicles could participate in tumor chemoresistance, as tumors treated with doxorubicin and cis-platin demonstrated shedding of microvesicles containing accumulated, high-concentrated drugs [495, 496].

Exosomes originate from reverse budding of the membrane of intracellular multivesicular bodies (MVB), and are released upon fusion with cellular membrane to extracellular fluid or circulation. They form round- or oval-shaped structures, and have 30–100 nm of diameter (reviewed in [497]). Release of exosomes is regulated by calcium ionophores, phorbol esters, and inositol 3-kinase inhibitors, as well as indirectly by p53 [498, 499]. Exosomes may contain numerous proteins, mRNA, miRNA, lipids, and other active molecules, and influence the cells locally in an autocrine and paracrine manner, as well as being able to regulate the function of distant cells. Exosomes may impact various cellular responses, and are engaged especially in regulation of inflammatory processes (reviewed in [457]). The presence of signal molecules on the exosomes' surface directs them to the target cells and provides their endocytosis or phagocytosis [500]. Endocytosis of exosomes is an energy-consuming process which may occur in a clathrin-dependent way and involve additional endocytosis mechanisms, and which needs both proteins included in exosome and proteins of target cell [501]. Exosomes are produced by various cancers, including melanoma, breast, prostate, and colorectal cancers, and contain specific proteins dependent on the cancer type. The presence of exosomes has been confirmed in vascular circulation, body fluids, and malignant ascites (reviewed in [497]). Studies performed on the mouse model of cancer demonstrated that transplantable breast tumors were capable of accelerating growth by releasing exosomes which decreased the number and cytotoxic activity of NK cells. The in-vitro effects of exosomes originating from human breast cancer and melanoma

on NK cells were identical [502]. FasL- and TRAIL-expressing exosomes were also shown to induce apoptosis in tumor-specific activated T effectors [503]. Treatment of immature mouse DCs with exosomes derived from breast cancer blocked maturation of DCs and stimulated pro-oncogenic cytokine response, as indicated by increase of IL-6 and activation of Stat3 pathway ([502], reviewed in [497]). Tumor exosomes containing PGE_2 and TGF-β also promoted MDSCs to decrease T cell cytotoxicity [504]. In-vivo studies showed the presence of exosomes in the sera of cancer patients, a fact that was correlated to the increased number of Tregs. It could be possible that exosomes containing suppressory cytokines IL-10 and TGF-β were involved in Tregs expansion in these patients, as a similar phenomenon was described in in-vitro studies (reviewed in [505]). Therefore, exosomes may be viewed as modulators of immune response and inducers of both local and peripheral tolerance towards tumor (reviewed in [486]). However, some studies have demonstrated that DC-derived exosomes could stimulate anti-tumor T cell responses and activate NK cells. Probably, a different composition of tumor-derived and DC-derived exosomes could be responsible for that discrepancy (reviewed in [497]).

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Chapter 9
Adoptive T-Cell Immunotherapy: Perfecting Self-Defenses

Katrina Shamalov, Yair Tal, Chen Ankri, and Cyrille J. Cohen

Abstract The unrivaled potential of T cells for targeted immune function is central to the eradication of cancer. While their natural anti-tumor response might sometimes be insufficient, several studies and importantly, multiple clinical trials in terminally-ill cancer patients have demonstrated that it is possible to design novel and efficient immunotherapeutic approaches based on the adoptive transfer of autologous tumor-specific T lymphocytes. Herein, we will expand on the development and the use of such strategies using tumor-infiltrating lymphocytes or genetically-engineered T cells. We will also comment on the requirements and potential hurdles encountered when elaborating and implementing such treatments as well as the exciting prospects for this kind of emerging personalized medicine therapy.

List of Abbreviations

ACT	Adoptive cell transfer
AICD	Activation-induced T-cell death
CAIX	Carboxy-anhydrase-IX
CAR	Chimeric antigen receptor
CEA	Carcino embryonic antigen
CT	Cancer/testis
EBV	Epstein–Barr virus
HLA	Human leukocyte antigen
hTERT	Human telomerase reverse transcriptase
HTLV-1	Human T-cell lymphotrophic virus type I
IL	Interleukin

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ITAM	Immunoreceptor tyrosine-based activation motif
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
PBL	Peripheral blood lymphocyte
RCC	Renal cell carcinoma
scFv	Single-chain variable fragment
TA	Tumor antigen
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
TIL	Tumor-infiltrating lymphocyte
Tregs	Regulatory T-cells

Contents

9.1 Tumor Antigens: Defining the Target

T cells play a central role in the immune response against cancer. Their activation is initiated by the interaction of the T-cell receptor (TCR) with its cognate MHC-peptide complex presented on the surface of the target cell, which activates them specifically [1]. Whether T cells could recognize endogenous tissues was a matter of debate during several decades, especially as T cells are supposed to be tolerant to self-antigens. Nevertheless, molecular and immunological advances in the 1990s led to the discovery of self-originated proteins that could be recognized

by T lymphocytes [2]. Accordingly, tumor-specific T cells have been shown to be activated through the binding of their TCR to specific epitopes derived from tumor antigens (TA) presented by a major histocompatibility complex (MHC) molecule [3]. TA are present on some tumor cells but also on normal tissues (in this case, they are termed tumor-associated antigens—TAA), and were shown to represent effective targets for T-cell-based cancer immunotherapy. They can be classified into several categories; this division pertains to the pattern of expression of these antigens (e.g., over-expressed, oncofetal, ...) and whether these antigens are "self" or mutated [4]. Several sources indicate different classifications, but five known classes of TA can be broadly described:

Cancer/testis antigens (*C/T*)—they are expressed in various human cancers, but also in normal testis tissues. Some evidences suggest that there may be some level of T-cell tolerance toward these antigens [5].

Tissue-specific differentiation antigens—these antigens are typically expressed only by the tumor and its tissue of origin. Known examples of tissue-specific differentiation antigens include the MART-1/Melan-A and gp100, which are expressed in both melanocytes and melanoma cells. These antigens have emerged as very promising target antigens for T-cell-based adoptive immunotherapy, but their presence on normal tissues can be the source of auto-immune manifestations.

Mutated self-proteins—usually when mutations occur in the initial cancerous cell (or one of its early daughter cells), this class of tumor antigens can potentially provide targets for T-cell-based immunotherapy of cancer, as they are to be expressed in most of the tumor tissues.

Over-expressed antigens—this type of antigens constitutes also an important TA class, which is relevant in both T-cell therapy and antibody-based treatments. Based on clinical data, it seems that their over-expression in several tumor tissues (e.g., Her2/neu) but then again their reduced levels in healthy cells may limit the potential for deleterious autoimmune side-effects [4].

Viral antigens—as it is believed that around 20 % of all cancer cases are linked to infectious agents [6], antigens derived from oncogenic viruses would provide a source of "non-self" targets, which would be recognized more efficiently than TAA due to a potential lack of tolerance against the viral epitopes.

9.2 Tumor-Infiltrating Lymphocytes

9.2.1 Presence of Intra-tumoral T Lymphocytes

For several decades, it has been demonstrated that tumor-specific T cells can massively migrate into tumor sites. Some of these tumor-infiltrating lymphocytes (TILs) have thus the ability to specifically recognize tumor antigens expressed on the surface of tumor cells, and may greatly influence directly or indirectly the anti-tumor immune responses and the progression of a variety of solid tumors [7]. The

presence of TILs in the tumor vicinity, and the nature of their interactions with target cells, contribute to determine whether a tumor is destroyed or grows unimpeded. It may also correlate with responses to chemotherapy/radiotherapy and disease prognosis. Indeed, high densities of $CD3^+$ T cells, $CD8^+$ cytotoxic T cells, and memory T cells into tumor sites could represent a reliable prognostic factor for the disease-free and overall survival of patients with different tumor types, such as melanoma, and head and neck, breast, bladder, urothelial, ovarian, colorectal, renal, prostatic, and lung cancer [8]. In contrast to the effects of cytotoxic T cells and memory T cells that are associated with a positive clinical outcome, the impact of $CD4^+$ T cell infiltration on survival and prognosis is unclear; for example, there are conflicting data about the role of regulatory T-cells (Tregs) in this context, and their effects on tumor progression have been a matter of debate for the past decade [7, 9]. Moreover, there is a great variability in the density and location of these infiltrating T cells between different patients bearing the same type of cancer [7].

9.2.2 Adoptive TIL Immunotherapy

Nonetheless, to harness the potential benefit of tumor-specific T cells in cancer treatment settings, pioneering therapeutic approaches (Fig. 9.1) were developed in the last three decades [10]. Adoptive immunotherapy using autologous TILs has become an appealing strategy for the treatment of mainly melanoma and renal cell carcinoma. This necessitated the development of techniques and systems to grow large numbers of anti-tumor lymphocytes. An important milestone in the development of this kind of immunotherapy occurred in 1987 when tumor-infiltrating lymphocytes from patients with metastatic malignant melanoma were successfully cultured and expanded using the T-cell growth factor interleukin 2 (IL-2) [11]. During this expansion process performed ex vivo, fragments from resected tumors were grown in culture vessels in conditions that favor T-cell growth (using for example high concentrations of IL-2). Tumor-specific T-cell populations can be identified on the basis of their reactivity with MHC-matched tumor cell lines or the autologous tumor. Reactive cultures can be then selected and expanded, and adoptively infused back into cancer patients. Furthermore, to facilitate the engraftment of this autologous T-cell transplant, patients receive high-dose intravenous bolus IL-2 [12, 13]. As exemplified in several studies, the transfer of these cells back into the patient led to dramatic partial or complete clinical responses and durable regression [14, 15].

The adoptive transfer of TILs is one of the most effective treatments for patients with stage IV melanoma. The first study aimed at directly targeting human tumor using autologous TILs to treat patients with metastatic melanoma was reported in 1988 by Rosenberg et al. at the National Cancer Institute [16], and a significant improvement in the response rate and durability of response was steadily reported in subsequent studies [15]. This improvement occurred when bulk cultures ($CD8^+$ and $CD4^+$) were transferred and more importantly, when a non-myeloablative

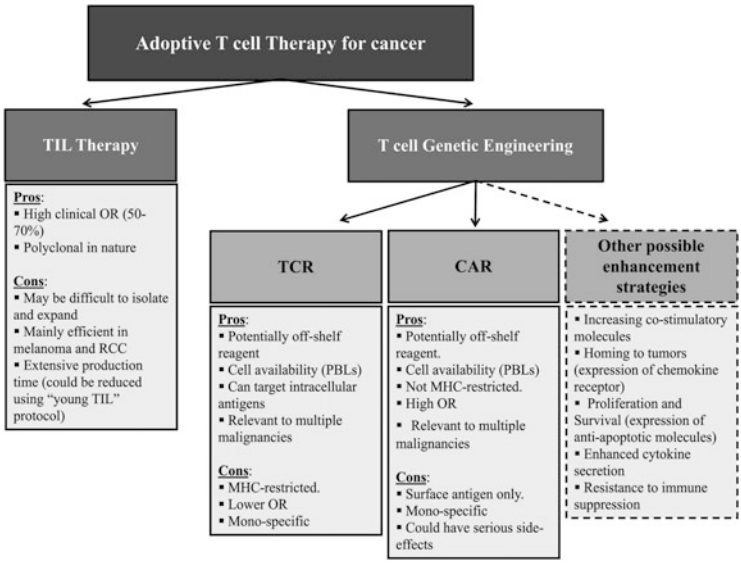

Fig. 9.1 A summary of different adoptive T-cell therapy approaches. *OR* objective response, *PBLs* peripheral blood lymphocytes

conditioning regimen (depleting chemotherapy or whole-body irradiation) was administered to the patient prior to T-cell transfer [12]. In that regard, studies reported a significant correlation between the intensity of lymphodepletion and the in-vivo anti-tumor effect of the infused cells [17]. It has been suggested that the positive impact of lymphodepletion prior to TIL transfer is based in part on the elimination of suppressive $CD4^+CD25^+$ Tregs as well as of normal endogenous lymphocytes that could compete with the transferred cells for homeostatic cytokines such as IL-7 and IL-15 [18, 19].

Recent results indicate that the objective clinical response observed in patients with metastatic melanoma that were treated with adoptively transferred autologous TILs ranges between 49 % and 72 % [15]. Importantly, objective response was highly associated with the persistence of the transferred cells [20]. Indeed, many patients in the recently reported trials display high levels of persistence, sometimes reaching up to 75 % of all of the circulating $CD8^+$ T cells. Still, it appears that persistence alone was not a sufficient requirement for an effective response [20, 21]. Studies have also shown that the state of differentiation of the transferred cells may be inversely correlated to the effectiveness of these cells in adoptive cell therapy (ACT) settings, and to their capacity to proliferate and persist [12, 22]. In other words, early effector T cells seem to mediate better anti-tumor response than intermediate and late effector T cells.

9.2.3 Tumor Microenvironment and Potential Hurdles

Solid tumors contain many other cell types, including cells derived from the innate and adoptive immune system, stromal cells, and myeloid-derived suppressor cells (MDSCs) [23, 24]. The latter are endowed with potent immunosuppressive properties, and their intratumoral presence at a high frequency correlates with a poor prognosis in patients with different tumor types. Recent findings indicate that targeting these cells, and the supportive environment (for the tumor) they promote, might represent an effective approach to promoting the destruction of cancer cells, leading to tumor elimination [25].

Despite its aforementioned success (especially in melanoma), adoptive cell transfer (ACT) therapy with autologous TILs bears some limitations which include, for example, the requirement to isolate and expand T cells with anti-tumor activity. Even if such cells are generated, adoptive T-cell therapy for some tumors will not necessarily be effective, as these may be poorly antigenic. Other tumors, such as colon and breast tumors, are infiltrated by T cells, but the specificities and functions of the latter are unclear [26, 27]. In this regard, a potential explanation as to why melanoma has been widely studied as a target for therapeutic TILs is that this type of cancer appears to be unique among human cancers because of its ability to promote elevated numbers of lymphocytes with anti-tumor activity. This might be due to the fact that melanoma tumors express a high number of mutated antigens that could help in breaking self-tolerance and were also shown to harbor class II-MHC molecules [10, 28]. Renal cell carcinoma (RCC) is also considered an immunogenic tumor that exhibits rich intra-tumoral lymphocytic infiltration. Still, it seems that T-cell activation is insufficient at the tumor site due to many immunosuppressive mechanisms induced in the microenvironment of RCC [29–32]. This may provide an explanation as to why previous clinical trials with TILs in RCC did not yield substantial benefit compared to melanoma. Nevertheless, current knowledge and experience with TIL generation from—and treatment of—melanoma patients could provide clues to elaborate an improved therapeutic regiment for ACT in RCC and other malignancies [33, 34].

9.2.4 TIL Treatment: Current Status and Future Promises

By utilizing current techniques today, tumor-infiltrating lymphocytes can be detected in approximately 80 % of melanoma patients [35]. However, in most cancer patients, those naturally-occurring TILs fail to destroy the tumor as they are outnumbered, subjected to constant immunosuppression, and due to other factors that are not fully understood. Additionally, the generation of a TIL culture (s) that prove reactive for each patient tumor is not always feasible and requires several weeks. The latter might be overcome, as exemplified in new clinical studies designed to improve the TIL anti-tumor activity, growth, and expansion by

generating "young TIL" cultures [36, 37]. In this method, tumor-infiltrating lymphocytes are grown and expanded briefly (around 2–3 weeks compared to 4–6 in the conventional TIL protocol) and are introduced back into patients without testing for selection. Thus, the "young TIL" protocol utilizes bulk unselected TIL which spend minimal time in culture by eliminating the individualized tumor reactivity screening step [38]. As no further selection process is required, all established "young TIL" cultures are technically eligible for treatment [37]. "Young TIL" protocols reduce labor time and can be implemented in most patients, but importantly, recent studies indicate that this approach leads to an objective response rate of 50 %, close to that observed in classical TIL protocols [36].

As immunomodulatory monoclonal antibodies show promise in the clinical trials recently conducted, the combination of T-cell transfer with antibodies blocking CTLA-4 or PD-1 function may help to overcome negative costimulatory signals, which may improve the function of the transferred T cells [39, 40]. In addition, it is possible to manipulate the T-cell differentiation state during culture/expansion to improve TIL-ACT for the treatment of human cancer, using, for example, molecules that may inhibit differentiation processes (e.g., GSK-3b [41]) or by subjecting TIL cultures to different cytokines, such as IL-7, IL-15, or IL-21 alone or in addition to IL-2 [42–47].

While TIL-based clinical trials have demonstrated impressive results in terminally-ill melanoma patients, they require dedicated facilities, and collaboration between surgical and cell therapy teams, which may have limited their implementation to a few clinical centers worldwide. Nonetheless, parallel approaches aimed at exploiting the unrivaled potential of T cells to mediate tumor regression are being developed, and are based on the genetic modification of T cells to express tumor-specific receptors.

9.3 Adoptive Immunotherapy Based on the Genetic Modification of Lymphocytes

9.3.1 TCR Gene Transfer

9.3.1.1 Development and Implementation of TCR Gene Transfer Approaches

As T-cell specificity is solely based on the nature of its TCR, TCR gene transfer therapy represents a promising approach based on the genetic modification of T cells engineered to recognize tumor antigens. A study by Steinmetz and colleagues back in 1986 demonstrated for the first time the feasibility of the TCR gene transfer approach. In this study, T cells were redirected by genetic engineering in order to study the receptor dynamics [48]. Since then, several studies have demonstrated

how human T cells can be redirected toward specific antigen by TCR gene transfer using a melanoma-specific TCR in vitro [49], followed by an in-vivo study using a mouse model [50]. In 2006, the first clinical trial involving TCR gene therapy was reported by Morgan et al. involving metastatic melanoma patients, who were treated with autologous peripheral blood lymphocytes (PBLs) retrovirally transduced with a MART-1 specific TCR following a lymphodepletion regimen. An objective clinical response was observed in two out of 17 patients treated in this trial (12 %), demonstrating dramatic tumor regression [51].

Three years later, the results of a second clinical trial were reported by the same group (led by Dr. Steven Rosenberg, NCI); in this trial, metastatic melanoma patients were treated with two high-affinity TCRs against the melanoma antigens MART-1 and gp100 [52]. The expression levels of the TCR and the persistence of modified T cells were markedly increased compared with the first trial, and an objective response rate of 30 % (six out of 20 patients) was reported. Since then, progress has been made towards the clinical testing of additional TCRs, specific to other antigens such as p53 [53], NY-ESO-1 [54], and CEA [55], in order to target cancers other than melanoma.

So far, TCR gene transfer has been proven to be an effective strategy to create specific tumor-reactive T cells, without the restrictions or the need of isolating natural tumor-reactive T cells from the patient. Factors that should be taken into account towards improving the clinical efficacy of this approach, and that will be discussed in part below are, for instance, the persistence of the TCR-modified T cells after infusion, the prolonged expression of the TCR genes, and the need to reach sufficient T-cell functional avidity.

9.3.1.2 How to Select the Appropriate (Suitable) Antigen?

As for other therapeutic treatments, two main factors should be considered to choose the proper target antigen for TCR gene therapy: safety and efficiency. By choosing a target antigen characterized by high levels of tumor-specific expression and lacking any expression levels in the normal tissue, one can limit the possibility of on/off-target effects and the possible dose-limiting toxicity which can result from the destruction of normal tissues that express the aimed target antigen [55].

Currently, over-expressed antigens, cancer-testis (CT) antigens, and differentiation antigens represent the most common target antigens for TCR-based adoptive immunotherapy. NY-ESO-1, a cancer-testis antigen (CT), is one of the most promising targets that have been the subject of a recent clinical trial for TCR gene therapy, which resulted in a 40–60 % objective response in melanoma and synovial cell sarcoma patients [54]. Many CT antigens have been identified in various human cancers is discussed above [5, 56], while they are normally expressed only in the human germ line. The restriction of CTs to cells that partially or do not express human leukocyte antigen (HLA) molecules (in healthy tissues) makes them unsusceptible to recognition by a TCR, thus preventing toxicity to normal tissues when targeting T cells to tumor-associated CT antigens. Two other

classes of tumor antigens that may be also taken into account as targets for TCR gene therapy are the mutation antigens and the neo-antigens [57, 58]. Indeed, it seems that the majority of these antigens are to be safe targets owing to their exclusive expression in tumor cells. While the first group is represented by antigens that are common not only to a variety of patients but also shared between several tumor types, the second group is constituted of patient-specific antigens that can be characterized using recent technological advancements such as individual tumor sequencing [57]. Still, as an immune selective pressure builds up, the down-regulation of target antigens could represent a concrete impediment to the therapeutic efficacy of TCR gene therapy [59, 60], especially as it is based on mono-specific T cells. Recently, the study of Kaluza and colleagues demonstrated tumor (B16/Ovalbumin) recurrence after adoptive transfer of specific (OT-1) effector cells, due to the loss of the target tumor antigen [61]. Possible solutions for the down-regulation of target antigen expression may consist in: (1) targeting of proteins that have an essential role in the survival of the tumor [4], (2) combining two (or more) different specificities expressed by the same T cell [61], or (3) using multiple populations of T cells, each expressing a different tumor-specific TCR.

9.3.1.3 Choosing the "Right" TCR for the "Right" pMHC Complex

Several approaches have been described in order to isolate the desirable TCR, which will not only recognize specifically the targeted peptide–MHC complex, but will also endow T cells with superior functional avidity. As mentioned above, the objective response rate observed in the first two clinical TCR-gene therapy trials, in which MART-1-specific TCRs were produced from a melanoma patient [51, 52], was low in comparison to that in TIL therapy trials [17, 38, 62]. This disparity could be due to: (1) low levels of TCR expression of the introduced TCR on the engineered T cells, (2) a diminished persistence of TCR-modified T cells after infusion, and/or (3) the induction of immunological self-tolerance that might hinder a proper response to target antigens with suboptimal affinity to their cognate TCR. Therefore, unmodified TCRs derived from melanoma patients may require further optimization steps to endow T cells with an improved performance.

High-affinity TCRs could be isolated from HLA-mismatched donors, since one does not expect that those TCRs would be subjected to any tolerance mechanism pertaining to the targeted MHC–peptide complex, which thus would be recognized as non-self [63–65]. Similarly, HLA-transgenic mice [66–69] and phage/yeast/T-cell display systems [70–73] also provide platforms that could be exploited to isolate "non-tolerized TCR." The TCR phage display technique, for example, yielded TCRs with high affinity specific for human telomerase reverse transcriptase (hTERT), human T-cell lymphotropic virus type 1 (HTLV-1), TAX antigen, and additional antigens [73, 74].

Additionally, a human-TCR repertoire transgenic mice system was recently established. In this system, the entire human TCR loci was cloned into HLA-A2-transgenic mice [75], and this resulted in the reconstitution of a potentially broad

human TCR repertoire in the mouse recipient which can provide a platform to isolate human high-affinity TCRs, provided the targeted epitope is not expressed by the mouse recipient.

9.3.1.4 TCR Expression Systems

In most of the clinical trials reported, TCR gene therapy made use of γ-retroviral vectors which are common viral expression systems that facilitate transgene integration into the genome of the host cells [76–78]. MFG/SFG-, MP71/SF91-, and MSGV1- are examples for such γ-retroviral vectors that in pre-clinical studies and clinical trials exhibit high transduction efficiency together with minimal vector-associated toxicity. Lentiviral vectors are another viral expression platform that, unlike γ-retroviral vectors, is largely independent from cells' dividing status and thus could successfully infect minimally activated T cells [79, 80]. Moreover, lentiviral vectors display a greater gene insertion capacity, allowing the transfer of larger and highly complex gene constructs into T cells.

There are also several non-viral alternatives for TCR-gene transfer into T-cells. One main advantage of the latter is that, unlike viral platforms, they require a minimal production and testing time from a regulatory standpoint. The *Sleeping Beauty* and the *piggyBac* are example of transposon-based systems that have been used to alternatively redirect T cells to express antigen-specific receptors [81, 82]. This approach relies on the expression of the transposase in the target cell, together with the transfer of the transposon that encodes the genes of interest [83, 84]. Transfer of mRNA molecules encoding TCR chains by electroporation may also be used as a non-viral expression system to modify T cells; it eliminates the risk of insertional mutagenesis. Still, the main downside of this approach is the short-term expression of the transgene (a few days), which necessitates repetitive injection of electroporated cells to achieve in-vivo effects [85].

9.3.1.5 Off-Target and Safety Risks Involved in TCR Gene Transfer Strategy

Off-target events following TCR gene therapy may be due to self/cross-reactivity of the transduced TCR and/or the formation of mixed dimmers between the two α and two β chains that are co-expressed in the transduced cell, which may potentially lead to new auto-immune specificity [86]. Four different TCR combinations can form when mixing the chains that originated from the exogenous α/β TCR with the two chains that originate from natural/endogenous α/β TCR. The two mispaired heterodimeric TCRs may result either in a non-functioning TCR or a receptor with a new specificity that can prove self-reactive. In this regard, a recent study demonstrated how the formation of mixed TCRs can result in self-reactive T cells that engendered autoimmune manifestations in a mouse model [87].

Several strategies have been devised to increase the expression of the introduced TCRs, which are often based on molecular approaches aiming for better pairing/association of the α/β chains of the introduced-exogenous TCR [86, 88]. For example, hybrid human TCRs that are composed of parts of/entire murine constant regions [89–93] mediated an improved expression of the transferred TCR. The inclusion of an additional disulfide bond within the constant region of the TCR [94, 95], molecular "knob into holes" inversions in the constant regions of the TCR chains [96], single-chain TCRs [97], and the use of TCR/CD3ζ fusion products [98] were also recently demonstrated as potential pairing-optimization strategies. Since α/β and γ/δ TCR chains cannot mutually pair [99], the use of γδ T cells that are transduced with an αβ TCR is also an alternative approach [100]. Silencing the endogenous TCRs is another strategy, which can be achieved by co-transferring siRNAs/shRNAs targeting the endogenous TCR [101] or by making use of zinc-finger nucleases (ZFNs) that are specific for the endogenous TCR chains [102].

9.3.1.6 How to Further Improve the Anti-tumor Efficacy of TCR Gene Transfer?

In addition to the aforementioned strategies to improve adoptive T-cell therapy (such as lymphodepletion and cytokine polarization), several approaches are being developed in order to enhance functional and durable responses by TCR gene therapy. TCR affinity enhancement, which is believed to lead to an improved functional avidity, could be achieved by introducing selective modifications in the CDR3 region of the TCR α or β chain, which has been shown to be crucial for the recognition and binding of the antigen [70, 73]. The use of pairing (see above) and codon optimization (to improve protein expression) may also contribute to enhancing antigen-specific reactivity in T cells [68, 103]. Additionally, it has been demonstrated that reduced TCR glycosylation can elevate functional avidity and prevent the internalization of the transduced TCRs [104]. Recently, we demonstrated that it is possible to greatly enhance T-cell functional avidity against tumor cells by mutating three transmembrane residues in the TCRα chain into hydrophobic amino acid, which led to increased TCR stability and expression and augmented TCR expression in the transduced T cells [105]. In addition, the design of the gene expression cassette may also influence TCR expression: the use of P2A or IRES elements, which link the α and β chains, has been shown to improve TCR expression and to reduce the risk of induced autoimmune pathology [87, 106].

Beyond the engineering of T-cell specificity using TCR transgenes, several genetic approaches to further amplify/generate important T-cell functions (such as co-stimulation, cytokine secretion, expression of chemokine receptors and homing factors) have been described (reviewed in [107]). For example, though the administration of IL-12 in tumor mouse models can improve host survival and tumor regression rate [108, 109], the associated toxicities are a major drawback. Engineering gene-modified T cells to produce IL-12 in vivo using an inducible retroviral vector demonstrated intensified anti-tumor activity against B16 murine

melanoma tumors [110]. Alternatively, the use of T cells that are conjugated to adjuvant cytokine-loaded nanoparticles is another potential way to lead to a local production/delivery of cytokines, while reducing toxicity [111]. The (sub-) type of T-cell to be transduced is also of importance; recent studies have demonstrated the superior properties of other kinds of lymphocytes, such as memory T cells, naïve T cells, memory stem cells and central-memory T cells [41, 112–114].

In addition to TCR signaling, T-cell function is controlled by both positive and negative regulation. The tumor microenvironment has been shown to greatly induce immune suppression. For example, the immunosuppressive role of transforming growth factor-β (TGF-β) involves the inhibition of proliferation and function of T cells [115, 116]. By expressing a non-functional TGF-β receptor, tumor cells may also escape the apoptotic effects of TGF-β [117, 118]. In order to diminish the inhibition induced by TGF-β, it is possible to express in the genetically engineered T cells a truncated (dominant negative) form of TGF-β receptor [119], or to use a decoy-soluble TGF-β receptor II [120]. Bollard et al. recently reported that human T cells transduced with a dominant negative form of TGF-β receptor were resistant to the anti-proliferative and anti-cytotoxic effects of exogenous TGF-β [121, 122]. More recently, several groups [120, 123] have shown that this strategy is also effective in vivo, though the sustained effects of this might not last as expected [123].

9.3.2 Chimeric Antigen Receptor Gene Transfer

In parallel to the TCR gene transfer approach, it is possible to redirect the specificity of T-cells using chimeric antigen receptors (CARs). These CARs, also known as "T-bodies" or "chimeric immune receptors" are fusion proteins that generally contain an extracellular targeting domains based on an antibody single-chain variable fragment (scFv) that is fused to intracellular signaling elements. As mentioned above for TCRs, transduction of peripheral blood T cells with CARs allows the redirection of T-cell specificity against tumor cell surface antigen.

9.3.2.1 CAR Development

The development of antibody-based chimer receptor, was first reported in 1989 in the pioneering studies by Gross and Eshhar [124]. They generated a chimeric T-cell receptor assembled from the TCR constant domains fused to the variable domains of an antibody specific for anti-2,4,6-trinitrophenyl (TNP). T cells that expressed this chimeric receptor successfully recognized TNP, which led to the production of IL-2 and cell-mediated cytotoxicity of TNP-expressing targets. Thus, the use of CARs enables the targeting of tumor in an HLA-independent manner, which

suggests the possibility, in theory, of treating a larger part of the population, compared to TCR-based therapies. Moreover, CARs allow the targeting of not only protein-based antigens but also carbohydrates and glycolipids, provided targeting moieties/monoclonal antibodies can be generated against these. Another advantage of the CAR approach, as these function in an MHC-independent way, is their ability to stimulate both $CD8^+$ and $CD4^+$ T cells, which have been shown to act synergistically in enhancing the T-cell anti-tumor effect [125]. Still, it is important to remember that technically CARs can target only surface expressed antigens (though intracellular antigens could be also detected by CARs based on antibodies that target a specific pMHC (peptide–MHC) complex, and thus can mimic the mode of action of the TCR [126, 127]).

9.3.2.2 CAR Structure

As mentioned earlier, the common design of CARs is based on a binding domain, an extracellular spacer/hinge element, a trans-membrane region, and an intracellular singling domain (Fig. 9.2). Most of the CAR targeting domains are scFv (i.e., the variable regions of heavy and light chains joined together by a short linker peptide). If the scFv is derived from a murine antibody, it is possible to "humanize" it by replacing the mouse framework regions by their human counterparts. Another possible design for the targeting moiety of CARs (instead of scFv) are protein receptor/ligands; such alternatives include, for instance, a vascular endothelial growth factor polypeptide [128], an integrin binding peptide heregulin [129], interleukin—13 mutein [130], NKp30 (NCR3/CD337) [131], and the NKG2D receptor [132].

The second component in this design is the hinge region that serves as spacer, which increases the distance of the binding domain from the transmembrane region, providing more flexibility for the binding domain. The nature of the hinge region can influence cytokine secretion and cell-mediated killing of target cells by CAR-modified T cells [133]. Some common examples for hinge region are immunoglobulin domains such as the fragment crystallizable (Fc) regions of antibodies, or immunoglobulin-like domains derived from CD8α and CD28 molecules. It has been found that the function of the hinge region in the CAR is dependent on the binding site on the antigen itself; if the binding site is a membrane-proximal epitope, the use of a hinge region will be beneficial. In contrast, when the binding site is a membrane-distal epitope, improved cytokine release and cytotoxicity will be higher in the absence of a hinge region [134].

The third component in the CARs is the transmembrane region: in most cases, it is based on transmembrane domains derived from co-receptor/costimulatory molecules such as CD8 and CD28.

The fourth module in the structure of the CARs is the intracellular signaling domain. Importantly, a lot of effort is being invested in order to develop optimal

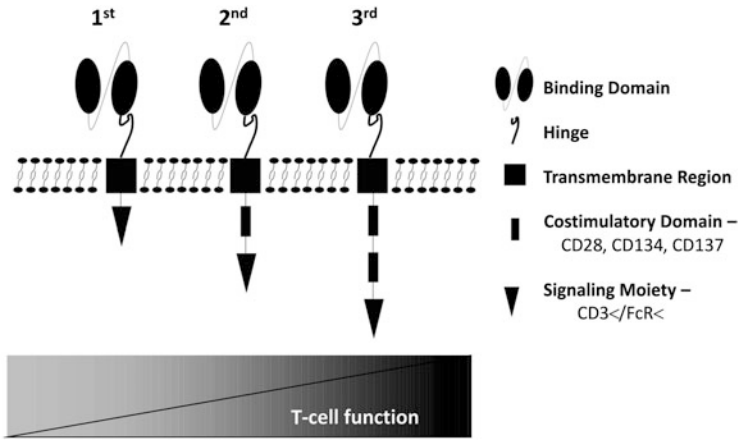

Fig. 9.2 Schematic representation of the different CAR generations

conformation of the intracellular signaling portions to achieve the best activation. The first generation of CARs included only one signaling domain (Fig. 9.2) derived either from the CD3ζ or FcRγ chains, which are the common signal-transducing subunits of the TCR or the immunoglobulin receptor respectively [135]. One main difference between these two subunits is the number of the immunoreceptor tyrosine-based activation motifs (ITAMs); while the CD3ζ chain contains three ITAMs, the FcRγ chain contains only one and this feature has been shown to impact on T-cell function and survival [136].

9.3.2.3 CAR Development and Generations

When first compared, the ζ and γ subunits were fused to single-chain variable domain chimeric receptors recognizing the carcinoembryonic antigen (CEA). Although similar levels of expression were detected after transduction, some significant functional difference was found after co-culture with target cells [137]. These assays demonstrated the superiority of the chimeric receptors that contained the CD3ζ, mainly in improved cytokine production and enhanced ability to mediate lysis of target cells. Additionally, it was revealed that CD3ζ-based chimeric receptors displayed a better ability to eradicate human tumors in vivo. While it has been postulated that the anti-tumor activity mediated by the CD3ζ moiety might result in activation-induced T-cell death (AICD) because of the numerous ITAMs (3), these claims have been refuted [138], and so far most of the CAR designs include a CD3ζ moiety as their main signaling domain.

Despite the encouraging results that were obtained in the studies with the first-generation CARs (that contained only the CD3ζ chain in the intracellular singling domain) and which demonstrated anti-tumor activity against a range of target cells

[139], the lack of co-stimulatory signals ("signal 2") led to inefficient cytokine production, reduced proliferation, and even a state of T-cell anergy [140, 141]. A second generation of CAR was designed to include a co-stimulatory portion in addition to the CD3ζ signaling domain. The most common co-stimulatory molecule that fills this role is CD28, the first isolated co-stimulatory molecule, which is essential to prevent anergy and to drive increased cytokine secretion [142]. Still, the possibility of generating two chimeras that express the ζ chain and the CD28 separately was explored, and this approach did mediate increased secretion of IL-2 in vitro [143]. More recently, a similar concept to reduce CAR side-effects made use of a first-generation CAR transduced in conjunction with a CCR (chimeric co-stimulatory receptor) specific for a second antigen, which enabled safer in-vivo targeting of tumors which expressed both cognate antigens [144]. So far, a more widespread concept is to combine both signaling moieties in the same receptor [145]. From a structural standpoint, a better surface expression of the CAR can be achieved by positioning the CD28 domain in proximity to the CD3ζ domain and immediately after the transmembrane region [146]. Several studies have demonstrated the improved function of second-generation CAR-modified T cells in mediating increased proliferation [147] and cytokine secretion (IL-2, interferon-γ, granulocyte–macrophage colony-stimulating factor) [148, 149]. Furthermore, this kind of design promoted the up-regulation of anti-apoptotic proteins such as Bcl-2 (which would contribute to reduce AICD) and better resistance to immunosuppressive conditions prevalent in the tumor microenvironment; studies have shown that second-generation CAR-modified T cells are less sensitive to TGF-β-mediated suppression [150], and could increase the expression of NFκB counteracting Tregs-induced inhibition [151].

There does not seem to be an optimal signaling moiety for CARs, and thus there is often a need to evaluate empirically several combinations for each given targeting moiety. Although most of the CARs use the CD28 signaling domain, alternative co-stimulatory molecules that were tested include the inducible T-cell costimulator (ICOS) B7 family member, and CD27, CD137 (4-1BB), and CD134 (OX-40) from the TNFR family members, which can enhance effector functions also in resting human T cells [152–154]. However, to further improve second-generation CARs, several studies have shown that it was possible to include another co-stimulatory moiety in addition to CD3ζ chain and CD28 in the signaling domain, leading to the design of third-generation CARs [155]. For example, a CAR for prostate-specific membrane antigen (PSMA), which contains $CD28^+$ $4\text{-}1BB^+$ CD3ζ signaling domain, showed an increased cytokine production and mediated an improved prostate tumor regression in vivo [154]. Furthermore, third-generation CARs can induce PI3Kinase/Akt activation and Bcl_{XL} expression and can help to reduce T-cell apoptosis. Another study showed that a CAR that contained the antigen-binding domain of the anti-GD2a fused to a CD28/OX40/ζ signaling domain endowed T-cells with improved proliferative capacity and anti-tumor function [156]. Still, the presence of the three activation/stimulation motifs in a single signaling domain may theoretically cause a lower sensitivity threshold, which should be taken into account when designing future clinical applications.

9.3.2.4 Driving the CARs into the Clinic

Results from in-vitro and in-vivo (in animal models) studies that show the potential of CARs in mediating tumor regression in several types of cancer—such as medulloblastoma, prostate [157] and colon carcinoma [158]—,facilitated their translation into the clinic. In the first clinical trial that made use of first-generation CAR-modified T cells, Lamers et al. treated three patients with metastatic renal cell carcinoma (RCC) using a CAR that recognizes carboxy-anhydrase-IX (CAIX), which is over-expressed by RCC tumors. All three patients were reported to suffer from liver toxicity, which was apparently caused by on-target effects of CAR-modified T cells against the $CAIX^+$ bile duct epithelial cells and no clinical responses were observed [159]. In another trial, 14 patients with metastatic ovarian cancer were treated with CAR-modified T cells against the ovarian cancer-associated antigen α-folate receptor (FR) [160]. Analysis of the CAR-modified T-cell presence in the circulation showed it quickly declined in the majority of the patients after 1 month, and also in this case no clinical response was observed in any of the patients treated.

Pule et al. engineered Epstein–Barr virus (EBV)-specific CTLs to express a first-generation CAR directed to the diasialoganglioside GD2 antigen, which is expressed on neuroblastoma cells. Infusion of these CAR-modified T cells seemed safe, and resulted in encouraging tumor regressions in half of the subjects tested [161]. Whereas these three clinical trials used retroviral transduction, in a clinical trial reported by Till et al., CAR-modified T cells were generated by electroporation with a vector plasmid encoding a CAR specific to CD20, to target indolent B-cell lymphoma (or mantle cell lymphoma). Out of seven patients treated, two achieved complete responses, one had a partial response, and four had stable disease [162]. Another notable clinical study was carried out recently by Kalos et al., in which three patients with advanced chronic lymphocytic leukemia (CLL) were treated with an anti-CD19 second-generation CAR that contained a CD3ζ chain coupled with CD137 domain. CAR-modified T-cells expanded over 1,000-fold in vivo, trafficked to the bone marrow and remained detectable 6 months post-infusion; a fraction of these cells even differentiated into memory T cells. Ten months after treatment, all the patients demonstrated an objective clinical response, with two of the three patients treated showing complete remission and one partial response [163]. A recent clinical trial using a third-generation CAR was conducted by Till et al. using a CAR targeting CD20 (which is expressed on indolent B-cell and mantle cell lymphomas) [164]. This third-generation CAR contained two co-stimulatory domains, CD28 and CD137, in addition to CD3ζ. CAR-modified T cells were detected for up to 1 year in patients' blood. Moreover, one out of four patients treated had an objective partial response (later relapsed a year after infusion), one patient developed transient infusional symptoms, and two patients remained progression-free for 12 and 24 months. Thus, some 20 years after they were initially developed, chimeric antigen receptors have entered the clinic and are showing promising results.

Nevertheless, one has to bear in mind that side-effects may arise, and unfortunately these may on rare occasions be lethal. In a trial that made use of a trastuzumab (Herceptin)-based third-generation CAR to target breast tumors, infusion of CAR-modified T cells led to the death of one patient. This was attributed to a "cytokine storm," possibly linked to the widespread expression of the targeted antigen, Her2/neu (ERBB2), by normal lung cells [165]. Another fatality was noted after using a second-generation CAR targeting CD19, in combination with cyclophosphamide lymphodepleting chemotherapy [166]. This treatment led to hypotension, dyspnea, and renal failure in the treated patient, and 4 days after the initial infusion the patient died. This suggests the need to include suicide genes in the CAR-bearing viral construct, or to use a dual-CAR/CCR design [144] to potentially provide another layer of safety. In addition, knocking down the expression of the endogenous TCR might prove valuable in order to prevent undesired/non-specific responses of CAR-activated T-cells [167].

9.4 Conclusions

In the past 25 years, adoptive T-cell transfer has established itself as a promising immunotherapeutic strategy for the treatment of advanced cancer. The basic idea, that the (autologous) immune system can be manipulated in order to promote tumor regression and remission, is appealing as it may provide long-lasting protection. Still, from the "bench-side" of things, additional targets/antigens have to be defined/characterized to provide safer treatments targeting a broad spectrum of tumors. From a clinical standpoint, there is a need to speed up processing times [168] and to ease regulatory requirements [169]. Improving the success rate of adoptive T-cell transfer will also require its combination with multi-modal therapies targeting, for instance, the tumor micro-environment as well as immunosuppressive agents. Much has to be done also to encourage partnership with the industry in order to commercialize this kind of immunotherapy that requires cell manipulation and conditioning [170]. Several studies also suggest that these concepts can be applied to treat other conditions than cancer [88]. Adoptive T-cell immunotherapy is certainly earning a respected place in the "Hall of Fame" of personalized medicine treatments.

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Chapter 10
Monoclonal Antibodies to CTLA-4 with Focus on Ipilimumab

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Abstract Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 or CD152) is a negative regulator of T-cell mediated immune responses, which plays a critical role in suppressing autoimmunity and maintaining immune homeostasis. Because of its inhibitory activity on T cells, CTLA-4 has been investigated as a drug target to induce immunostimulation, blocking the interaction with its ligands. The antitumour effects mediated by CTLA-4 blockade have been attributed to a sustained active immune response against cancer cells, due to the release of a brake on T cell activation. Ipilimumab (Yervoy, Bristol–Myers Squibb) is a fully human monoclonal IgG1κ antibody against CTLA-4 approved by FDA and EMA in 2011 for the treatment of advanced (unresectable or metastatic) melanoma, based on the increase of overall survival demonstrated in a phase III clinical trial. Further development of ipilimumab includes its use in other refractory and advanced solid tumours, either as monotherapy or in combination with additional immunostimulating agents or molecularly targeted therapies.

Keywords Ipilimumab • Immunotherapy • Cancer

List of Abbreviations

APC	Antigen-presenting cells
CTLA-4	Cytotoxic T-lymphocyte antigen-4
FoxP3	Transcription factor forkhead box protein P3
HRQL	Health-related quality of life

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ICOS	Inducible costimulator
IDO	Indoleamine 2,3-dioxygenase
irAEs	Immune-related adverse effects
irRC	Immune-related criteria
LAT	Linker for activation of T cells
MHC	Major histocompatibility complex
mWHO	Modified World Health Organization
NIBIT	Italian Network of Tumour Biotherapy
NSCLC	Non-small-cell lung cancer
PD-1	Programmed death-1
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PP2A	Serine–threonine protein phosphatase 2A
PSA	Prostate-specific antigen
RECIST	Response Evaluation Criteria in Solid Tumours
REMS	Risk Evaluation and Mitigation Strategy
SCLC	Small-cell lung cancer
SHP2	src homology 2 domain-containing tyrosine phosphatase 2
TCR	T-cell receptor
Tregs	Regulatory T cells

Contents

10.1 CTLA-4

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 or CD152) is a negative regulator of T cell-mediated immune responses, which plays a critical role in suppressing autoimmunity and maintaining immune homeostasis. The inhibition of the effector T-cell function is induced by CTLA-4 using both effector T-cell "intrinsic" (i.e., transducing a cell-intrinsic negative signal directly in effector T cells) and "extrinsic" mechanisms.

CTLA-4 acts as a negative regulator of CD28-dependent T-cell responses (Fig. 10.1). After the binding of T-cell receptor (TCR) with an antigen bound to the major histocompatibility complex (MHC) on the surface of antigen-presenting cells (APC), T-cell activation is completed by a second co-stimulatory signal, represented by the interaction between CD28 on T cells and the B7 molecules on APC (Fig. 10.1). The main effects of CD28 signalling are to augment and sustain T-cell responses, favour survival of T cells and direct the production of cytokines required for clonal expansion and differentiation of T cells. CTLA-4 is closely related to CD28 and shares with it the same ligands, B7-1 (CD80) and B7-2 (CD86), with CTLA-4 exhibiting higher affinities than CD28, in particular for CD80 [1, 2]. Like CD28 and the other costimulatory receptor inducible costimulator (ICOS), CTLA-4 is a transmembrane protein bearing a single extracellular immunoglobulin variable domain linked to a stalk region, containing a unique cysteine residue responsible for the formation of disulfide-linked homodimers, and a transmembrane segment followed by a short cytoplasmic tail endowed with tyrosine-based signalling motifs [3]. Despite their structural and sequence similarities, CD28 and CTLA-4 differ in their localization in T cells, the former being expressed at the cell surface both in resting and activated cells. CTLA-4 is, instead, up-regulated on the surface of activated T cells in response to TCR/CD28 costimulation [3]. In resting T cells, CTLA-4 has a primarily intracellular distribution that is dependent on motifs contained within its C terminal cytoplasmic tail. Upon T-cell stimulation, CTLA-4 is mobilized to the cell surface but not stabilized at the plasma membrane; in fact, it continues to undergo clathrin-mediated endocytosis, recycling, and degradation [4]. Once expressed on plasma membrane of activated T cells, CTLA-4 outcompetes with CD28 for the binding to B7 complex inhibiting T-cell activation, as a result of decreased proliferation and impairment of CD28-mediated interleukin 2 (IL-2) secretion [3]. CTLA-4 inhibits signal-transduction pathways downstream of TCR through the interaction of its cytoplasmic tail with serine–threonine protein phosphatase 2A (PP2A) and src homology 2 domain-containing tyrosine phosphatase 2 (SHP2) [5]. Moreover, it stimulates T-cell survival through the binding of phosphoinositol-3 kinase (PI3K), inducing T-cell anergy in the absence of T-cell death [5]. The CTLA-4 induced PI3K activation generates phosphatidylinositol 3,4-biphosphate (PIP2) which recruits PH domain kinase 1 (PDK1), a kinase capable of activating serine–threonine protein kinase B (PKB/AKT). The latter enzyme, in turn, phosphorylates and inactivates the pro-apoptotic protein BAD, which is degraded

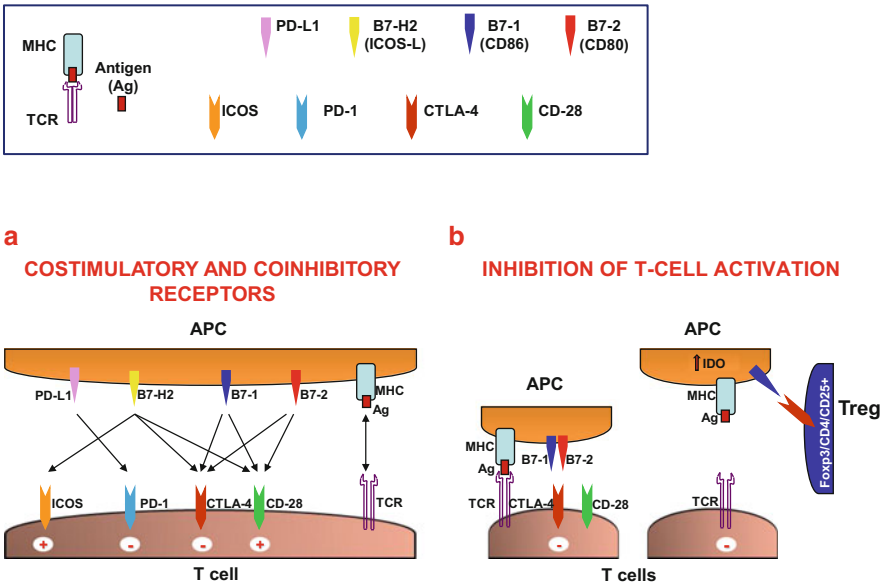

Fig. 10.1 CTLA-4 is a negative modulator of T cell activation. **a** Costimulatory and coinhibitory molecules. T-cell activation is triggered when TCR binds to an antigen bound to the MHC on the surface of APC, and it is completed by a second co-stimulatory signal, represented by the interaction between CD28 on T cells and its ligands B7-1 (CD80) or B7-2 (CD86) expressed on APC. PD-1 and CTLA-4 are negative regulators of T-cell-mediated immune responses. CTLA-4 shares with CD28 the same ligands, B7-1 and B7-2. ICOS is a costimulatory receptor and its ligand, B7-H2 (ICOS-L), has recently been proposed to bind also CD28 and CTLA-4. (The *plus sign* represents a positive/activating signal; the *minus sign* indicates a negative/inhibitory signal). **b** Inhibition of T-cell activation. Following T-cell activation, CTLA-4 is up-regulated in activated effector T cells, and functions as an inhibitory co-stimulatory molecule, outcompeting with CD28 for the binding to B7 complex. CTLA-4 is constitutively expressed on Tregs surface, and its interaction with B7 molecules triggers a reverse signalling in APC that leads to up-regulation in APC of IDO, reducing the supply of tryptophan in the local tissue microenvironment and producing kynurenines, with consequent inhibition of T-cell proliferation. Other mechanisms involved in CTLA-4 inhibitory effects on T-cell activation are described in the text

by 14-3-3 proteins, preventing its interaction with the anti-apoptotic Bcl-xL and Bcl-2 proteins, and causes up-regulation of Bcl-xL expression. In this way, Bcl-xL and Bcl-2 are free to mediate mitochondrial-dependent cell survival [6]. Through this pathway, CTLA-4 favours T-cell survival under condition of anergy induction, thus ensuring the maintenance of a long-term tolerance in the immune system.

Other intrinsic mechanisms by which CTLA-4 inhibits T-cell activation rely on the ability of CTLA-4 to increase T-cell motility, overriding the TCR-mediated "stop-signal" (i.e., the arrest of T-cell motility), which is required for a stable conjugate formation between T cells and APC [7]. In this way, CTLA-4 decreases the contact period between T cells and APC, reduces the efficiency of MHC-peptide presentation, and raises the threshold for T-cell activation conferring protection against autoimmunity. Moreover, CTLA-4 inhibits the expression of lipid rafts, a

clustering of glycosphingolipid-enriched microdomains that is considered as an essential component of the immunologic synapse [8]. Lipid rafts form, on the T-cell surface, a "platform" for signalling proteins crucial for proper TCR-mediated signalling. After TCR engagement, molecules such as Lck, Fyn, protein kinase C (PKC)θ, phospholipase C (PLC)γ, and linker for activation of T cells (LAT), are recruited to the raft aggregates at the T cell–APC contact area. During CTLA-4 interaction with the rafts, its associated phosphatases might dephosphorylate important signal components and then cause dissociation of the raft associated molecules such as Lck, Fyn, LAT, and TCR chain [8]. Finally, CTLA-4 also blocks the formation of microclusters containing TCR and molecules needed for an effective transmission of signals from TCR [9].

A well-characterized extrinsic mechanism by which CTLA-4 may act as negative regulator of T-cell responses is through the action of regulatory T cells (Tregs) (Fig. 10.1), where CTLA-4 is constitutively expressed [10]. Tregs are a subset of TCR $\alpha\beta^+$ $CD4^+$ T cells, which behave as immunosuppressive regulators both through the production of cytokines and by direct cell–cell contacts [11]. They are characterized by surface expression of IL-2 receptor alpha chain (CD25) and intracellular expression of the X-chromosome–linked transcription factor forkhead box protein P3 (FoxP3). In Tregs, CTLA-4 expression is controlled by Foxp3 and further up-regulated by TCR stimulation. These $Foxp3^+CD4^+CD25^+$ Tregs suppress naïve T-cell activation (referred to as "suppression"), have impaired TCR signal transduction ("TCR hyposignalling"), scarcely produce IL-2 and are anergic in vitro ("anergy"), although they are highly proliferative when provided with an exocrine source of IL-2 [12]. Recently, it has been found that Treg suppression and anergy require the external domain of CTLA-4, which binds to costimulatory ligands on APCs, whereas TCR hyposignalling only requires CTLA-4 internal domain [12]. Suppression of the activation of naïve T cells associated with Treg externalization of CTLA-4 can be mediated by its interaction with CD80/CD86, which triggers a reverse signalling in APC, causing up-regulation of the indoleamine 2,3-dioxygenase (IDO), an enzyme involved in the catabolism of tryptophan. The increase in IDO activity limits the available tryptophan in the local tissue microenvironment, required for T-cell proliferation, and enhances the formation of kynurenines which induce apoptosis in T cells [13–16]. The tryptophan starvation and the presence of kynurenines can also stimulate the conversion of naïve $CD4^+CD25^-$ T cells into highly suppressive $CD4^+CD25^+FoxP3^+$ Tregs, further expanding the Treg cell compartment [17].

CTLA-4 proteins have been shown to induce costimulatory blockade either by sequestering or removing costimulatory ligands from the APC surface. In fact, Tregs expressing CTLA-4 on the surface can induce the down-regulation of CD80 and CD86 on APC, limiting the activation of naïve T cells via CD28 [18]. CTLA-4 expressed in Tregs or in activated T cells is able to capture and remove co-stimulatory ligands (i.e., CD80 and CD86) from opposing cells by trans-endocytosis. Following removal, these costimulatory ligands are degraded inside CTLA-4-positive cells, depriving T cells of CD28-mediated co-stimulation [19].

10.2 CTLA-4 as Pharmacological Target for Immunosuppression or Immunostimulation

Because of its inhibitory activity on T-cell-mediated responses, CTLA-4 has been investigated as a drug target either to induce immunosuppression, using agents that mimic its function, or, conversely, to induce immunostimulation, blocking the interactions with its ligands (Fig. 10.2). With regard to immunosuppressive compounds that amply the CTLA-4 function, abatacept and belatacept are recombinant soluble homodimeric fusion proteins composed by the extracellular domain of CTLA-4 fused with the hinge region, and CH2 and CH3 Fc portions of human IgG1 [20, 21]. Via their CTLA-4 portion, these recombinant proteins act as competitors in the binding of CD28 to CD80/86 with CD28 on T cells, thus inhibiting full T-cell activation (Fig. 10.3). The Fc portion of both recombinant proteins has been deliberately mutated at three sites so that it lost the complement binding and Fc receptor-binding capabilities. For this reason, the Fc portion present in abatacept and belatacept cannot trigger complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity. Abatacept (Orencia, Bristol–Myers Squibb) was approved in 2006 by FDA and in 2007 by EMA for rheumatoid arthritis and polyarticular juvenile idiopathic arthritis [20]. Belatacept (Nulojix, Bristol–Myers Squibb), which differs from abatacept in two amino acid residues in the CTLA-4 part and binds with greater avidity to CD80/86 compared with abatacept, received approval in 2011 by FDA and EMA to prevent rejection of kidney transplantations [21] (Fig. 10.2).

In contrast, since it is known that tumours have developed numerous ways to suppress and evade the immune system, the blockade of CTLA-4 signalling was expected to prolong T-cell activation and to amplify T-cell-mediated immunity against cancer cells. Preclinical evidence that abrogation of CTLA-4 function would have resulted in increase of T-cell activation and proliferation came from CTLA-4 knock-out mice, which showed a massive CD28-dependent expansion of autoreactive T cells in lymph nodes, spleen, and other peripheral tissues, causing severe myocarditis and death by 3–4 weeks of age [22, 23]. In-vivo preclinical studies in the murine model indicated that administration of antibodies to CTLA-4 resulted in the rejection of tumours of different tissue origin, such as colon, prostatic, and renal carcinomas, fibrosarcoma, and lymphoma [24–28].

Two monoclonal antibodies (tremelimumab and ipilimumab) that block the inhibitory signal of CTLA-4 have been developed for clinical use (Fig. 10.2). The antitumour effects mediated by CTLA-4 blockade have been attributed to a sustained active immune response against cancer cells, due to the release of a brake on T-cell activation. The increase of the antitumour immune response appears to derive from a combination of direct enhancement of effector T cell function and concomitant inhibition of Treg activity through blockade of CTLA-4 on both cell types (Fig. 10.4) [29].

Tremelimumab (CP 675206; CP-675; CP-675,206; CP-675206; ticilimumab; Pfizer) is a fully human non-complement-fixing IgG2 monoclonal antibody.

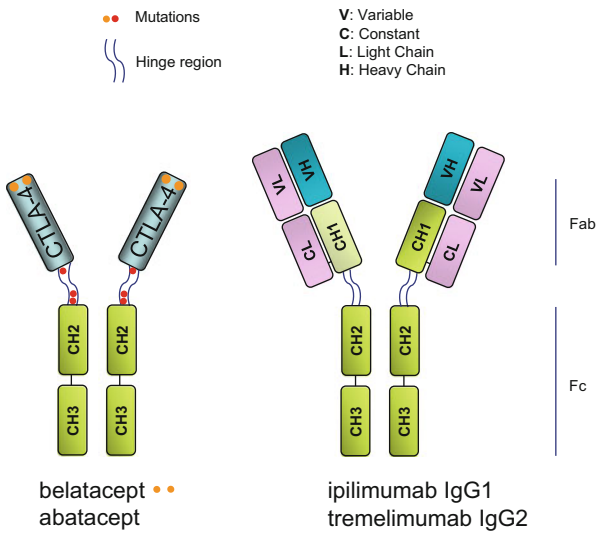

Fig. 10.2 CTLA-4 as a target for immunosuppressive or immunostimulating agents. Abatacept was generated by fusing the extracellular domain of human CTLA-4 to the Fc portion of human IgG1. The Fc portion is mutated at three sites (*red dots*), to eliminate effector functions of the Fc part. Belatacept was generated by inserting two mutations (*orange dots*) in the CTLA-4 portion of abatacept to increase the binding avidity to B7-1 and B7-2. Ipilimumab is a fully human monoclonal IgG1*k* antibody against the CTLA-4. Tremelimumab is a fully human monoclonal non-complement-fixing IgG2 antibody against CTLA-4

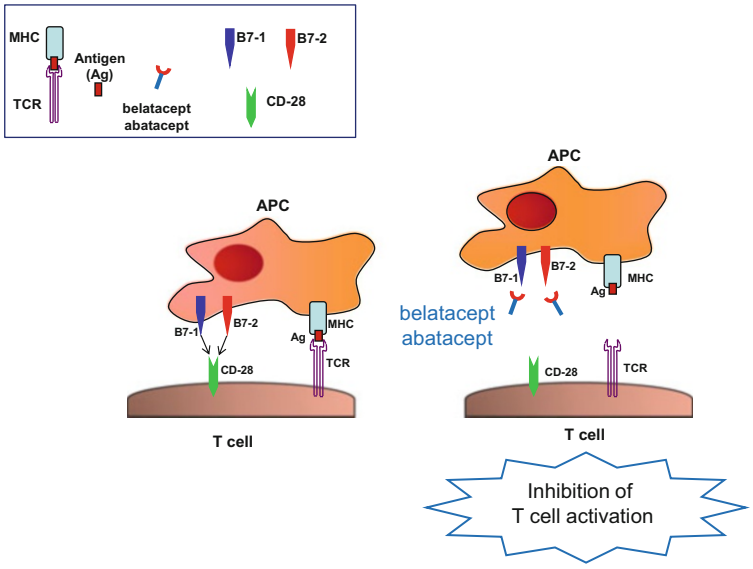

Fig. 10.3 Enhancement of CTLA-4 function. Belatacept or abatacept interfere with CD28/B7 pathway by binding to B7 molecules. Via their CTLA-4 portion, these recombinant proteins prevent the interaction of B7 with CD28 on T cells, thus inhibiting full T-cell activation

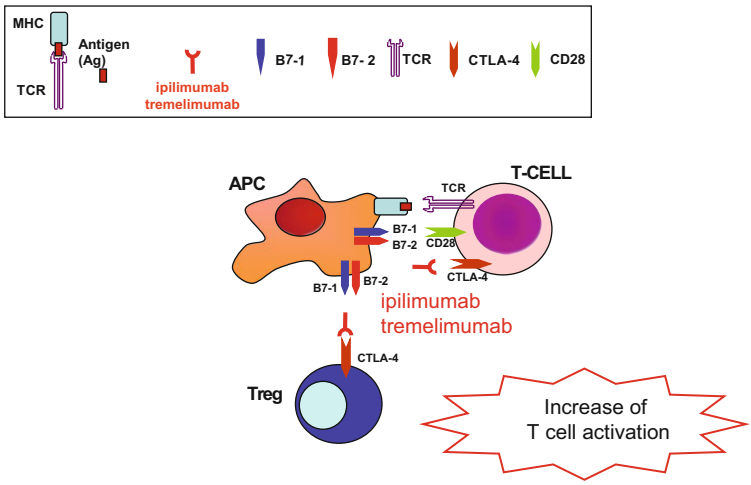

Fig. 10.4 Inhibition of CTLA-4 function. The monoclonal antibodies ipilimab and tremelimumab block CTLA-4 inhibitory signals prolonging T-cell activation and amplifying T-cell-mediated immunity against tumours

Currently, it is in phase I/II clinical trials in combination with short-term androgen deprivation for prostate-specific antigen (PSA)-recurrent prostate cancer without radiographic evidence of metastatic disease, or with the CD40 agonist monoclonal antibody CP-870,893 for metastatic melanoma, and, as single agent, for advanced hepatocellular carcinoma, refractory metastatic colorectal cancer and mesothelioma ([30], http://www.clinicaltrials.gov). In a previous phase III study, tremelimumab monotherapy, as first-line treatment in patients with advanced melanoma, failed to demonstrate an improvement in overall survival with respect to temozolomide or dacarbazine [31]. A recently concluded phase II study, in which 37 patients with metastatic melanoma received tremelimumab in combination with high doses of interferon α-2b, showed that this treatment has an acceptable toxicity profile and promising antitumour efficacy that warrant further testing in randomized trials [32].

The following sections will focus on the pharmacological properties of ipilimumab and on the main results of clinical trials with this agent.

10.3 Ipilimumab

Ipilimumab (BMS734016, MDX 101, MDX-010, MDX-CTLA-4, MDX-CTLA4, Yervoy, Bristol–Myers Squibb) is a fully human monoclonal IgG1κ antibody that specifically binds to human and cynomolgus CTLA-4. Ipilimumab was originated by the University of Berkeley (CA, USA) and licensed to Medarex, which was then acquired by Bristol–Myers Squibb. The antibody was initially produced by immunizing, with the extracellular domain of CTLA-4, Medarex's proprietary transgenic HuMAb mice (strain HC2/KCo7), which express the human genes

encoding heavy and light antibody chains and have the corresponding murine genes inactivated. Spleen cells from immunized animals were then fused with a murine myeloma cell line (P3X63Ag8.653) to produce hybridomas, which were screened for IgGκ production and CTLA-4 reactivity. The hybridoma 10D1 was selected for further development based on binding specificity, affinity, and ability to block ligand binding [33]. This product was used for phase I studies; for phase II studies and beyond, ipilimumab was produced from a recombinant Chinese hamster ovary (CHO) cell line, transfected with a vector containing the coding sequences for both heavy and light chains of ipilimumab and expressing the same sequence of the antibody produced by the 10D1 hybridoma (EMA/CHMP/557664/2011). The antibody is purified using standard chromatography and filtration steps.

Ipilimumab was approved by FDA in March 2011 for the treatment of unresectable or metastatic melanoma, and in July 2011 by EMA for advanced (unresectable or metastatic) melanoma in adults who have received prior therapy. The recommended dose of ipilimumab is 3 mg/kg administered intravenously every 3 weeks for a total of four doses.

The pharmacokinetic profile of intravenous ipilimumab was studied in three monotherapy trials on a total of 498 patients with advanced melanoma treated with four doses of 0.3, 3, or 10 mg/kg every 3 weeks. The values of peak concentration (C_{max}), trough concentration (C_{min}), and area under the curve (AUC) were found to be dose-proportional within the dose range examined. The steady state concentration was reached by the third dose. The C_{max} with the 3 mg/kg approved regimen ranges between 72 ± 33 μg/ml and 84.5 μg/ml, according to different studies [34–37]. Since the maximal blockade of the binding of CD80 and CD86 to human CTLA-4, induced in vitro by ipilimumab, is observed at 6–20 μg/ml and 1–3 μg/ml respectively, the target C_{min} concentration is 20 μg/ml. Prior to the second dose of 3 mg/kg the mean C_{min} is 12 ± 7 μg/ml, and the concentration at steady-state is 21.8 ± 1.2 μg/ml [36, 37]. The terminal half-life of ipilimumab is 14.7 days [35, 36]. The mean (percentage coefficient of variation) systemic clearance is 15.3 ml/h (38.5 %) and the volume of distribution at steady-state is 7.21 L (10.5 %) [36].

10.3.1 Clinical Efficacy Studies with Ipilimumab

10.3.1.1 Malignant Melanoma

Melanoma is the most aggressive form of skin cancer that, if detected at an early stage, before dermis invasion, can be cured by surgery in 99 % of patients. In contrast, the median overall survival of patients with metastatic melanoma is low (about 6–9 months), and the expected 2-year survival rate is 10–20 %. The first chemotherapeutic agent approved by FDA in 1975 for the treatment of metastatic melanoma was the DNA methylating compound dacarbazine, which is still considered the reference drug. The response rates with intravenous administration of dacarbazine are 15–25 %, with median response durations of 5–6 months, but

complete responses are less than 5 %. Dacarbazine is unable to cross the blood–brain barrier; thus, it is ineffective against brain metastases that at autopsy can be identified in up to two thirds of patients with metastatic melanoma [38]. The oral dacarbazine analogue temozolomide and the chloroethylating agent fotemustine have also been compared with dacarbazine, but none of these agents were found to be more efficacious [39, 40]. Temozolomide has been approved by FDA and EMA only for the treatment of newly diagnosed glioblastoma multiforme and recurrent anaplastic astrocytoma. However, it is frequently used off-label for the treatment of metastatic melanoma, especially in the presence of brain metastases, due to its higher brain penetration with respect to dacarbazine. The overall response rates with temozolomide, alone or in combination with whole brain irradiation, in patients with brain metastases from melanoma were up to 9 % [41]. Unfortunately, in a phase III study with 149 patients the global and 1-year incidence of CNS metastases in melanoma patients was not significantly reduced by temozolomide, in combination with cisplatin and IL-2, with respect to the same combination with dacarbazine [42]. A number of studies are currently evaluating temozolomide in combination with other chemotherapeutic agents or with modulators of DNA repair, such as inhibitors of poly(ADP-ribose) polymerase activity ([43], http://www.clinicaltrials.gov). In some European countries, fotemustine is used for the treatment of brain metastases in melanoma patients; the reported overall response rate was 5.9 % versus 0 % with dacarbazine [40]. However, the bone-marrow toxicity induced by fotemustine is more severe than that caused by temozolomide.

In 1998 high doses of IL-2 have been also approved by FDA in USA, but not by EMA in Europe, for the treatment of the metastatic disease, based on the results of phase II studies showing its ability to induce durable responses in 5–7 % of patients [44]. The IL-2 antitumour activity is dependent on its ability to modulate immune responses in the host. The high toxicity (including hypotension, vascular leak syndrome, cardiac dysrhythmias) restricts the use of this cytokine to carefully selected and younger patients with preserved performance status and absence of cardiovascular disease.

The 1-year survival of patients with unresectable melanoma treated with a variety of chemotherapeutic protocols is about 25 %, as indicated by the meta-analysis of a large number of phase II trials [45]. Before the recent approval of ipilimumab and of the BRAF inhibitor vemurafenib, no other agents have demonstrated better results than dacarbazine in phase III studies. Vemurafenib (Zelboraf, Hoffman–La Roche) was approved by FDA in August 2011 and by EMA in February 2012 for unresectable or metastatic melanoma with the BRAF V600E mutation as detected by an FDA-approved test. BRAF is a threonine/serine protein kinase that activates the mitogen activation protein (MAP) kinase–ERK pathways. Mutations of BRAF (resulting in about 90 % of cases in glutamic acid substitution for valine at amino acid 600, BRAF V600E) are present in 50 % of melanoma patients, and cause an over-activation of the downstream MAP kinase/ERK pathway, involved in cell proliferation and survival. Vemurafenib is a small-molecule kinase inhibitor that selectively targets activated BRAF V600E and lacks activity against melanoma cell lines with wild-type BRAF. Differently from ipilimumab, which is given intravenously for a total of four doses, treatment with

vemurafenib requires continuous daily doses *per os*. In a phase III trial enrolling untreated patients with metastatic melanoma carrying the BRAF V600E mutation, the overall survival at 6 months was 84 % in the vemurafenib arm and 64 % in the group treated with dacarbazine, and the response rates were 48 % and 5 % respectively [46]. In previously treated patients with BRAF V600E-mutant metastatic melanoma, vemurafenib induced clinical responses in more than half of patients, with a median overall survival of 16 months [47]. The most commonly reported adverse effects of vemurafenib include arthralgia, rash, photosensitivity, fatigue, pruritus, alopecia, diarrhoea, nausea, and cutaneous squamous-cell carcinoma [46, 47]. Evidence on the clinical efficacy deriving from targeting BRAF V600E also derives from the results of a phase III trial with the other BRAF inhibitor dabrafenib that led to drug approval in 2013 (GSK-2118436, GlaxoSmithKline) [48]. Unfortunately, responses to BRAF inhibitors are short-lived due to the development of different mechanisms of acquired tumour drug resistance that lead to the recovery of the MAPK signalling. Among these resistance mechanisms, switching between BRAF isoforms or secondary activating NRAS mutations are frequently described [49]. Interestingly, the cutaneous squamous-cell carcinomas and keratoacanthomas that develop in 15–30 % of patients treated with vemurafenib or dabrafenib frequently show RAS mutations [50].

The approval of ipilimumab by FDA was based on its ability to increase the overall survival with respect to vaccine with gp100 peptide in a phase III study (NCT00094653/CA184-002) that recruited 676 patients with unresectable stage III or IV melanoma, whose disease had progressed after at least one prior systemic treatment with chemotherapy [51]. This phase III study is the first randomized clinical trial showing increased overall survival in patients with metastatic melanoma (about 70 % of the patients had visceral metastases), and the first reporting efficacy as second-line treatment of melanoma. The patients were randomly in a 3:1:1 fashion to receive: ipilimumab (3 mg/kg) plus gp100 (1 mg each of two modified peptides) every 3 weeks for four doses, ipilimumab plus placebo, and gp100 plus placebo. All patients were HLA-A*0201-positive, since the cancer vaccine consists of a nine amino acid synthetic peptide derived from the melanosomal glycoprotein 100 (gp100) that is presented to the immune system in the context of HLA-A*0201. Before ipilimumab approval, no accepted standard of care for second-line therapy of metastatic melanoma was available, and enrolment in a clinical trial was recommended. The median overall survival with ipilimumab alone was 10.1 months, while with gp100 alone it was 6.4 months. The rationale of evaluating ipilimumab in combination with gp100 was based on the hypothesis that the addition of the cancer vaccine might have enhanced T-cell responses compared with ipilimumab alone. However, ipilimumab did not synergize with the vaccine, since the overall survival of the combined treatment was identical to that of ipilimumab alone [51]. On the other hand, gp100 was recently found to increase the efficacy of IL-2 in patients with locally advanced stage III or IV melanoma [52].

Ipilimumab, as single agent or in combination with gp100, almost doubled the 1- or 2-year survival rate for patients with stage III or IV melanoma. In fact, the rates of overall survival in the ipilimumab plus gp100 group, the ipilimumab-alone group

and the gp100-alone group, respectively, were 43.6 %, 45.6 %, and 25.3 % at 1 year, and 21.6 %, 23.5 %, and 13.7 % at 2 years [51]. A retrospective analysis of pooled efficacy data stratified by HLA-A*0201 status showed that ipilimumab-treated patients had similar outcomes regardless of their HLA-A*0201 status [53]. Despite the fact that the NCT00094653 study was done exclusively in patients who had failed prior therapy, FDA approved ipilimumab, at the dose of 3 mg/kg, for all patients affected by metastatic melanoma, both those who were treatment-naïve and those who had failed previous therapy. Approval almost coincided with the announcement by Bristol–Myers Squibb Company that a phase III study (NCT00324155/CA184-024) in 502 previously untreated patients, comparing the efficacy of 10 mg/kg ipilimumab plus dacarbazine versus monotherapy with dacarbazine, had met the primary endpoint of improving overall survival. The results, published 3 months later, indicated that ipilimumab every 3 weeks for four doses in combination with dacarbazine (850 mg/m^2) significantly improved overall survival compared to dacarbazine plus placebo (11.2 months versus 9.1 months) as the front-line metastatic setting [54]. After the induction phase, eligible patients received a maintenance therapy with ipilimumab every 12 weeks. The survival rates in the ipilimumab–dacarbazine arm were higher than in the dacarbazine arm, being 47.3 % and 36.3 % at 1 year, 28.5 % and 17.9 % at 2 years respectively. In the ipilimumab–dacarbazine group, prolonged survival was observed in patients monitored for 4 years [54]. A randomized double-blind phase III study (NCT01515189/CA184-169) is presently comparing 3 mg/kg with 10 mg/kg ipilimumab in patients with previously treated or untreated unresectable or metastatic melanoma. Moreover, a phase II study (NCT01119508/2009-0408) is evaluating the efficacy and safety of 10 mg/kg ipilimumab in combination with temozolomide (200 mg/m^2) on day 1–4, every 3 weeks for four courses, followed by a maintenance therapy with ipilimumab every 12 weeks and temozolomide on day 1–5 every 4 weeks until the occurrence of disease progression or unacceptable toxicity. The results on 64 patients indicated that the treatment was well-tolerated and efficacious in this clinical setting [55]. Moreover, a prospective phase I/II dose-escalation trial is investigating the safety of the combination of ipilimumab plus vemurafenib in patients with metastatic melanoma containing the BRAF V600E mutation (NCT01400451/CA184-161) [56].

Apart from the phase III registration trial used by FDA for ipilimumab approval (NCT00094653/CA184-002) in which 10–15 % of patients in each arm presented CNS involvement at baseline [51], in most of the clinical trials with ipilimumab, patients with brain metastases were excluded. The outcomes among these patients are quite poor; in fact, after diagnosis of brain metastases the median overall survival is only 4 months [57]. Previous case reports showed clinical benefits of ipilimumab for brain metastases from melanoma [58, 59]. Moreover, a recent phase II trial specifically designed to enrol patients with brain metastases (NCT00623766/CA184-042) indicated that 10 mg/kg ipilimumab has activity in this clinical setting, particularly when metastases are stable, asymptomatic, and do not need glucocorticosteroid treatment [60]. Moreover, the Italian Network of Tumour Biotherapy (NIBIT) has evaluated the efficacy of ipilimumab (10 mg/kg every 3 weeks for four

doses and once every 12 weeks from week 24) in combination with fotemustine (100 mg/m^2 weekly for 3 weeks and every 3 weeks from week 9) in a phase II study (NIBIT-M1) for patients with metastatic melanoma, with or without brain metastases [61–63]. Of the 86 patients enrolled in this study, 20 showed brain metastases, and combination of ipilimumab with fotemustine was found to be active regardless of prior treatment, warranting further investigation in a subsequent phase III NIBIT-M2 trial [62].

Conventional treatment options for melanoma brain metastases consist of surgical resection, whole-brain radiation and stereotactic radiotherapy. An effect observed when ipilimumab was combined with radiotherapy is the abscopal effect, a phenomenon related to activation of the immune system, in which local radiotherapy is associated with the regression of metastatic cancer at a distance from the irradiated site. The regression of non-irradiated lesions in melanoma patients treated with radiotherapy and ipilimumab suggests a potential synergism between these two therapeutic approaches [64, 65]. Indeed, several phase I/II clinical trials are evaluating the combination of ipilimumab with radiation therapy for the treatment of unresectable stage III or stage IV melanoma (http://www.clinicaltrials.gov). According to the experiences of the Italian Medical Oncology and Immunotherapy Unit at the University Hospital of Siena, in the context of an ipilimumab–ocular melanoma expanded access program, and of the Memorial Sloan Kettering cancer centre, ipilimumab monotherapy has shown promising activity also for uveal melanoma [66, 67]. Trials are currently recruiting patients for this clinical setting [NCT01585194/2011-0919, NCT01355120/DeCOG-MM-PAL11].

Ipilimumab is also being tested in phase III trials as adjuvant therapy after surgical removal of melanoma for patients with high-risk stage III or IV, versus high-dose interferon α-2b (NCT01274338/ECOG-E1609) or versus placebo (NCT00636168/CA184-029). A neoadjuvant use of ipilimumab monotherapy (NCT00972933/08-144) or in combination with high doses of interferon α-2b (NCT01608594/NCT01608594) is currently under evaluation in patients with stage IIIB/C melanoma before surgery. These studies also aim at comparing immunological parameters at baseline and after treatment. Data on 30 patients indicated that ipilimumab induced a significant increase in the frequency of circulating Tregs at 6 weeks, and that greater increases in Tregs were associated with improved progression-free survival [68].

Phase II combination studies are currently testing ipilimumab with other immunostimulating agents, such as nivolumab (BMS-936558), a fully human monoclonal antibody against programmed death-1 (PD-1), an inhibitory receptor expressed on activated T cells (NCT01927419/CA209-069), or various cancer vaccine. One of the vaccines combined with ipilimumab is TriMix-DC, formed by of autologous dendritic cells, transfected with mRNA encoding CD40 ligand, constitutively active toll-like receptor 4, and CD70. The dendritic cells have been further co-electroporated with mRNA encoding the melanoma-associated antigens MAGE-A3, MAGE-C2, tyrosinase, and gp100 [69], in order to induce a T-cell repertoire able to recognize in a HLA-restricted way these melanoma antigens (NCT01302496/2010-023058-35).

10.3.1.2 Hormone-Sensitive and -Resistant Prostate Cancer

The standard of care for hormone-sensitive metastatic prostate cancer is androgen deprivation therapy via medical [i.e., with the gonadotropin-releasing hormone (GnRH) agonist/analogues leuprolide or goserelin or with the GnRH antagonist degarelix] or surgical castration. However, most recurrent prostate cancers that initially responded to androgen deprivation therapy eventually become castration-resistant. Once the prostate cancer becomes refractory to hormonal therapy, the disease course is uniformly fatal, since the treatment options available so far only modestly extend survival. Docetaxel-based regimens are regarded as the standard first-line chemotherapy for metastatic castration-resistant prostate cancer. Recently, cabazitaxel, a semisynthetic taxane derivative, and abiraterone, a pregnenolone derivative that irreversibly inhibits CYP17A (a key enzyme in androgen synthesis), have been approved for patients previously treated with a docetaxel-containing regimen. In addition, immunotherapy with sipuleucel-T, an autologous antigen-presenting cell vaccine loaded with prostate acid phosphatase conjugated with granulocyte–macrophage colony-stimulating factor (GM-CSF), was approved for men with asymptomatic metastatic disease [70]. Ipilimumab has shown some activity in several phase I/II clinical trials in metastatic prostate cancer, as single agent [71] and in combination with GM-CSF [72] or radiotherapy [73]. A phase II study with ipilimumab given alone or in combination with docetaxel has been recently completed (NCT00050596/CA184-019). Two multicentre randomized phase III studies, both with overall survival as primary endpoint, are currently underway in chemotherapy-naïve or post-docetaxel patients with metastatic castration-resistant prostate cancer. One of these studies is comparing radiotherapy followed by ipilimumab (10 mg/kg) versus radiotherapy plus placebo in patients who have received prior treatment with docetaxel (NCT00861614/CA184-043) [74], based on data supporting a role for irradiation to enhance the immune responses, whereas the other is testing the same dose of ipilimumab versus placebo in asymptomatic or minimally symptomatic chemotherapy-naïve patients (NCT01057810/CA184-095) [75].

Phase II studies with ipilimumab in combination with either GnRH analogues (leuprolide, goserelin) or the GnRH antagonists degarelix plus androgen deprivation therapy in castrate-sensitive prostate carcinoma (NCT01377389/2009-0378), or as neoadjuvant therapy before surgery (NCT01194271/2009-0135), are ongoing.

Based on the previously reported synergy between the anti-CTLA-4 antibody in combination with GM-CSF secreting tumour-cell vaccines, a phase I trial with GMCF-transduced allogeneic prostate cancer cells vaccine (GVAX) plus 3 mg/kg ipilimumab has been undertaken in patients with metastatic castration-resistant prostate cancer (NCT01510288/G-0016) [76]. Moreover, another phase I study (NCT00113984/NCT00124670) has been carried out with escalating doses of ipilimumab plus PSA-Tricom vaccine, a poxviral-based vaccine targeting PSA and containing three T-cell co-stimulatory molecules (CD58, CD80, and ICAM1) [77].

10.3.1.3 Lung Cancer

About 85–90 % of all lung cancers are non-small-cell lung cancers (NSCLC); at an advanced stage, standard chemotherapy only marginally improves overall survival. Platinum-based combination therapies are the standard of first-line care for patients with advanced NSCLC, with a median overall survival of 8–12 months. In 203 chemotherapy-naïve recurrent or stage IIIb/IV patients with NSCLC, 10 mg/kg ipilimumab was administered concomitantly with (concurrent ipilimumab) or sequentially (phased ipilimumab) to carboplatin and paclitaxel, and compared to chemotherapy alone (NCT00527735/CA184-041). The results of this phase II trial indicated that phased ipilimumab plus paclitaxel and carboplatin improved progression-free survival (phased ipilimumab 5.1 months and concurrent ipilimumab 4.1 months versus chemotherapy alone 4.2 months). Median overall survival were 12.2, 9.7, and 8.3 months respectively [78]. A phase III trial has been recently planned to test the impact of paclitaxel/carboplatin followed by ipilimumab on overall survival in NCSLC with squamous histology (NCT01285609/CA184-104) [79]. Similar results to those obtained with NCSLC were reported also in patients with extensive-disease small-cell lung cancer (ED-SCLC) who were enrolled onto the same phase II study NCT00527735/CA184-041 [78]. For newly diagnosed ED-SCLC, a phase III trial (NCT01450761/CA184-156) is recruiting patients to compare the efficacy of ipilimumab plus etoposide/cisplatin or carboplatin, which represent the standard treatment for metastatic SCLC [80].

10.3.1.4 Other Cancers

Ipilimumab is in phase I/II clinical trials for a variety of solid tumours. In renal cell cancer, immunotherapy with IL-2 induces 15–25 % objective response rate. In a phase II trial (NCT00057889/NCI-03-C-0094) with 61 patients affected by metastatic renal cell cancer, refractory to or ineligible for treatment with IL-2 treatment, single-agent ipilimumab (1 mg/kg and 3 mg/kg) induced an overall response rate of 12.5 % in the group receiving the higher dose of ipilimumab, and responses were seen in patients previously not responding to IL-2 [81]. Another phase II study (NCT01524991/GU10-148) has been designed to assess the efficacy of ipilimumab in combination with gemcitabine and cisplatin against metastatic urothelial carcinoma, which is regarded as an immunogenic tumour and is generally treated with first-line platinum-based combinations [82]. A small phase I study has also evaluated the tolerability of ipilimumab as neoadjuvant treatment for urothelial carcinoma before surgery (NCT00362713/CA184-027) [83]. Phase I trials are testing the safety of ipilimumab in combination with gemcitabine (NCT01473940/NU 10I02) or with a pancreatic cancer vaccine, consisting of allogeneic pancreatic tumour cells transfected with a GM-CSF gene (NCT00836407/J0834), for locally advanced, unresectable, or metastatic pancreatic adenocarcinoma.

10.4 Immune-Related Response Criteria

The clinical experience with ipilimumab has indicated that the Response Evaluation Criteria in Solid Tumours (RECIST) or modified World Health Organization (mWHO) criteria, typically used by oncologists to define tumour response and disease progression, are not suitable for assessing the clinical responses to imuunotherapy. In fact, patients treated with ipilimumab may have a delayed yet durable response and obtain long-term survival benefit despite an initial tumour growth. On the contrary, the cytotoxic activity of chemotherapeutic agents generally causes tumour shrinkage within a few weeks from the beginning of drug administration. A decrease in tumour size after the initial cycle of chemotherapy is predictive of improved survival, whereas an early increase of the primary tumour or the appearance of new lesions is indicative of progressive disease and drug failure. On the other hand, ipilimumab, due to its particular mechanism of action that relies on activation of T-cell mediated immune responses against the tumour, may induce four distinct response patterns, all of them associated with a favourable survival: (a) a shrinkage in baseline lesions, (b) a stable disease followed by a slow decline in tumour burden, (c) a response after an increase of tumour burden, or (d) a response in the presence of new lesions [84]. The progression during treatment might indicate an actual tumour growth occurring before an adequate immune response is raised against cancer cells. Alternatively, the progression may reflect an active immune response with infiltration of cytotoxic T lymphocytes and inflammatory cells within the tumour, which will cause an increase in the size of the lesion [85]. Therefore, RECIST or mWHO criteria might underestimate the clinical benefit of ipilimumab, since an increase in tumour size or the appearance of new lesions would be considered progressive disease, leading to an unwanted early cessation of treatment in potential responders.

This unusual pattern of treatment responses has led to the development of new immune-related criteria (irRC) that may help in the decision-making regarding continuation of therapy [84]. Patients with new lesions, but with a decrease in size of baseline lesions, will not necessarily be considered to have progressive disease. They will, instead, be considered responders and continue to receive ipilimumab, with possible long-term benefits. Nevertheless, the value of irRC has to be tested in prospective clinical trials.

10.5 Adverse Effects

The adverse effects of ipilimumab are related to increased immune-reactivity against normal tissues (immune-related adverse effects or irAEs). The most common irAEs include rash and pruritus, colitis and diarrhoea, vitiligo, endocrinopathies involving pituitary, thyroid, or adrenal gland, hepatitis, and uveitis. Indeed, the prescribing information of ipilimumab includes a boxed warning about the risk of severe and fatal irAEs due to T-cell activation and proliferation [36]. Moreover, the

FDA required a Risk Evaluation and Mitigation Strategy (REMS) from the manufacturer to ensure that the benefits of ipilimumab outweigh its risks. The REMS program consists in a communication plan for healthcare providers and patients to facilitate early identification of the risks deriving from treatment with ipilimumab, and to provide an overview of recommended management of patients with moderate or severe irAEs (http://www.yervoy.com/hcp/rems.aspx).

A retrospective review of safety data from 14 completed phase I–III trials of ipilimumab in 1,498 patients with advanced melanoma indicated that irAEs occurred in 64.2 % of patients, and confirmed that the gastro-intestinal tract and the skin were the most common sites of adverse effects [86]. In the registration phase III trial (NCT00094653/CA184-002), the most common irAE was diarrhoea at any grade in 27–31 % of the patients receiving ipilimumab [51]. Interestingly, health-related quality of life (HRQL) outcomes demonstrated that ipilimumab with/without gp100 vaccine did not have a significant negative HRQL impact during the treatment induction phase relative to gp100 alone in melanoma patient [87]. Analysis of the safety profile of patients alive after 2 years of the phase III trial NCT00324155/CA184-024, in which ipilimumab plus dacarbazine was compared to dacarbazine plus placebo, indicated a low rate of irAE in the ipilimumab-containing arm, and indicated that irAE were medically manageable according to established guidelines [88]. Indeed, algorithms are available for the correct management of irAEs, which depends on the severity of adverse effects [89]. Frequencies of dose-limiting ipilimumab-related irAEs increased with dose. Grade 3 and 4 irAE have been reported in 25 % of patients treated with 10 mg/kg, and in 7 % of those treated with 3 mg/kg [34]. The majority of irAEs resolve with systemic administration of glucocorticosteroids; for grade $\geq$2 irAEs or in patients experiencing symptomatic endocrinopathy, ipilimumab should be held. Once side-effects improve to grade 0–1, steroids should be gradually tapered within at least 1 month. The influence of high-dose systemic glucocorticosteroids on ipilimumab antitumour efficacy has not been established in large-scale trials. Retrospective studies or case reports did not show so far unfavourable effects of steroid treatments on the antitumour efficacy of ipilimumab [90–92]. Several trials have reported a possible correlation between grade 3 and 4 irAEs with the clinical efficacy of ipilimumab [93, 94], suggesting that tumour regression is associated with the development of autoimmunity. However, clinical benefit has been observed also in patients who did not develop irAEs [94].

10.5.1 Skin Toxicity

Maculopapular rash and pruritus have been observed in 47–68 % of patients receiving ipilimumab, generally appearing 3–4 weeks after the beginning of treatment. Histological analysis of affected skin revealed perivascular lymphocytic infiltrate in the dermis and epidermis and immunohistochemical staining showed the presence of $CD4^+$ and melan-A specific $CD8^+$ T lymphocytes in the proximity of apoptotic melanocytes [95]. Skin eruptions and pruritus usually do not require

skipping a dose or discontinuation of ipilimumab, and resolve with topical glucocorticosteroids or urea-containing creams and antipruritic agents.

10.5.2 Colitis and Diarrhoea

Diarrhoea has been observed in 31–46 % of patients, after about 7 weeks, and can be associated with colitis, which can lead to obstruction and bowel perforation (<1 %). In ipilimumab-related colitis, the descending colon is more often affected than ascending colon, sigmoid colon, or rectum. Colon biopsies show neutrophilic infiltrate in 46 % of patients, lymphocytic infiltrate in 15 %, and neutrophilic-lymphocytic infiltrate in 38 % [96]. Treatment of mild diarrhoea is symptomatic, with loperamide, oral hydration, and electrolyte substitution. For persistent or grade ≥2 diarrhoea, infection must be excluded by stool cultures, and sigmoidoscopy or colonoscopy is indicated to confirm or rule out colitis [97]. Ipilimumab must be suspended, and budesonide, a locally acting glucocorticosteroid with low bioavailability after oral administration, or 1 mg/kg prednisone are used. Unfortunately, the prophylactic use of budesonide did not reduce the rate of grade ≥2 gastro-intestinal irAEs [98]. In patients with severe diarrhoea or colitis (grade ≥3), ipilimumab should be permanently discontinued. These patients require high-dose intravenous steroids (e.g., methylprednisolone or dexamethasone) or, in case of no improvement after a week, infliximab. Refractory or severe cases of colitis may require ileostomy or colectomy.

10.5.3 Hepatitis

Hepatotoxicity (3–9 %; after 6–7 weeks) is usually revealed by an asymptomatic increase in transaminases and bilirubin or by an immune-mediated hepatitis. Disease progression with metastases in the liver, as well as viral hepatitis must be ruled out. The histologic changes observed with ipilimumab-related hepatitis are similar to those with acute viral and autoimmune hepatitis [99]. For grade 3 and 4 liver toxicity, ipilimumab should be discontinued, and high doses of intravenous glucocorticosteroids given, followed by an oral steroid taper with dexamethasone. If serum transaminase levels do not decrease within 48 h after the beginning of systemic steroids, oral mycophenolate may be required [97].

10.5.4 Endocrinopathies

Among the endocrine dysfunctions provoked by ipilimumab (4–6 %, after about 9–11 weeks), hypophysitis is the most frequently reported. The presenting clinical symptoms relate to a pituitary mass effect and hormonal deficiencies. The enlargement of pituitary gland causes symptoms which mimic intracranial hypertension caused by brain metastases, which need to be excluded. Most patients present with

headache, fatigue, asthenia, lethargy, nausea, vertigo, behaviour change, loss of libido, or visual disturbances. Typically, low levels of thyroid, adrenal, and gonadal hormones may be found, and clinical symptoms depend on the prevalent suppression of endocrine axes (thyroid, adrenal glands, or gonads). The majority of male patients (83–87 %) have hypogonadotrophic hypogonadism [100]. Treatment of endocrine irAEs includes high-dose steroid therapy and appropriate hormone replacement, which should be undertaken in consultation with an endocrinologist [89, 97]. Unlike most of the other irAEs, hypophysitis takes a long time to resolve and in many cases persists, requiring lifelong therapy.

10.5.5 Other irAE

Immune-related pancreatitis has been reported in less than 1.5 % of treated patients, and generally manifested as an asymptomatic increase of amylase and lipase [93]. Diffuse lymphadenopathy and a sarcoid-like syndrome have been reported anecdotally [101–103]. Transient peripheral neuropathies, both sensory and motor, associated with ipilimumab have been noted in less than 1 % of patients [97]. A case of acquired hemophilia A was diagnosed in a patient with metastatic melanoma 2 months after the introduction of ipilimumab, and was related to ipilimumab therapy [104].

10.6 Conclusions

About one third of melanoma patients achieve clinical benefit from ipilimumab treatment, and some of the responses are long-lasting, with follow-up >5 years for the earliest studies. The most impressive property of ipilimumab is represented by the ability of a short-course treatment (four doses) to increase the overall survival in a subset of heavily pre-treated patients with metastatic melanoma [51].

The immune-related toxicity of ipilimumab needs a prompt diagnosis and treatment according to product-specific guidelines to adequately manage irAE, which sometimes can be also life-threatening. The use of a specified treatment algorithm has substantially reduced the drug-related deaths to <1 % of patients, and requires an accurate training of physicians who will use this agent.

The clinical experience with ipilimumab indicates that patients receiving ipilimumab should not have treatment terminated prematurely (unless severe toxicity occurs) because of early progressive disease. In fact, lack of objective response evaluated by standard criteria might not always reflect treatment failure, due to the peculiar kinetics of response deriving from the immune-mediated mechanism of action of ipilimumab. This highlights the importance of identifying biomarkers capable of recognizing those patients who will behave as late responders, in order to spare non-responder patients unnecessary toxicity.

Despite the dramatic effects in a subgroup of patients, the majority of patients with metastatic melanoma do not obtain long-lasting clinical benefit from ipilimumab.

Thus, combination therapies with other novel immunomodulating agents, targeted therapies, or anti-angiogenic agents need to be evaluated principally to enhance the percentage of long-term survivors, and to improve ipilimumab efficacy.

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