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Magdalena Klink
Izabela Szulc-Kielbik *Editors*

Interaction of Immune and Cancer Cells

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Preface

The microenvironment of solid tumor is a complex structure consisting of a heterogeneous population of tumor cells and various non-tumor cells including immune cells, fibroblast, mesenchymal cells, endothelial cells, stem cells, and others. Moreover, the tumor microenvironment (TME) also contains extracellular matrix proteins, soluble factors (i.e., chemokines and cytokines), extracellular vesicles, and vascular and lymphatic networks. Every element of this complex structure strongly affects the malignant cells' growth, survival, and ability to metastasize. The dynamic processes occurring in TME between tumor and non-tumor cells, as well as stromal factors, are known as cancer immunoediting which consists of three phases: elimination, equilibrium, and escape. However, a full understanding of the mechanisms underlying tumor progression requires the thorough study of abovementioned factors and cells within TME.

The first purpose of this book is to present current knowledge and the most important aspects of interactions between several types of cells present in the TME. Infiltrating immune cells such as various subsets of lymphocytes, dendritic cells, macrophages, neutrophils, natural killer cells, and myeloid-derived suppressor cells are the most important players involved in a cross talk with the tumor cells. Depending on the phenotype and repertoire of secreting signals, these cells can either suppress or promote tumor growth and metastasis. Moreover, the tumor-infiltrating lymphocytes, macrophages, and neutrophils are potential prognostic or predictive biomarkers in cancer and are also components of a promising therapeutic strategy. Another important element, taking part in tumor development and progression, is stromal tissue which consists of fibroblasts, myofibroblasts, endothelial cells, and extracellular matrix proteins. The stromal cells, mainly fibroblasts, secrete various factors (e.g., transforming growth factor β) that affect tumor cells and result in a more aggressive cancer phenotype. Currently, the main focus is on cancer stem cells also known as tumor-propagating cells which are capable of self-renewal, which is an important mechanism of tumor proliferation, differentiation, metastasis, and chemoresistance. In general, the immune and non-immune cells as well as other components of TME (cytokines/chemokines, growth factors, and extracellular

vehicles) are the best known to participate in (i) development of immunosuppression of adaptive immunity, (ii) induction of epithelial–mesenchymal transition of cancer cells allowing them to leave primary tumor and colonize secondary sites, (iii) stromal remodeling, (iv) angiogenesis, and (v) cancer escape from immunosurveillance.

The second aim of this book is to focus on the currently ongoing preclinical and clinical studies concerning immunotherapy. The most advanced research concerns targeted therapy to immune checkpoints, the cytotoxic T lymphocyte-associated antigen 4 and anti-programmed cell death protein 1. What is more, the full human monoclonal antibodies against these both proteins (ipilimumab and nivolumab) are approved for the treatment of metastatic melanoma and are further extensively studied in other malignant diseases. Another promising approach for immunotherapy is the one based on the adoptive transfer of autologous tumor-specific T lymphocytes or genetically engineered T cells that express an exogenous cancer-specific T-cell receptor or chimeric antigen receptor. The third strategy is based on dendritic cells. The most studied are methods concerning the targeting of lectin/scavenger receptors or using tumor antigen-loaded dendritic cells as a vaccine.

Presented collective work features the comprehensive summary of the interaction between various types of cells present in the solid tumor microenvironment as well as the most advanced strategies of immunotherapy. This second edition of previously published book is strongly updated and expanded with two new chapters describing stem cells and natural killer cells. We hope that the presented work is describing, in sufficient detail, why tumor cells can survive and spread in the host organism, despite of anti-tumor activity of immune cells, and how the activity of immune cells can be used to develop anticancer therapeutic strategies.

Finally, we would like to take an opportunity to express our gratitude for 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
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Chapter 1
Cancer Immunoediting: Elimination, Equilibrium, and Immune Escape in Solid Tumors

Jacek R. Wilczyński and Marek Nowak

Contents

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 how the three phases of cancer immunoediting functionally evolve and how tumor shaped by the host immune system gets finally resistant phenotype. The elimination, equilibrium, and escape have been described in this chapter in details, including the role of immune surveillance, cancer dormancy, disruption of the antigen-presenting machinery, tumor-infiltrating immune cells, resistance to apoptosis, as well as the function of tumor stroma, microvesicles, exosomes, and inflammation.

Keywords Cancer immunoediting · Immunosurveillance · Cancer dormancy · Cancer escape mechanisms

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Abbreviations

AKT	protein kinase B
APCs	antigen-presenting cells
BMP	bone morphogenetic protein
CAFs	cancer-associated fibroblasts
CCR	C-C chemokine receptor
COX	cyclooxygenase
CSCs	cancer stem cells
CSF-1	colony-stimulating factor-1
CTCs	circulating tumor cells
CTLA-4	cytotoxic T lymphocyte-associated antigen-4
CTLs	cytotoxic T lymphocytes
CXCR	C-X-C motif chemokine receptor
DCs	dendritic cells
DTCs	disseminated tumor cells
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal transition
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
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
HSP	heat-shock protein
IAPs	inhibitor of apoptosis proteins
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
MCP-1	monocyte chemotactic protein-1
M-CSF	macrophage colony stimulating factor
mDCs	mature dendritic cells
MDCs	myeloid dendritic cells
MDSCs	myeloid-derived suppressor cells
MICs	metastasis-initiating cells

MMPs	metalloproteinases
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cells
NKG2D	activating receptor of NK cells
NKT	natural killer T cells
NO	nitric oxide
NOTCH	neurogenic locus notch homolog protein
NR2F1	nuclear receptor subfamily-2 group-F member-1
PD-1	programmed death-1
PDCs	plasmacytoid dendritic cells
PD-L1	programmed death-1 ligand (also called B7-H1)
PGE_2	prostaglandin E_2
PI3K	phosphatidylinositol 3-kinase/phosphatase
RANTES	Regulated on Activation, Normal T-cell Expressed and Secreted (CCL5)
RNS	reactive nitrogen species
ROI/ROS	reactive oxygen intermediates/species
STAT	signal transducer and activator of transcription
TAA	tumor-associated antigen
TAMs	tumor-associated macrophages
TANs	tumor-associated neutrophils
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
uPAR	urokinase plasminogen activator receptor
VEGF	vascular-endothelial growth factor

1.1 Introduction

The idea of cancer immunosurveillance has been built on the hypothesis that cancer cells are recognized as nonself 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 noncancer

cells. However, during subsequent investigations, antigens primarily thought as TSA have been found also on normal human cells. Actually, 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. alpha-fetoprotein (AFP) in germ cell tumors, carcinoembryonic antigen (CEA) in colorectal cancer), and viral antigens (e.g. human papilloma virus—HPV in cervical cancer) (reviewed in Liu et al. 2010). At present, more than 1000 human tumor antigens have been described (Cancer Immunome Database). Conceptually, TAAs may be divided into three groups: self-antigens or embryonic antigens overexpressed or respectively aberrantly expressed on cancer cells, self-antigens modified by posttranslational tumor-specific disturbances, and neoantigens originating from mutations, chromosomal aberrations, and viral transformation (Töpfer et al. 2011).

Thus, the intact immune system may recognize TAAs and prevent the development of cancer in a process initially termed immunological surveillance (Burnet 1970). 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 developed variety of strategies to evade the host immune system. They shed surface antigens and downregulate 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 (Poggi and Zocchi 2006, Whiteside 2006). 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 (Lin and Karin 2007; Liu et al. 2010).

Emphasizing the dynamic processes between cancer and host immune system, the concept of cancer immunosurveillance (Burnet 1970) has been replaced by the current concept of cancer immunoediting (Dunn et al. 2002) 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. Developing tumor creates proinflammatory and immunosuppressive microenvironment leading to the impairment of the host immune function and escape from immunosurveillance resulting in tumor growth and metastases.

1.2 Immunosurveillance of the Host Against Cancer—Elimination

The main effectors of cancer immunosurveillance are natural killer (NK) cells, natural killer T cells (NKT), γδ T cells, cytotoxic T lymphocytes (CTLs), interferon (IFN) γ, perforins, and system Fas/FasL. Their role in the cancer immunosurveillance was firstly confirmed and described in immunologically manipulated mice (reviewed in Kim et al. 2007, Wilczyński and Duechler 2010). 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, esophageal, gastric and colorectal cancer, and malignant melanoma. Moreover, the presence of both tumor-specific cellular (T cells) and humoral (antibodies) response was connected with better prognosis in cancer patients (Whiteside 2010).

Elimination process is initiated when growing tumor cells, and also macrophages and stromal cells present in cancer site release inflammatory cytokines what recruits and activates other innate effector cells like NK, NKT, or γδ T cells. They recognize and destroy neoplastic cells by meaning of perforins, Fas/FasL, TNF-related apoptosis-inducing ligand (TRAIL), and IFN-γ (Smyth et al. 2000). Secreted IFN-γ exerts cytotoxic effects and induces apoptosis of the cancer cells. Necrotic tumor cells release tumor antigens which evolve 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 tumor site and eliminate the rest of cancer cells expressing tumor antigens (Kim et al. 2007). 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 like uric acid, heat shock proteins, and extracellular matrix derivatives which may induce proinflammatory response activating innate immune system (Powell and Horton 2005, Shi et al. 2003). 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 like interleukin (IL) 10 and transforming growth factor (TGF) β as a feedback loop (Kim et al. 2005). Moreover, genetic instability of cancer cells under host immunologic pressure creates less immunogenic types of cells (Whiteside 2010). 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.

1.3 Cancer Dormancy and Cancer-Immune Equilibrium

Cancer dormancy defined as 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 (Uhr and Pantel 2011). The relapse of breast cancer 10–20 years after the primary treatment has been noticed in relatively steady population of 1.5% of patients. It was also shown that circulating tumor cells were present in 36% of breast cancer patients after mastectomy as long as 7–22 years after the surgery (Marches et al. 2006).

Clinical dormancy is probably connected to the existence of several partially overlapping functionally populations of cells called metastasis-initiating cells (MICs), circulating tumor cells (CTCs), disseminated cancer cells (DTCs), and cancer stem cells (CSCs). MICs are a population of either early-stage disseminating or late-stage disseminating cancer cells, usually considered to be in a quiescent or dormant status. MICs are present among CTCs and DTCs residing inside metastatic niche. Quiescence and dormancy are similar states, but when dormancy is a more stable and passive state, quiescence is rather an active and transient program of cell behavior regulated by both occurrence of new or lack of typical signals from the cell environment. CSCs are considered to be a considerable part of a population of quiescent cells in many tumors. Quiescent cells are slow-cycling CSCs possessing increased repopulating ability and capable to resist against a spectrum of unfavorable conditions. Their presence is usually linked to hypoxic, acidic, and necrotic areas of tumor. Quiescent CSCs show expression of genes responsible for activation of both hypoxic (hypoxia-inducible factor-1α—HIF-1α, glucose transporter-1—GLUT1) and dormant (nuclear receptor subfamily-2 group-F member-1—NR2F1, p27) regulatory pathways (reviewed in: De Angelis et al. 2019). Activation of mammalian target for rapamycin (mTOR) pathway is necessary for survival of quiescent CSCs and dormant DTCs (Hen and Barkan 2019).

Increasing evidence seems to support the notion that DTCs quiescent/stem cells are able to disseminate from the early primary tumors as CTCs. This possibility was, between others, raised by the studies suggesting that mammary ductal carcinoma in situ (DCIS) produced disseminated cells. CTCs go through epithelial-to-mesenchymal transition (EMT), enter the bloodstream, and are able to survive in circulation, being the marker of adverse clinical outcome. In the blood, CTCs circulate in the form of cell clusters or circulating tumor microemboli (CTMs) composed of the mixture of cancer cells, platelets, epithelial cells, fibroblasts, and immune cells. The latter contain more cells and therefore have usually a greater metastatic potential than clusters (Liao et al. 2014). Both CTCs and DTCs are cancer cells that either have acquired stemness traits, or alternatively they are true CSCs. Significant similarity in biology of CTCs, DTCs, and CSCs supports this notion. Therefore, metastases may originate from CSC-like cells or true CSCs. Not all CTCs or DTCs are capable of forming micro- and macrometastases, as their metastatic potential is dependent on interactions with premetastatic and metastatic niche. It was 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 (Marches et al. 2006). There are also "early DTCs" produced by premalignant lesions which are not able to initiate a metastatic growth in target organs due to their insufficient genetic alterations and suppressive signals from the environment. They similarly to normal DTCs enter the state that prevents apoptosis but maintains dormancy (Bragado et al. 2012).

An important observation made in the XIX century by Paget contributes to the contemporary understanding of clinical 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 hostile or hospitable environment in the target organ ("seed and soil" theory) (Ossowski and Aguirre-Ghiso 2010). CTCs disseminated to distant organs cross the vascular barrier more frequently in organs which have more permissive vessels, like bone marrow or lungs. It was 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. Squamous carcinoma cells were 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. Moreover, murine models indicated that cancer cells disseminated to the bone marrow failed to expand unless they were transplanted into irradiated recipients. 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 (Taylor et al. 2008).

In target organs, cancer cells reside in premetastatic niches which are actively created by both cancer cells and local cells recruited from stroma (cancer-associated fibroblasts—CAFs, myeloid-derived suppressor cells—MDSCs) and immune system (tumor-associated macrophages—TAMs, T regulatory cells—Tregs). Organ-specific niches protect dormant DTCs from environmental stressors and drug-dependent toxicity (De Angelis et al. 2019, Klein 2009, Sosa et al. 2014). The premetastatic niche is regulated by several signals from activated cells including growth factors, cytokines, chemokines, and exosomes. This is a hypoxic milieu that promotes survival of cancer cells. Inside niche dormant DTCs are unable to acquire proangiogenic activity and are described by high expression of angiogenesis inhibitors, like angiostatin, endostatin, and trombospondin-1 (reviewed in: Jahanban-Esfahlan et al. 2019). Acidic conditions enhance extracellular matrix (ECM) degradation and inhibit anticancer immune response. TAMs are extensively recruited to premetastatic niche through tumor-derived colony-stimulating factor-1 (CSF-1), vascular-endothelial growth factor (VEGF), CCL2, and CXCL12, and they inhibit host defense against cancer cells via programmed cell death PD-1/PD-L1 checkpoint molecules. Immature DCs and neutrophils being components of premetastatic niche play also important role in deviating of antitumor response (reviewed in: Ingangi et al. 2019). The role of CAFs in promotion of DTCs is practically the same as for CSCs niche [described in the chapter devoted to CSCs]. In

premetastatic niche, DTCs/CSCs with acquired quiescence or dormancy wait until the moment when signals from local environment change the niche into mature metastatic niche. These niche alterations occurring frequently as a result of inflammation could "wake up" DTCs from dormancy, activate angiogenic pathways ("angiogenic switch"), and initiate metastatic growth (reviewed in: Jahanban-Esfahlan et al. 2019; Ingangi et al. 2019). Also others components of metastatic niche are responsible for regulatory switch from quiescence/ dormancy to metastatic growth. Acquisition of stemness, self-renewal, and proliferation properties by DTCs is dependent on EMT. Inhibitors of dormancy mediated by signals from ECM are collagen type I, fibronectin, activation of focal adhesion kinase (FAK)/nonreceptor tyrosine kinase Src/MEK pathway, and aurora kinase-A (AURKA). DTCs are not only responders to signals emitted from metastatic niche components, but also are capable to interfere with them actively. Breast cancer DTCs were shown to stimulate niche stromal cells to release ECM components like periostin and tenascin C, which in turn activated stemness pathways in DTCs mediated by Wnt/β-catenin, NANOG, and octamer-binding transcription factor-4 (Oct4) leading to their metastatic outgrowth (Malanchi et al. 2012, Oskarsson et al. 2014).

There are two forms of dormancy: tumor and cellular dormancy. Tumor dormancy is based on the balance between tumor proliferation and apoptosis dependent mostly on vascular deficit ("angiogenic dormancy") (reviewed in: Hen and Barkan 2019). Angiogenic dormancy is one of the reasons for dormancy of a small micrometastatic tumor (reviewed in: Ossowski and Aguirre-Ghiso 2010). Tumor can slowly proliferate, but is avascular both because of the lack of angiogenic factors expression and the upregulation of angiogenesis inhibitors. Due to this and ongoing apoptosis, the tumor has stable dimensions (Naumov et al. 2006). Escape from angiogenic dormancy triggers the growth of macrometastases which show significantly higher proliferation potential and vascularity. Another cause for dormancy of small micrometastatic tumors is a balance between proliferation and apoptosis dependent on effective immune surveillance of the host against tumor cells. It is called "immune dormancy" (Shiozawa et al. 2013, reviewed in: Hen and Barkan 2019). The problem of tumor immune dormancy is closely connected to the status of cancer-immune equilibrium. 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 NKG2D and TRAIL pathways had no effect (reviewed in: Teng et al. 2008). 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. The equilibrium between T $CD8^+$ cells and small skin tumors was also observed in another murine studies. These findings strictly indicate that adaptive T effectors, IFN-γ, and IL-12 play an important role in controlling tumor growth (reviewed in: Teng et al. 2008). Tumors in cancer-immune equilibrium were slowly proliferating tumors with increased ratio of dying cells and the presence of host immune effectors (reviewed in: Teng et al. 2008). Clinical observations support tumor dormancy hypothesis. It was shown that the late lung cancer remissions

occurred mostly in immunodefective persons, as well as small nondetected tumors transplanted unintentionally with the organs of immunocompetent donors became clinically evident in immunosuppressed recipients (Stewart et al. 1991). The small clinically "silent" tumors were 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 (Feldman et al. 1986).

The second form of dormancy is called cellular dormancy. Most of dormant solitary cells were isolated from bone marrow of various cancer patients and showed G_0/G_1 arrest with overexpression of p21 and p27. There are plenty of known inducers of cell dormant status, including hypoxia, starvation, components of ECM, cellular stress, activation of signaling pathways, or epigenetic regulation. Cancer cells subjected to metabolic stressors like hypoxia or starvation are prone to dormancy. Disturbances of lipid metabolism, reactive oxygen species, and oxidative DNA damage are inducers of metabolic dormancy of DTCs, while inhibitors comprise mitochondrial dysfunction and activity of mitochondrial serine-beta-lactamase-like protein (LACTB) (reviewed in: Jahanban-Esfahlan et al. 2019).

Unappropriate interactions with ECM of metastatic niche may trigger mechanisms leading to DTC dormancy (reviewed in: Páez et al. 2012, Barkan et al. 2010). Inducers of ECM-dependent dormancy include expression of kisspeptin gene *KISS-1*, urokinase receptor u-PAR, cytokine TGF-β2, E-selectin, SDF-1/CXCR4, WnT5a, insulin growth factor-1 (IGF1)/protein kinase B (AKT) pathway, and GTP-binding RAS-like-3 family molecules (DIRAS3). Expression of *KISS-1* inhibits motility and proliferation of melanoma cells. Receptor u-PAR is one of the key molecules for long-standing survival of cancer cells in bone marrow. Downregulation of urokinase plasminogen activator receptor (uPAR), β1-integrins, FAK, and 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 imbalance between p38 and extracellular signal-regulated kinase (ERK) in the cancer cells, which activates endoplasmic reticulum (ER) stress-like reaction (reviewed in: Ranganathan et al. 2006). Overbalance of the $p38^{high}/ERK^{low}$ status promotes dormancy, while overbalance of $p38^{low}/ERK^{high}$ triggers mitogenesis. Moreover, p38-dependent activation of p53 and inhibition of c-Jun protein, as well as activation of p38/ER chaperone BiP/protein R-like ER kinase (PERK) pathway induces dormancy and quiescence/chemoresistance of cancer cells, respectively (Ranganathan et al. 2006). The interactions between fibronectin and α5β1 integrin were also uPAR-dependent and modulated the ECM functions (reviewed in: Laufs et al. 2006). Impaired signaling through integrins and adhesion signal transducers has been noticed in DTCs of squamous and breast cancers. Disturbed interactions with ECM may also trigger autophagy. The presence of both autophagy and dormancy was confirmed in ovarian cancer cells upon stress conditions (Lu et al. 2008).

Notch and Wnt/β-catenin signaling so important for maintenance of CSCs also control the balance between dormancy and proliferation in DTCs. Cytokine TGF-β2 induces dormancy by protection against cellular adhesion of cancer cells and is

highly expressed in the bone marrow. TGF-β 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 (Bragado et al. 2012). In the lung, another member of TGF-β family, bone morphogenic protein (BMP) 4, augments dormancy of breast cancer cells. E-selectin and SDF-1/CXCR4 pathway help breast cancer cells to home into premetastatic niche in bone marrow. Similarly, Wnt5a/receptor tyrosine kinase Ror2 (ROR2)/E3 ubiquitin-protein ligase (SIAH2) signaling is engaged in induction of prostate cancer dormancy inside bone marrow. DIRAS3/ERK/AKT signaling induces dormancy via activation of autophagy (Allgayer and Aguirre-Ghiso 2008, Mao et al. 2019). Another recognized inducers of DTCs dormancy are: N-cadherin, Notch, aminopeptidase N (CD13), BMP7, osteonectin (SPARC), sex-determining region-Y box-2 transcription factor (Sox2), TANK-binding kinase-1 (TBK1), p53, and paired related homeobox-1 (PRRX1). Transcription factor HES-1, which induced dormancy but prevented from cell senescence and terminal differentiation, was identified in melanoma cells (Jia et al. 2019, Jiang et al. 2019).

Epigenetic upregulation of NR2F1 nuclear receptor increases expression of NANOG and chromatin repression, which promotes dormancy in breast and prostate cancer. Cells entering dormancy have also epigenetically increased expression of mitogen and stress-activated kinase-1 (MSK1) and transcription factor PCL1 (Sosa et al. 2015, Gawrzak et al. 2018). Another example of epigenetic regulation of DTCs is dormancy-miRNA (called DmiRs). Their transfer inside of exosomes from metastatic niche cells into DTCs promotes quiescence and dormancy, and chemoresistance, and prevents apoptosis. The most known DmiRs are miR-222/223, miR-34a, miR-190, miR-100-5p, miR-200, and miR-125b (Almog et al. 2013, Tiram et al. 2016, Watson et al. 2018).

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: Bragado et al. 2012). 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 CAFs to switch dormant cells into proliferative malignant phenotype (reviewed in: Castaño et al. 2011).

1.4 Cancer Escape Mechanisms

1.4.1 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 posttranslational tumor-specific disturbances, and neoantigens

originating from mutations, chromosomal aberrations, and viral transformation (reviewed in: Töpfer et al. 2011). As most of solid tumors express self- or modified self-antigens, T effectors are unable to recognize them properly due to the 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 receptor (TLR) receptors. 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) (Tesniere et al. 2010, Scaffidi et al. 2002). Low tumor-induced expression of TLR9 receptor on plasmacytoid DCs was observed in head and neck squamous cancer. In colon cancer patients, loss of functional TLR4 resulted in short progression-free survival (Tesniere et al. 2010). 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. Observations in cancer patients revealed the presence of soluble forms of human leukocyte antigen (HLA)—sHLA. The data concerning the concentration of sHLA in cancer are not consistent and depend on the tumor type and HLA allotypes. Increased, not changed or decreased, sHLA levels were described in pancreatic, melanoma, and gastric cancers, respectively. sHLA may downregulate activity of CTL and NK cells (reviewed in: Campoli and Ferrone 2008). The mechanism of tumor recognition by T effectors is also disturbed by abnormalities in antigen presentation machinery, including loss or downregulation of HLA class I antigens due to gene mutations, loss of heterozygosity, and disturbed transcriptional regulation (reviewed in: Töpfer et al. 2011). The presence of such mechanisms was confirmed in esophageal, prostate, and lung cancer. Tumors are capable to loose 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 (Dunn et al. 2004). In melanoma and colon cancer, the mutation of β2-microglobulin was observed. Tumors are also characterized by an acquired deficits in antigen peptide transporter (TAP) and low-molecular mass polypeptide (LMP)2 and LMP7 immunoproteasome subunits (Seliger et al. 2000). In melanoma and renal cancer, decreased expression of HLA class I antigen was caused by methylation of TAP-1 and -2 (Seliger 2008). Interferon is capable of upregulation of HLA molecules, but defects in IFN-γ signaling such as mutations of Janus kinases (JAK-1 and -2) may also decrease their expression. In head and neck squamous cancers, downregulation 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: Duray et al. 2010).

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 nonclassical HLA class I antigen

HLA-G on their surface (reviewed in: Campoli and Ferrone 2008). Epigenetic changes like demethylation or histone acetylation may be responsible for ectopic HLA-G expression on cancer cells. Unfortunately, it seems that host immunosurveillance against tumor accounts for initiating HLA-G, as IFN-producing immune effectors upregulate HLA-G expression. Moreover, tumor-infiltrating immune cells also acquire the HLA-G-positive phenotype, producing strongly immunosuppressive environment inside 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 Urosevic and Dummer 2008). 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 to inhibit NK cytotoxicity, but also to modulate DCs' activity, followed by inhibition of proliferative T-cell responses (reviewed in Urosevic and Dummer 2008, Sheu and Shih 2007, Pistoia et al. 2007). Through inhibitory ILT-2 receptor, HLA-G disturbs T-cell activation and decreases CD3ζ phosphorylation and IL-2 secretion. In addition to expression of membrane-bound HLA-G, tumors are capable to secrete 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 autoenhancing regulatory loop. HLA-G could be also present in exosomes disseminated into the circulation from the tumor (Urosevic and Dummer 2008). Inside established tumors, there are several factors that trigger and support HLA-G expression, including hypoxia (via HIF-1α), chronic inflammation (via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)), and immunosuppressive IL-10 (reviewed in: Duechler and Wilczyński 2010). Activators of NF-κB transcription factor stimulate also the sHLA-G shedding from cancer cells (Mouillot et al. 2007, Urosevic and Dummer 2003). The presence of HLA-G molecules was confirmed in many cancers, especially these associated with inflammation (Urosevic and Dummer 2008). Concentration of sHLA-G correlates with tumor size. Besides HLA-G, some other nonclassic HLA molecules like HLA-E and HLA-F have been described in tumors, including lung cancers, and their expression indicates bad prognosis. HLA-E exerts additional suppressive signals to lymphocytes through CD94/NKG2A KIR, and HLA-G has stabilizing effect on this molecule (Mouillot et al. 2007, Urosevic and Dummer 2003).

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: Campoli and Ferrone 2008). Tumors are capable to disturb the recognition of surface ligands by

NKG2D receptors through several mechanisms (Raulet 2003). Firstly, constant overexpression of NKG2D ligands results in downregulation of NKG2D expression. Moreover, by TGF-β production, cancer can directly downregulate NKG2D expression (Coudert et al. 2005). Soluble MIC molecules released from cancer cells could further disturb CTLs and NK-cell cytotoxicity by downregulation of activating NKG2D receptor, natural cytotoxicity receptor NKp44, and chemokine receptors CCR7 and CXCR1. 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. Expression of NKG2D ligands was observed in human colorectal tumors, however varied between different tumor types, and became progressively less frequent in more advanced tumors. High expression correlated with improved survival and NK-cell infiltration (McGilvray et al. 2009).

Costimulatory molecules which transfer positive or negative signals necessary to initiate T-cell responses belong either to classic B7 family (CD80, CD86) or to the family of B7 homologs containing B7-H2, B7-H3, B7-H4, and some others members. Absence of classic costimulatory molecules CD80 and CD86 on the surface of tumor cells produces anergy in T $CD4^+$ lymphocytes recognizing HLA class II antigens (Byrne and Halliday 2003). Recently, B7-H4 homolog, transferring a negative signal for T-cell activation, deserved greater attention, due to its abundance both on the tumor and immune cells in cancer patients (reviewed in: He et al. 2011). 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. To date, expression of B7-H4 has been confirmed in variety of solid tumors including colon, prostate, lung, gastric, ovarian, pancreatic, uterine cancer, and melanoma (reviewed in: He et al. 2011). Tregs were reported to induce molecules B7-H4 on the surface of DCs and TAMs, where it functioned as an inhibitor of T-cell activation and cytotoxicity (reviewed in: Palucka et al. 2011). Moreover, B7-H4 mediated inhibitory effects on the growth of neutrophils. Besides regulatory effects on the function of immune system, B7-H4 influenced the tumorigenesis by enhancing the proliferation, migration, and invasiveness, and protecting cancer cells from apoptosis, as was shown in ovarian cancer murine model (Cheng et al. 2009). 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: He et al. 2011). Similarly in breast cancer, the overexpression of B7-H4 was connected with negative receptor status and HER-2/neu positivity. In bladder cancer, B7-H4 promoted EMT and NF-κB signaling pathway. Another group of costimulatory proteins that are functioning as immune response downregulators (so called checkpoint proteins) are cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1). Both show an immunosuppressive activity and inhibit an excessive immune responses, thus possessing tumor-promoting properties. The CTLA-4 regulates the T-cell priming and activation by binding to CD80 or CD86 molecules. The PD-1 modulates the activity of antigen-primed T effector cells (Gaillard et al. 2016) and acts through binding to one of its ligands (PD-L1; B7-H1) or PD-2 ligand-2 (PD-L2;

B2-DC). PD-1 inhibits T-cell activity by inhibition of the T-cell receptor downstream signaling. It also enhances Treg proliferation and suppressive activity and inhibits both B- and NK-cell activity (Francisco et al. 2009). In the tumor environment, overexpression of PD-L1 could result from activity of oncogenic signaling pathways. Tumors are capable of using the PD-1/PD-L1 pathway to escape from host immunosurveillance. Expression of PD-L1 ligand was described in many cancers, including renal, stomach, bladder, breast, and lung cancers, and was associated with poor prognosis (McDermott and Atkins 2013, Wang et al. 2016). Higher PD-L1 expression was found in malignant tumors compared to benign/borderline tumors (Maine et al. 2014). In advanced ovarian cancer, TILs are abundant population inside tumor; however, they frequently express PD-1 molecule and seem to be functionally incompetent. A soluble form of PD-L1 has been also observed in aggressive renal cancer. Blockade of PD-1/PD-L1 pathway resulted in increased frequency of T CD8 + CD4-CD45RO+ effector memory lymphocytes, B lymphocytes, and MDSCs in tumors (Ribas et al. 2016).

1.4.2 Tumor-Infiltrating Lymphocytes and Immune Escape

Tumor-infiltrating lymphocytes are the heterogeneous population of immune cells, which upon existence of immunoregulatory conditions in tumor environment acquire in most circumstances immunosuppressive or regulatory phenotype and lose at least partially an antitumor 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.

Effector T CD8$^+$ cells in TIL population have been considered to be a good prognostic sign in ovarian cancer (Curiel et al. 2004b); however, there are suggestions that the T CD8$^+$/Tregs ratio could be a better indicator of good prognosis. The presence of T CD8$^+$ effectors capable of recognition of tumor-associated antigens was 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. Similar observations were done for colorectal cancer (Hamann et al. 1997). However, the antitumor 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: Nagorsen et al. 2003). 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. Moreover, distinct regulatory mechanisms are probably engaged in control of TILs' function in different intratumor localizations. In ovarian cancer, increased intraepithelial T CD8$^+$ lymphocyte density was correlated with better prognosis, while the intensity

of stromal T $CD8^+$ infiltrate did not indicate such correlation. It was 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 VEGF, indicate immunosuppressive actions against effector TILs (Bamias et al. 2008). Inside the tumor, effector TILs are functionally impaired as was indicated by downregulation of CD3ζ chain, decreased proliferation and expression of activation antigens (CD25, CD69, and HLA-DR), and low secretion of stimulatory cytokines, like IL-2, IL-4, and IFN-γ (Chen et al. 1999b, Santin et al. 2001, reviewed in: Frey and Monu 2006). The mechanisms of effector TILs inhibition include also tolerance-inducing plasmacytoid DCs, B7-$H4^+$ macrophages, TAMs, and MDSCs (Piver et al. 1984, Serafini et al. 2004). Expression of galectins by tumor cells is another mechanism of effector TILs' 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, correlated with apoptosis of TILs. Expression of galectin-1 (Gal-1) in the tumor cells and in its stroma was correlated with malignancy and poor patient's 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: Camby et al. 2006). Another immunoregulatory molecule influencing negatively effector function is indoleamine 2,3-dioxygenase (IDO), which expression was noticed in 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, overexpression of IDO correlated with the abundance of Treg 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: Godin-Ethier et al. 2011). However, in different conditions and in certain tumor types, the infiltration of effector TILs may be more vigorous than in most cancers. Tumors showing overexpression of chemokines CCL2, CCL5, CXCL9, and CCL22, activatory cytokines IL-2 and IFNγ, and parallel low concentration of VEGF were infiltrated with significantly increased T-cell number (Bamias et al. 2008). The state of TIL effectors' anergy is not permanent, as cells tested outside the tumor hostile environment presented *in vitro* conditions, expression of activation marker (HLA-DR), and costimulatory molecules (CD28, CD80, and CD86) and indicated cytotoxicity against cultured ovarian cancer cells (Santin et al. 2001, Freedman et al. 2004). Not only TILs but also peripheral blood lymphocytes (PBLs) may be functionally impaired in cancer patients. The functional impairment and downregulation of JAK3, signal transducer and activator of transcription (STAT) 3, and CD3-zeta signaling molecules in PBLs of ovarian cancer patients were noted (Klink et al. 2012a).

$CD4^+CD25^+Foxp3^+$ T regulatory cells 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 repetitively noted (reviewed in: Wilczynski et al. 2008). Consistent with these observations, the patients with gastric and esophageal cancers showed increased numbers of circulating peripheral blood natural Tregs. Population of Tregs-infiltrating tumors was also present inside tumors themselves and was more abundant in advanced tumors compared to early-stage disease, with it being a poor outcome predictor in certain tumors (Curiel et al. 2004). Accumulation of Tregs was observed in variety of solid tumors including lung, pancreatic, breast, liver, ovarian, gastrointestinal, and head and neck cancers (reviewed in: Töpfer et al. 2011). It seems that expansion of Tregs includes both population of natural circulating and local induced Tregs (reviewed in: Janikashvili et al. 2011). Tumor-derived TGF-β correlated with the intensity of Tregs' infiltrate in gastric cancer and was the inducer of local population of Tregs from naïve T $CD4^+CD25^-$ cells. In 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: Amedei et al. 2012, Janikashvili et al. 2011). The way 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 (Cannon et al. 2011, Sharma et al. 2009, Inaba et al. 2009). Similarly, tumors secreting increased levels of TGF-β were characterized by increased Tregs infiltrate and disturbed T $CD8^+$ and T $CD4^+CD25^-$effector activity evidenced by a low secretion of IL-2, IFN-γ, and TNF-α (Curiel et al. 2004). The potent sources of TGF-β are also intratumoral immature DCs. TGF-β induces in T cells an intracellular Smad-2 and -3 signaling pathway and STAT3 and STAT5 activation which result in switch into Tregs phenotype. Another regulators of Tregs expansion are mechanisms engaging interactions of T-cell CTLA-4 and glucocorticoid-induced tumor necrosis factor receptor (GITR) with corresponding ligands on DCs, as well as interactions between PD-1 on T cells with B7-H1 expressed on DCs and TAMs (reviewed in: Janikashvili et al. 2011). Immunoregulatory Tregs could effectively inhibit host defense against cancer based on cytotoxic effectors like $CD8^+$ lymphocytes, NK, NKT cells, and antigen-specific T $CD4^+CD25^-$ lymphocytes, as well as could reversely block maturation of DCs. *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-cell activity and IFN-γ secretion. Both low number of circulating NK cells and downregulation of NKG2D expression on NK cells were poor prognostic factors in colon cancer patients (Ghiringhelli et al. 2005a). It was also presented that $CCR4^+$ Tregs utilized galectin-1 to inactivate NK cells in metastasizing breast cancer. Tregs could also upregulate expression of B7-H3 and B7-H4 immunosuppressive molecules on DCs, which contributed to DC-mediated inhibition of T effectors activity (reviewed in: Janikashvili et al. 2011). Murine studies indicated that Tregs were capable to impair 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. Tregs closely cooperate with MDSCs to promote tumor growth; however, they might have different roles. Tregs could protect tumors in early stages of proliferation and metastases when host antitumor defense is still effective, while MDSCs augment tumor progression and induce systemic suppression (reviewed in: Biragyn and Longo 2012). 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 to the GITR-ligand (GITR-L). It was shown that gastrointestinal tumor cell lines indicated the expression of GITR-L. The GITR/GITR-L signaling downregulated the CD40, CD54, and epithelial cell adhesion molecule (EpCAM), as well as induced TGF-β secretion by tumor cells. Constitutive expression of GITR-L by cancer cells diminished antitumor NK-cell activity (Baltz et al. 2007). Independently on 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 gastrointestinal tumors through downregulation of inflammation (Erdman et al. 2010). In familial ovarian cancer, the observation that high Tregs density correlated with better prognosis was consistent with clinical observation that patients with familial ovarian cancer and carriers of BRCA mutations have better outcome, although their tumors are usually more aggressive (Mhawech-Fauceglia et al. 2013).

Tr1 T lymphocytes represent another group of regulatory IL-10-producing cells generated upon immature DC stimulation. The detailed profile of secreted cytokines specific for Tr1 cells includes IL-10, TGF-β, and trace amounts of IFNγ. The possible role of type 1 regulatory T cells (Tr1 cells) for human pathology and unfavorable outcome was confirmed in studies of different types of tumors (Moore et al. 2001). It was shown that Tr1 cells primed by cyclooxygenase (COX) 2 were associated with inhibition of DC maturation and contributed to increased growth of head and neck squamous cancer. Moreover, murine model revealed that IL-10-knockout or Tr1-depleted mice showed improved antitumor immunity. The population of regulatory T cells with similarity to Tr1 cells' profile of secreted cytokines makes Th3 cells. In addition to TGF-β and IL-10, they are able to produce IL-4 (MacDonald 1998). The importance of Tr1/Th3 infiltrate for progression of B16 melanoma was 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 (Seo et al. 2002).

T $CD4^+$ Th17 cells are the next population of lymphocytes engaged in immunoregulatory mechanisms existing inside the tumor, which upon stimulation by IL-23 produce IL-17 (Castellino and Germain 2006, Steinman 2007, Bi et al. 2007). In murine model, Th17 cells promoted growth of transplanted cervical cancers into the nude mice. Increased number of Th17 lymphocytes was noted in several solid tumors, including melanoma, breast, colon, and hepatocellular carcinoma, with some of them having a bad prognostic factor. Similarly, 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: Amedei et al. 2012). 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 (Su et al. 2010). Tumor cells, cancer-associated fibroblasts, TAMs, T cells, and antigen-presenting cells (APCs) produce proinflammatory cytokines (IL-1β, IL-6, IL-23, TNF-α) that facilitate the expansion of Th17 cells in tumor environment. The Th17 upregulation 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 antibody reduced serum IL-17 levels in EOC patients. Chemoattraction of Th17 cells by both tumor- and CAFs-derived chemokines monocyte chemotactic protein-1 (MCP-1 also CCL2) and Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES—CCL5) was demonstrated. TAMs could participate in Th17 expansion by production of proinflammatory cytokines. The role of Th17 cells for enhancement of tumor growth is probably based on their vasculogenic abilities (Numasaki et al. 2003, reviewed in: Amedei et al. 2012). However, the results of studies concerning the role of Th17 cells and IL-17 have been inconclusive, as have indicated its functional ambiguity both for promotion and rejection of tumors (Langowski et al. 2006, Numasaki et al. 2003, Bettelli et al. 2006). It was shown that Th17 cells secreting IFN-γ and IL-17 were able to upregulate CXCL9 and CXCL10 chemokines, thus leading to chemoattraction of NK and T cytotoxic cells (Kryczek et al. 2009). 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: Amedei et al. 2012).

Natural killer T lymphocytes 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: Terabe and Berzofsky 2008). Both 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: Terabe and Berzofsky 2008). Low circulating number of NKT I cells in head and neck squamous cancer was 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. 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 (Crowe et al. 2002). 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 an intermediary anergic plasmacytoid DCs. In another network presented in murine model, Tregs seemed to reduce the number, proliferative response, and cytokine secretion of NKT I cells. Activated NKT I cells were shown to produce IFN-γ and IL-2 which together with IL-12 secreted by APCs activated NK cells (Eberl and MacDonald 2000). They also induced maturation of

DCs by upregulation of costimulatory molecules, expression of class II MHC, and IL-12 secretion. On the other hand, myeloid DCs (MDCs) in the peripheral blood of melanoma and renal cancer induced NKT I cells reversible dysfunction mediated by TGF-β and IL-10. The suppressive NKT II cells activity is based on function of IL-13 which promotes the expansion of M2-type macrophages and stimulates IL-13 receptor-positive Gr-1^{+}CD11b^{+}MDSC cells to inhibit T CD8^{+} effectors by secretion of TGF-β (Terabe and Berzofsky 2008).

Lymphocytes B are a heterogeneous population of cells which, according to the 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: Biragyn and Longo 2012). Production of immunoglobulins by B cells initiates creation of immune complexes which could initiate FcR- and complement-dependent chronic inflammation promoting cancer (de Visser et al. 2005). Tumor-infiltrating B cells produce lymphotoxin α/β, which through activation of STAT3 in prostate cancer cells sustains their growth. Moreover, immunoglobulins could function as a carrier for immunosuppressive TGF-β. 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: Biragyn and Longo 2012). B-cell-deficient mice were shown to be resistant to syngeneic tumors including colon carcinoma and some types of melanoma, whereas partial B-cell depletion was correlated with reduced tumor growth in mouse model of colorectal cancer (reviewed in: DeNardo et al. 2010). However, it seems that the precise role of B cells depends on B-cell subpopulation studied, the tumor type, and particular immune situation inside, as is syngeneic mouse melanoma model depletion of B cells' enhanced tumor growth and metastases (Schreiber et al. 2000). 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 (Olkhanud et al. 2011).

1.4.3 Immunoregulatory Function of Tumor-Associated Myeloid Cells (TAMCs)

Tumor-associated myeloid cells (TAMCs) constitute the heterogenic population of cells of common myeloid lineage and include at least four cell subpopulations: MDSCs, TAMs, tumor-associated neutrophils (TANs), and the angiogenic

monocytes/macrophages expressing endothelial kinase-2 (Tie-2) called TEMs (reviewed in: Sica et al. 2012).

Myeloid-derived suppressor cells characterized in mice by $CD11b^+/Gr\text{-}1^+$ 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 (Bennaceur et al. 2009). In humans, MDSCs are characterized as $CD14^-CD11b^+$ cells or alternatively $CD33^+$ cells lacking the expression of mature myeloid or lymphoid markers (Serafini et al. 2006; Nagaraj and Gabrilovich 2008). It seems however that in humans, precise phenotype of the MDSCs depends on the tumor type (reviewed in: Sica et al. 2012). Similarly like in mice, human MDSCs could also belong to either monocytic or granulocytic line. Monocytic 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 (ARG1) activity. Granulocytic G-MDSCs suppress immune responses via direct cell-to-cell contact and reactive oxygen intermediates (ROI)/reactive nitrogen species (RNS) (reviewed in: Sica et al. 2012). 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 (Serafini et al. 2006; Nagaraj and Gabrilovich 2008). Receptor CCR2, C5a component of the complement, and proinflammatory S-100 proteins are responsible for chemoattraction of MDSCs into tumor (reviewed in: Sica et al. 2012). This unique cell population possesses the common feature of suppressing in both antigen-specific and nonspecific manner of host antitumor responses mediated by T $CD8^+$ CTLs, NK cells, and NKT cells, as well as of blocking DCs' maturation (Serafini et al. 2006). The pleiotropic effects of MDSCs are mediated through production of ARG1 and ROI/RNS (Serafini et al. 2006; Rodriguez and Ochoa 2006; Kusmartsev and Gabrilovich 2006), 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 (Sinha et al. 2007a). 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) (Gabrilovich and Nagaraj 2009). Cytokines IL-1β, IL-6, and PGE_2 increase accumulation and suppressive activity of MDSCs (Bunt et al. 2006; Sinha et al. 2007b). In the tumor site, the main activity of MDSCs is based on nonspecific inhibition of immune effectors mediated by NO and ARG1 production. NO inhibits T effectors by interfering with intracellular JAK3 and STAT5 pathways, induction of T-cell apoptosis, and downregulation of MHC class II expression. ARG1 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 (Nagaraj and Gabrilovich 2008). Action of MDSCs against T $CD8^+$ CTLs is probably based on modification of TCR-binding activity caused by peroxynitrite activity (Nagaraj et al. 2007). A correlation between high peroxynitrite concentration and immunosuppression was demonstrated in

various cancers including pancreatic, head and neck, breast cancers, mesothelioma, and melanoma (Nagaraj and Gabrilovich 2008). MDSC-inhibited T $CD8^+$ cells are unable to secrete IFN-γ and IL-2, and to kill the target cells (Kusmartsev et al. 2005). It was also found that MDSCs inhibited T cells by depletion of cysteine which is essential for T-cell activation. Moreover, they were capable to downregulate CD62L selectin expression on T cells, thus reducing their migration into regional lymph nodes (reviewed in: Srivastava et al. 2012). Myeloid-derived suppressor cells are also capable of inducing tumor mutations and thus augmenting the tumor metastatic potential (Bennaceur et al. 2009). By production of IL-10, MDSCs could also skew the function of TAMs into protumoral M2-type activity (Sinha et al. 2007a). They promote the formation of a new blood vessel by expressing metalloproteinases and increasing the bioavailability of VEGF (Murdoch et al. 2008). Circulating MDSCs may differentiate in hypoxic tumor environment into Gr1-$F4/80^+$ macrophages (Kusmartsev and Gabrilovich 2006). The expansion and functional activation of MDSCs are regulated by NF-κB, as IL-1β signaling crucial for recruitment of MDSCs into gastric cancer was found to be NF-κB-dependent. The STAT system also regulates MDSCs' function. STAT1 is responsible for MDSCs' interferon-dependent activation, and STAT5 is engaged in MDSCs' survival (reviewed in: Sica et al. 2012).

Macrophages constitute one of the major immune cell population responsible for both tumor rejection and promotion (Ostrand-Rosenberg 2008; Sica et al. 2008; Siveen and Kuttan 2009), but their function is determined by the way they are activated. There are two groups of macrophages: one are tissue-resident macrophages derived from embryonic yolk sac, and second are infiltrating macrophages recruited from bone marrow monocytes. In the tumor microenvironment, they are converted into TAMs (tumor-associated macrophages). The presence of IFNγ, 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 (Mills et al. 2000). Tumor MDSCs in murine breast cancer model were shown to contribute to M2 switch of TAMs, similarly like cancer-associated fibroblasts (reviewed in: Sica et al. 2012). It was 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 (DeNardo et al. 2010). Lymphocyte B also participates in skewing activity of TAMs into M2 phenotype by stimulating Fcγ receptors on resident myeloid cells. Several additional signals switching the differentiation of macrophages into M2-type were identified, including hormones, growth factors, and bacterial products (reviewed in: Sica et al. 2012). However, it seems that polarization into M1 and M2 phenotypes is somehow artificial and represents the extremal differentiation status, while many cells indicate a functions being a mixture of M1/M2 phenotypes with balance slightly pushed toward M1 or M2 type (Mantovani et al. 2002). Different signals present in tumor environment could be the source of heterogeneous 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: Sica et al. 2012). Macrophages of M1 type could effectively

destroy tumor cells through production of Th1 cytokines and stimulation of T $CD8^+$ CTLs (Ostrand-Rosenberg 2008). 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 and stimulate tissue healing and remodeling as well as angiogenesis. This cell subset constitutes the vast majority of TAMs, which play a discreditable role in tumor progression (Ostrand-Rosenberg 2008, Sica et al. 2008). 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. And in contrast, p50 NF-κB-deficient mice, which are unable to mount M2 polarization, show resistance to transplantable tumors. It was shown that most aggressively growing tumors were infiltrated by large numbers of TAMs. Recruitment of macrophages into tumors is regulated by Th2 cytokines, chemokines (Sica et al. 2008, Mantovani et al. 2006, Ben-Baruch 2006), urokinase plasminogen activator (uPa), microbial defensins, and hypoxia. 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: Sica et al. 2012). CSF-1 and TGF-β are major cytokines that are believed to play important role for recruitment of macrophages into the tumors. Both of them are expressed constitutively on the surface of solid tumors (Wojtowicz-Praga 2003) correlated with intensity of TAMs infiltrate and poor prognosis for the patients (Sapi 2004). The chemokines CCL2 (MCP-1) and CCL5 (Regulated on Activation, Normal T-cell Expressed and Secreted—RANTES) were found to be expressed predominantly by the solid tumors (Zhou et al. 2004). Their overexpression correlated with intratumor TAMs' content as well as with bad survival ratio. They were also shown to regulate migration of peripheral blood monocytes into the tumor. Upon the tumor-derived M-CSF, attracted monocytes differentiate to macrophages. High M-CSF production correlates with poor outcome in ovarian, breast, and endometrial cancers (reviewed in: Allavena and Mantovani 2012). 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 self-enhancing loop. IL-6 secreted by TAMs plays 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: Allavena and Mantovani 2012). 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 (Mhawech-Fauceglia et al. 2013). 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 (Gordon and Freedman 2006). 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 (Raghunand et al. 2003). 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 proangiogenic and prometastatic TAMs activity. Clinical studies seem to confirm that there is an enhancement of invasiveness and peritoneal metastatic activity in ovarian cancer under hypoxic conditions. Tumor-associated macrophages secrete Th2 cytokines, enhance intratumor 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 and resulting in increased tumor metastatic potential (Ostrand-Rosenberg 2008, Sica et al. 2008, reviewed in: Wilczyński and Duechler 2010). TAMs also secrete some specific molecules like semaphorin 4D (Sema4D) and growth-arrest specific-6 (Gas6) which promote cancer neoangiogenesis and proliferation. Subsets of TAMs not completely biased toward M2-type activity may secrete some amounts of Th1 cytokines, for instance TNF-α. Although TNF-α is considered to be an antitumor cytokine, it has also some protumor activities. It might contribute to DNA damage, induce angiogenic factors, and act as a growth factor for cancer cells (Balkwill 2002). Investigations performed on ovarian cancer indicated that TAMs were also able to inhibit host T effectors by expression of B7-H4 costimulatory molecule, which was identified as a negative regulator of T-cell activation. 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 nitric oxide synthase (NOS) and production of ROI, and that their activity is related to TAMs (Malmberg 2004, MacMicking et al. 1997, Bogdan 2001, Thomsen and Miles 1998). Defective M1-type functions showed by TAMs are probably caused by disturbed activation of NF-κB in response to proinflammatory stimuli present in advanced tumors, including TNF-α. Factor NF-κB is responsible for regulation of transcription of many genes including those for cytokines, chemokines, and antiapoptotic molecules (Ostrand-Rosenberg 2008). 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: Sica et al. 2012).

Tumor-associated neutrophils are a population of $CD11b^{+}Ly6G^{+}$ cells which have 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: Sica et al. 2012). The presence of TANs was 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: Sica et al. 2012). TANs contribute to tumor growth by promoting the angiogenesis, proliferation, and metastases, and on contrary, their depletion inhibits the 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 was suggested that N1-type TANs are fully activated neutrophils, whereas N2-type TANs are immature ones (reviewed in: Sica et al. 2012). TANs could secrete hepatocyte growth factor (HGF) and oncostatin which augmented invasiveness of cancer cells and upregulated expression of CXCR4 (reviewed in: Reiman et al. 2007). Upon activation, neutrophils secrete fibers composed from proteins and chromatin, called neutrophil extracellular trap (NET), and used for entrapment and killing 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 (Berger-Achituv et al. 2013). The peripheral blood neutrophils could also participate in tumor growth promotion, as IL-8 secreted by neutrophils together with upregulation of CD11b/CD18 on their surface facilitated melanoma cell arrest on endothelium and tumor cell extravasation (Dong and Robertson 2009). Moreover, *in vitro* studies have shown that ovarian cancer cells could participate in potentiation of peripheral blood neutrophils inflammatory responses (enhancement of reactive oxygen species (ROS) formation) by the direct cell-to-cell contact (Klink et al. 2008). 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 (Klink et al. 2012).

Tie-2-expressing monocytes/macrophages are a population of $CD11b^{+}/Gr1^{low/-}/Tie\text{-}2^{+}$ cells which express endothelial kinase-2 (Tie-2) receptor for angiopoietin. They originate from peripheral blood $Tie\text{-}2^{+}$ 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. 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: Sica et al. 2012). TEMs are related to M2-type TAMs and have however a more M2-skewed functional signature, with pronounced expression of ARG1, scavenger receptors, and lowered expression of IL-1β, COX2, IL-12, TNF-α, and iNOS. They also express proangiogenic molecules, like VEGF and MMPs (reviewed in: Sica et al. 2012). TEMs play a crucial role in tumor angiogenesis. They are seen mainly in the hypoxic areas of the tumor in the proximity of the vessels. Mouse studies confirmed that ablation of $Tie\text{-}2^{+}$ 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.

1.4.4 Dendritic Cells as Tumor Growth Enhancers

Dendritic cells are professional antigen-presenting cells of myeloid or plasmacytoid origin (Colonna and Liu 2004; O'Neill and Bhardwaj 2004). MDCs are characterized by $CD11c^{+}CD33^{+}CD45RA^{-}CD123^{-}$, whereas plasmacytoid DCs (PDCs) by CD11c-$CD4^{+}CD45RA^{+}CD123^{+}$ phenotype, respectively. PDCs show exclusively expression of TLR7 and TLR9, as well as IFN secretion upon viral stimulation. On the contrary, MDCs indicate the expression of a broad spectrum of TLRs, excluding TLR7 and -9, and are not capable to secrete 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: Fricke and Gabrilovich 2006; Palucka et al. 2011). Presence of competent mature DCs (mDCs) is very rare in the tumors, which was confirmed in ovarian, prostate, breast, and renal cancers (reviewed in: Fricke and Gabrilovich 2006). If present, they occupy the peritumoral tissues. On the contrary, progressive tumors usually contain DCs having immature $CD4^{-}CD8^{-}$ phenotype (iDCs). Opposite to mature DCs, these cells indicate protolerogenic functions and are unable to effectively stimulate cytotoxic responses (Liu et al. 2005). Moreover, they are able to inhibit tumor-specific T $CD8^{+}$ cytotoxic responses even in chemotherapy pretreated mice, by capturing $CD8^{+}$ CTLs into DCs reach areas of the tumor. 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 DCs' maturation (reviewed in: Fricke and Gabrilovich 2006). Tumor environment also contains many other cytokines and immunoregulatory factors that modulate DC function, and among them are cytokines such as VEGF, IL-10, IL-6, TGF-β, and PGE_2, factors like IDO and ROI, and finally tumor antigens and metabolites (reviewed in: Bennaceur et al. 2009). The meaning of VEGF for DCs function was shown in murine studies, where use of VEGF-neutralizing antibody stimulated DCs' differentiation and raised the number of mDCs, while in the presence of VEGF, the DCs showed disturbed antigen-presentation capacity. Murine studies found the presence of functionally immature $CD11c^{+}$DCs expressing low levels of costimulatory CD86 and CD40 molecules in tumor and tumor-draining lymph nodes. Depletion of these DCs in tumor-bearing mice retarded significantly tumor progression. Studies in human gastric and nonsmall lung cancer confirmed that differentiation of DCs was negatively affected by VEGF (Takahashi 2004). 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 (Coukos et al. 2005). Interleukin-10 is responsible for downregulation of costimulatory molecules on DCs, thus cooperating with VEGF in worsening of APC function of DCs. It also blocks DCs' differentiation. The source of IL-10 is tumor itself and TAMs. Similar effects showed exposition of DCs to TGF-β function (reviewed in: Fricke and Gabrilovich 2006). Renal cancer cell lines were shown to produce IL-6 and GM-CSF which

inhibited DCs' differentiation. The blocking effect of IL-6 was also observed in myeloma. Retention of DCs inside tumors and downregulation of their migratory potential are probably mediated by CXCL8 (IL-8) produced by tumors, including hepatocellular, pancreatic, and colon cancers, which act through CXCR1 and -2 receptors on DCs (reviewed in: Fricke and Gabrilovich 2006). Expression of IDO on DCs deprives tryptophan to the T cells and promotes T-cell apoptosis or anergy. The presence of IDO-positive DCs was 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 (Munn et al. 2002). Population of cells which mediated entirely all IDO-dependent suppression in lymph nodes was population of $CD19^+B220^+$ plasmacytoid DCs (Munn et al. 2004). Expression of IDO on DCs is probably upregulated by PGE_2 present in tumor environment. IDO +DCs are capable of inducing $CD4^+CD25^+Foxp3^+$ Tregs. Immature DCs also exert other activating T $CD4^+CD25^+Foxp3^+$ Tregs effects, mediated through TGF-β and IL-10, thus promoting tumor growth (Ghiringhelli et al. 2005; reviewed in: Palucka et al. 2011). Interactions between DCs and Tregs mediated through CTLA-4 could compromise antitumor immunity in an IDO-dependent way. DCs can also modulate the trafficking of Tregs into tumor site and lymph nodes, thanks to CCR4/CXCL22 interactions (reviewed in: Palucka et al. 2011). Tregs were shown to direct back regulatory signals toward DCs, mainly by downregulation of costimulatory molecules on DCs, inhibition of their maturation, and impairment of APC functions by TGF-β and IL-10. Tregs were also reported to induce immunosuppressive molecules B7-H3 and B7-H4 on the surface of DCs (reviewed in: Palucka et al. 2011). Accumulation of ROI in tumor localization creates a constant stress which has profound impact on DCs functions and vulnerability to apoptosis, through modulation of NF-κB and c-Jun N-terminal kinases (JNK) pathways (reviewed in: Fricke and Gabrilovich 2006). Molecule CD200 is a membrane protein belonging to costimulatory 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 toward Th1 activity. Moreover, tumors themselves (including ovarian cancer) are capable of expressing CD200 molecules, thus influencing DCs' function. Myeloid DCs isolated from ovarian tumors also exhibited the expression of programmed cell death-1 ligand 1 (PD-L1, B7-H1). Accumulation of $PD\text{-}1^+B7\text{-}H1^+$ DCs in the tumor was associated with suppression of $TCD4^+$ helper, T $CD3^+CD8^+$ cytotoxic/regulatory cell activity, decreased infiltration of T cells, and expansion of Tregs (Krempski et al. 2011; reviewed in: Palucka et al. 2011). In ovarian cancer, plasmacytoid DCs accumulate in tumor environment, preferentially in ascites, where they are attracted by CXCL12 (Curiel et al. 2004). Similarly to MDCs, ascitic PDCs have immature phenotype. Plasmacytoid DCs promote the generation of immunoregulatory $IL\text{-}10^+$ T $CD8^+$ suppressors, which independently from T $CD4^+CD25^+FoxP3^+$ Tregs downregulate IFN-γ secretion mediated by T effectors and prevent them from proliferation. They also secrete TNF-α and IL-8, thus being

capable of promoting angiogenesis (Curiel et al. 2004). Tumor-associated PDCs were found to have different phenotype compared to ascitic PDCs, and expressed semimature 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 (Labidi-Galy et al. 2011). The DCs intercellular machinery exposed to tumor-derived regulatory molecules inhibits their differentiation to mature phenotype via induction of STAT3 signaling. Moreover, activation of STAT3 in tumors blocks secretion of proinflammatory factors and enhances DCs immaturity (reviewed in: Palucka et al. 2011).

1.4.5 Inflammation and Cancer Escape

Chronic inflammation may account for about 15% of cancers, due to the fact that inflammation mediators like TNF-α could initiate tumor growth by stimulation of NO synthase and ROI production, both being capable of DNA damage (Balkwill and Mantovani 2001; Li and Karin 2007; Hussain et al. 2003). During progressive tumor growth, chronic inflammation caused by tumor-infiltrating immune cells contributes to cancer progression (Ben-Baruch 2006). Oxidative stress seems to play pivotal role in this process by stimulating 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 (reviewed in: Reuter et al. 2010). Moreover, stromal cells could also contribute to chronic inflammation and initiate or promote tumor growth. Upon senescence, fibroblasts acquire "senescence-associated secretory phenotype" (SASP) characterized by activation and production of proinflammatory cytokines (IL-6, IL-1β), chemokines (IL-8, MCP-1, GRO-1/α), MMPs, adhesion molecules, and integrins (Shan et al. 2009). The senescent stromal fibroblasts were detected in specimens of ovarian tumors in areas adjacent to malignant epithelium. The chronic inflammation and oxidative stress also promote the generation of heat-shock proteins (HSPs), which prevent cells from apoptosis and enhance their survival. Overexpression of HSP90 was found on the 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: Goldstein and Li 2009).

Toll-like receptor polymorphisms in genes encoding TLR6 and TLR10 increased risk of some cancers. Activation of TLR receptors both on macrophages and on the cancer cells enhanced tumor growth by various mechanisms like stimulation of growth-promoting cytokines or protection against apoptosis (Medzhitov 2001). In ovarian cancer, stem-like slow-growing cell population initiates tumor regrowth after surgery or chemotherapy by activation of TLR4-pathway, which regulates proinflammatory phenotype of these cells characterized by high NF-κB, IL-6, IL-8, MCP-1, and GRO-1/α activity (Mor et al. 2011). Therefore, the $TLR4^{+}$

phenotype of ovarian cancer cells was correlated to chemoresistance. Similarly, the expression of TLR9 was connected to high metastatic potential of ovarian tumors.

Tumor necrosis factor-α is one of the proinflammatory cytokines stimulated by TLRs, which promotes tumor survival by stimulation of NF-κB-dependent pathways regulating antiapoptotic molecules, tumor proliferation, neoangiogenesis, and metastatic properties (Elgert et al. 1998). Polymorphisms leading to overproduction of TNF-α were connected with greater risk of cancer, including breast and gastric tumors (Mocellin et al. 2005). Increased TNF-α concentrations were observed in ovarian cancer patients in serum and cyst fluid, as well as in cancer tissues and ascites. Cancer patients were also characterized by overexpression of receptor TNF-R2, which was further correlated with tumor stage and patients prognosis (Dobrzycka et al. 2009). TNF-α expressed on tumor cells orchestrates the paracrine "TNF network" and together with IL-6 and CXCL12 regulates tumor growth (Kulbe et al. 2012). 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. Moreover, prostate tumors were characterized by increased TNF-α, TNFR1, and TNFR2 levels, which correlated with poor prognosis (Tse et al. 2012).

Interleukin-6 is another proinflammatory cytokine which through activation of intracellular STAT3 pathway regulates cell proliferation, induces epithelial–mesenchymal transition and appearance of cell migratory phenotype, and upregulates resistance to apoptosis and chemoresistance (Hodge et al. 2005). Polymorphisms of the *IL6* gene promoter region could influence the risk of certain tumors (Berger 2004). *In vitro* investigations in ovarian cancer showed that *p53* overexpression could regulate IL-6 secretion (Nash et al. 1999). 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 produce a cooperative network for promotion of tumor growth (Lane et al. 2011; Kulbe et al. 2012). IL-6 could induce suppressive Th2 phenotype in tumor-infiltrating T cells and M2-type activity in TAMs. *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. Increased IL-6 expression was related to 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 (Waldner et al. 2012). Women with advanced ovarian cancer had significantly higher IL-6 levels both in the serum and ascites (Clendenen et al. 2011; Nowak et al. 2010a). In these patients, IL-6 was engaged in neoangiogenesis, spread of peritoneal metastases, and ascites production. In several prostate cancer cell lines, IL-6 inhibited apoptosis and enhanced survival by activation of phosphatidylinositol-3-kinase signaling (Culig and Puhr 2012).

Transforming growth factor-β despite its antitumor activity in early tumors might also enhance tumor escape and contribute for 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. On the contrary, some

polymorphisms of *TGF* gene make individuals less prone for development of lung cancer (reviewed in: Jadus et al. 2012). The source of TGF-β could be both tumor cells and M2-type TAMs (Ostrand-Rosenberg 2008). Lung cancers overexpress TGF-β and are characterized by several mutations of TGF-β receptors, which prevent cancer cells from negative autocrine regulation of growth by this cytokine. As a result, high TGF-β concentration produces suppressory environment inside the tumor (reviewed in: Jadus et al. 2012). In advanced tumors, TGF-β is engaged in Th17 cell differentiation, inhibition of DCs maturation, and 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 (Moutsopoulos et al. 2008; Yu et al. 2006; Gavalas et al. 2010). In breast cancer, chemotherapy-induced TGF-β signaling enhances tumor recurrence through IL-8-dependent expansion of CSCs, while TGF-β pathway inhibitors prevent the development of drug-resistant CSCs. TGF-β signaling induces mTOR complex 2 in cancer cells and regulates epithelial–mesenchymal transition (Moutsopoulos et al. 2008; Yu et al. 2006).

Interleukin-10, similarly to TGF-β, exerts both antitumor and protumor activity, which seems to be dependent on the tumor type and advance of the disease. IL-10 was 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 intratumor and peripheral blood immunosuppressive phenotype by stimulation of M2-type TAMs and Th2-type lymphocytes (Rabinovich et al. 2010; Seo et al. 2002; Moutsopoulos et al. 2008; Yu et al. 2006). Autocrine activation of the STAT3 pathway by IL-10 in tumor cells upregulates expression of Bcl-2 and HLA-G, thus protecting cancer cells from host effectors and apoptosis (Urosevic and Dummer 2008). In ovarian cancer patients, IL-10 concentrations were increased in peritoneal fluid and serum compared to benign ovarian disease (Nowak et al. 2010). Moreover, the expression of IL-10 was found to correlate with tumor aggressiveness, the presence of metastases, and shorter progression-free survival (Matte et al. 2012). High levels of IL-10 in TAMs significantly correlated with stage, tumor size, lymph node metastasis, lymphovascular invasion, or histologic poor differentiation in nonsmall cell lung cancer. 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 (Itakura et al. 2011).

Cyclooxygenase-prostaglandin E_2 inflammatory pathway is important for tumor development, as revealed by studies showing antitumor effects of selective COX2 inhibitors in colorectal cancer (Wang and DuBois 2006). Activity of *cox2* gene was proved to participate in ovarian carcinogenesis both in sporadic and in BRCA 1/2-conditioned cancers. Upregulation of COX2-PGE_2 in tumor cells and TAMs results from hypoxia and HIF-1α, and influences several regulatory and signaling pathways including Ras/mitogen-activated protein kinases (MAPK), phosphatidylinositol 3-kinase/phosphatase (PI3K)/AKT, and NF-κB-mediated pathway (Wang and DuBois 2006). COX2 overexpression stimulates VEGF and neoangiogenesis, and its raised levels predict poor survival in some cancers (Zhang and Sun 2002). In

murine model, COX inhibitors administered together with taxol decreased the expression of VEGF and reduced microvessel density (MVD) of transplanted ovarian tumors. Overexpression of COX2 in ovarian cancer also correlated with resistance to platinum-based chemotherapy. COX2, 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 the COX/PGE_2 signaling. Lung cancers also overexpress COX2 and produce several prostanoids and leukotrienes. The presence of COX2 overexpression seems to be the key factor in promotion of lung cancer growth, as the pharmacologic inhibition of COX2 reduced tumor growth in lung cancer murine model. COX2 was capable to modulate MDSCs' activity through PGE_2-mediated ARG-1 expression and to enhance expansion of Tregs also by PGE_2 (reviewed in: Srivastava et al. 2012). PGE_2 inhibits DCs' maturation and migration toward regional lymph nodes, upregulates IL-4 and IL-10 cytokines, and finally increases tumor migratory and metastatic potential (Wang and DuBois 2006; Bennaceur et al. 2009). 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 stimulator of lung cancer growth by augmenting angiogenesis and proliferation, and simultaneously inhibits T and NK effector cells (reviewed in: Jadus et al. 2012). Peroxisome proliferator-activated receptor-γ (PPARγ) is an inhibitor of COX-dependent inflammatory reaction, and in mouse studies produced decrease of PGE_2 levels, reduction of MVD, enhanced tumor apoptosis, and improved mice survival.

Proinflammatory cytokine IL-23 also documents the relationship between the cancer and inflammation. In ovarian cancer, particularly high level of expression of genes regulating proinflammatory pathway including IL-23 was detected. Moreover, *IL-23* receptor gene polymorphism was shown to correlate with advancement of tumors. Upon stimulation by tumor-derived IL-23, Th17 cells release IL-17 and other inflammatory mediators like IL-1, IL-8, TNF-α, and PGE_2 which produce protumor inflammatory environment. The increased expression of both IL-23 and IL-17 was observed in many malignant tumors and correlated with angiogenesis, expression of MMPs, and decrease of cytotoxic antitumor immune response (Langowski et al. 2007; Whiteside 2010).

Interleukin-18 is a proinflammatory cytokine which activates immune CTL and NK cells and induces IFN-γ, thus is capable to exert antitumor effects. However, IL-18 was also found to potentiate tumor growth (reviewed in: Park et al. 2007). The expression of IL-18 was demonstrated on melanoma, squamous skin, breast, and gastric cancers, and was connected with the presence of distant metastases in breast and gastric cancers. *In vitro* studies showed that transfection of poorly metastatic lung cancer cells with IL-18 construct enhanced their invasion ability and downregulated E-cadherin, thus increasing metastasis potential (Jiang et al. 2003). In murine melanoma model, the prometastatic IL-18 action was mediated by upregulation of vascular cell adhesion molecule 1 (VCAM-1). Also the proangiogenic properties of IL-18 were noticed in gastric cancer, where IL-18-dependent stimulation of thrombospondin-1 was discovered. Moreover, IL-18

induced Fas ligand expression on melanoma cells and makes them less susceptible for effector destruction (reviewed in: Park et al. 2007).

Interleukin-8 (also CXCL8) is a chemokine secreted by macrophages, neutrophils, endothelial, and tumor cells, mediating its biological effects through binding to CXCR1 and CXCR2 receptors present on both tumor and endothelial cells (Walz et al. 1987; Murdoch et al. 1999; Xu and Fidler 2000). Hypoxia and oxidative stresses are strong inducers of IL-8 expression on cells of several malignancies, including ovarian cancer, via *Ras* gene overexpression and activation of PI3K/AKT and p38 MAPK signaling (Xu et al. 2004). Some *IL-8* gene polymorphisms are correlated with the overall risk of developing the intestinal type of gastric cancer (Xue et al. 2012). Increased IL-8 was found in ascites and serum of ovarian cancer patients, while IL-8 overexpression was observed on tumor cells, both correlated with advancement, vascularity of tumors, and short patient's survival (Uslu et al. 2005, Merritt et al. 2008). IL-8 is engaged in blocking of TRAIL-induced cancer cells apoptosis and in recruiting certain immune cells into peritoneum, where they contribute to tumor spread and formation of ascites (Wang et al. 2006, Abdollahi et al. 2003). It was shown that chemoresistant ovarian cancers were characterized by increased expression of IL-8 (Duan et al. 1999). IL-8 and CXCR1 were found to be overexpressed in pancreatic cancer, and *in vivo* studies showed that tumors from patients who had higher IL-8 levels grew faster (Chen et al. 2012). *In vitro* studies of gastric cancer revealed that IL-8 increased NF-κB and AKT signaling and adhesion molecules intercellular adhesion molecule (ICAM-1) and VCAM-1 expression in cancer cells, thus increasing their migration, adhesion, and invasion (Kuai et al. 2012). 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 (Ning et al. 2011). Overexpression of CXCR2 receptor inhibited cancer apoptosis, upregulated VEGF on tumor cells, and was an indicator of poor prognosis (Yang et al. 2010).

Hedgehog (Hh) signaling pathway plays important role in human development. The expression of Hh ligands and the intensity of Hh signaling are upregulated by hypoxia and inflammation (Bijlsma et al. 2009, Pratap et al. 2010). Classical activation way requires binding of one of 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 upregulate target genes (reviewed in: Harris et al. 2011). During embryonic development, Hh signaling promotes cell proliferation, angiogenesis, EMT, and stem cell regrowth, all under hypoxic conditions; thus, situation according to the Hh function resembles in some circumstances that inside solid tumors. Inhibition of Hh signaling was found to decrease the proliferation of cancer cells (Berman et al. 2002). The Hh-GLI-mediated increase of proliferation was observed in melanoma cells (Stecca et al. 2007). Target genes responding to Hh-GLI regulation include proliferation activators including cyclins, IGF-BP6, and osteopontin. Moreover, Hh-GLI pathway upregulates the expression of Bcl-2 antiapoptotic molecule (in brain, gastric, and pancreatic cancers) and regulates stability of p53 (in breast cancer) (Yoon et al.

2002, Wang et al. 2010, Das et al. 2009, Han et al. 2009, Abe et al. 2008). In ovarian and endometrial cancer, Hh signaling downregulates the p21 and p27 inhibitors of cell-cycle progression, and correlates with advancement of the tumors (Feng et al. 2007, Liao et al. 2009). The Hh-GLI pathway is also engaged in angiogenesis via upregulation of VEGF, and enhances invasiveness and migration in several tumors including skin, breast, ovarian, pancreatic, prostate cancers, and melanoma (reviewed in: Harris et al. 2011). It also represses E-cadherin expression, enhances MMPs, and activates stromal fibroblasts, thus inducing EMT (Li et al. 2007b; Yoo et al. 2008; Dunér et al. 2011). One of the most important functions of Hh signaling is the maintenance of the CSCs, with slow-proliferating, self-renewing population of cells being the reservoir for tumor regrowth (Li et al. 2007). The stimulatory effect of Hh on viability of CSCs was observer in variety of tumors including breast, brain, ovarian, and colon cancers (reviewed in: Harris et al. 2011).

1.4.6 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 (Kerr and Harmon 1991). Resistance to apoptosis or its reduced efficacy has been repeatedly reported as one of the escape mechanisms observed in the cancer development. The background for these phenomena could originate from disturbances of merely all steps of apoptotic pathway inside tumors, including disrupted pro- and anti-apoptotic signaling, impaired caspase activity, and defective death receptor function (reviewed in: Wong 2011). 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 (Balkwill 2002, Lai et al. 2003, Sun et al. 2004). On the contrary, presence of certain *DR4* and *CASP8* polymorphisms could have a protective effect against bladder and breast cancers, respectively (Hazra et al. 2003, MacPherson et al. 2004). Downregulation 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 apoptotic pathway.

Apoptosis-regulatory proteins that have been extensively studied in solid cancers belong to Bcl-2 family proteins or inhibitors of apoptosis. The Bcl-2 family of proteins is engaged in intrinsic pathway of apoptosis and acts in mitochondria-dependent way (Gross et al. 1999). The mutations of proapoptotic proteins and overexpression of antiapoptotic 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 (Cory et al. 2003). Hence, although Bcl-2 mutation is

causally connected with 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 (Zhivotovsky and Orrenius 2006). 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 (Jager et al. 1997). Overexpression of Bcl-2 protein was shown in prostate and breast cancers, and led to inhibition of TRAIL-mediated apoptosis (Raffo et al. 1995, Fulda et al. 2000). Bcl-2 is also highly expressed in small cell lung cancer and to a lesser extent in squamous lung cancers (reviewed in: Jadus et al. 2012). 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 (O'Reilly et al. 2001, Lee et al. 2003). Colorectal cancers characterized by microsatellite instability demonstrated the presence of mutations in the *bax* gene resulting in impaired function of proapoptotic Bax protein (Miquel et al. 2005). The stable tumor cell lines overexpressing Bcl-xL protein were found to be apoptosis- and drug-resistant (Minn et al. 1995).

Inhibitor of apoptosis proteins (IAPs) are endogenous inhibitors of caspases. Amplification of chromosomal regions which encompass the IAPs-coding sequences was observed in various tumors including esophageal squamous cell carcinoma (Zhivotovsky and Orrenius 2006). The upregulation of IAPs family members' expression was documented in various cancers, including pancreatic cancer and glioma, and was responsible for chemoresistance (Lopes et al. 2007, Chen et al. 1999b). Overexpression of survivin, another extensively studied member of IAPs family, was demonstrated in nonsmall cell lung carcinoma (Krepela et al. 2009). In neuroblastoma, expression of survivin was correlated with more aggressive and unfavorable disease (Adida et al. 1998).

Another example of apoptosis-regulatory protein that has been studied is p53 suppressor protein, due to its multidirectional function frequently called the "guardian of the genome" (Wong 2011, Lane 1992). The p53 protein, found to be downregulated in numerous cancers, functions as a regulator of some target genes involved in apoptosis resistance and increased proliferation activity of melanoma (Avery-Kiejda et al. 2011). It was also shown that silencing of p53 mutants in cancer cell lines resulted in reduced cellular growth due to increased apoptosis (Vikhanskaya et al. 2007). Point mutations of p53 occurring frequently in lung cancers caused upregulation of Bcl-2 with concomitant Bax hypoexpression (reviewed in: Jadus et al. 2012).

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 stop codon, missense mutation at the codon 96, and the deletion of the leucine 62, were found in head and neck cancer, neuroblastoma, and vulvar squamous cancer, respectively (Mandruzzato et al. 1997, Takita et al. 2001, Liu et al. 2002). 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 (Catchpoole and Lock 2001, reviewed in: Jadus et al. 2012). Loss of caspase-1 mRNA was observed in gastric cancer and metastatic melanoma, and in both tumors correlated with clinical stage and bad prognosis (Jee et al. 2005, Mouawad et al. 2002). Both downregulation 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 (Shen et al. 2010, Devarajan et al. 2002). A deficiency of caspase-8 was described in small cell lung cancer and neuroblastoma (Joseph et al. 1999, Fulda et al. 2001). And conversely, high levels of caspase-3 inside the tumors cells correlated with low malignancy and good outcome in pancreatic and lung cancers (Volm and Koomagi 2000; Koomagi and Volm 2000). 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 clinicopathological features of breast cancer (Grigoriev et al. 2002), and active caspase-6 concentrations were increased in progressive melanoma and its metastases compared to nonmalignant naevi (Woenckhaus et al. 2003). 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 (Zhivotovsky and Orrenius 2006).

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 important role in the regulation of extrinsic apoptosis pathway. Tumors are able to inhibit the death receptor signaling at several steps. The spectrum of possible disturbances covers the downregulation or impairment of receptor function and the reduced level of the death signals (Wong 2011). Loss of Fas was attributed to mutations in *ras* and *TP53* genes (Peli et al. 1999, Volkmann et al. 2001). 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: Khong and Restifo 2002). Deletions and mutations of TRAILR1 and -R2 receptors were detected in many tumors, including nonsmall cell lung cancer (reviewed in: Igney and Krammer 2002). Lack of cytoplasmic signaling domains of Fas and TRAILR1 and -R2 was found in many tumors, including myeloma, gastric, and breast cancers (reviewed in: Töpfer et al. 2011). Inactivating mutations of downstream Fas signaling molecules like FADD and caspase-10 were found in nonsmall cell lung cancer (Shin et al. 2002). The low expression of Fas and both FasL and TRAIL was documented in neuroblastoma and precancerous cervical lesions, respectively (Fulda et al. 1998, Reesink-Peters et al. 2005). High levels of antiapoptotic regulator FLICE inhibitory protein (c-FLIP) were demonstrated to correlate with TRAIL-mediated apoptosis in melanoma cells (Griffith et al. 1998). Overexpression of c-FLIP was confirmed in several tumors in mice and humans and, in some of them, was correlated to bad prognosis (reviewed in: Töpfer et al. 2011; Igney and Krammer 2002).

Activation of T cells during immune response is a self-limiting phenomenon, as activated T cells upregulate 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: Töpfer et al. 2011, Kim et al. 2004). Expression of FasL on their surface is either constitutive or induced by chemotherapy (reviewed in: Igney and Krammer 2002). A significant reduction in TILs and apoptosis of Fas-positive TILs was observed in esophageal cancer and metastatic gastric carcinoma, respectively. Similar correlation was 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: Kim et al. 2004). The meaning of FasL for tumor escape is sustained by observation that downregulation of FasL expression in colon cancer cells significantly reduced tumor growth in syngeneic mice and stimulated T-cell antitumor response (Ryan et al. 2005). 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 (Andreola et al. 2002, reviewed in: Kim et al. 2004). Significantly increased number of $CD3^+Fas^+$ apoptotic T cells was 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 (Dworacki et al. 2001, Hoffmann et al. 2002). These mechanisms were called FasL "counter attack" (Hahne et al. 1996). 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: Rabinovich et al. 2007, Khong and Restifo 2002). Moreover, human metastatic melanoma cells are capable to engulf and ingest T lymphocytes in a process called "tumor cannibalism" (Lugini et al. 2006). However, the function of FasL can also accelerate the rejection of tumor by induction of proinflammatory and antitumor effects mediated *in vivo* by activated neutrophils (Arai et al. 1997). In addition, screening of the melanoma cell lines by RT-PCR and functional assays did not reveal expression of functional FasL (Chappell et al. 1999). 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 antitumor T responses 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 proinflammatory proteins and chemoattractants (Igney and Krammer 2002). 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 downregulate Fas expression (reviewed in: Kim et al. 2004). The effects of FasL/Fas signaling might also

depend on the local environment, which through the action of some immunoregulatory molecules may create an appropriate condition to tumor escape. Upregulation of FasL on tumor cells resulted from proinflammatory cytokines TGF-β, IL-10, prostaglandins, and reactive oxygen species (reviewed in: Rabinovich et al. 2007, Kim et al. 2004).

Other molecules including RANTES and receptor-binding cancer antigen expressed on SiSo cells (RCAS1) could augment FasL "counter attack" by inducing cycle arrest and apoptosis of antitumor-activated T cells (reviewed in: Rabinovich et al. 2007, Khong and Restifo 2002). Tumor cells also showed ability to use a transmembrane or soluble decoy receptors with nonfunctional or absent death domain to avoid T-cell-mediated apoptosis. Decoy receptors, like soluble Fas (sFas) or various TRAIL receptors (−R3, −R4), have been described in tumors (reviewed in: Töpfer et al. 2011). Increased serum level of sFas was detected in various tumors, and correlated with poor outcome in melanoma patients (reviewed in: Igney and Krammer 2002). T cells can also eliminate target cells by the perforin/granzyme pathway. It was 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 patients outcome (Medema et al. 2001, van Houdt et al. 2005). 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, similar to tumor-infiltrating myeloid cells in nonsmall cell lung cancer (Jadus et al. 2012). 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 (Rabinovich et al. 2007, Keir et al. 2008, Chemnitz et al. 2007). Overexpression of PD-L1 on ovarian cancer epithelial cells is a mechanism of possible importance for intraepithelial T $CD8^+$ depletion and deactivation (Hamanishi et al. 2007). Lung tumors possessing high expression of PD-L1 showed less TILs compared to B7-H1-negative tumors (reviewed in: Jadus et al. 2012). The precise mechanism of PD-1/PD-L1 interactions is probably based on upregulated 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 (Xiao et al. 2012). Tumor cells subjected to apoptosis generate apoptotic bodies, a structure 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 antitumor T $CD8^+$ lymphocytes (reviewed in: D'Souza-Schorey and Clancy 2012).

1.4.7 The Role of Tumor Stroma in Immune Escape

Solid tumors are composed not only of neoplastic cells, but also of stroma containing fibroblasts, extracellular matrix, endothelial cells, and tumor-infiltrating immune cells. One of the most important population of cells which are residents in tumor stroma are CAFs. These cells met with growing interest, due to their capabilities to initiate and promote tumor growth (reviewed in: Östman and Augsten 2009). The population of CAFs gathers distinct subpopulations of fibroblasts; however, their precise functions and differences between them still await investigation. Another interesting question is 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 EMT mechanism (reviewed in: Franco et al. 2010). 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 a cooperation with surrounding tissues, cancer cells cannot form an aggressive tumor. The interaction between the fibroblasts and ECM in cancer reminds processes of tissue repair, however, disturbed during carcinogenesis (reviewed in: Franco et al. 2010). CAFs produce growth factors exerting tumor-promoting activity, like epidermal growth factor (EGF), FGF, TGF-β, platelet-derived growth factor (PDGF), or IGF (Kalluri and Zeisberg 2006, Östman and Heldin 2007). The population of CAFs also showed expression of chemokines CCL5, CXCL12, and CXCL14, which are responsible for tumor metastatic potential (Karnoub et al. 2007), increased angiogenesis (Orimo et al. 2005), and influx of macrophages into the tumor (Augsten et al. 2009). Previous studies showed that CAFs are an alternative source of VEGF-A capable of compensating the lack of tumor-derived VEGF-A (reviewed in: Ferrara 2010, Kammertoens et al. 2005). These factors act in 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 upregulation of NF-κB transcription factor (reviewed in: Franco et al. 2010). 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 (Akashi et al. 2008). IGF-1 expressed by prostate tumor stroma stimulates proliferation of epithelial cells by upregulation of MAPK, AKT, and cyclin D1. In 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 TGF-β intercellular Smad pathway and blocks apoptosis of epithelial cancer cells (Saikali et al. 2008). 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 (Hayward et al. 2001, Macintosh et al. 1998). Similarly, the murine studies of breast cancer indicated that implantation of tumor cells together with fibroblasts not responding to

TGF-β into laboratory animals augmented growth and metastases of implanted cancer (Cheng et al. 2005). 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: Franco et al. 2010). 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 proinflammatory cytokines (Aoki et al. 2006).

During some physiologic processes, like embryonic development and wound repair, there is a temporal 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 (EMT) and occurs also in pathological conditions during cancer development and progression. The EMT is an active process during which epithelial cells loose intercellular connections and acquire migratory capacities (Bates and Mercurio 2005). Cell adhesion molecule epithelial E-cadherin belongs to the key negative regulators of EMT, which are 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: Bates and Mercurio 2005; Srivastava et al. 2012). 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 (Becker et al. 1994; Hirohashi 1998). The initiating signal for EMT is delivered by both tumor- and stroma-derived TGF-β which cooperates with activated Ras pathway (Bhowmick et al. 2001; Fujimoto et al. 2001). EMT accelerates significantly upon TNF-α costimulation with TGF-β (Bates and Mercurio 2005). Following the changes of E-cadherin functions, the alterations in expression of integrin αvβ6 receptor for fibronectin and tenascin occur. The inflammation and tissue repair mechanisms are both the stimulators of this change (reviewed in: Bates and Mercurio 2005). Upregulation 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 the self-perpetuating loop (Busk et al. 1992; Kemperman et al. 1995). As a result of EMT, a single cancer cell migrates in the absence of any intercellular contact, and their survival depends on the autocrine VEGF/Flt1 interactions (Bates et al. 2003). Snail transcription factor expression was confirmed in nonsmall cell lung cancer and melanoma, and correlated with shorter survival and predisposition to metastases, respectively (Yanagawa et al. 2009; Kudo-Saito et al. 2009). Murine studies indicated that snail expression affects the function of MDSCs, as snail-knockout mice were characterized by reduced number and arginase activity (reviewed in: Srivastava et al. 2012).

1.4.8 *Microvesicles and Exosomes—Mediators of Tumor Escape*

Microvesicles are small membrane-enclosed structures shed from the variety of cells, including cancer, which are present in both physiological and pathological conditions in body fluids, like 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 (Muralidharan-Chari et al. 2010). 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 (Muralidharan-Chari 2010; Dainiak and Sorba 1991). However, compared to normal cells, tumor cells could shed microvesicles from entire surface, especially from the invading cellular edges (Giusti et al. 2013). The function and contents of microvesicles depend on the cell type which they originate from (Piccin et al. 2007). Tumor-shed microvesicles contain cytokines, miRNA, mRNA, FasL, chemokine receptors, tissue factor, EGFR, Her-2, metalloproteinases, or other molecules (reviewed in: Muralidharan-Chari 2010). 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: D'Souza-Schorey and Clancy 2012). 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 (Valenti et al. 2007). It was demonstrated in mouse model that microvesicles shed from highly metastatic melanoma cells were able to change the phenotype of weakly metastatic melanoma cell line into aggressive phenotype capable of metastasizing (Poste and Nicolson 1980). Similarly, the oncogenic receptor EGFRvIII found on the aggressive gliomas was transferred to a nonaggressive population of tumors (Al-Nedawi et al. 2008). Moreover, the number of microvesicles was shown to correlate with invasiveness of tumor *in vitro* and *in vivo* (Ginestra et al. 1999). Similarly, early stages of ovarian cancer were characterized by lower number of microvesicles in malignant ascites compared to advanced disease (Graves et al. 2004). Microvesicles containing mRNA, miRNA, or fragments of genomic DNA could influence the transcriptome of the target cells and augment tumor invasiveness (reviewed in: D'Souza-Schorey and Clancy 2012). Tumor-derived microvesicles stimulate endothelial cells and stromal fibroblasts to promote neoangiogenesis 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 esfingomielin which in autocrine manner further promoted endothelial

invasion to the stroma. Those processes were stimulated by hypoxic conditions (reviewed in: Muralidharan-Chari et al. 2010). 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 (Castellana et al. 2009; Wysoczynski and Ratajczak 2009). 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 $CD14^{+}HLA\text{-}DR^{-}$ phenotype, indicated lack of costimulatory molecules upregulation, and started to secrete TGF-β (Valenti et al. 2006). Fas-containing cancer-derived microvesicles induced apoptosis of T cells and abrogated their killing abilities (Wysoczynski and Ratajczak 2009). Tumor cells can escape effector immune cells-mediated apoptosis by preventing the intracellular accumulation of caspase-3, and abrogating of microvesicles production was shown to increase of caspase-3 and apoptosis of tumor cells (reviewed in: Giusti et al. 2013). 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 hypoxic environment and played probable role in upregulation of tumor-metastasizing capacity (reviewed in: Muralidharan-Chari et al. 2010). Association between the presence of tissue factor (TF)-containing microvesicles shed from the tumor and increased risk of thromboembolism suggests their role in hypercoagulative state observed in cancer patients (Zwicker et al. 2009). And finally, microvesicles could participate in tumor chemoresistance, as tumors treated with doxorubicin and cisplatin demonstrated shedding of microvesicles containing accumulated, high-concentrated drugs (Shedden et al. 2003; Safaei et al. 2005).

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: Zhang et al. 2012). Release of exosomes is regulated by calcium ionophores, phorbol esters, and inositol 3-kinase inhibitors, as well as indirectly by p53 (Clayton et al. 2001, Yu et al. 2009). Exosomes may contain numerous proteins, mRNA, miRNA, lipids, and other active molecules, and influence the cells locally in autocrine and paracrine manner, as well as can regulate the function of distant cells. Exosomes may impact various cellular responses and are engaged especially in regulation of inflammatory processes (reviewed in: D'Souza-Schorey and Clancy 2012). The presence of signal molecules on the exosomes' surface directs them to the target cells and provides their endocytosis or phagocytosis (Thery et al. 2002). Endocytosis of exosomes is energy-consuming process which may occur in clathrin-dependent way and additional endocytosis mechanisms, and which needs both proteins included in exosome and proteins of target cell (Escrevente et al. 2011). 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 was confirmed in

vascular circulation, body fluids, and malignant ascites (reviewed in: Zhang et al. 2012). Studies performed on the mouse model of cancer demonstrated that transplantable breast tumors were capable to accelerate growth by releasing exosomes which decreased the number and cytotoxic activity of NK cells. The *in vitro* effects of exosomes originated from human breast cancer and melanoma on NK cells were identical (Liu et al. 2006). FasL- and TRAIL-expressing exosomes were also shown to induce apoptosis in tumor-specific activated T effectors (Abusamra et al. 2005). Treatment of immature mouse DCs with exosomes derived from breast cancer blocked maturation of DCs and stimulated prooncogenic cytokine response, as indicated by increase of IL-6 and activation of STAT3 pathway (Liu et al. 2006, reviewed in: Zhang et al. 2012). Tumor exosomes containing PGE_2 and TGF-β also promoted MDSCs to decrease T-cell cytotoxicity (Xiang et al. 2009). *In vivo* studies showed the presence of exosomes in cancer patients' sera, the 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: Whiteside et al. 2011). Therefore, exosomes may be viewed as modulators of immune response and inducers of both local and peripheral tolerance toward tumor (reviewed in: Valenti et al. 2007). However, some studies demonstrated that DC-derived exosomes could stimulate antitumor T-cell responses and activate NK cells. Probably different composition of tumor-derived and DC-derived exosomes could be responsible for that discrepancy (reviewed in: Zhang et al. 2012).

1.5 Conclusions

Cancers are capable not only to escape from host immune surveillance, but also to modulate it in order to improve conditions for tumor growth and metastasizing. To achieve this, tumors use a complex and diversified combination of mechanisms. Therefore, treatment based simply on either enhancement of tumor antigenicity or patient's immune response, although effective in many circumstances, still lacks satisfactory accuracy. Management based on multidirectional disorganization of tumor growth or a combination of biological and chemical drugs seems to show more optimistic results; however, a much deeper knowledge about tumor biology is needed to achieve more satisfactory results.

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Chapter 2
Tumor: Stroma Interaction and Cancer

Michael P. Rogers, Zhiyong Mi, Neill Y. Li, Philip Y. Wai, and Paul C. Kuo

Contents

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.

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Keywords Cancer · Tumor microenvironment · Immunoediting

Abbreviations

bFGF	Basic fibroblast growth factor
BM	Basement membrane
BSP	Bone sialoprotein
CAF	Cancer-associated fibroblasts
CDE	Cancer-associated fibroblast-derived exosome
CSC	Cancer stem cell
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
GM-CSF	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–Darby canine kidney
MDSC	Myeloid-derived suppressor cell
MEPE	Matrix extracellular phosphoglycoprotein
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

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 (Hanahan and Weinberg 2000). 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, 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 includes 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 (Hanahan and Weinberg 2011).

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 (Kalluri and Neilson 2003; Kalluri and Weinberg 2009). 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 Nonimmune 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 turnover is required, there is a common predisposition to exposure to injurious toxins, infectious agents, growth factors, or hormones (Table 2.1; (Siegel et al. 2012)). Injury and cellular turnover can lead to the accumulation of genetic alterations required for cancer cell development (Vogelstein and Kinzler 1993). The myriad molecular mutations and

Table 2.1 Types and incidence of epithelial cancers in the United States (Siegel et al. 2012)

Epithelial Cancer	New Cases in US (2012)	Deaths in US (2012)
Anal	6230	780
Bladder	73,510	14,880
Breast	226,870 (female), 2190 (male)	39,510 (female), 410 (male)
Cervix	12,170	4220
Colorectal	103,170 (colon), 40,290 (rectal)	51,090 (colon and rectal)
Endometrial	47,130	8010
Esophageal	17,460	15,070
Gallbladder	9810	3200

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 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 (Kalluri and Weinberg 2009), 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 (Shattil et al. 2010). 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 noncellular components including water, proteins, and polysaccharides that fill interstitial spaces to provide scaffolding and cushioning against external forces and protection of interstitial cells (Frantz et al. 2010). 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 attracts 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 (Sanderson et al. 2005). Hyaluronic acid also contributes to tumor growth by binding to CD44 receptors located on malignant cells, promoting cell differentiation and migration (Naor et al. 2002; Timar et al. 2002). 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 (Csiszar 2001). Engagement between integrins, collagen, and elastin fibers enables 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 (Levental et al. 2009; Hoechst et al. 2009). 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 (Bhowmick et al. 2004). 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 (Lum et al. 2005). In the TME, increased growth factor release enhances heterotypic signaling between stromal cells and malignant cells or among the malignant cells themselves. For example, mitogens that stimulate cell division are overproduced in cancer cells to produce an autocrine proliferative signal pattern (Gruss et al. 2003). Cancer cells can also enhance their sensitivity to growth factors by upregulating growth-factor receptors so that available ligands transmit a greater and more efficient response (Bhowmick et al. 2004). Important growth factors that drive tumor progression within the TME are listed in Table 2.2 (Elenbaas and Weinberg 2001).

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 (Mantovani et al. 2008). 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 pathways 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 parallel the tissue remodeling processes that occur in cancer progression. Dvorak (2019) 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

Table 2.2 Key growth factors found within the TME (Elenbaas and Weinberg 2001)

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-α—TGF-α	Epithelial development can bind EGF receptor by close homology	Macrophages, keratinocytes
Transforming Growth Factor-β—TGF-β	Epithelial–mesenchymal transition, epithelial motility, cellular survival, antiproliferative factor in epithelial cells at early stages of oncogenesis	Mesenchymal stem cells, macrophages
Tumor Necrosis Factor-α—TNF-α	Inflammation, immune cell regulation	Macrophages
Vascular Endothelial Growth Factor—VEGF	Angiogenesis, vasculogenesis, endothelial cell differentiation	Endothelial cells, tumor cells, pericytes

through the extracellular matrix, and 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 (Schafer and Werner 2008). Different cell types arrive into this niche during specific phases in a highly coordinated fashion. Important proinflammatory 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 (Schafer and Werner 2008). Release of

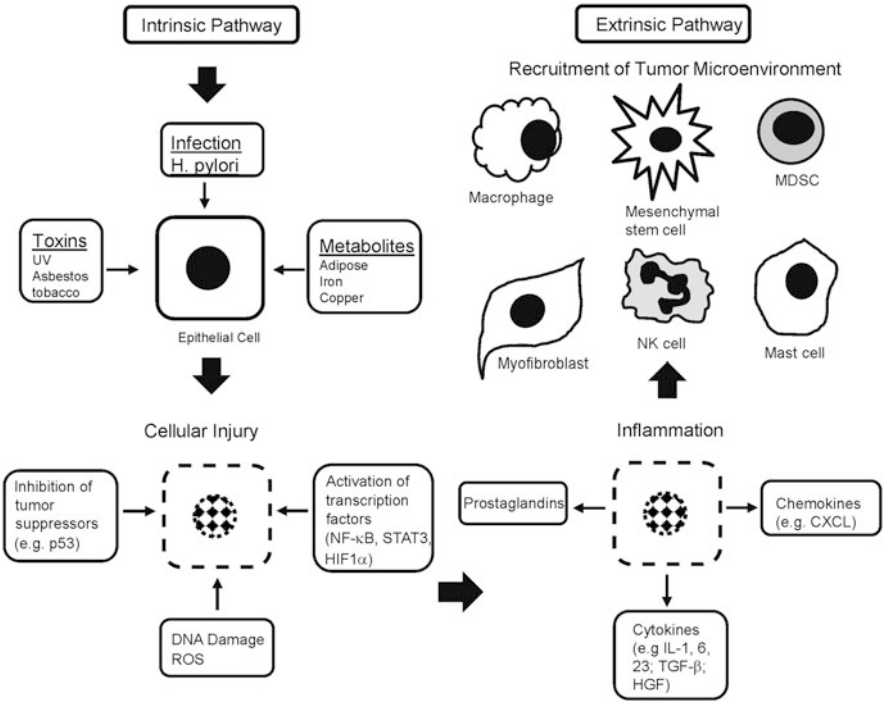

Fig. 2.1 The intrinsic and extrinsic pathways combine to create a local microenvironment around the injured and transformed hepatocyte to augment tumor-promoting mechanisms. *ROS* reactive oxygen species, *HIF1*α hypoxia-inducible factor 1 alpha, *NK cell* natural killer cell, *MDSC* myeloid-derived suppressor cell, *IL* interleukin, *TGF*-β transforming growth factor Beta, *HGF* hepatocyte growth factor, *NF*-κ*B* nuclear factor kappa-light-chain-enhancer of activated B cells, *CXCL* chemokine ligand, *STAT3* signal transducer and activator of transcription 3

cytokines, chemokines, and prostaglandins to recruit additional inflammatory cells, the production of reactive oxygen species (ROS) to destroy infectious vectors, the generation of proangiogenic factors, and modulation of apoptosis represent other essential, activated functions.

Physiological inflammation is often self-limiting through downstream release of anti-inflammatory regulators (IL-10, IL-11, IL-13) which temper the proinflammatory 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" (Dvorak 2019). For example, in chronic disease states of the liver, an environment is often created that enables 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 nonalcoholic 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 enables 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 upregulated expression of protumor cytokines. For example, dendritic cells in neoplastic infiltrates are regulated by tumor-derived granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4 and are frequently immature, less effective at capturing antigens, and defective in T-cell stimulatory capacity (Coussens and Werb 2002). IL-10 released into the TME is a potent inhibitor of dendritic cell activation and differentiation, allowing evasion of host adaptive immunity (Mantovani et al. 2008). Increased serum levels of IL-10 are associated with poor prognosis and reduced survival in patients with various types of cancer (Beckebaum et al. 2004; Chau et al. 2000; Hattori et al. 2003). IL-10 exerts immunosuppressive effects in a variety of ways (Moore et al. 2001), including inhibition of dendritic cell maturation and differentiation, downregulation of costimulatory molecules and major histocompatibility complex (MHC) class I and II, inhibition of antigen priming of näıve T cells (Allavena et al. 1998; Buelens et al. 1995, 1997; McBride et al. 2002), induction of tolerance and promotion of regulatory T cells (Mocellin et al. 2003), and reduction of tumor recognition by cytotoxic lymphocytes (Kundu and Fulton 1997; Zheng et al. 1996). Experimental studies have shown that IL-10 administration before anticancer vaccination results in tumor progression (Berman et al. 1996; Fujii et al. 2001; Groux et al. 1999). 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 (Wang et al. 2012). Glycyrrhizae polysaccharide treatment of HCC in H22 hepatoma-bearing mice decreased tumor burden through downregulation of regulatory T cells, decreased lymph node IL-10 mRNA expression, and decreased serum IL-10 (Berdiel-Acer et al. 2011). 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 antitumor interferon (IFN)-γ-producing capability in peripheral blood lymphocytes (Shiraki et al. 2011). 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* (Kuang et al. 2010).

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 antitumor immune responses, while M2 macrophages are induced *in vitro* by IL-4, and IL-13, and consequently downregulate MHC class II and IL-12 expression while increasing IL-10, scavenger receptor A, and arginase, among other cytokines. M2 polarization is associated with a tumor-permissive environment producing tumor-associated macrophages (TAM) (Aris et al. 2012; Mantovani et al. 2002). 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 (Hagens et al. 2017). 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 (Bhowmick et al. 2001). 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 (Bhowmick et al. 2001). In addition to these mechanisms, the inhibition of tumor-suppressor pathways represents 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 (Hudson et al. 1999). 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 (Mantovani et al. 2002; De Palma et al. 2005) (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 includes the myeloid-derived suppressor cells (MDSCs). These cells are abundant in tumors and strongly inhibit antitumor immunity (Schafer and Werner 2008). 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 (Ostrand-Rosenberg and Sinha 2009). Although no definitive molecular characterization

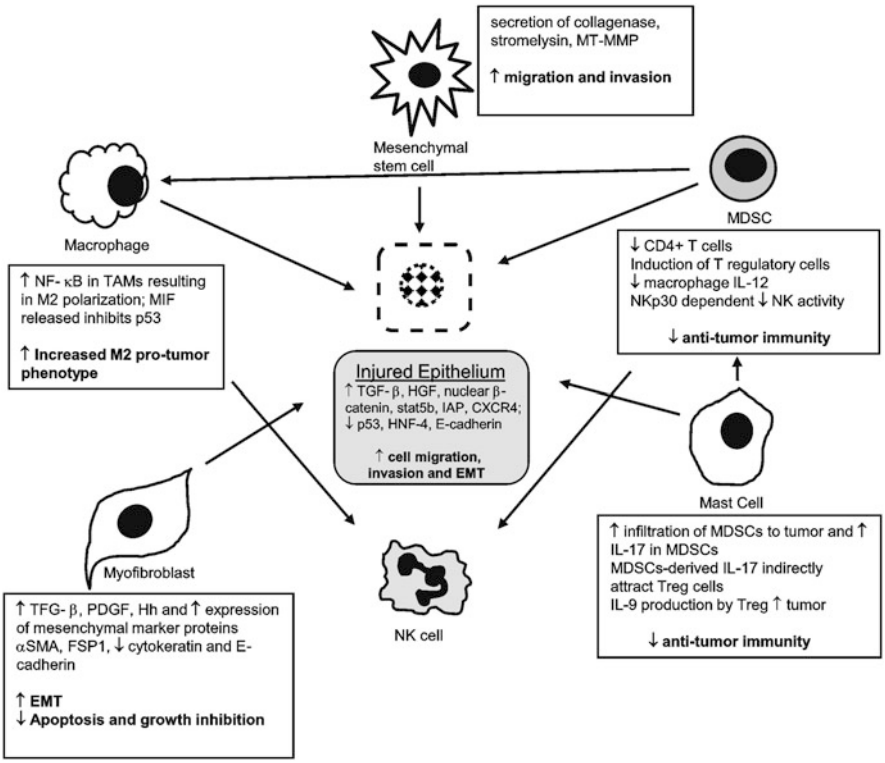

Fig. 2.2 The complex cellular network in the tumor microenvironment mediated by chemokines, cytokines, and cellular transcription factors. *NK cell* natural killer cell, *MDSC* myeloid-derived suppressor cell, *IL* interleukin, *TGF*-β transforming growth factor Beta, α*SMA* alpha smooth actin, *FSP-1* 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

exists, many investigators have found human MDSCs to express CD33, CD11b, and CD15 cell surface markers (Ostrand-Rosenberg and Sinha 2009). MDSC inhibition of antitumor immunity is mediated by suppression of $CD4^+$ T cells (Sinha et al. 2005), inducing T regulatory cells (Huang et al. 2006), by downregulating macrophage production of the type 1 cytokine, IL-12 (Sinha et al. 2007), and potentially suppressing natural killer (NK) cell cytotoxicity (Hoechst et al. 2009). In tumor models, trafficking and accumulation of MDSCs appear to be gp130-dependent, and downregulation of NK-cell cytokine production to be NKp30-dependent (Hoechst et al. 2009). Recent studies have also focused on the 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 (Schafer and Werner 2008). 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 (Direkze et al. 2004). 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 (Gabbiani 2003). 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 (Dvorak et al. 1984). 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 (Shao et al. 2000). 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") (Mani et al. 2008).

Cancer-associated fibroblasts (CAFs) are important contributors to the TME (Berdiel-Acer et al. 2011). 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 (Alison et al. 2007). CAFs have been isolated from a variety of malignant tissues, including prostate, lung, breast, gastric, colorectal, and pancreatic cancers (Kanzaki and Pietras 2020). Reported markers of CAFs have demonstrated partially overlapping cell populations but also show distinct expression profiles (Kanzaki and Pietras 2020). Recent efforts at identifying CAF heterogeneity have garnered significant interest as a potential target for novel therapeutics in CAF subsets. Ongoing research aims to leverage this understanding for clinical benefit. Friedman and colleagues defined Podoplanin marker in a specific subtype of CAFs with significant interest as a potential target for therapy (Friedman et al. 2020). CAFs expressing Podoplanin are reported to be a prognostic indicator in breast and lung cancer and are functionally responsible for the promotion of tumor formation in mouse subcutaneous tissue (Ishii et al. 2016). Consequently, potential therapeutics that target Podoplanin are under current investigation as a targeted therapy in this subgroup of CAFs (Kanzaki and Pietras 2020). Other research into potential therapeutic targets, including immunecheckpoint blockade of $LRRC15^{+}$ CAFs associated with poor response to

anti-PD-L1 therapy, is ongoing and demonstrates a potentially novel therapeutic pathway (Dominguez et al. 2020).

Recently, a Consensus Statement on the basis of a meeting of experts in CAF biology put forth a framework for characterizing CAF origination, markers, activation mechanisms, functions, and subtypes within the TME (Sahai et al. 2020). Reductionist cell culture experiments and mouse models have allowed for further CAF characterization, including their ability to deposit and remodel the ECM. A diverse set of mechanisms has been found to contribute to CAF activation and includes inflammatory signaling (IL-1, IL-6, TNF), physiologic stress (ROS and disrupted metabolism), TGF-β, DNA damage, physical changes in ECM composition, and loss of contact signals (i.e., Notch) (Sahai et al. 2020). Activated CAFs promote local tumor invasion and are able to enhance cancer cell metastasis in experimental models (Biswas et al. 2017; D'Inzeo et al. 2012; Zhou et al. 2011). Cancer cell dissemination further promotes *de novo* activation of fibroblasts at secondary sites which allow for the establishment of macrometastases (Dooley et al. 2008). The secretome of CAFs includes numerous cytokines and chemokines which influence the TME and act on a host of physiologic mechanisms to promote cancer cell development, including influencing angiogenesis, local immunosuppression, and the exchange of metabolites and amino acids (Sahai et al. 2020).

Given the expansive research into CAF biology, a framework for nomenclature has become necessary. Identification of markers to delineate unique CAFs in different carcinoma types is crucial to the development of therapeutic strategies (Dongre and Weinberg 2019). To this end, suggestions have been made to appropriately characterize cancer-associated fibroblasts on the basis of function, cell lineage, and immunomodulation (Sahai et al. 2020). Further, the recommended reporting of CAF metadata and assay standardization is poised to homogenize results and allow for increased applicability. Several studies have identified distinct populations through the use of single-cell RNA sequencing, fluorescence-activated cell sorting, and immunohistochemistry (Kanzaki and Pietras 2020). Five CAF subtypes have been identified in lung cancer with αSMA^{High} and EMT signature subtype defining markers corresponding to angiogenesis and ECM production (Kanzaki and Pietras 2020). Breast cancer patient samples have shown CAF subtypes CAF-S1, CAF-S2, CAF-S3, and CAF-S4 with FAP^{High}, αSMA^{+}, $CXCL12^{+}$, and $IL6^{+}$ defining markers with immunosuppressive and ECM-producing functions (Kanzaki and Pietras 2020). Melanoma has shown immune CAF1, desmoplastic CAF2, and contractile CAF3 with expressions of $CD34^{High}$, $CXCL12^{+}$, $C3^{+}$, $CD34^{low}$, $CTGF^{+}$ TNC^{+}, $PDGFR\alpha^{+}$, αSMA^{High}, and $RGS5^{+}$ subtypes with immunosuppressive, ECM-producing, and contractile signature putative functions. Other CAF subtypes have been identified in head and neck cancers, colon cancer, and pancreatic ductal adenocarcinoma, among others. These proposed classifications will allow for uniformity in the identification of novel CAF subtypes and reporting of results across research groups. Indeed, further understanding of the components of the TME will promote significant breakthroughs in cancer research and in detailing classification of CAF origin, function, subtypes, and future potential therapeutic targets.

Exosomes are an important part of the TME and act as effective signaling molecules between cancer cells and the surrounding cells that comprise the TME (Dai et al. 2020). Recent investigation into exosomes' role in the TME has gained significant attention. CAF-derived exosomes (CDEs) are recognized as a key factor in oncogenic transformation and promote growth of cancer cells through inhibition of mitochondrial oxidative phosphorylation, causing an increase in glycolysis and glutamine-dependent reduction carboxylation in cancer cells (Dai et al. 2020). Evidence also suggests that CDEs promote drug resistance and tumor metastasis. CDEs could also promote neoplastic angiogenesis and tumor development and may also induce the dedifferentiation of cancer cells through the Wnt pathway, promoting chemical resistance (Dai et al. 2020). CAF-produced exosomes have high levels of TGF-β1 (Biswas et al. 2017), which is essential for CAF-induced EMT and metastasis in breast cancer cells. Additionally, tumor cell-derived exosomes (TDEs) provide a source of cellular components that stimulate the immune response through alarmins (mRNA, CD9, CD63, CD81, HSPs, major histocompatibility complex I molecules) and tumor-associated antigens (Ramos-Zayas et al. 2019). TDEs may then contribute to the recruitment and reconstruction of the tumor microenvironment and induce immunosuppression (Jan et al. 2019). As biomarkers, exosomes may be used in early cancer detection, prognostic indicators, or therapeutic monitoring. Exosomes implicated in signal transduction pathways for tumor development, invasive, and metastasis may be targets for specific therapeutic intervention. Further work is ongoing to identify the molecular mechanisms of exosome production and areas of diagnostic and therapeutic development.

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 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 (Barrallo-Gimeno and Nieto 2005; Klymkowsky and Savagner 2009; Polyak and Weinberg 2009; Thiery 2009). 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 (Kalluri and Weinberg 2009). 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 (Gruss et al. 2003; Dooley et al. 2008; Kokudo et al. 2008; Niessen et al. 2008) arises 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 (Mani et al. 2008; Micalizzi et al. 2010; Taube et al. 2010). 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 (Peinado et al. 2004). 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 (Hlubek et al. 2007).

Understanding the molecular cascades that regulate cancer EMT becomes important, as the modulation of this process can potentially reverse the cancer-activating programs. Currently, there are at least more than 11 signaling pathways that have been characterized to activate EMT. For example, the TGF-β pathway through phosphorylation of SMAD2/SMAD3 to form an active complex with SMAD4, in turn activating ZEB1/Snail/Twist transcriptional factors to initiate EMT. The Wnt pathway through Wnt ligand binding to its Frizzled family receptors allows activated β-catenin to translocate inside the nucleus and act as a transcriptional coactivator of TCF/LEF transcriptional factors to initiate EMT. The Notch pathway through which Notch ligands (Delta like and Jagged family) bind with Notch receptors and cause the extracellular domain, cleaved through γ-secretase or TACE (TNFα-Adam metalloprotease converting enzyme), to undergo endocytosis to activate CSL transcriptional factor to initiate EMT. Moreover, some mitogenic growth factor (EGF) or cytokines (IL-6) can also active mTOR/NF-kB or JAK-STAT signal pathways to active EMT. Further, efforts to identify molecular mechanisms underlying EMT-induced immunosuppression may help identify novel immunomodulatory markers to predict tumor progression and response to potential treatment (Dongre and Weinberg 2019). 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, though evidence is mounting (Bakir et al. 2020). Recent work by Panchy and colleagues demonstrating tumor cell plasticity used transcriptomic analysis to demonstrate the high degree to which cells can be along the epithelial to mesenchymal transformation spectrum (Panchy et al. 2019). Confirmation that this transformation is required for metastasis also remains elusive, though it has been suggested in the context of squamous cell carcinoma (Tsai et al. 2012). MET is thought to occur due to cell-intrinsic changes in signaling cascades and epigenetic alterations leading to repression of mesenchymal properties and re-expression of epithelial markers, including E-cadherin (Dongre and Weinberg 2019). 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 coexpressing both epithelial and mesenchymal genes and traits. The stability and precise contextual signals that mediate an intermediate EMT state remains elusive. 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 as intravasation, transport through the circulation, extravasation, and formation of micrometastases (Kalluri and Weinberg 2009; Brabletz et al. 2001; Fidler 2001; Thiery 2002). Paradoxically, cancer cells established at distant secondary sites often resemble the primary tumor from which they were derived, prior to EMT. Partial EMT programs have also been observed during fibrosis as well as carcinoma progression. These observations suggest that the metastasizing cancer cells must be capable of reversing their mesenchymal phenotype via MET during the course of secondary tumor formation (Zeisberg et al. 2005). Current and future technologies focusing around single-cell genomics will help to further elucidate tumor cell temporal and spatial plasticity and dynamics.

2.4.1 TGF-β Signaling

Although the molecular regulation of EMT involves a variety of signals, including Wnt, Notch, mitogenic growth factors, and others 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) (Bhattacharya et al. 2012). 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 (Koinuma et al. 2009). Ubiquitination by E3 ligases and Smurf family proteins contributes to degradation of TGF-β pathway constituents. In this context, Smurf 1 and 2 often interact with Smad7 to regulate ubiquitin-mediated degradation (Meulmeester and Ten Dijke 2011). 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, noncanonical 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

(Bierie and Moses 2006; Hata et al. 1998; Oft et al. 1998) as well as inducing cancer EMT (Kalluri and Weinberg 2009; Song 2007). 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 (Postigo et al. 2003).

TGF-β provides a vital role in activating pro-EMT signals (Miettinen et al. 1994; Tian et al. 2011). The downstream transcriptional activation of Snail, Slug, ZEB1, Twist, and BHLH (Leptin 1991; Wendt et al. 2012; Li et al. 2009) results in the dismantling of cell–cell tight junctions and rearrangement of the actin cytoskeleton (Wendt et al. 2012). 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 (D'Inzeo et al. 2012; Bhattacharya et al. 2012). 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 (Biswas et al. 2017), and ionizing radiation was found to promote TGF-β-related EMT and associated increases in invasiveness and migration in six different cancer cell types (Zhou et al. 2011). 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 (Dooley and ten Dijke 2012). 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 (Kanzler et al. 2001). Alternatively, transgenic mice with upregulated Smad7 expression restricted to hepatocytes demonstrate significantly diminished liver damage and fibrosis, suggesting that TGF-β signaling in hepatocytes is required for fibrogenesis progression (Dooley et al. 2008). 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 (Mantovani et al. 2008). Smad7 activation or RNA interference against Smad4 decreases TGF-β signaling and attenuates the expression of profibrotic genes (Dooley et al. 2008; Kaimori et al. 2007). 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 (Nitta et al. 2008). 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 nontransformed human fetal hepatocytes, while transdifferentiation into a mesenchymal stem cell-like phenotype with increased expression of Snail, decreased E-cadherin expression, and increased

vimentin and N-cadherin expression (protumor) is also TGF-β-mediated (Caja et al. 2011). 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 impair TGF-β apoptosis in cancer cells (Franco et al. 2010).

In other signaling pathways, β-catenin and lymphoid enhancer factor (LEF) also cooperate with Smads in inducing EMT (Kalluri and Weinberg 2009; Yang et al. 2006; Eger et al. 2000; Stockinger et al. 2001). 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 (Bhowmick et al. 2001). Fibulin-5, an ECM molecule, augments TGF-β-induced EMT in an MAPK-dependent mechanism (Korpal et al. 2008). Other MAPK-related mechanisms included TGF-β induction of an EMT in Ras-transformed hepatocytes, mammary epithelial cells (via MAPK), and Madin–Darby canine kidney (MDCK) cells (Gotzmann et al. 2002; Lehmann et al. 2000; Oft et al. 1996). Interestingly, in mouse models of skin carcinoma and human colon cancer, the absence of TGF-β receptor expression confers improved prognosis (Cui et al. 1996; Watanabe et al. 2001). Loss of E-cadherin expression by cancer cells and passage through an EMT has also been shown to be TGF-β dependent (Edelman et al. 1983; Tepass et al. 2000). 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 (Stockinger et al. 2001; Gottardi et al. 2001). β-Catenin accumulation in the nucleus is often associated with loss of E-cadherin expression (Thiery 2002; Kim et al. 2002). Noncoding microRNAs including microRNA 200 (miR200) and miR205 inhibit the repressors of E-cadherin expression, ZEB1, and ZEB2, and maintain epithelial cell characteristics, thereby forming a double-negative feedback loop (Korpal et al. 2008; Gregory et al. 2008). TGF-β is also known to induce the expression of a subset of lncRNAs that promote an EMT in carcinogenesis, fibrosis, and development (Dongre and Weinberg 2019).

2.5 Osteopontin and EMT

Osteopontin (OPN) was initially discovered as an inducible tumor promoter, is overexpressed 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 (Hattori et al. 2003; Berdiel-Acer et al. 2011; Bhattacharya et al. 2012). OPN was initially characterized in 1979 as a phosphoprotein secreted by transformed, malignant epithelial cells (Senger et al. 1979). Investigators have since independently detected this molecule as secreted phosphoprotein I (Spp1), 2ar, uropontin, and early T-lymphocyte activation-1 (Eta-1) (Wai and Kuo 2008). 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) (Fisher and Fedarko 2003). 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 (Denhardt and Guo 1993). 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 (Wai and Kuo 2008). 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 (Bautista et al. 1996; Senger et al. 1988, 1989). 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 (Wai and Kuo 2008).

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 (Merszei et al. 2010). Using the animal model for unilateral ureteral obstruction (UUO), OPN-null mice demonstrated less interstitial fibrosis in comparison with wild-type mice (Yoo et al. 2006). The primary function of OPN is the recruitment, regulation, and differentiation of fibroblasts and myofibroblasts (Lenga et al. 2008). Acting as a chemoattractant 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 (Liaw et al. 1998). 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 (Lee et al. 2008). 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 (Lee et al. 2008). In comparison, investigators using a corneal injury model showed that wound closure was delayed in conditions where OPN function was lost (Miyazaki et al. 2008). 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.

Our laboratory sought to determine whether OPN represents a target for altering EMT induction mediated by TGF-β. Using a cocultured 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. 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 cocultured 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 (Hattori et al. 2003). Medico et al. (Medico et al. 2001) 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. (Hattori et al. 2003) 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 (Bhattacharya et al. 2012). 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. (2008) 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. (Nitta et al. 2008) used lentiviral delivery of microRNA against OPN, and suppressed *in vitro* proliferation and *in vivo* tumor growth of HCCLM3.

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 upregulated in the injured mouse lens before initiation of EMT (Saika et al. 2007). Using OPN-null mice, these authors found that absence of OPN was associated with inhibition of EMT as measured by SMA, TGF-β, and

collagen type 1. In nonsmall cell lung cancers, OPN expression was associated with increased expression of the EMT markers, matrix metalloproteinase-2, Snail-1, Snail-2, TGF-β1-R, matrix metalloproteinase-9, N-cadherin, vimentin, SOX-8, and SOX-9 (Goparaju et al. 2010). 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 (Bhattacharya et al. 2012). Using an animal model, OPN and EMT markers were significantly increased in the metastatic cohort. OPN-aptamer inhibition decreased tumor adhesion, migration/invasion, EMT protein markers, SMA, vimentin, and tenascin-c. *In vivo* treatment with OPN-aptamer inhibition decreased HCC growth by more than tenfold (Bhattacharya et al. 2012).

2.6 Summary

Tumor progression, invasion, and metastasis are 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 have transformed our understanding of how tumors can simultaneously adopt invasive properties while also house themselves at distant metastatic sites. Exciting innovations are ongoing into characterizing CAF subtypes, EMT signaling pathways, and exosome function in TME signaling. 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
Tumor-Infiltrating Lymphocytes and Their Role in Solid Tumor Progression

Theresa L. Whiteside

Contents

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. Today, emphasis has shifted to beneficial effects of TIL for the host and to therapies optimizing the benefits by reducing immune suppression in the tumor microenvironment. Evidence indicates that when TILs are present in the tumor as dense aggregates of activated immune cells, tumor prognosis and responses to therapy are favorable. Gene signatures and protein profiling of TIL at the population and single-cell levels provide clues not only about their phenotype and numbers but also about TIL potential functions in the tumor. Correlations of the TIL data with clinicopathological tumor characteristics, clinical outcome, and patients' survival indicate that TILs exert influence on the disease progression, especially in colorectal carcinomas and breast cancer. At the same time, the recognition that TIL signatures vary with time and cancer progression has initiated investigations of TIL as potential prognostic biomarkers. Multiple mechanisms are utilized by tumors to subvert the host immune system. The balance between pro- and antitumor responses of TIL largely depends on the tumor microenvironment, which is unique in each cancer patient. This balance is orchestrated by the tumor and thus is shifted toward the promotion of tumor growth. Changes occurring in TIL during tumor progression

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appear to serve as a measure of tumor aggressiveness and potentially provide a key to selecting therapeutic strategies and inform about prognosis.

Keywords Cancer · Tumor-infiltrating cells · Lymphocytes · Prognosis

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
CTL	Cytolytic T cell
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
DC	Dendritic cells
EVs	Extracellular vesicles
ICIs	Immune checkpoint inhibitors
IFN-γ	Interferon γ
IGKC	IgG kappa chain
IL	Interleukin
MHC	Major histocompatibility complex
NK	Natural killer cells
NKG2D	nk2G gene
NSCLC	Nonsmall cell lung cancer
PD-1	Programmed cell death protein-1
TAA	Tumor-associated antigen
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
Th	T helper cell
TIL	Tumor-infiltrating lymphocytes
TME	Tumor microenvironment
Treg	Regulatory T cells

3.1 Introduction

The immune cells present in the tumor microenvironment belong to both 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 two decades, largely because of the perception that TIL might play a critical role in carcinogenesis and also might be therapeutically useful. In fact, inflammatory infiltrates into tumors have achieved the status of one of the "Hallmarks of Cancer" by Hanahan and Weinberg (2011) in recognition of the role they play in tumor

progression and in tumor escape from the host immune system. Recent 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 (TME). Phenotypic and functional characteristics of TIL, their localization *in situ*, and their interactions with the tumor cells or nonmalignant cells residing in the tumor have become a subject of intense investigations worldwide. These studies are aimed at the confirmation and validation of prognostic and predictive significance of TIL in patients with cancer. It has also become clear that cancer cells have a complex relationship with the immune system, and that even subtle differences in immune cell infiltrates into the tumor can result in the eradication of cancer cells or in enhancement of their growth.

The dynamic relationship existing between TIL and the tumor has been extensively evaluated in mouse models of tumor growth (Allen et al. 2020) as well as human tumor tissues (Thommen and Schumacher 2018). The TME is formed as a result of prolonged and constantly changing interactions between the developing tumor and the host immune system responsible for immune surveillance (Fouad and Aanei 2017). From its inception, the tumor protects itself from elimination by immune cells and gradually develops mechanisms for suppression of their functions. As tumor progresses, TILs accumulating in the TME become dysfunctional and fail to arrest the tumor progression. The mechanisms of tumor-induced immune suppression include a variety of cellular elements, soluble factors, and subcellular components and are unique in every tumor (Whiteside 2010). The key role tumor-derived factors, including extracellular vesicles (EVs) or exosomes, play in regulating intercellular interactions in the TME has emerged as the major theme of cancer research. The results suggest that every tumor creates its own TME and establishes its own ways for disarming the immune system. While the molecular pathways leading to immune suppression in the TME might be the same, the constellation or mix of various suppressive factors seems to be distinct for each tumor. Thus, interactions between the tumor and TIL are unique for each tumor, even for the tumors of the same origin and histology. Further, the heterogeneity in immunoregulatory pathways may exist within the same tumor, depending on regional or local environmental stimuli. The term "tumor heterogeneity" implies that within the tumor mass, there are considerable differences in cellular as well as molecular and genetic characteristics.

In this brief review, I will summarize the current perception of the role TILs play in tumor progression or responses to oncologic therapies and describe immunoregulatory mechanisms that exist in the TME. I will focus on T cells, B cells, and natural killer (NK) cells. While other leukocytes, M1 and M2 macrophages, dendritic cells (DC), and neutrophils (PMN) are all important components of the TME, it is TILs that remain in the highlights. This is due to newly acquired insights into potential of TIL as potential prognostic or predictive biomarkers in cancer and also as components of a promising therapeutic strategy, in which *in vitro*-expanded TILs are adoptively transferred to patients with cancer.

3.2 Studies of the Intratumor Immune Landscape

Technological advances in cellular, molecular, and genetic evaluation of TIL populations or single infiltrating immune cells have provided a wealth of novel information about the spatial distribution of TIL in the tumor, frequency of various TIL subsets, and their functional attributes. Given the heterogeneity of human tumors and the complexity of personalized cellular and molecular interactions in the TME, it is not surprising that monitoring of the TME has been a difficult task, and that biomarkers of tumor progression or response to therapy are not readily identifiable. Dissecting the complex interplay between immune and tumor cells to identify such biomarkers requires the integration of multiple currently available approaches into a "systems biology" approach (Bracci et al. 2020). Systems biology employing multiomics technologies represents a combination of genetic, epigenetic, transcriptional, proteomic, and metabolomic methodologies with immunological insights to provide a comprehensive view of the tumor immune landscape (Bracci et al. 2020). Systems biology employing multiomics technologies is most likely to characterize mechanisms underlying cellular interactions in the TME and to define biomarkers of response to therapy (Bracci et al. 2020). Today, while various multiomics technologies are slowly being applied to studies of immune-tumor interactions, the integrative analyses of TILs *in situ* supported by bioinformatics, computational science, and clinical correlations are still not widely available and require implementation.

Despite the existing barriers, studies of TIL *in situ* have rapidly progressed from immunohistology profiling of immune phenotypes or definition of immunoregulatory cell subsets, to highly sophisticated, multiparameter genetic, and immunological analyses of the TME, where interaction of TIL with tumor cells and each other takes place. A broad variety of monitoring strategies is now available for studies of TIL and tumor cells *in situ* (Yadav et al. 2014). These include sequencing of the whole genome, defining of gene signatures, epigenetic modifications, and changes in protein expression of tumor and immune cells. Further, in TIL, we can define the immune score, T- or B-cell receptor repertoires, identify different types of immune cells by flow cytometry or CyTOFF-based mass spectrometry, and perform multispectral immunocytochemistry (Galon et al. 2012; Giraldo et al. 2019; Maby et al. 2020). Using these strategies, human tumors can be categorized into immune cell-rich ("hot") or immune cell-depleted ("cold") tumors (Giraldo et al. 2014). The former are considered to be immunologically responsive, or "hot," and the latter immunologically unresponsive ("cold") tumor types (Giraldo et al. 2014). Thus, the extent of infiltration of immune cells into the TME emerges as a general measure of the tumor response to immunotherapy. "Sterile" or poorly infiltrated tumors might not be suitable candidates for immune therapy.

The mutational tumor load might be a promising predictive measure of therapeutic response, whereby tumors with a high mutational burden, and consequently enriched in neoantigens, are viewed as immunogenic and potentially more responsive when treated with immune therapies (Snyder and Chan 2015; Strickler et al. 2021). Efforts made to correlate mutational tumor loads with immune cell landscapes

to reinforce the predictive algorithm of response to therapy are ongoing and remain inconclusive. Whole-genome sequencing and RNAseq of formalin fixed and paraffin embedded (FFPE) or fresh-frozen tumor tissues are routine procedures that are widely used to define the mutational landscape of tumors and to identify the potential driver mutations in individual tumors (Snyder and Chan 2015; Duan et al. 2014; Robins 2013). The availability of The Cancer Genome Atlas (TCGA) database with its extensive roster of gene profiles for different tumors or types has been a valuable resource for identifying mutations as well as immune subtypes and functional gene modules, including immune cell-specific genes (Thorsson et al. 2018). Next-generation sequencing (NGS) in combination with newly developed bioinformatic programs offers the means for establishing gene signatures/patterns not only for tumor cells but also for TIL. The intratumoral signatures of these T cells can be determined on a patient-specific basis (Fridman et al. 2017). Further, NGS data can be applied to the neoantigen prediction pipeline that evaluates antigen processing, binding to MHC class I and gene expression to generate a map of mutation-associated neoantigens (MANAs) specific to the patient's HLA haplotype. Neoantigen expression and immune signatures can then be further interrogated by RNAseq.

Single-cell sequencing of tumor cells as well as immune cells is readily applicable to fresh human tumor specimens. Tumor tissues are enzymatically digested and single tumor or single immune cells are isolated by flow cytometry for single-cell (sc)RNAseq (Tirosh et al. 2016). This approach provides gene profiles of both tumor and immune cell types and allows for testing of correlations between the mutational tumor landscape and immune cells in the TME. A search for T cells which are naïve, regulatory, cytotoxic, or exhausted, based on differentially expressed genes typifying these T-cell subsets, identifies distinct clusters of the T cells and allows for heat maps to be constructed and for the estimation of their abundance in the tumor tissue. Special computational algorithms are available to do so, and the immune signatures of TILs can be identified and chartered (Wang et al. 2016). Specifically, signatures of immune dysfunction-associated genes, such as, e.g., elevations in the FOXP3 gene expression characterizing Tregs or in genes for exhaustion markers in $CD8^+$ T cells, can be established. Overexpression of genes that mediate immune dysfunction in the TME (e.g., TGFβ, CTLA-4, PD-L1) is often a sign of neoplastic progression. Although these analyses performed at the RNA level may be potentially skewed because of the presence of posttranscriptional modifications in proteins that mediate cellular functions, studies of transcriptomes from tumors have been useful in defining the TME in individual tumors (i.e., personalized analysis) or in tumors with a common histologic type.

Protein-based phenotypic and functional analyses of immunoinhibitory ligands associated with immune dysfunction, such as PD-L1, CTLA4, or TGF-β, are an important tool. Based on results of these analyses, it may be possible to establish an association between the signature of immune dysfunction in the tumor, the immunomodulatory ligands expression in the TME, and the genetic alterations identified by NGS. The next critical step would be to link these findings to clinical endpoints, including a patient's response to therapy and outcome. This type of assessment, which is applicable to FFPE tissue samples and is largely based on genetic profiling

of the tumor and of immune cells found in the TME, is slowly eliminating the dependence on conventional pathological examinations. Phenotypic and functional assessments of isolated TILs without mechanistic and genetic insights that shape their physiology have become obsolete. The above-described analyses of TIL in tumor tissues have resulted in the recognition of TIL as a biomarker of prognosis and response to therapy (Fridman et al. 2017). Further, TIL and their antitumor potential are being explored in adoptive immunotherapy of cancer.

3.3 Immune Score in the TME

Favorable associations of dense T-cell infiltrates with improved prognosis of many human cancers have been reported for decades. Immunohistochemistry of fresh-frozen or FFPE tumor sections has been instrumental in establishing the grading scale for immune cell infiltrations into the tumor now referred to as "immune score" (Galon et al. 2012). In 2006, Galon and colleagues demonstrated the prognostic significance of these TILs (Galon et al. 2006). The immune score uses systems biology and an objective scoring system to measure the type, density, and localization of immune cells within the TME. In a series of studies in colorectal carcinoma (Mlecnik et al. 2011) and later in other solid tumors (Fridman et al. 2011), Fridman et al. performed immunostaining of hundreds of tumor specimens and showed that a strong local immune reaction, including $CD3^+CD8^+$ and memory $CD45RO^+$ T cells, correlated with a favorable prognosis regardless of the regional tumor involvement or the tumor stage (Fridman et al. 2011). In subsequent independent studies, the prognostic role of infiltrating T cells was confirmed and has led to the proposal for routine evaluation of the TME for density, location, phenotype, and function of immune cells as a part of the standard pathological examination (Galon et al. 2014). The globally collected data strongly support the predictive value of the immune score (Van den Eynde et al. 2018), which is currently widely employed for testing its predictive value for response to immunotherapies, including immune checkpoint inhibitors (ICIs).

3.4 Antitumor Effects of TIL

Traditionally, T lymphocytes, and especially $CD8^+$ cytolytic T cells (CTL), have been considered the major antitumor immune effector cells. They are MHC class I-restricted and when specific for cognate tumor-associated antigens (TAA) become activated, produce perforin, granzymes, and cytokines which induce death of tumor cells but spare nonmalignant 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 (Fregni et al. 2012). These lymphocytes are mediators of cellular antitumor immunity. B cells, which upon Ag-specific activation give rise to antibody (Ab)-producing plasma cells, mediate humoral antitumor 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 antitumor 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 antitumor responses. The presence of B cells, which often form follicular-like structures in the TME, has been recently recognized as a potential prognostic biomarker, and the involvement of infiltrating NK cells in cooperative antitumor effects has been confirmed (Freud et al. 2017). These antitumor effects of TIL are being actively explored in cancer therapy (Freud et al. 2017).

3.4.1 CD8$^+$ Cytolytic T Cells

The presence and effector functions of T cells in the tumor remain the major interest of most studies. Analyses of the diversity in cellular composition of immune infiltrates in various tumor types can define unique tumor "immune signatures" that correlate TIL with outcome, providing prognostically relevant immune classification of human cancer potentially equal to or better than the conventional tumor-node-metastasis (TNM) classification (Hendry et al. 2017). In addition to the overall TIL immune score, the presence, frequency, or *in situ* localization of CD8$^+$ T cells in immune tumor infiltrates is of critical importance as is functional evaluation of their antitumor activity. The availability of standardized single-cell assays able to detect tumor antigen-specific T cells (ELISPOT, cytokine flow cytometry, and tetramer binding) among TIL has greatly facilitated evaluations of their potential value as prognostic biomarkers in cancer (Britten et al. 2011). 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 the Trail/TrailR pathways (Whiteside 2008) or indirectly through the release of tumor-derived exosomes carrying death receptor ligands (Whiteside 2013). 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 (Whiteside 2008). 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 PD-L1 or TGF-β in exosomes, could contribute to apoptosis of antitumor effector T cells among TIL and thus to tumor escape from the host immune system (Ferrone and Whiteside 2007). In aggregate, these studies suggest that the presence of death-inducing ligands on tumor cells or carried by tumor-derived exosomes contributes to

elimination of TIL responsible for antitumor effects in the TME (Mittendorf and Sharma 2010). Thus, antitumor effector $CD8^+$ T cells accumulating in the TME and expected to eliminate tumor cells become dysfunctional or "exhausted" due to immunosuppressive activities of the tumor. TIL exhaustion in the TME favors tumor progression. For this reason, the "immune score" when used as a biomarker of outcome should contain estimates of tumor-induced suppression, e.g., numbers and disposition of exhausted T cells. The exhausted T cells overexpress various inhibitory surface receptors, such as PD-L1, lymphocyte activation gene-3 (LAG-3), T-cell immunoglobulin and mucin domain-3 (TIM-3); secrete interferon (IFN) γ and low levels of the effector cytokine, tumor necrosis factor (TNF) α. In the TME, where ligands that stimulate signaling via these receptors are commonly present, suppression of antitumor responses is profound. These receptors are therapeutic targets for checkpoint inhibition aimed at restoration of antitumor activity of T cells (Pardoll 2012).

Although activated $CD8^+$ T cells are present in many human tumors, these tumors fail to undergo spontaneous regression. This is likely due to regulatory mechanisms which inhibit T-cell responses in the TME (Mittendorf and Sharma 2010). These mechanisms can operate at the level of tumor cells inducing, e.g., loss of tumor antigens or downregulation of class I MHC molecules rendering the tumor invisible to CD 8^+ effector T cells (Ferrone and Whiteside 2007). Alternatively, as suggested above, T cells upregulate 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 upregulate CTLA-4, an inhibitory receptor that counteracts the stimulatory receptor, CD28 (Pardoll 2012). 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 (Hugo et al. 2016). Still another regulatory break is the presence in the tumor microenvironment of suppressor cells, such as Treg (see below) or myeloid-derived suppressor cells. These regulatory cells produce inhibitory cytokines (e.g., IL-10, TGF-β) or suppressive factors which dampen or abrogate antitumor immunity (Groth et al. 2019; Whiteside 2012).

Today, in the checkpoint inhibitor era, much attention has been paid to T-cell activation or reinvigoration in the periphery and in the TME after immunotherapy. It appears that patients with solid tumors who respond to ICIs have greater $CD8^+$ T-cell density at the tumor margin and their numbers/phenotypes are associated with the gene inflammation signature and high tumor mutational burden (Linette and Carreno 2019). However, the specificity of $CD8^+$ TIL for tumor-associated antigens or neoantigens remains poorly defined representing a significant challenge for cancer immunologists (Linette and Carreno 2019). NGS of TCR-Vβ repertoire in TILs can reveal different levels of TCR diversity and prevalence in the tumor as compared to peripheral blood, suggesting that antigen-driven proliferation of cognate T cells occurs in the tumor (Lucca et al. 2021). In some cases, T-cell diversity appears to correlate with the mutational burden of the tumor (Van Allen et al. 2015). Newer data suggest that neoantigen-specific $CD8^+$ T cells are the major effector cells that

mediate tumor regression following checkpoint inhibition (Linette and Carreno 2019).

A subset of $CD8^+$ T cells present in tumors, and relatively recently identified using transcriptome analysis as tissue resident memory T cells (T_{RM}), is a heterogeneous T-cell population with functions of effector and memory T cells (Okla et al. 2021). T_{RM} downregulate the expression markers that regulate their exit from tissue and overexpress markers for tissue retention. This phenotype enables them to traffic to, reside in and patrol, various tissues, exercising a long-term protective role. In tumors, T_{RM} infiltration was shown to correlate with enhanced patients' responses to immunotherapy and associates with favorable prognosis. T_{RM} in the tumor undergo a unique, hybrid effector cell-memory cell differentiation program of effector cells by expression of PD-1, IFN-γ, perforin, and granzymes and of memory cells by their stem-like properties (Okla et al. 2021). Tumor-specific T_{RM} preferentially reside in the tumor milieu, where they proliferate in response to TAA and combat tumor cells or eliminate transformed cells *in situ* (Okla et al. 2021). The reportedly potent antitumor effects of T_{RM} cells suggest they represent potential therapeutic targets for enhancing responses to immunotherapy.

3.4.2 $CD4^+$ Helper T Cells

This subset of T cells is present in solid tumors with the frequency that equals or exceeds that of $CD8^+$ T cells. Several subsets of helper T cells (Th) are recognized, including Th1, Th2, Th17, and Treg. The well-known "Th1/Th2" paradigm (Romagnani 1997) refers to the balance that exists between the functionally distinct subsets of T helper cells (Th). Th1 cells produce cytokines, notably IL-2 and IFN-γ, which play a role in activating and enhancing expansion as well as effector functions of $CD8^+$ T cells and NK cells (Kalams and Walker 1998). Th1 cells also influence the antigen-presenting capacity of DC, thus shaping CTL responses (Knutson and Disis 2005). 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 (Zhu and Paul 2010). 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 (Tatsumi et al. 2002). 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 (Teschendorff et al. 2010). 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 (Teschendorff et al. 2010). 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 serves as 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 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 antitumor responses (Yang et al. 2012). 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 (Kryczek et al. 2009). However, experiments in mouse models of cancer indicate that Th17 may also be involved in protumor functions by promoting angiogenesis (Silva-Santos 2010). IL-17 has been shown to induce expression of proangiogenic factors such as vascular endothelial growth factor, angiotensin, IL-8, and prostaglandin E_2 in stromal, endothelial, and tumor cells (Silva-Santos 2010). The exact cellular mechanisms that determine pro- vs. antitumor 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.

3.4.3 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 33]. The presence and functional competence of Treg inversely correlates with outcome in many, but not all, human tumors (Whiteside 2012; Lanca and Silva-Santos 2012). The existing conflicting reports in 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. It appears that the $CD4^+CD25^{high}FOXP3^+$ natural (n) Treg, normally responsible for maintaining peripheral tolerance, control cancer-associated inflammation (Whiteside et al. 2012), while another subset of Treg, inducible (i) Treg which may or may not be $FOXP3^+$ but produce adenosine and TGF-β, arises by tumor-driven conversion of conventional $CD4^+$ T cells to highly suppressive, therapy-resistant cells. These iTreg appear to be responsible for downregulating antitumor immune responses *in situ* (Whiteside et al. 2012). 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 (Lanca and Silva-Santos 2012). 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. On the other hand, Treg maintain a strong suppression of effector cells in the TME, and their functional attributes might serve as markers of suppression levels existing in the TME. Treg possess a metabolic profile that is distinct from that of effector T cells (Watson et al. 2021). Recent studies showed that glucose uptake by Treg correlates with their poor suppressor function and their long-term instability. In contrast, Treg upregulate lactic acid metabolism, withstand high lactate conditions, and successfully proliferate in the TME. These metabolic differences in utilization of the glycolytic pathway by Treg illustrate their flexibility for survival in the hostile TME by excluding glucose uptake in favor of lactic acid (Watson et al. 2021). Treg exploit the metabolism in the TME and, unlike effector T cells, thrive in the lactate-rich milieu and mediate high levels of immunosuppression. Additional studies evaluating the role of Treg present in the tumor microenvironment as an independent predictor of prognosis in cancer are necessary.

3.4.4 B Cells

B cells 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 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 (Schmidt et al. 2008, 2012). 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, nonsmall cell lung cancer (NSCLC), and colorectal cancer (CRC) (Schmidt et al. 2012; Whiteside and Ferrone 2012). In this multiinstitutional study, the 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 (Schmidt et al. 2012). 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 (Schmidt et al. 2008, 2012). 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 and 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 (Whiteside and Ferrone 2012).

In support of this key role of the B-cell signature, Nielsen et al. (2012) reported that among TIL present in high-grade serous ovarian carcinomas, $CD20^+$ B cells colocalized 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 (Nielsen et al. 2012).

Recently, the role of tertiary lymphoid structures (TLS), which are ectopic cellular aggregates, resembles secondary lymphoid organs in the cellular content and structural organization (Jacquelot et al. 2021). TLS are formed in nonlymphoid tissues in response to local inflammation and are found in solid tumors (Jacquelot et al. 2021). Composed of the antigen-specific B cells and T cells as well as dendritic cells, TLS drive the antitumor immune responses and have an impact on tumor progression. Formation of TLS in the tumor and abundance of TLS associates with favorable clinical outcome (Sautes-Fridman et al. 2019).

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 (Biragyn and Lee-Chang 2012). Thus, human B cells were found to express CD39 and CD73, the ectoenzymes hydrolyzing exogenous ATP to adenosine (Saze et al. 2013). 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 (Saze et al. 2013).

3.4.5 Natural Killer (NK) Cells

NK cells mediate innate immune responses and can mediate direct cellular cytotoxicity without a need for prior sensitization (Freud et al. 2017). NK cells play a key role in cancer immunosurveillance. 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 several others (Freud et al. 2017), which calibrate antitumor 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, MICB, and UL16-binding proteins, which are minimally

or not expressed in nonmalignant cells or tissues. These ligands are promptly and efficiently induced by stress, including malignant transformation, and their overexpression on activated NK cells is regarded as the "danger signal" marking cells for immune elimination. There is little evidence for an association of the NK-cell presence in the TME 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 ADCC. NK cells are also strong IFN-γ producers (Vivier et al. 2011). Unfortunately, NK-cell functions are often found to be downregulated in cancer, and in a study of highly aggressive NSCLC, NK cells were found to have an altered phenotype and were impaired in the ability to secrete IFN-γ (Melaiu et al. 2019). Tumor- and peripheral blood-derived NK cells in patients with cancer are frequently compromised, and in many cases, this impairment has been linked to the tumor progression and poor prognosis (Platonova et al. 2011). Recently, it has been reported that EVs produced by tumor cells play a key role in regulating of immune surveillance by NK cells, which is dependent on receptor–ligand interactions driven by MICA expression in the tumor-derived EVs (Wu et al. 2021). Thus, another mechanism of tumor-induced immune suppression is revealed, and the focus on this mechanism might provide evidence for an association of inhibitory ligand carrying EVs with cancer progression in the near future.

3.5 Summary and Conclusions

The antitumor 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, but this is now being extended to other solid tumors (Fridman et al. 2017). Patients with large infiltrates of T or B cells or increased expression of genes encoding T-cell or B-cell signatures (i.e., high immune score) tend to have better survival compared to those with few tumor-infiltrating immune cells (Fridman et al. 2017). 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, soluble factors, and membrane-bound EVs. Tumor cells themselves also produce immunosuppressive cytokines, a variety of soluble and masses of EVs decorated with immunoinhibitory ligands, which have direct as well indirect effects on immune cells recruited to the TME (Marar et al. 2021). Therefore, cellular composition of the TME and interactions of cells residing within the tumor determine the outcome of antitumor immune responses. As neither the cellular composition nor the cytokine milieus in the microenvironment are constant, because they undergo changes as tumors progress from premalignant to malignant and eventually metastatic phenotype, the impact TIL may have on outcome is highly 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 molecular mechanisms that are responsible for the influence TIL exert on cancer patient

outcome. Understanding of the cellular and molecular mechanisms involved in creating and maintaining this balance is, therefore, necessary for determining of how TIL contribute to survival of patients with cancer and for the selection of therapeutic strategies that could improve patient survival.

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Chapter 4
Tumor-Associated Macrophages: Reasons to Be Cheerful, Reasons to Be Fearful

Izabela Szulc-Kielbik and Michal Kielbik

Contents

Abstract Tumor microenvironment (TME) is a complex and constantly evolving entity that consists not only of cancer cells, but also of resident host cells and immune-infiltrating cells, among which macrophages are significant components, due to their diversity of functions through which they can influence the immune response against tumor cells. Macrophages present in tumor environment are termed as tumor-associated macrophages (TAMs). They are strongly plastic cells, and depending on the TME stimuli (i.e., cytokines, chemokines), TAMs polarize to antitumoral (M1-like TAMs) or protumoral (M2-like TAMs) phenotype. Both types of TAMs differ in the surface receptors' expression, activation of intracellular signaling pathways, and ability of production and various metabolites release. At the early stage of tumor formation, TAMs are M1-like phenotype, and they are able to eliminate tumor cells, i.e., by reactive oxygen species formation or by presentation of cancer antigens to other effector immune cells. However, during tumor progression, TAMs M2-like phenotype is dominating. They mainly contribute to angiogenesis, stromal remodeling, enhancement of tumor cells migration and invasion, and immunosuppression. This wide variety of TAMs' functions makes them an excellent

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subject for use in developing antitumor therapies which mainly is based on three strategies: TAMs' elimination, reprograming, or recruitment inhibition.

Keywords Tumor-associated macrophages · Tumor cells · M1/M2 macrophages · Polarization · Angiogenesis · Metastasis · TAMs targeting therapies

Abbreviations

AKT	serine-threonine protein kinase
Ang-2	angiopoietin-2
APCs	antigen-presenting cells
ARG1	arginase-1
CCL	C-C chemokine ligand
CTLs	cytotoxic T lymphocytes
CXCL	C-X-C motif chemokine ligand
DCs	dendritic cells
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial–mesenchymal transition
GM-CSF/CSF-2	granulocyte-macrophage colony-stimulating factor
HIF	hypoxia-inducible factor
ICB	immune-checkpoint blockade
IFN	interferon
IL	interleukin
JAK	Janus kinase
M1	classically activated macrophages
M2	alternatively activated macrophages
M-CSF/CSF-1	macrophage colony-stimulating factor
MHC	major histocompatibility complex
MIF	migration inhibitory factor
MMP	metalloproteinase
NF-κB	nuclear factor kappa B
NK	natural killer
PD-1	programmed cell death protein 1
PDGF	platelet-derived growth factor
PD-L1	programmed cell death ligand 1
PI3K	phosphatidylinositol 3-kinase
PlGF	placenta growth factor
RNS	reactive nitrogen species
ROS	reactive oxygen species
STAT	signal transducer and activator of transcription
TAMs	tumor-associated macrophages
TEMs	TIE-2-expressing monocytes

TGF	transforming growth factor
TIE	Tek tyrosine kinase receptor
TLR	Toll-like receptor
TME	tumor microenvironment
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

4.1 Introduction

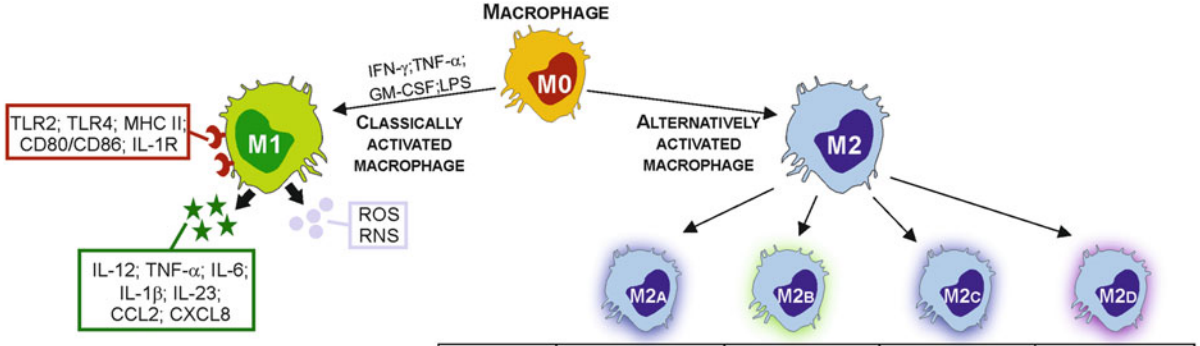
Tumor is not simply a group of cancer cells, as it is a heterogeneous collection of resident host cells, infiltrating cells, multiple secreted factors, and extracellular matrix. Together, they create the tumor microenvironment (TME), which is a complex and constantly evolving entity (Anderson and Simon 2020). It is dominated by cancer cells, which aim to control molecular and cellular events within TME and surrounding tissues via various signaling networks. These intercellular cross-talks are mainly based on the secretion of cytokines, chemokines, growth factors, inflammatory mediators, and matrix-remodeling enzymes. However, there are also other mechanisms of cell-to-cell interactions, such as circulating tumor cells, exosomes, cell-free DNA (cfDNA), or mediators of horizontal gene transfer (HGT) like apoptotic bodies (Balkwill et al. 2012; Denisenko et al. 2018). The consequence of these interactions is reflected in the formation of tumor, and TME being actively involved in working for the benefit of the cancer cells by helping in their maintenance and progression (Hanahan and Coussens 2012; Truffi et al. 2020).

The composition of TME varies between different tumor types, but it generally consists of cellular and noncellular components. Proliferating tumor cells, stromal cells, blood vessels, and immune cells fall into the first category, while exosomes and extracellular matrix to the latter (Baghban et al. 2020). Immune cells are very important component of the tumor microenvironment, and it has been well documented that solid tumors are generally infiltrated by inflammatory cells (Balkwill and Coussens 2004). Both adaptive and innate types of immune response can be observed within tumor microenvironment. Mediators of adaptive immunity are represented by T lymphocytes (T cells) and (occasionally) B lymphocytes (B cells), whereas effectors of innate immunity include polymorphonuclear leukocytes (mainly neutrophils), dendritic cells (DCs), macrophages, and (very rarely) natural killer (NK) cells (Whiteside 2008). Among all of the immune infiltrates, macrophages are one of the most interesting due to their diversity of functions, through which they can influence the immune response against tumor cells.

4.2 Classification of Macrophages

Macrophages, discovered by Élie Metchinkoff in the late nineteenth century, are a type of white blood cells of the mononuclear phagocytic lineage (Mosser and Edwards 2008). Their multifaceted role assumes the maintenance of tissue homeostasis and protection of human body through the detection, engulfment, and destruction of all harmful matter including dead cells, cellular debris, pathogens, and cancer cells. Macrophages are the part of both, innate immunity, comprising the first line of defense against any foreign molecules, and adaptive immunity, by orchestrating inflammatory processes such as other immune cells (i.e., lymphocytes) recruitment, various cytokines secretion, antigen presentation, or complement system activation (Prenen and Mazzone 2019; Zhou et al. 2020). Part of macrophages originates in the bone marrow and enters the blood system as monocytes. Circulating monocytes in the face of inflammation process undergo a series of changes, differentiating to macrophages when they leave the bloodstream and travel to various tissues and organs becoming tissue-specific macrophages (Varol et al. 2015). However, recent studies showed that a majority of macrophages are derived from yolk sac during embryonic development, referred to as tissue-resident macrophages, strategically placed in tissues and organs where microbial invasion or foreign material accumulation is frequent. Alveolar macrophages in lungs, Kupffer cells in liver, osteoclasts in bones, epidermal Langerhans cells, brain microglia, histiocytes in spleen, and the interstitial connective tissue or intestinal macrophages in guts are the examples of tissue-resident macrophages that characterize with self-renewal ability and a lifespan of about several months or even years (significantly longer than in the case of circulating blood monocytes—which is about a day). They fulfill a great variety of functions, acting not only as phagocytes fighting pathogens, but they are also the guardians of homeostasis in the body, secreting various factors important for tissue regeneration and recruitment of additional macrophages when needed (Mass et al. 2016; Davies et al. 2013; Epelman et al. 2014).

Regardless of the origin, macrophages can exhibit different effector functions in immune defense and surveillance. Depending on how they are stimulated by the surrounding environment, macrophages roughly differentiate into two main populations with varied physiological functions: classically activated macrophages (M1) and the various forms of alternatively activated macrophages (M2) (Fig. 4.1) (Gordon 2003; Martinez et al. 2006; Mills et al. 2000). M1 macrophages characterize with high proinflammatory properties. They are mainly promoted by Th1-related cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α), or granulocyte-macrophage colony-stimulating factor (GM-CSF also CSF-2) secreted by other immune cells, but also activation of Toll-like receptors (TLR) by bacterial products like lipopolysaccharide-induced polarization to M1 phenotype. Functionally, classically activated macrophages are aggressive phagocytes that participate in the elimination of invading microbes during infection. They are able to produce and secrete multiple proinflammatory cytokines and chemokines, such as interleukin (IL)-12, TNF-α, IL-6, IL-23, IL-1β, C-C chemokine ligand (CCL) 2, or C-X-C motif



STIMULI	IL-4; IL-13	IC; TLRs; IL-1R	IL-10; TGF-β; glucocorticoids	LIF; CSF-1; adenosine; IL-6
EXPRESS	MR; SR; ARG1; IL-1RII; dectin-1	MHC II; CD80; CD86	MR; SR; CD14; CD150	SR
PRODUCE	IL-10; TGF-β; IL-1ra; CCL17; CCL18; CCL22; CCL24	IL-10↑ ; IL-6; IL-1β; TNF-α; CCL1	IL-10↑; TGF-β↑; CCL16; CCL18; CXCL13	IL-10; TGF-β; VEGF
FUNCTION	• stimulate Th2 response • stimulate type II inflammation • stimulate allergy • parasite killing	• metastasis control • tumor growth suppression • Th2 activation	• tissue repair • immune suppression • matrix remodeling	• angiogenesis induction • tumor progression

Fig. 4.1 Classification of macrophages. Depending on environmental stimuli, macrophages (M0) differentiate into two main populations with varied phenotype and physiological functions: classically activated macrophages (M1) and alternatively activated macrophages (M2), which are represented by four different subsets, including M2a, M2b, M2c, and M2d phenotypes

chemokine ligand (CXCL) 8, thereby promoting a Th1 response. Moreover, their important feature is the ability to generate large amounts of reactive oxygen and nitrogen species (ROS/RNS), highly toxic molecules that can kill pathogens, as well as tumor cells. M1 macrophages mediate ROS-induced tissue damage, contributing to tissue destruction and impairing the process of wound healing (Redente et al. 2010; Biswas et al. 2012; Sica and Mantovani 2012; Sica et al. 2015; Murray 2017). On their surface, classically activated macrophages have high expression of TLR2 and TLR4, major histocompatibility complex (MHC) class II, costimulatory molecules CD80/CD86, and receptor for IL-1 (IL-1R) (Redente et al. 2010; Biswas et al. 2012).

To maintain the balance and protect the organism against tissue damage, the chronic inflammatory response caused by M1 macrophages is regulated and inhibited by the action of anti-inflammatory M2 macrophages, involved in homeostatic processes. In general, they play an important role in inducing the Th2 response, tissue repair and remodeling, wound healing, dampening of inflammation, or parasite clearance. Unfortunately, M2 macrophages are also responsible for the tumor formation and progression. Alternatively activated macrophages characterize with: (1) enhanced production of anti-inflammatory cytokines like IL-10 and transforming growth factor β (TGF-β); (2) the expression of arginase-1 (ARG1), an enzyme which supports fibrosis and tissue remodeling functions; and (3) the upregulation of scavenger receptors, while downregulation of MHC II molecules makes them unable to efficiently present antigen (Shapouri-Moghaddam et al. 2018; Lopez-Castejón et al. 2011; Mantovani and Sica 2010). Furthermore, M2 macrophages are represented by four different subsets, including M2a, M2b, M2c, and M2d phenotypes; however, it was recently proposed to define these subsets more precisely, using the particular activator/inducer for each class of macrophages (Murray et al. 2014). Each of these subsets varies a bit in its functions and is induced by different set of cytokines (Fig. 4.1).

- M2a macrophages, induced by IL-4 and IL-13, express high levels of surface molecules (i.e., CXCR1, CXCR2, dectin-1), receptors (i.e., mannose receptor (CD206), decoy IL-1RII, scavenger receptor (CD163)), and proteins (i.e., ARG1, Fizz1, Ym1/2). They produce IL-10, TGF-β, IL-1ra, CCL17, CCL18, CCL22, and CCL24. M2a macrophages mainly stimulate Th2 response, type II inflammation, and allergy and take part in killing and encapsulation of parasites.
- M2b subset exerts immunomodulatory functions, as it controls metastasis, suppresses tumor growth, and is involved in Th2 activation. M2b cells are induced by combined exposure to immune complexes (IC) and TLR or IL-1R ligands, characterized with production of both anti- and proinflammatory cytokines like IL-10 (large amounts), IL-6, IL-1β, TNF-α, and chemokine—CCL1. On their surface, they express CD80, CD86, and MHC II molecules.
- M2c macrophages (also described as deactivated) are induced by IL-10, TGF-β, or glucocorticoids and characterized with anti-inflammatory activities, with these being involved in immune suppression, tissue repair, and matrix remodeling. They release large amounts of IL-10 and TGF-β and chemokines such as CCL16,

CCL18, or CXCL13. On their surface, there is high expression of mannose receptors, scavenger receptors, and CD14 and CD150 molecules.

- M2d subset is induced by adenosine, leukemia-inhibitory factor (LIF), macrophage colony-stimulating factor (M-CSF also CSF-1), and IL-6. These cells secrete mainly anti-inflammatory cytokines such as IL-10 and TGF-β, and on their surface, they express scavenger receptors (i.e., CD163). Importantly, M2d are able to produce high levels of vascular endothelial growth factor (VEGF), thereby promoting angiogenesis what makes them helpful in tumor progression (Shapouri-Moghaddam et al. 2018; Benoit et al. 2008; Cheng et al. 2019; Mantovani et al. 2004; Weagel et al. 2015).

Macrophages present in tumor environment are termed as tumor-associated macrophages (TAMs), and their characteristic properties are similar to M2d subset. However, polarization of TAMs is not so definite, as they are highly plastic cells able to change their polarization after receiving particular signals from the surrounding microenvironment. During the cancer progression, macrophages can modulate their phenotype, and in TME, we can observe both, M1- and M2-like TAMs' populations, that can cross-regulate each other's functions, although in great advantage, TAMs display rather M2 phenotype with tumor-supporting functions (Sica and Mantovani 2012).

4.3 Characterization of Tumor-Associated Macrophages

Tumor-associated macrophages are the major infiltrating leukocytes of TME, especially abundant in solid tumors, and the key cells of the immune system that determine the interactions of cancer cells with the immune components present in the microenvironment (Belgiovine et al. 2016; Noy and Pollard 2014; Raggi et al. 2016). The origin of TAMs is currently the topic of debate, and numerous studies have shown their two main sources. In great advantage, macrophages in TME originate from circulating $Ly6C^{+}CCR2^{+}$ monocytes that are derived from bone marrow hematopoietic stem cells. These inflammatory monocytes are recruited from blood to the tumor site by factors present in TME: particularly by chemokine CCL2, also known as monocyte chemotactic protein-1 (MCP-1), but also other factors: CCL5, CSF-1, CCL20, or VEGF (Liu and Cao 2015; Yin et al. 2019; Larionova et al. 2019). As many recent studies reported, besides circulating monocytes, the second source of TAMs constitutes the long-living, embryonically derived, tissue-resident macrophages. The presence of TAMs derived from these both sources has been proved in some mouse models of brain tumor (Chen et al. 2017; Bowman et al. 2016), pancreatic ductal adenocarcinoma (Zhu et al. 2017), breast tumor (Tymoszuk et al. 2014; Franklin et al. 2014), or lung cancer (Loyher et al. 2018).

TAMs can either positively or negatively affect the growth and behavior of malignant cells; thus, they display dual effect on tumor environment, depending

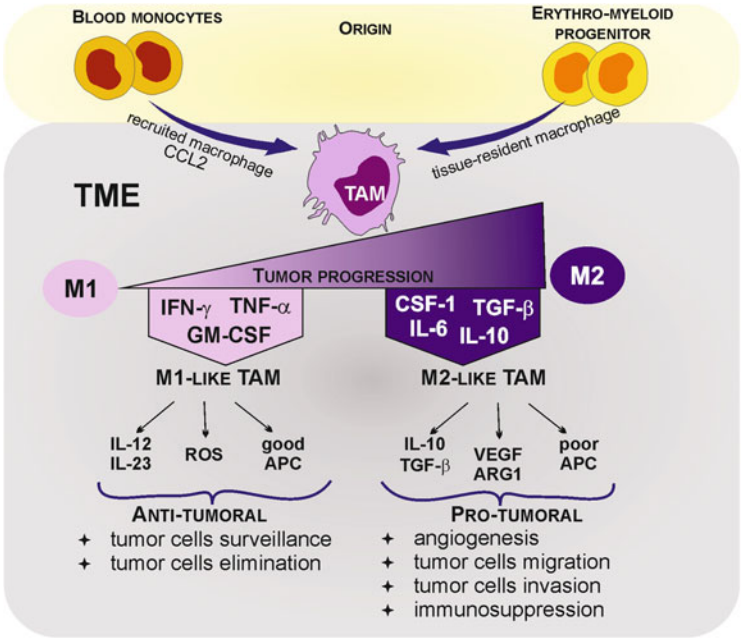


Fig. 4.2 Tumor-associated macrophages (TAMs)—origin and functions. Tumor-associated macrophages (TAMs) originate from both blood monocytes and erythro-myeloid progenitor cells. TAMs can either positively or negatively affect the behavior of malignant cells and the whole tumor microenvironment, displaying dual, antitumoral (M1-like TAMs) or protumoral (M2-like TAMs) effect

on the biological context. Especially at the early stage of tumor formation, TAMs are M1-like phenotype before transferring to the M2-like type (Fig. 4.2). It has been demonstrated in many reports that higher infiltration of M1-like TAMs in the tumor site correlates with a better survival prognosis. Macciò et al. (2020) showed that prevalence of M1-like TAMs and the higher M1/M2 ratio corresponds with longer overall survival and progression-free survival of patients with ovarian cancer. Similar results were shown by Zhang et al. (2014), where ovarian cancer patients with increased M1/M2 TAMs ratio had better 5-years prognosis. Another examples showing that higher M1/M2 ratio and/or higher density of M1-like TAMs was correlated with better patients' survival were reported for neuroblastoma (Liu and Joshi 2020), lung cancer (Ma et al. 2010), breast cancer (Honkanen et al. 2019), gastric cancer (Pantano et al. 2013), or colorectal cancer (Edin et al. 2012).

TAMs with M1-like phenotype, if appropriately stimulated, are able to eliminate tumor cells. They produce large amounts of IL-12 and IL-23, generate ROS and characterize with high capacity to recognize the malignant cells, and present their antigens to the effector cells of the immune system, providing in consequence Th1-type responses toward cancer cells (Belgiovine et al. 2016; Allavena et al. 2008). Important feature of M1-like TAMs, that is essential in tumor surveillance

and elimination, is macrophage-mediated programmed cell removal (PrCR). Activation of macrophages by proinflammatory cytokines (IFN-γ; CSF-2) leads to the induction of TLR signaling, that in turn activates the Burton's tyrosine kinase (Btk) signaling pathway, providing further activation and secretion of calreticulin (CRT) from endoplasmic reticulum and its cell-surface exposure. CRT, previously shown as an "eat-me" signal on cancer cells, exposed on or secreted by macrophages plays a crucial role in mediating the recognition and phagocytosis of adjacent tumor cells, even if they themselves do not express CRT (Feng et al. 2015, 2018). To prevent phagocytosis, tumor cells express "do-not-eat-me" signal (CD47 molecule), inhibiting the whole process. Therefore, blocking CD47 on tumor cells can synergize with the activation of TLR signaling pathways in macrophages to enhance PrCR (Zhou et al. 2020; Feng et al. 2015).

Once tumor is established, TAMs are educated to become supportive for cancer cells (Pollard 2004; Qian and Pollard 2010). The changes taking place in TME during the transition from the benign growth to an invasive cancer are mainly dominated by the profile of cytokines and growth factors present in TME. Secretion of proinflammatory cytokines is reduced in favor of suppressive ones (Noy and Pollard 2014). These include CSF-1, IL-10, IL-6, and TGF-β produced by many types of tumor cells but also by TAMs themselves (Mantovani et al. 2002). Numerous clinical observations and experimental data demonstrated that macrophages assist cancer development and malignant progression. High density of M2-like TAMs correlates with poor prognosis in many types of human cancers: breast (Tsutsui et al. 2005), kidney (Hamada et al. 2002), gastric (Yan et al. 2016), lung (Sumitomo et al. 2019), prostate cancer (Lissbrant et al. 2000), or melanoma (Jensen et al. 2009). Additionally, meta-analysis report prepared by Zhang et al. (2012) showed that high density of TAMs displayed negative effect on overall survival of patients with gastric, breast, ovarian, bladder, oral, or thyroid cancer. During tumor progression, TAMs promote angiogenesis, lymphangiogenesis, and stromal remodeling, enhance tumor cells migration and invasion, and suppress anticancer immunity (Fig. 4.3). M2-like TAMs are poor antigen-presenting cells and are unable to secrete IL-12, but on the other hand, they produce large amounts of immunosuppressive IL-10 and TGF-β, which in turn block T-cell proliferation, suppress cytotoxic T lymphocytes (CTLs) response, and activate T regulatory cells (Treg) (Sica and Mantovani 2012; Belgiovine et al. 2016). At metastatic sites, TAMs take part in preparing tissue for influx of cancer cells and contribute to their extravasation, survival, and later growth (Larionova et al. 2019; Qian and Pollard 2010). Moreover, TAMs are able to support functions of cancer stem cells, a subset of tumor cells able to initiate tumor progression, dissemination, and relapse (Raggi et al. 2016).

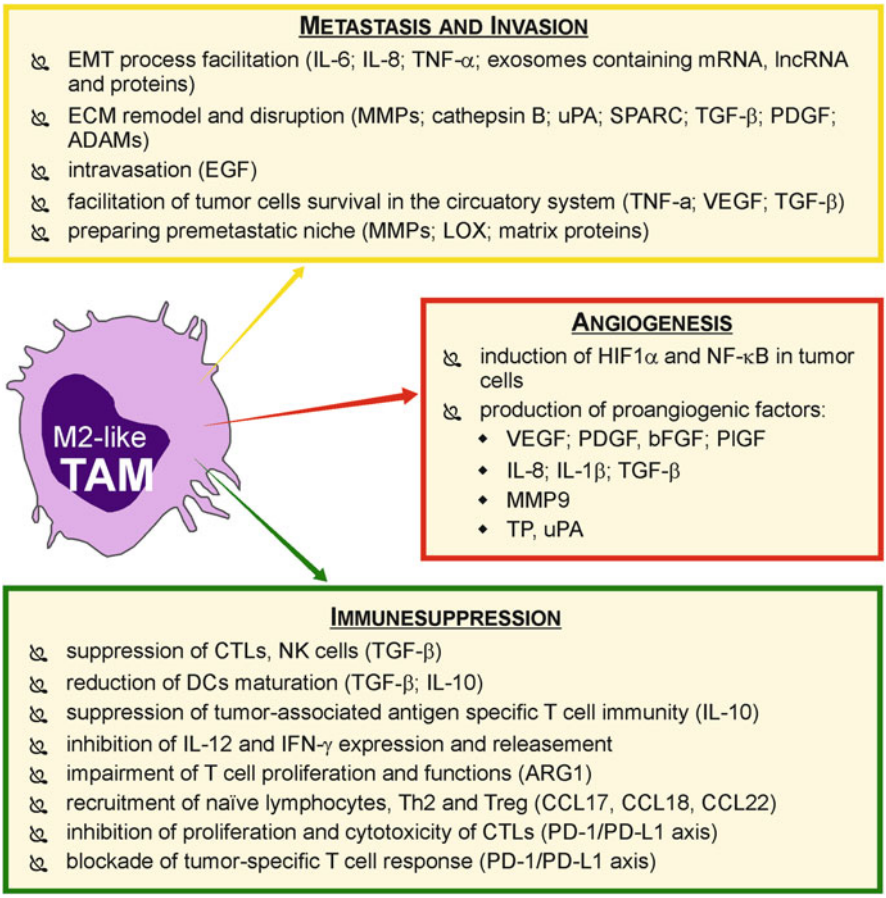


Fig. 4.3 Protumoral features of M2-like TAMs. M2-like TAMs play crucial roles in metastasis, invasion, angiogenesis, and immunosuppression

4.4 Role of TAMs in Cancer Initiation, Promotion, and Progression

4.4.1 TAMs in Chronic Inflammation

The inflammation process is one of the hallmarks of cancer. Chronic inflammation characterizes with sustained tissue damage, damage-induced cellular proliferation, and tissue repair. There are two pathways causing cancer inflammation: (1) an intrinsic one, driven by genetic changes resulting in inflammatory processes and neoplasia; (2) an extrinsic one, driven by inflammatory factors produced by host cells in the context of chronic infections or persistent inflammatory conditions that foster the increased risk of cancer (Erreni et al. 2011). In general, TAMs are

considered to be a linker between cancer and inflammation process, as chronic inflammatory microenvironment is predominated by macrophages that cooperate with other leukocytes, providing tumor development. Generally, it is well recognized that at the early stage of tumor formation, TAMs polarize to M1 phenotype that generates high levels of ROS and RNS (i.e., peroxynitrite anion). These molecules when released continuously induce tissue damage, cause DNA breaks, and lead to mutations in proliferating epithelial and stromal cells (Pollard 2004). Furthermore, migration inhibitory factor (MIF) and TNF-α, released by macrophages, exacerbate DNA damage. TNF-α derived by M1-like TAMs can promote ROS accumulation in latent tumor cells that can damage different proto-oncogenes as well as antioncogenes, such as p53 (Wang et al. 2019). Also MIF acts as a negative regulator of p53-mediated growth arrest and apoptosis, thus causing the augmentation of oncogenic mutations and sustaining normal and malignant cells' growth (Calandra and Roger 2003; Singh et al. 2019). IL-12, IL-23, TNFα, as well as IL-1β are essential in the initiation of chronic inflammation, and by activation of nuclear factor kappa B (NF-κB) signaling pathway in cancer cells, they enhance metastatic potential of tumor cells, promote their proliferation, and inhibit apoptosis (Karin and Greten 2005; Cho et al. 2018). Recent data indicate that another inflammatory cytokine—IL-6—promotes chronic inflammation and eventually tumor formation via signal transducer and activator of transcription (STAT) 3 signaling pathway, as was established in hepatocellular carcinoma (Kong et al. 2016) as well as colon cancer (Bromberg and Wang 2009).

4.4.2 TAMs in Metastasis and Premetastatic Niche

Metastasis is a complex process of solid tumor progression that can be divided into five major stages: (1) invasion of the basement membrane and cell migration; (2) intravasation into blood and lymphatic vessels; (3) survival in the circulation; (4) extravasation from vasculature to reach new niche; and (5) settlement and growth in new niche (Hapach et al. 2019). TAMs are implicated in almost every step of metastasis, providing factors that enhance this process. The first stage starts with a morphological event called epithelial–mesenchymal transition (EMT), when tumor cells acquire the ability to escape the primary tumor site and invade the surrounding stroma. During EMT, molecular and phenotypical changes are observed in tumor cells—they lose cell–cell junctions and apical-basal polarity as a result of downregulation of adhesion molecules (E-cadherin, laminin) and acquire a motile cell phenotype which is connected with the upregulation of mesenchymal markers such as N-cadherin, vimentin, fibronectin, β-catenin, ZEB1, ZEB2, Slug, and Snail (Lin et al. 2019; Chen et al. 2019). TAMs by the secretion of cytokines like IL-6, IL-8, and TNF-α contribute to activation of signaling pathways such as Janus kinase (JAK)/STAT3 and NF-κB in tumor cells, thus facilitating the process of EMT, mainly by downregulation of E-cadherin and upregulation of N-cadherin (Song et al. 2017). It has been shown that TAMs are engaged in the regulation of EMT

process in numerous cancers, including pancreatic cancer (Liu et al. 2013), colorectal cancer (Cai et al. 2019; Li et al. 2017), hepatocellular carcinoma (Fan et al. 2014), breast cancer (Su et al. 2014), ovarian cancer (Cortés et al. 2017), or head and neck squamous cell carcinoma (Gao et al. 2018).

It is worth to mention that TAMs-derived exosomes help macrophages to communicate with tumor cells. Zheng et al. (2018) have discovered that TAMs are able to enhance metastatic potential of gastric cancer cells via delivery of exosomes containing miRNA, lncRNA, and specific proteins. These M2-like TAM-derived vesicles are rich in apolipoprotein E (ApoE) that can activate phosphatidylinositol 3-kinase/serine-threonine protein kinase (PI3K/AKT) pathway in tumor cells, inducing their EMT and cytoskeleton rearrangement.

Intense cross-talk between macrophages and neoplastic cells causes continuous process of matrix deposition and remodeling. First of all, M2-like TAMs abundantly secrete various enzymes that are necessary to remodel and disrupt extracellular matrix (ECM) proteins, what is crucial in tumor cell metastasis, allowing them to escape from the confines of the basic membrane and to migrate through the dense stroma. These enzymes include proteases such as matrix metalloproteinases (MMP2, MMP7, and MMP9), cathepsin B, and urokinase-type plasminogen activator (uPA), able to degrade most of ECM proteins: fibronectin, collagens, elastin, or laminin. Other important factors secreted by TAMs are: secreted protein acidic and rich in cysteine (SPARC) that increases tumor extracellular matrix deposition and interaction by modulating collagen density and leukocyte and blood vessel infiltration; platelet-derived growth factor (PDGF), which upregulates MMP2/MMP9 expression; TGF-β that promotes MMP9 expression by tumor cells, thus enhancing their invasiveness; A disintegrin and metalloproteinase (ADAM) 10 and 17 proteases that activate signaling pathways important in oncogenic development, enhance VEGF-A secretion, and increase bioavailability of epidermal growth factor receptor (EFGR) ligands; and VEGF-A, which stimulates angiogenesis and then provides nutrient for tumor growth (Larionova et al. 2019; Lin et al. 2019; Jeon et al. 2007; Sangaletti et al. 2008; Wang et al. 2011; Ireland and Mielgo 2018; Huang et al. 2017; Saha et al. 2019; Schumacher et al. 2020).

Intravasation is another critical step in metastasis process. TAMs help tumor cells to penetrate the basement membrane and invade blood and lymphatic vessels, through which they reach distinct sites, where they settle down and grow. Wyckoff et al. (2007), in their experiment using multiphoton microscopy, showed that TAMs are involved in mammary tumor cell intravasation. Visualization in this experiment gave a direct evidence that tumor cell is always accompanied by a macrophage within one cell diameter. The important role in this process fulfills the paracrine loop signaling between tumor cells that produce CSF-1 and TAMs which release epidermal growth factor (EGF). The first factor promotes the proliferation, differentiation, and polarization of macrophages toward M2-like phenotype and also stimulates them to release EGF. EGF, in turn, signals to tumor cells and mediates their proliferation and chemotactic migration toward blood vessels. Moreover, EGF provokes carcinoma cells to release CSF-1 (Wyckoff et al. 2004; Goswami et al. 2005; Laoui et al. 2014). Once tumor cells enter into the vasculature, they need to survive

in suspension and resist detachment-induced cell death or anoikis. TAMs due to their secreted chemokines and cytokines, i.e., TNF-α or IL-6 that activate NF-κB and STAT3 signaling pathways in tumor cells, facilitate their survival in the circulatory system (Grivennikov et al. 2010). Other studies showed that recruited macrophages triggered the PI3K/AKT survival signaling pathway in breast cancer cells by engaging vascular cell adhesion molecule-1 (VCAM-1) via α4 integrins (Chen et al. 2011; Lu et al. 2011). Once tumor cells are settled in the capillaries of targeted organs, they try to attach and extrude through the vessels, and TAMs assist this process. Qian et al. (2009) using intact lung imaging system visualized and analyzed the process of extravasation, proving the existence of an intact contact between tumor cells and macrophages during this phenomenon.

In the last step of metastasis process, tumor cells reach the new tissue, where they settle and proliferate, creating a new tumor site. However, before tumor cells' dissemination, primary tumors can prepare future metastatic site for colonization and "prime" the secondary organs, creating so-called premetastatic niches. One of the key factors involved in its formation are TAMs. Primary tumor cells produce various factors, such as CCL2, CSF-1, placenta growth factor (PlGF), tissue inhibitor of metallopeptidase (TIMP)-1, or miRNA-rich exosomes that mobilize macrophages to the bloodstream and then induce their accumulation in the premetastatic sites (Nielsen and Schmid 2017; Joyce and Pollard 2009). Moreover, TNF-α, VEGF, and TGF-β secreted by TAMs in the primary cancer tissue are believed to be transported through the bloodstream to destination organs, and here they stimulate tissue-resident macrophages to produce S100A8 and serum amyloid A3. These factors are able to recruit macrophages and tumor cells to the secondary sites, promoting the formation of premetastatic niches (Sanchez et al. 2019). Both bone marrow-derived and tissue-resident macrophages are called metastasis-associated macrophages (MAMs), and their presence provides a road map for the homing of circulating tumor cells into the PMNs. MAMs prepare niche before the lodging of tumor cells, as they release matrix proteins, remodel ECM mainly by secreting enzymes like MMP, integrins, or lysyl oxidase (LOX), and through VEGF production, they foster extravasation (Kaplan et al. 2005, 2006; Erler et al. 2009; Sceneay et al. 2013). Additionally, macrophages can aid metastatic growth of newly settled tumor cells by inhibiting immune response of T cells and DCs, attenuating their tumoricidal and antigen-presenting properties (Lin et al. 2019).

4.4.3 TAMs in Angiogenesis

The growth of the tumor largely depends on angiogenesis, the mechanism of new blood vessels' formation from existing ones surrounding the growing tumor mass. Angiogenesis is crucial for tumor development by providing the nutrients and oxygen for fast growing cancer cells and by contributing to metastasis process (Wang et al. 2019). There are numerous evidences proving that level of TAMs is closely related to the number of vessels in human cancers, including melanoma

(Torisu et al. 2000), breast cancer (Leek et al. 1996), glioma (Nishie et al. 1999), gastric cancer (Wu et al. 2012), colon cancer (Badawi et al. 2015), or pulmonary adenocarcinoma (Takanami et al. 1999). During intensive proliferation and growth of tumor tissue, oxygen demand is much higher than available oxygen supply, what in turn leads to tumor hypoxia. Hypoxia induces the activation of different signaling pathways in TAMs, such as the major hypoxia-inducible factor 1 (HIF-1) pathway, PI3K/AKT/mammalian target of rapamycin (mTOR) pathway, and NF-κB pathway. However, in cancers, these signaling pathways may be activated also in a hypoxia-independent manner—by growth factors, cytokines, chemokines, or mutations of particular proteins of these pathways. This results in the production and release of proangiogenic factors (Prenen and Mazzone 2019). Abundant TAMs have been located in the hypoxic regions of malignant tumor, especially in necrotic tissues, where HIF expression is high. Here, they function as the primary producers of proangiogenic factors, particularly VEGF, the key mediator of angiogenesis in cancer. VEGF, by binding to the two receptors, VEGFR1 and VEGFR2, expressed on vascular endothelial cells, initiates angiogenesis, but this process also requires the participation of other signaling molecules, i.e., angiopoietin-2 (Ang-2) and delta ligand-like 4 (De Palma and Naldini 2011). The production of VEGF and other growth factors, as well as hypoxia in tumor microenvironment, results in the "angiogenic switch," where new blood vessels are formed in and around the tumor, allowing it to grow exponentially. Tumor blood vessels are often abnormal, twisted, swollen, irregular, and leaky, have dead ends, and are unorganized. These features make tumor blood flow suboptimal, resulting in further hypoxia and VEGF production (Carmeliet 2005).

Apart from VEGF, TAMs also secrete range of factors that play important role in angiogenesis, including cytokines that alter the VEGF production, i.e., IL-1β, which induces VEGF release from cancer cells; TGF-β, which gives rise to VEGF expression via an autocrine effect; basic fibroblast growth factor (bFGF), which promotes VEGF expression; and MMP9, which mediates the release of bioactive VEGF from matrix (Qian and Pollard 2010; Goswami et al. 2017). Moreover, bFGF acts as a chemoattractant for monocytes, and it decreases endothelial adhesion molecules. Yet, another factor—PDGF, released by TAMs—helps in macrophage recruitment and migration, as well as causes vessel stabilization. Additionally, IL-8 secreted by TAMs increases microvessel density, acts as the chemoattractant for monocytes and macrophages, enhances their recruitment to the tumor site, and influences the promotion toward M2-like phenotype. Another cytokine—TNF-α—affects the angiogenesis by upregulating IL-8, VEGF, bFGF, and angiogenin, increasing the expression of their receptors and upregulating the expression in cancer cells (Goswami et al. 2017). Other proangiogenic factors released by TAMs are thymidine phosphorylase (TP) and uPA. The first one stimulates the migration of endothelial cells, while the latter causes extracellular matrix degradation and increases vascular invasion (Riabov et al. 2014). M2-like TAMs, but also tumor cells, release PlGF, another key molecule in angiogenesis, that contributes to vessel disorganization and acts as the chemoattractant for TAMs, while also playing role in their abnormal polarization (Hedlund et al. 2009; Rolny et al. 2011).

It is worth to mention about the engagement of monocytes that are not only the precursors of macrophages, but can also promote angiogenesis. Precisely, a unique subset of monocytes, expressing the Tek tyrosine kinase receptor TIE-2 (TEMs: TIE-2-expressing monocytes), was identified by De Palma and collaborators in 2005. TEMs comprise a functionally distinct myeloid lineage that is able to induce angiogenesis and tumor growth (De Palma et al. 2005). *In vitro*, TEMs are attracted to tumor site by Ang-2, a ligand for TIE-2, that is upregulated on activated endothelial cells and angiogenic vessels, suggesting a homing mechanism for TEMs to tumors (Venneri et al. 2007). TEMs have been detected in different human tumors, including those of colon, kidney, pancreas, lungs (Venneri et al. 2007), breast (Guex et al. 2015; Bron et al. 2015), or hepatocellular carcinoma (Matsubara et al. 2013), but they were excluded from surrounding healthy tissues. Additionally, Ang-2 produced by endothelial cells induces the secretion of IL-10 and VEGF by TEMs, contributing to the angiogenesis (VEGF) and suppression of T-cell proliferation and promotion of Treg (IL-10), therefore enabling tumor cells to escape from immune response (Coffelt et al. 2011; Ibberson et al. 2013).

4.4.4 TAMs in Antitumor Immune Response Suppression

First of all, as described above, TAMs in a great manner contribute to tumor development, by their implication in chronic inflammation, tumor metastasis, or angiogenesis. Another important role of TAMs in tumor progression is their ability to suppress antitumor immune response in TME. M2-like TAMs, but not M1, are poor antigen presenters, and they secrete an array of chemokines (i.e., CCL2, CCL5, CCL17, CCL18, and CCL22), cytokines (IL-10, IL-4, TGF-β, HGF, VEGF, and prostaglandin), and enzymes (i.e., ARG1, MMP, COX-2, and cathepsin K) that exert immunosuppressive effect on host immune system and downregulate the activation of numerous immune cells. On the other hand, M2-like TAMs produce low levels of immune-stimulating cytokines, like IL-12, IL-1, or TNF-α, mainly due to defective NF-κB activation, especially in TAMs of advanced cancer (Chen et al. 2019; Sica et al. 2006).

It is reported that TAMs, isolated from human and mouse tumors, are able to directly suppress T-cell responses *in vitro* (Ruffell and Coussens 2015). M2-like TAMs significantly overexpress IL-10, which alone or together with IL-6 upregulates B7-H4 expression in macrophages, a molecule that is responsible for the suppression of tumor-associated antigen-specific T-cell immunity (Sica et al. 2006; Kryczek et al. 2006). Moreover, TAM-derived IL-10 in TME restrains the expression of IL-12 and inhibits release of IFN-γ by other immune cells. Another immunosuppressive cytokine secreted by TAMs—TGF-β—suppresses the functions of CTLs and cytolytic activity of NK cells, as it inhibits gene expression of granzymes A and B, IFN-γ, or FAS ligand. Additionally, TGF-β by reducing DCs' maturation and enhancing their apoptosis fosters downregulation of the adaptive immune response (Ito et al. 2006; Thomas and Massagué 2005). Also

chemokines released by TAMs contribute to impairment of immune response. CCL2 not only acts as a chemoattractant for macrophages, but is also secreted by TAMs and fosters Th2-polarized immunity (Balkwill 2004). In addition, TAM-secreted CCL17, CCL18, and CCL22 are responsible for attraction of T-cell subsets devoid of cytotoxic functions: They recruit naïve, Th2, and Treg lymphocytes, promoting ineffective immune response and also causing T-cell anergy (Erreni et al. 2011; Solinas et al. 2009). M2-like TAMs produce high amounts of ARG1, an enzyme responsible for conversion of L-arginine to L-ornithine and urea. L-arginine is needed for the activation of T-cell response; however, by expressing ARG1, TAMs lead to degradation of extracellular arginine, thus providing metabolic starvation of T cells, generally impairing their proliferation and functions (Sica and Mantovani 2012).

Another mechanism that M2-like TAMs use to regulate T-cell activity is their influence on programmed cell death protein 1 (PD-1), an immune checkpoint upregulated on activated T cells. In normal conditions, its ligand—programmed cell death ligand 1 (PD-L1)—is expressed by antigen-presenting cells (APCs), and its axis PD-1/PD-L1 guarantees that T cells will not launch an attack (Boussiotis et al. 2014). However, tumor cells frequently overexpress PD-L1, thus preventing from being killed by T cells, escaping from the immune system. Additionally, recent studies showed that TAMs also express PD-1 (Gordon et al. 2017). The interaction of TAMs with cytotoxic T cells via the PD-1/PD-L1 axis inhibits T-cell proliferation, cytotoxicity, and production of cytokines, and causes the suppression of T-cell receptors and/or costimulatory signaling, which in turn leads to blockade of tumor-specific T-cell response. Moreover, PD-1/PD-L1 signaling pathway can limit the functions of NK cells, DCs, and also TAMs, i.e., by inhibiting their phagocytic properties (Chen et al. 2019; Katsuya et al. 2016; Qin et al. 2019).

A wide variety of TAMs' functions makes them an excellent subject for use in developing antitumor therapies. Below, we tried to give a short overview regarding an employment of TAMs in cancer treatment.

4.5 TAMs in Cancer Therapy

As it has been described above, TAMs are a major component of immune cells within tumor microenvironment, and they have a dominant role as orchestrators of immune response and tumor-related inflammation (Yang and Zhang 2017). Numerous studies have shown that TAMs interfere with most antitumor therapies commonly used in clinical oncology, such as conventional (classical) chemotherapy, radiotherapy, antiangiogenic treatment, and antibody-based immunotherapy (De Palma and Lewis 2013).

TAMs can exert dual effects on standard chemotherapy by occasionally enhancing efficacy of treatment but more often by mediating chemoresistance. The positive impact of TAMs on chemotherapy has been observed by Mantovani and Allavena (2015) and Kroemer et al. (2013) who pointed that macrophages contributed to

doxorubicin-based treatment either by inducing the differentiation of myeloid cells into APCs and activation of immune response or by inducing immunogenic cell death (ICD). It has been also reported that M1-like differentiation of macrophages and enhancement of their cytotoxic potential against cancer cells was stimulated by specific drugs, such as actinomycin D or gemcitabine, in human sarcoma and pancreatic adenocarcinoma (Colotta et al. 1984; Di Caro et al. 2016). Similarly, it was observed in B-cell leukemia model that cyclophosphamide induces the secretion of CCL4, IL-8, and TNF-α by treated tumor cells which stimulated macrophage infiltration and their phagocytic activity (Pallasch et al. 2014).

On the other hand, TAMs are able to diminish the effectiveness of chemotherapy. Induction of macrophage-dependent chemoresistance was described in human lung carcinoma, breast carcinoma, and metastatic bone lesions (Hughes et al. 2015). There are three suggested mechanisms of action, by which TAMs hamper the effectiveness of chemotherapeutics: (1) increased recruitment of immune-suppressive myeloid cells; (2) suppression of adaptive antitumor immune responses; and (3) activation of antiapoptotic programs in cancer cells. The first mechanism has been described in breast cancer model, where chemotherapy-induced tissue damage promoted secretion of IL-34 and CSF-1 by cancer cells and led to the recruitment of immune-suppressive myeloid cells in the attempt to heal injured tissues (DeNardo et al. 2011). The second type of mechanism has been reported in ovarian carcinoma, in which macrophages indirectly regulated T-cell response with Treg via secretion of CCL2 (Curiel et al. 2004). Moreover, in mouse bearing mammary carcinomas treated with paclitaxel or carboplatin, TAMs manifested increased secretion of IL-10, which downregulated IL-12 production by DCs and inhibited $CD8^+$ T cells' antitumor activity (Ruffell and Coussens 2015). Third mechanism was observed in colorectal cancer treated with 5-fluorouracil (5-FU), which promoted secretion of diamine putrescine by macrophages and prevented cancer cell apoptosis (Zhang et al. 2016).

Similarly to chemotherapy, also in radiotherapy (RT), it is possible to observe controversial impact of TAMs. In glioblastoma treated with X-ray radiation, increased number of M2 macrophages was observed (Leblond et al. 2017). Moreover, it has been documented that irradiated macrophages may sustain colon cancer cells invasion (Pinto et al. 2016). On the other hand, some studies have demonstrated that low doses of radiation treatment of pancreatic carcinoma may reprogram macrophages toward iNOS+/M1 phenotype (Klug et al. 2013; Nadella et al. 2018). The protumoral effect of macrophages in RT may be explained by the fact that M2-like phenotype is more resistant to radiation than M1-like one. The antitumoral effect of macrophages is based on the fact that radiation kills cancer cells in a similar way to ICD activators, leading to the release of danger signals and triggering effective immune responses (Leblond et al. 2017; Pinto et al. 2016).

Since TAMs are known to be important mediators of angiogenic switch in tumors and produce factors promoting creation of a new vessel network, it is no surprise that they may interfere with antiangiogenic drugs. The anti-VEGF therapy of mice bearing refractory tumors revealed that resistant tumors were characterized with higher number of TAMs in comparison to sensitive tumors (Shojaei et al. 2007).

Similarly, the treatment of murine glioblastoma with vatalanib (protein kinase inhibitor that blocks angiogenesis) was associated with increased TAMs infiltration which diminished the efficacy of therapy. However, the therapy was significantly improved with the coadministration of anti-CSF-1R antibody that impaired TAMs' recruitment (Achyut et al. 2015). Interestingly, interrupting interaction of TEMs with Ang-2 by administration of blocking antibody resulted in reduced angiogenesis but increased recruitment of macrophages in murine models of breast and pancreatic cancers (Mazzieri et al. 2011).

The latest approach in cancer immunotherapy is mainly based on the use of antibodies targeting immune checkpoints on the surface of T cells and is called immune-checkpoint blockade (ICB) therapy. These immune checkpoints are a family of proteins, which interact with specific ligands on APCs or cancer cells and inhibit TCR-mediated activation of naïve T cells (Ribas and Wolchok 2018). Since anticheckpoint antibodies prevent this interaction, they have become the holy grail of cancer immunotherapy and have shown great clinical responses in some types of cancer (melanoma, lung, or renal cancer) (Quaranta and Schmid 2019). Unfortunately, ICB has limited effectiveness in certain tumor types, such as pancreatic, colorectal, or ovarian (Kalbasi and Ribas 2020). TAMs have demonstrated ability to reduce the efficacy of ICB therapy by expressing various molecules, such as PD-L1/2, CD80, CD86, or VISTA (V-domain immunoglobulin suppressor of T-cell activation), which serves as additional ligands for checkpoint receptors and could mediate $CD8^+$ T-cell dysfunction (Chen et al. 2013; Kuklinski et al. 2018). Moreover, Arlauckas et al. (2017) have shown that TAMs can bind anti-PD-1 antibody with their Fc receptor, significantly hampering its binding with PD-1 on T cells. On the other hand, it has been demonstrated that TAMs can contribute to clinical efficacy of Rituximab, which targets and kills B cells via antibody-dependent cellular cytotoxicity (ADCC) mechanism (Uchida et al. 2004). This could be potentially advantageous in therapies with anti-PD-L1 antibodies targeting cancer cells.

4.6 Therapeutic Approaches of Targeting TAMs—From Experimentation to Clinical Trials

As it was described above, TAMs' involvement in antitumor therapies is significant and may result in cancer resistance to particular treatment. Therefore, there is a considerable interest focused on therapeutic targeting of TAMs in order to synergize with current therapies. Generally, strategies of targeting TAMs can be divided into three groups (Fig. 4.4): (1) elimination of TAMs already present in TME; (2) inhibition of TAMs recruitment and infiltration; and (3) reprogramming of TAMs' protumor polarization and activation of their antitumor functions (Mantovani et al. 2017; Cassetta and Pollard 2018; Anfray et al. 2019).

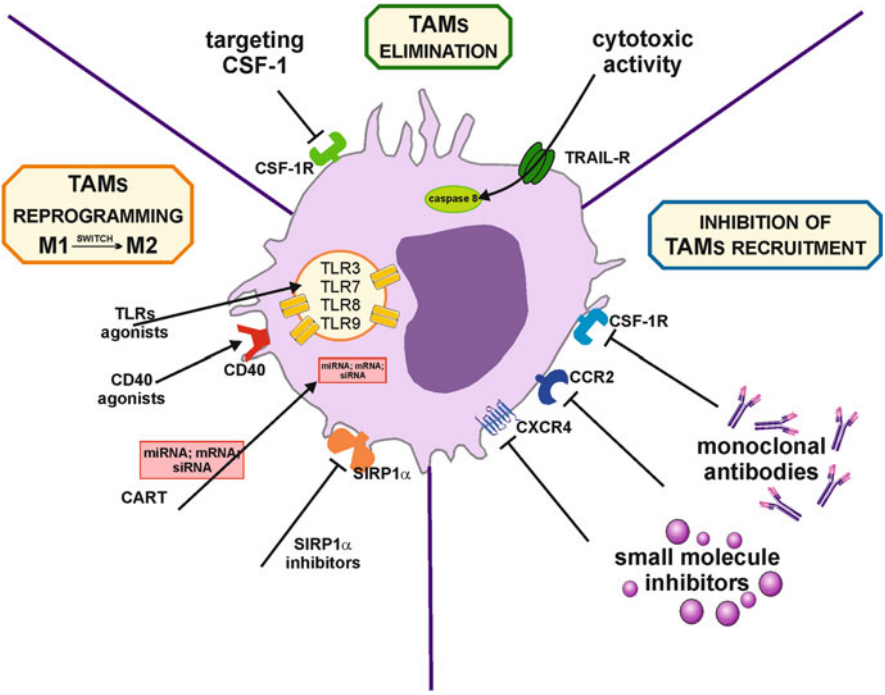


Fig. 4.4 Three groups of strategies targeting TAMs. Three main groups of strategies targeting TAMs include elimination of TAMs already present in TME; inhibition of TAMs' recruitment and infiltration; and reprogramming of TAMs' protumor polarization and activation of their antitumor functions

The first strategy focuses on depletion of TAMs and is considered to be a promising option to counter their negative effects, as well as enhance anticancer treatment. Generally, there are two types of approach within this strategy: (A) Targeting factors involved in the proliferation differentiation and survival of monocytes/macrophages; (B) Using drugs with selective cytotoxicity toward macrophages. The first type of approach (A) is focused on the CSF-1, since it plays crucial role in the growth of monocytes and macrophages (Jones and Ricardo 2013). High level of CSF-1 or its receptor (CSF-1R) has been associated with poor prognosis for patients with lymphoma, breast cancer, or hepatocellular carcinoma (Goswami et al. 2005; Koh et al. 2014; Zhu et al. 2008). Thus, several inhibitors of CSF-1/CSF1R axis have been developed and are being investigated in clinical trials either as monotherapy or in combination with chemotherapeutics/ICB therapy. For example, **Emactuzumab** (humanized antibody), used alone or in combination with paclitaxel, has shown to significantly reduce number of TAMs in TME (with a good safety profile) in patients with advanced solid tumors (phase I trials) (Gomez-Roca et al. 2019). Another interesting compound is small drug **PLX3397 (Pexidartinib)**, which proved to effectively deplete TAMs, enhance $CD8^+$ T-cell infiltration in

TME, and improve response to therapy in murine model of breast and prostate cancer (DeNardo et al. 2011; Xu et al. 2013). Application of PLX3397 in combination with ICB or standard chemotherapy led to tumor regression and increased sensitivity to therapy of pancreatic and peripheral nerve sheath tumors (phase I and II trials) (Zhu et al. 2014; Patwardhan et al. 2014).

As it was mentioned before, another type of approach (B) within this strategy involves drugs with preferential cytotoxic activity toward monocytes/macrophages. Compounds typically used in this regard belong to the family of inorganic bisphosphonates, which falls into two categories: non-nitrogen or nitrogen-containing (Roelofs et al. 2006). **Clodronate** is a first category drug, which application combined with nanoparticles (usually liposomes) depleted TAMs and reduced tumor growth of metastatic liver cancer in preclinical trials (Zhang et al. 2010). Similarly, the use of **Zoledronate**, second category bisphosphonate with selective cytotoxicity toward MMP9-expressing macrophages, has demonstrated noticeable depletion of macrophages and decreased angiogenesis and inhibition of tumor progression in various preclinical models (Zhang et al. 2010; Zhou et al. 2017; Lv et al. 2020). Moreover, **Zoledronate acid** has shown some potential in enhancing treatment of kidney cancer and lung metastases by successfully completing phase I clinical trials (Xiang et al. 2021). Yet another compound, not belonging to the family of bisphosphonates, is **Trabectedin**—a registered antineoplastic drug, which can be successfully used to target macrophages. The mechanism of this compound's action is based on inducing apoptosis of monocytes and macrophages via TRAIL-dependent pathway (Germano et al. 2013). The effectiveness of Trabectedin in depleting TAMs has been demonstrated in preclinical trials of prostate cancer, pancreatic cancer, and melanoma (Jones et al. 2019; Carminati et al. 2019; Borgoni et al. 2018). While a strategy to eliminate TAMs is valid and looks promising, the major possible barrier of this approach is the fact that depletion of monocytes/macrophages is not selective to TAMs only. The overall loss of resident macrophages in other organs may disturb tissue homeostasis and diminish bacterial clearance (Krenkel and Tacke 2017).

The second TAM-targeting strategy aims to limit macrophage accumulation within TME by cutting off their recruitment from circulation. This approach is designed around using monoclonal antibodies or small-molecule inhibitors targeting tumor-derived factors (TDFs) or their receptors. TDFs are the key players in TAMs' replenishment, since they act as mediators in the cross-talk between monocytes and cancer cells. The major factors in this group are considered to be: CSF-1, VEGF, CCL2, and CXCL12—also known as stromal cell-derived factor 1 alpha (SDF-1α) (Xiang et al. 2021; Argyle and Kitamura 2018). It has been demonstrated in preclinical studies that antibodies targeting CCL2 or CCR2 antagonists have not only downregulated recruitment of circulatory monocytes but also enhanced function of $CD8^+$ T cells and NK cells (Schmall et al. 2015). Clinical trials with anti-CCL2 antibodies **CNTO 888 (Carlumab)** were successfully performed in patients with prostate cancer (phase I), with some noticeable efficiency. Similarly, the use of CCR2 antagonist **PF-04136309** either as monotherapy or in combination with chemotherapy (FOLFIRINOX) in pancreatic cancer or advanced solid tumors

proved to be effective, however with overall limited results (phase I and II) (Anfray et al. 2019). Other approach in this strategy involves CXCL12/CXCR4 axis, which contributes to recruitment of M2 macrophages (Chen et al. 2014). This pathway is induced by hypoxia and HIF-1α; thus, it is of great importance in solid tumors. The study of CXCR4 antagonist **AMD3100** in breast cancer model demonstrated its capability to reduce tumor progression and formation of metastasis (Boimel et al. 2012). Application of AMD3100 and another CXCR4 antagonist—**Plerixafor**—is currently being evaluated in clinical trials for patients with head and neck squamous cell carcinoma, acute myeloid leukemia (AML), and advanced solid tumors (phases I and II) (Anfray et al. 2019; Xiang et al. 2021) The strategy to inhibit TAMs' recruitment can enhance effectiveness of standard therapies (especially immunotherapies); however, a possible mechanism of resistance involving rapid compensation of macrophage depletion by tumor-associated neutrophil (TANs) should be taken into consideration (Nywening et al. 2018).

The last strategy of targeting TAMs is based on the pharmacological reprograming of macrophages to induce their selective polarization toward M1 type. Switching protumor M2-like TAMs into antitumor M1-like TAMs allows to use their potential as major phagocytes and professional APCs within TME (DeNardo and Ruffell 2019). There are several approaches to conduct such reprogramming, and these include the use of TLR agonists, application of monoclonal antibodies, and delivery of nucleic acids (RNA, miRNA, or siRNA).

TLRs belong to the family of pattern recognition receptors, which stimulate macrophages and activate M1-like polarization upon engagement with their ligands (Mantovani et al. 2017). It has been demonstrated that TLRs located in endosomal compartment of APCs (TLR3, TLR7, TLR8, or TLR9) are more effective in triggering antitumor immune response than extracellular TLRs (TLR1, TLR2, TLR4, or TLR6) (Huang et al. 2021). Therefore, multiple studies have focused on evaluating the capacity of intracellular TLR agonists to induce TAMs reprograming. Some success has been already achieved in this regard, since **Imiquimod** (TLR7 agonist) passed phase III clinical trials and is approved by Food and Drug Administration for administration in squamous and basal cell carcinoma (Keshavarz-Fathi and Rezaei 2021). Moreover, Maeda et al. (2019) have recently shown that stimulation of macrophages with **Poly I:C** (TLR3 agonist) is more effective alternative to Imiquimod. Currently, the ongoing clinical trials aim to evaluate the potency of Poly I:C, distributed alone or in combination with ICB, in the treatment of melanoma, sarcoma, as well as head and neck cancer (phases I and II) (Anfray et al. 2019; Zhao et al. 2018). It is also worth to mention that in the last few years, much attention has been attracted by the agonist to TLR7/8—**Resiquimod** (**R848**)—which is an analog to Imiquimod. Several experimental studies have demonstrated that it has the ability to trigger stronger antitumor response than Imiquimod; however, it is burdened with toxicity (Thauvin et al. 2019; Huang et al. 2018; Hasham et al. 2017). Another formulation of R848—MEDI9197—has been developed in order to limit the systemic cytotoxicity (Mullins et al. 2019). The application of TLRs' agonists seems to be a promising approach for the treatment of cancer. Some of these compounds have been already used for the vaccination purposes (Bocanegra

Gondan et al. 2018; Da Silva et al. 2019); however, the information regarding their efficiency *in vivo* is still very limited.

The second approach to reprogram TAMs focuses on the use of monoclonal antibodies to either restore macrophages phagocytic ability or unleash their immune-stimulatory capacity. The phagocytosis is regulated by signal regulatory protein alpha (SIRPα), inhibitory receptor expressed on the macrophages. It recognizes CD47, a "do-not-eat-me" signal, overexpressed on the tumor cells (Feng et al. 2019; Willingham et al. 2012). The interaction of CD47–SIRPα axis is the main mechanism of resistance to phagocytosis, and many studies have proved that pharmacological inhibition of CD47 restored the ability of macrophages to kill tumor cells in various preclinical cancer models (Yang et al. 2019; Noman et al. 2018; Gu et al. 2018). So far the promising results have been obtained in clinical trials of **Hu5F9-G4** monoclonal antibody, which was administrated either alone or in combination with Rituximab (anti-CD20 antibody) in patients with myeloid leukemia and lymphoma (Anfray et al. 2019; Advani et al. 2018). The ability of macrophages to stimulate other immune cells, such as T cells, is dependent on CD40. It is a surface receptor belonging to TNF receptor superfamily, which is expressed primarily on APCs. Interaction of CD40 with its ligand (CD40L) upregulates the expression of MHC molecules and promotes secretion of proinflammatory cytokines (like IL-12) (Zhang et al. 2018). The experimental data indicate that agonistic anti-CD40 antibodies led to the recovery of tumor immune surveillance and effective antitumor activity by TAMs in murine tumor model (Beatty et al. 2011; Perry et al. 2018). Currently, there is an ongoing clinical evaluation of **RO7009789** (CD40 agonist antibody) used in combination with chemotherapy or checkpoint immunotherapy in the treatment of patients with advanced solid tumors (phase I) (Anfray et al. 2019).

The technological advancement in molecular biology regarding cell transfection allowed to develop new strategy of reprograming TAMs, which is based on the delivery of mRNA, miRNA, or siRNA. Novel charge-altering releasable transporters (CARTs) combined with oligo (carbonate-b-alfa-amino ester) as dynamic carriers are capable to protect and deliver polyanionic mRNA through controlled degradation and facilitation of cytosolic release of functional mRNA (McKinlay et al. 2017). This method has been used to deliver mRNA encoding CD80, CD86, and OX40L into two-tumor model of lymphoma and colon carcinoma. Results of this experiment indicate that CARTs have successfully transfected tumor-infiltrating cells, including TAMs (at the level of 28% of their population), and induced a systemic antitumor immunity (Haabeth et al. 2019). Another study has shown that encapsulation and administration of two mRNAs—first encoding interferon regulatory factor 5 (IRF5), second encoding serine kinase IKKβ—in biodegradable polymeric nanoparticle led to increased number of M1-like macrophages by downregulation of M2 genes expression (like CCL12) and upregulation of M1 genes (like CCL5) in murine ovarian tumor model (Zhang et al. 2019). Alteration in genes level is also performed with microRNA (miRNA). These small noncoding RNA molecules are capable of regulating gene expression at posttranscriptional level (O'Brien et al. 2018). It has been shown in mouse sarcoma model that delivery of miRNA-155, facilitated by

lipid-coated phosphonate nanoparticles, successfully reprogrammed TAMs toward M1 phenotype (Cai et al. 2012). Delivery of small interfering RNA (siRNA) on the other hand aims at silencing genes involved in immune-suppressive functions of TAMs. In the study of Song et al., two siRNAs—targeting VEGF and PlGF—were loaded into mannosylated dual pH-responsive nanoparticles. These two growth factors are overexpressed in cancer cells and TAMs, promoting tumor cell proliferation and immunosuppression. The designed nanoparticles were used in murine breast cancer model, which resulted in silencing of targeted genes, inhibition of tumor growth, and metastasis (Song et al. 2018). Up to this point, there are phase I and II clinical trials evaluating the use of liposomes loaded with **mRNA-2416**, encoding human OX40L, in combination with anti-PD-L1 therapy in patients with advanced tumors; however, to our best knowledge, there are no new clinical trials initiated based on RNA delivery technology (Anfray et al. 2019).

4.7 Conclusions

To summarize, TAMs have significant impact on tumor development due to their multifaceted functions in tumor microenvironment. They can display both antitumoral (M1-like TAMs) and protumoral (M2-like TAMs) activities. M1-like TAMs' role is based on inflammatory cytokines production, cytotoxic molecules generation, and enhancement of other immune cell activity. Presence of high M1-like TAMs' quantity in TME is related to better prognosis of the patients' overall survival rate. On the other hand, M2-like TAMs, more frequently observed in cancer patients, contribute to metastasis, angiogenesis, creation of premalignant niche, and suppression of host's antitumor immune response. Therefore, they contribute to poor outcome of the disease. Because of a wide range of TAMs' properties, they comprise an excellent therapeutic target. Many approaches to use TAMs in a battle with cancer have been proposed, among which the most promising are strategies involving macrophage depletion, inhibition of their recruitment, or reprogramming of their functions. Hopefully, TAM-oriented therapies will give scientists' reasons to be cheerful.

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Chapter 5
Polymorphonuclear Neutrophils and Tumors: Friend or Foe?

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Contents

Abstract Tumor microenvironment (TME) is a dynamic network that apart from tumor cells includes also cells of the immune system, e.g., neutrophils, which are recruited from blood circulation. In TME, neutrophils are strongly implicated in the direct and indirect interactions with tumor cells or other immune cells, and they play roles in both preventing and/or facilitating tumor progression and metastasis. The dual role of neutrophils is determined by their high plasticity and heterogeneity. Analogous to the macrophages, neutrophils can express antitumoral (N1) and protumoral (N2) phenotypes which differ substantially in morphology and function. N1 phenotype characterizes with a high cytotoxic and proinflammatory activities, while N2 phenotype with immunosuppressive and prometastatic properties. The antitumoral effect of neutrophils includes for example the production of reactive oxygen species or proapoptotic molecules. The protumoral action of neutrophils relies on releasing of proangiogenic and prometastatic mediators, immunosuppressive factors, as well as on direct helping tumor cells in extravasation process. This chapter summarizes the heterogeneity of neutrophils in TME, as well as their dual role on tumor cells.

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Keywords Tumor-associated neutrophils · Tumor cells · Reactive oxygen species · Death receptors · Neutrophil elastase · Arginase-1 · Cathepsin G · Neutrophil extracellular traps · Metalloproteinases · Tumor cells extravasation

Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
ARG-1	Arginase 1
BMs	Basement membranes
CG	Cathepsin G
CTCs	Circulatory tumor cells
ECM	Extracellular matrix
EMT	Epithelial–mesenchymal transition
FGF	Fibroblast growth factor
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDNs	High density neutrophils
huGCP-2	Human granulocyte chemotactic protein 2
ICAM-1	Intracellular adhesion molecule 1
IFN	Interferon
IL	Interleukin
IRS-1	Insulin receptor substrate-1
LDNs	Low-density neutrophils
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases
MIP-1 α	Macrophage inflammatory protein-1 α
MMPs	Metalloproteinases
NE	Neutrophil elastase
NETs	Neutrophil extracellular traps
NK	Natural killer
OSM	Oncostatin M
PD-1	Programmed cell death protein 1
PD-L1	Ligand for programmed cell death protein 1
PI3K	Phosphoinositide-3 kinase
PMA	Phorbol-12-myristate-13-acetate
ROS	Reactive oxygen species
sLeX	Sialyl Lewis X
TAMs	Tumor-associated macrophages
TANs	Tumor-associated neutrophils
TGF-β	Transforming growth factor β
TLR	Toll-like receptor
TME	Tumor microenvironment

TNF-α	Tumor necrosis factor-α
TRAIL	TNF-Related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor

5.1 Introduction

Neutrophils are the most common cells' population found in the human peripheral blood, and they constitute approximately 50–70% of circulating leukocyte (Ng et al. 2019). It is generally accepted that they are short-living cells, with half-life of approximately 7 h, after which they undergo spontaneous apoptosis and are cleared by macrophages (Rankin 2010). Nevertheless, data indicating on half-life more than 5 days are also published (Pillay et al. 2010). In contrast to neutrophils' well-established role in host defense against infection (Teng et al. 2017), definitely less is known about their involvement in the development, growth, and progression of human tumors and/or in the induction of antitumor immune response and tumor destruction. Moreover, in comparison to tumor-associated macrophages or tumor-infiltrating T cells, neutrophils were reputed as marginally important. However, increasing number of studies has evidenced that they are the key player in the whole tumor biology. Due to neutrophils' ability to extravasate from peripheral blood into tissue, they willingly infiltrate many types of solid tumors, e.g., renal (Jensen et al. 2009), gastric (Caruso et al. 2002; Zhao et al. 2012), lung (Teixidó and Rosell 2017), melanoma (Jensen et al. 2012), liver (Li et al. 2011; Kuang et al. 2011), bladder (Mandelli et al. 2020), and pancreatic (Reid et al. 2011). In the tumor microenvironment (TME), they present both antitumoral and protumoral functions, depending on stage of disease, kind of tumor, and even individuality of patient. What is more, these contrasting activities are a result of high plasticity and heterogeneity of neutrophils infiltrating tumor tissue (Sionov et al. 2015; Treffers et al. 2016; Shaul and Fridlender 2018). As an important creator of TME behavior, neutrophils are involved in a complex (direct or indirect) cross-talk with both tumor and stromal cells (Sionov et al. 2015; Carnevale et al. 2020). Here, we will focus on the phenotypic heterogeneity of neutrophils in TME, as well as on their double role in the facilitation and prevention of tumor progression and metastasis.

5.2 Characterization of Tumor-Associated Neutrophils

5.2.1 Requirement of Neutrophils to the Tumor Tissue

To reach the tumor, neutrophils must leave the circulation in the process called extravasation. This step requires influx of neutrophils to the site of tumor, coordinated interactions between them, and endothelial cells allowing to leave the

circulation. Various molecules expressed on neutrophils (e.g., CD11a/CD18, CD11b/CD18) and on blood vessels' endothelial cells (e.g., ICAM-1), as well as changes in neutrophils' shape and polarization let in firm cell–cell interaction and passing the blood–endothelial cell barrier into tumor tissue (Filippi 2019). Numerous tumor-derived soluble factors are known to induce the migration of neutrophils into TME and their later intratumoral accumulation. The neutrophils-attracting factors are produced by tumor cells, immune cells (including neutrophils), epithelial cells, and other stromal cells. The most effective and the best-known chemoattractant is an interleukin 8 (IL-8/CXCL8), a chemokine belonging to the CXCL family which binds to CXCR1 and CXCR2 receptors highly expressed on circulating neutrophils. Through the CXCR1 and CXCR2, neutrophils can also be attracted by other CXCL-type chemokines such as CXCL1, 2, 5, 6, and 7. Especially, CXCL2–CXCLR2 axis induces their intensified extravasation. The second most recognized stimulus recruiting neutrophils to the tumor tissue is granulocyte-macrophage colony-stimulating factor (GM-CSF). Another critical factor that mobilizes the neutrophils' influx is IL-17, which also upregulates the expression of chemoattractant—GM-CSF. Apart from mentioned above, the chemokines and cytokines affecting the influx of neutrophils into TME are also: macrophage inflammatory protein-1 α (MIP-1α), human granulocyte chemotactic protein 2 (huGCP-2), tumor necrosis factor-α (TNF-α), granulocyte colony-stimulating factor (G-CSF), and CCL2 (Shaul and Fridlender 2018; Fridlender and Albelda 2012; Uribe-Querol and Rosales 2015; SenGupta et al. 2019; Wu et al. 2019). Moreover, noncytokine factors connected with inflammation and highly expressed in tumors, like leukotriene B_4 (LTB_4) or the exosomal proteins (S100A8 and S100A9), are also able to recruit neutrophils (Shaul and Fridlender 2018; SenGupta et al. 2019; Masucci et al. 2019). One more factor enhancing neutrophils' influx into TME is hypoxia. Interestingly, neutrophils usually localize in highly hypoxic regions of tumor tissue (SenGupta et al. 2019).

5.2.2 Heterogeneity of Tumor-Associated Neutrophils

When neutrophils reach the tumor, they are termed tumor-associated neutrophils (TANs) and are defined by the surface markers $CD11b^+/CD14^-/CD66^+/CD15^{hi}$ in human and $CD11b^+$/Ly-6Ghi/Ly-$6C^{int}$ in mice. Moreover, surface CD10 molecule proved to be a key marker for the maturation and suppressive potential of neutrophils (Eruslanov 2017; Lecot et al. 2019). In the tumor tissue, TANs display functional and phenotypic heterogeneity. Fridlender et al. (Fridlender et al. 2009) have provided evidence for the existence of N1 (antitumoral) and N2 (protumoral) TAN phenotypes (Fig. 5.1) analogous to the polarization of tumor-associated macrophages (TAMs) toward a protumoral (M2) or antitumoral (M1) phenotype. The neutrophil polarization is predominantly regulated by transforming growth factor β (TGF-β), which induces the accumulation of N2 TANs and strongly prevents the generation of N1 neutrophils. It was found that TGF-β inhibition markedly increases the number of N1 TANs in TME (Fridlender and Albelda 2012; Fridlender et al.

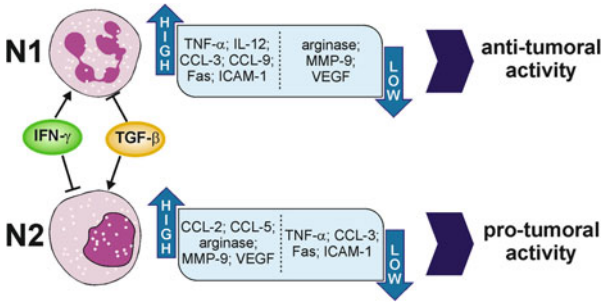

Fig. 5.1 Polarization of tumor-associated neutrophils. Tumor-associated neutrophils (TANs) undergo polarization into an antitumoral (N1) phenotype or a protumoral (N2) phenotype. N1 TANs produce high level of proinflammatory and chemotactic cytokines and highly express molecules such as Fas and ICAM-1. On the other hand, they produce low amounts of immunosuppressive arginase, proangiogenic factors, MMP-9, and VEGF. By contrast, N2 TANs produce low amounts of proinflammatory and chemotactic cytokines and have a low level of surface Fas and ICAM-1 expression. However, N2 TANs release large amounts of acute inflammatory cytokines, arginase, MMP-9, and VEGF

2009; Piccard et al. 2012). The study of Andzinski et al. (Andzinski et al. 2016) and Pylaeva et al. (Pylaeva et al. 2016) clearly demonstrated that type I interferon (type I IFN/IFN-β) polarizes neutrophils into N1 phenotype, while the inhibition of this cytokine production results in the accumulation of N2 TANs. Various other cytokines have also been described as significant factors influencing polarization of neutrophils. For example, both IL-6 and IL-35 induce the generation of protumoral N2, while IL-12 polarizes neutrophils into N1 phenotype (Shaul and Fridlender 2018; Zou et al. 2017).

The N1 and N2 TANs differ substantially in morphology and functional activity. Highly activated neutrophils with N1 phenotype are short-living mature cells that produce high levels of reactive oxygen species (ROS), as well as proinflammatory and chemotactic cytokines (tumor necrosis factor α, TNF-α, CCL3, CXCL9, IL-12, and GM-CSF) that are responsible for stimulation of NK cells and cytotoxic T cells. Type N1 TANs also characterize with elevated level of Fas and ICAM-1 molecules on their surface and exhibit low levels of arginase 1 (ARG-1), matrix metalloproteinase-9 (MMP-9), as well as vascular endothelial growth factor (VEGF). Functionally, N1 TANs can kill cancer cells and promote the recruitment and activation of cytotoxic $CD8^+$ T cells. In contrast, N2 TANs are long-living, low cytotoxic cells that do not produce considerable level of cytokines capable of activating immune cells. However, they produce a large amount of ARG-1, which is immunosuppressive for T cells and inactivates their effector function via downregulation of T-cell receptor (TCR). In addition, N2 TANs express high level of neutrophil elastase (NE) and proangiogenic and prometastatic factors such as MMP-9 and VEGF and are characterized by upregulation of chemokines (CCL2, 3, 4, 5, 8,12 and CXCL1, 2, 8). Furthermore, the nuclei of N1 and N2 neutrophils differ in shape; N1 TANs have a hypersegmented nucleus, whereas N2 TANs have

circular one (Sionov et al. 2015; Masucci et al. 2019; Piccard et al. 2012; Rakic et al. 2018). The differences between both phenotypes of cells are also observed at the level of genes. In-depth transcriptomic analysis of neutrophils has revealed that N1 cells characterize with upregulation of genes associated with actin polymerization, secretory vesicles, MHC class I antigen presentation, and chemokines—CXCL10, CCL2, 3, 7. In contrast, the same genes are markedly downregulated in N2 TANs (Shaul et al. 2016).

Numerous papers evidence that neutrophils present in circulatory system of cancer patients are also heterogeneous. Generally, they are divided into two subpopulations: high-density neutrophils (HDNs) and low-density neutrophils (LDNs). HDNs are mature, segmented, and characterized with cytotoxic activity against tumor cells and possess high phagocytic ability. LDNs are larger in size and can be further divided into the mature (segmented) and immature (banded nuclei) populations. All LDNs display low phagocytic and low oxidative burst activities, and hence, they are characterized by reduced antitumor activity. Immature LDNs are also known as immature myeloid-derived suppressor cells of granulocytic origin (G-MDSC), which exhibit strong immunosuppressive effect toward $CD8^+$ T cells and generally display protumoral functions (Treffers et al. 2016; Shaul and Fridlender 2018; Wang et al. 2018). Since this chapter is focused on the functional activity of neutrophils present in TME, the detailed characterization of circulating neutrophils will not be described here. Moreover, another chapter of this book is strictly dedicated to the MDSC. It is not clear whether TANs come from G-MDSC, LDNs, or HDNs. However, HDNs are functionally similar to N1 phenotype, while functional likeness of mature LDNs and N2 TANs suggests that they belong to the same population. Nevertheless, it is difficult to definitely confirm this possibility due to the lack of specific markers of distinct neutrophils' populations (Wang et al. 2018; Rosales 2018). What is more, based on the genomic profile, some data indicate that G-MDSC can be separate population of cells (Masucci et al. 2019). It should be underlined that LDNs, HDNs, and N1 and N2 TANs are defined with high plasticity, and under treatment with cytokines, e.g., TGF-β or IFN-β, they can be converted into other phenotype. Therefore, future investigation is required to understand the relationship between N1/N2 TANs and HDN/LDN subpopulations.

5.3 Dual Role of TANs in TME

TANs are fully capable of modifying tumor growth and invasiveness, and their presence in TME may indicate either a better or a worse host antitumoral response. Generally, the prognostic value of the presence or absence of TANs varies between types of tumors (Treffers et al. 2016). Nevertheless, most authors have reported that increased number of TANs in TME constitutes as an independent factor indicating the unfavorable survival and frequent recurrence in various human tumors (Treffers et al. 2016; Shaul and Fridlender 2018; Shen et al. 2014; Moses and Brandau 2016). Association of TANs with poor prognosis was reported, e.g., with renal carcinoma

(Jensen et al. 2009), gastric carcinoma (Zhao et al. 2012), melanoma (Jensen et al. 2012), pancreatic adenocarcinoma (Reid et al. 2011), and head and neck cancer (Trellakis et al. 2011). However, in the case of colorectal cancer, the potential role of intratumoral neutrophils, as a factor influencing the survival of patients, is controversial with both favorable prognosis (Droeser et al. 2013; Galdiero et al. 2016) and adverse prognosis (Rao et al. 2012). On the other hand, it is also possible to find report indicating that a higher number of TANs has reduced the mortality of female patients with advanced gastric carcinoma (Caruso et al. 2002). Moreover, study in animal model has also demonstrated the antitumoral activity of neutrophils. First, these cells isolated from the peripheral blood of healthy rats have been shown to have a highly cytotoxic and antiproliferative effect on Walker 256 carcinoma cells (W256). Second, such neutrophils administered at the site of tumor in rats bearing W256 tumors significantly prolong the survival of animals and increase tumor regression (Zivkovic et al. 2007; Jaganjac et al. 2008, 2010).

The anti- or protumoral function of TANs has also been connected with the tumor stage. Studies of lung tumor in mice model, as well as on neutrophils isolated from patients' tumor tissue (e.g., gastric cancer, lung cancer) have indicated that in the late stage of disease, the immunosuppressive N2 phenotype predominates in tumor tissue. In contrast, TANs with antitumoral function are found in TME at the early stage of disease (Lecot et al. 2019; Wang et al. 2018). However, it should be underlined that neutrophils can fluently change their polarization state due to alternations (cytokines, hypoxia) occurring in the tumor microenvironment.

5.4 Antitumoral Effect of TANs

Neutrophils *per se* are not capable of recognizing tumor cells specifically. Tumor cells are also too large to be ingested by these phagocytes. However, recruited neutrophils produce several cytotoxic mediators, including ROS, membrane-perforating agents, and soluble factors, as well as express various proapoptotic molecules, which are involved in the induction of tumor cells' dysfunction and finally in tumor destruction (Fig. 5.2).

5.4.1 ROS Production

Activated neutrophils produce and release a variety of powerful ROS. Radical species, such as superoxide anion (O_2^-) and hydroxyl radical (OH), as well as nonradical species, such as hydrogen peroxide (H_2O_2), are generated by neutrophils during the complex series of reactions named "respiratory burst." It is assumed that a plasma membrane-bound NADPH oxidase complex (NOX-2) catalyzes a one-electron reduction of oxygen to $^{\cdot}O_2^-$, which is then converted into H_2O_2 spontaneously or via the action of superoxide dismutase (SOD). In the reaction of

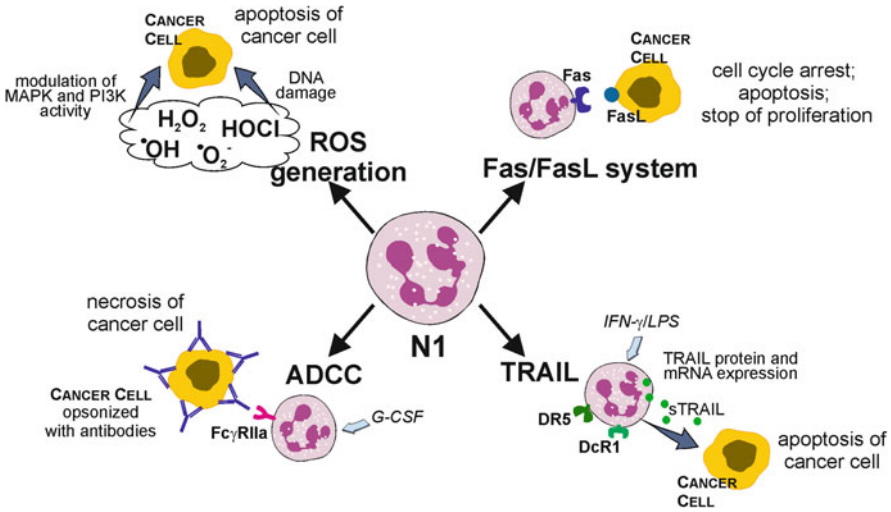

Fig. 5.2 Antitumoral activity of N1 phenotype of tumor-associated neutrophils

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 (Jones et al. 2000; Babior 2004; Brandes et al. 2014).

ROS are known to exert dual effect on cancer cells. On the one hand, they possess a genotoxic activity resulting in tumor establishment. On the other hand, their cytotoxic action leads to the killing of tumor cells and as a consequence to tumor regression. The cytotoxicity of $^{\bullet}O_2^-$ and $^{\bullet}OH$ is related to various types of DNA damages such as oxidation, depurination, methylation, deamination, and single- and double-strand breaks. Especially the former damage is very danger for the whole genome stability. HOCl, another member of ROS, is known to induce a DNA–protein cross-links, and chlorination of DNA bases, as well as a pyrimidine oxidation (Kulcharyk and Heinecke 2001; Knaapen et al. 2006). All mentioned damaged in the DNA, if intensified due to permanent presence of oxygen radicals and not properly repaired, lead to the cell death. The involvement of ROS produced by neutrophils in the lysis of tumor cells has been proven by Zivkovic et al. (2007) and Dallegri et al. (1991). They have demonstrated that phorbol-12-myristate-13-acetate (PMA)-activated neutrophils induce tumor cell lysis (melanoma B16-F16 cells, B lymphoblasts) via ROS. Moreover, it seems that high amount of ROS generated by TANs in TME is sufficient to resolve cytotoxic effect on tumor cells, and direct cell-to-cell contact is not necessary (Sionov et al. 2015).

ROS, particularly H_2O_2, can also act as second messenger regulating the activity of signaling proteins, e.g., nuclear factor kappa B, kinases belonging to the family of mitogen-activated protein kinases (MAPKs), or proteins of phosphoinositide-3-kinase (PI3K)/Akt-regulated signaling cascade (Liou and Storz 2010; Reczek and Chandel 2017). Modulation of MAPK activity induces cell-cycle arrest, prevents cancer cell growth and division, and finally induces cell apoptosis (Reczek and

Chandel 2017). Another action of ROS released by TANs is the modulation of immune cells' activity. Mensurado et al. (Mensurado et al. 2018) clearly demonstrated that TANs via ROS inhibit the proliferation of highly immunosuppressive murine γδ17 T cells.

5.4.2 *Fas/FasL System*

The Fas/Apo-1 (CD95)/Fas ligand (FasL) system plays an important role in the immune surveillance against cancer cells through the induction of their apoptosis. The Fas molecule is a death receptor that belongs to the TNF superfamily of receptors. Its primary and best-known function is the induction of apoptotic cell death after interaction with its physiological ligand FasL (Nagata 1999; Strasser et al. 2009). The presence of Fas molecule on the surface of N1 phenotype of TANs (Sionov et al. 2015; Piccard et al. 2012), as well the presence of membrane-bound FasL (mFasL) on colorectal (Pryczynicz et al. 2010), colon (Peduto Eberl et al. 1999; Zhang et al. 2005), renal (Peduto Eberl et al. 1999), liver (Shiraki et al. 1997), pancreatic (Kornmann et al. 2000), and breast cancer cells (O'Connell et al. 1999) has been well documented. The involvement of Fas/FasL system in the cytotoxic activity of neutrophils against tumor cells was evidenced in the mice model of hepatoma (Shimizu et al. 2001) or melanoma (Chen et al. 2002) tumors. It was also noted that neutrophils via Fas/FasL axis arrest the cell cycle of human lung carcinoma cell line and stop their proliferation, *in vitro* (Sun et al. 2018a). However, opposite findings have shown that interaction of neutrophils with human glioma cell lines via Fas/FasL is not enough to induce tumor cells' apoptosis (Hor et al. 2003). Other authors have reported that soluble FasL (sFasL), which is generated by cleaving mFasL, is a potent neutrophil chemoattractant but not a neutrophil activator (Ottonello et al. 1999; Dupont and Warrens 2007). However, it should be noted that the interaction of FasL on tumor cells with Fas on neutrophils can also initiate neutrophil apoptosis, which is considered to be one of mechanisms of tumor escape from immune surveillance (Chen et al. 2003).

5.4.3 *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 the anticancer immunity, such as NK cells, and activated cytotoxic T cells. In humans, five receptors for TRAIL are known: DR4, DR5, DcR1, DcR2, and OPG (MacFarlane 2003; Thorburn 2007; James and Griffith 2015). It was reported that neutrophils have been shown to express both TRAIL mRNA and surface protein as well as the TRAIL receptors DR5 and DcR1. TNF-α is known to downregulate,

while IFN-γ to upregulate the TRAIL surface level on neutrophils. Additionally, these phagocytes can release soluble TRAIL and DR5, particularly after stimulation with IFN-γ or lipopolysaccharide (LPS) (Kamohara et al. 2004; Cassatella 2006; Jablonska et al. 2008; Sag et al. 2019).

The potential antitumor significance of neutrophil-derived TRAIL has been examined and published. Koga et al. (2004) have demonstrated that IFN-γ-stimulated neutrophils employ TRAIL to exert cytotoxic effect on leukemic cells. Tecchio et al. (2004) have reported that soluble TRAIL, present in supernatants harvested from IFN-α-activated neutrophils, has remarkable proapoptotic impact against TRAIL-sensitive cells (Jurkat J32 clone and MEG-01). It was also evidenced that TRAIL expression on neutrophils surface and its release into cellular milieu greatly improve the efficacy of therapeutic effect of *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) in the treatment of patients with urothelial carcinoma of the bladder. The BCG accelerates the requirement of neutrophils to the tumor tissue and enhances TRAIL expression and its release from neutrophils, which results in the induction of cancer cells apoptosis (Ludwig et al. 2004; Rosevear et al. 2009; Brincks et al. 2013).

5.4.4 Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

The neutrophils can kill tumor cells via ADCC when specific antibodies are used to target malignant cells. This type of killing was described in the case of glioma, squamous cell carcinoma, neuroblastoma, melanoma, and ovarian cancer (Sionov et al. 2015). Neutrophils express several types of receptor for Fc fragment of antibody (FcγRI, FcγRIIa, FcγRIIIa, and FcγRIIIb), although not all of them are needed to develop ADCC. Although the expression of high-affinity FcγRI is very low or undetectable on resting neutrophils, its surface level arises rapidly after cells' stimulation with G-CSF, which concentration is very high in TME. Moreover, the contribution of FcγRI (even expressed) in neutrophil-related ADCC is still controversial. The principal receptor required for neutrophil-related ADCC is FcγRIIa, while FcγRIIIb serves as a decoy receptor and restricts neutrophils-dependent ADCC (Sionov et al. 2015; Uribe-Querol and Rosales 2015; van Egmond and Bakema 2013; Treffers et al. 2019). The mechanism, by which neutrophils kill tumor cells opsonized with antibodies, is called trogoptosis and was described by Matlung et al. (Matlung et al. 2018). This cytotoxic way is based on disruption of plasma membrane of tumor cell leading to its necrosis. Moreover, the authors evidence that direct interaction between neutrophils and tumor cells (before initiation of ADCC) is mediated through CD11b/CD18 integrin.

5.5 Protumoral Effects of TANs

Neutrophils are strongly involved in keeping tumor cell alive and in its ability to metastasize. The effect of TANs in this aspect is related to (1) release of various proangiogenic and protumoral products of granules; (2) a direct help to tumor cells extravasation; and (3) interaction with other immune cells to induce immunosuppression (Fig. 5.3).

5.5.1 Neutrophil Elastase (NE)

Neutrophil elastase (NE) is neutral serine protease produced by neutrophils that is stored in azurophilic granules. It is released into the extracellular space through degranulation or during neutrophil extracellular trap (NET) formation. It was first identified as an enzyme with bactericidal activity. Currently, it is well established that NE has various biological functions including an ability to destroy extracellular matrix (ECM) components. This serine protease has specificity against elastin, fibronectin, proteoglycans, and type IV collagen. NE is known to be implicated in

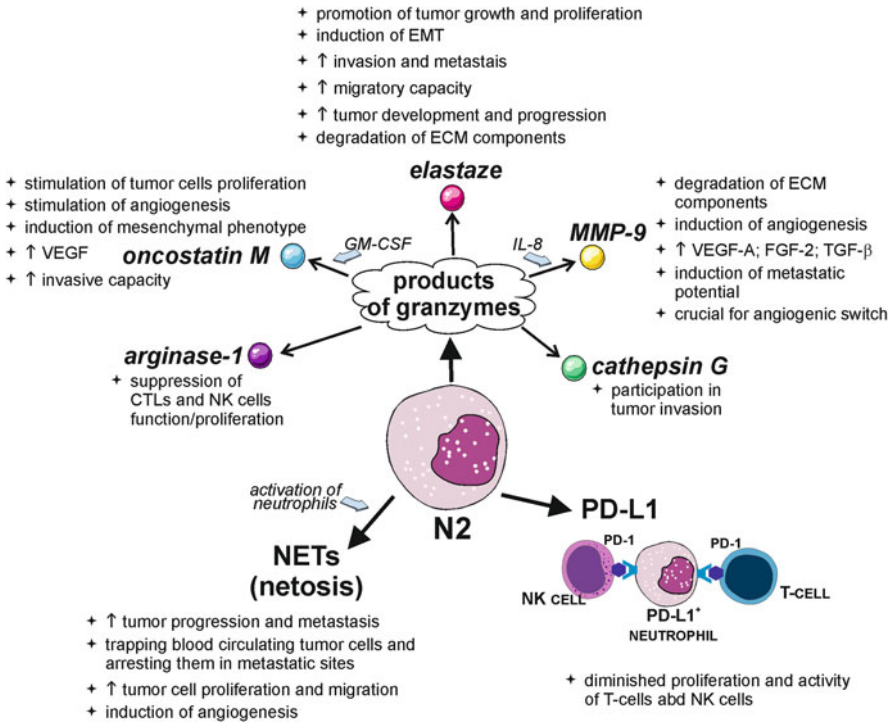

Fig. 5.3 Protumoral activity of N2 phenotype of tumor-associated neutrophils

a variety of inflammatory diseases including: chronic obstructive pulmonary disease, acute respiratory distress syndrome, ischemic-reperfusion injury or arthritis, as well as in various cancers (Pham 2008; Korkmaz et al. 2008). In neoplastic disease, NE promotes the development, progression, and metastasis of many tumors. Its protumorigenic role was clearly described in breast, gastric, and esophageal cancers (Treffers et al. 2016; Sun and Yang 2004). NE was also considered as a factor, which can indicate on patients' survival. Some reports have showed that breast cancer patients with high concentration of NE in tumor tissue were associated with rapid relapse and poor overall survival compared to individuals with low level of NE in TME (Foekens et al. 2003; Akizuki et al. 2007). Moreover, NE expression in tumor tissue predicted a poor clinical outcome and lymph node metastasis in oral squamous cell carcinoma (Jaiswal et al. 2019).

Several mechanisms have been described for protumoral activity of NE. First, it degrades the basement membrane (BM) and the ECM proteins, both of which are crucial for the invasion and metastasis of malignant cells. Second, NE can activate the membrane receptors such as epidermal growth factor receptor (EGFR) and toll-like receptor 4 (TLR4) resulting in the activation of MAP kinases allowing for the tumor cell proliferation (Lerman and Hammes 2018). Moreover, NE promotes tumor cell growth via direct activation of their prosurvival signaling pathway. It is possible because TANs secrete NE near the tumor cell surface; thus, it can enter 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). In the absence of IRS-1, the activity of PI3K is increased, leading to the phosphorylation of the serine/threonine kinase B (PKB), also known as AKT (Lerman and Hammes 2018; Houghton et al. 2010; Metz and Houghton 2011; Gregory and Houghton 2011). Activated AKT phosphorylates a variety of substrates that are crucial in maintaining cell growth and survival, and it also regulates glucose metabolism (Paez and Sellers 2003). Another important role of NE in tumor growth is related to an epithelial–mesenchymal transition (EMT) process of tumor cells. Gaida et al. (2012) have demonstrated that NE degrades E-cadherin on pancreatic tumor cells resulting in a significant increase of their migratory capacity and tumor invasion. Grosse-Steffen et al. (2012) have evidenced that NE cleaves E-cadherin on human pancreatic cancer cell line and effectively induces EMT in these cells.

The NE is multifunctional enzyme, and except its direct effect on tumor cells, this serine protease can also be involved in the modulation of the functional activity of whole TME. For example, NE appears to play the proangiogenic role through the regulation of metalloproteinases' activity. It targets the conversion of pro-MMP-9 and pro-MMP-8 into their biologically active forms, as well as inactivate TIMP-1, an inhibitor of these metalloproteinases (Lerman and Hammes 2018).

5.5.2 Matrix Metalloproteinase-9 (MMP-9)

Tumor invasion, metastasis, and angiogenesis require the controlled degradation of ECM through variety of MMPs. All metalloproteinases have been divided by researchers into several groups based on their structure and substrate specificity. The MMP-9 also called gelatinase B primarily hydrolyzes components of the basal lamina, including gelatin and collagen IV. The biological activity of MMP-9 also includes cleavage of cell surface proteins (e.g., cell adhesion molecules) and proteins present in extracellular environment (e.g., polypeptides) (Löffek et al. 2011; Huang 2018). Within neutrophils, MMP-9 is stored inside secondary granules and released upon stimulation with IL-8 (Xie 2001; Faurschou and Borregaard 2003; Chakrabarti et al. 2006). Neutrophils, opposed to other cells, secrete the unique form of MMP-9 which is free of TIMP-1, an endogenous inhibitor, poising it for activation and allowing for rapid and effective reveal of its catalytic activity (Ardi et al. 2007, 2009).

The TANs have been identified as a major source of MMP-9 in TME (Tazzyman et al. 2013; Deryugina et al. 2014). Moreover, *in vitro* and *in vivo* models (cell lines, mouse model, and human tumor tissue of various cancers) have clearly showed that neutrophil-derived MMP-9 is crucial for angiogenic switch and induction of metastatic potential of tumor cells. High angiogenic potency of neutrophil-derived MMP-9 is primarily related to its unique way of production (without TIMP-1), leading to an instant and rapid degradation of ECM components (Kuang et al. 2011; Ardi et al. 2007; Nozawa et al. 2006; Deryugina and Quigley 2010; Bausch et al. 2011). Apart from cleavage of ECM proteins, the proangiogenic function of neutrophil-derived MMP-9 is also linked with proteolytical release of VEGF-A and fibroblast growth factor-2 (FGF-2) usually sequestered in an inactive form within ECM (Bergers et al. 2000; Bekes et al. 2011; Deryugina and Quigley 2015), as well as with stimulation of the production and activation of proangiogenic factor TGF-β (Kobayashi et al. 2014). It was also noted that strong interplay exists between MMP-9 and VEGF-A, and the latest is known to regulate the MMP-9 production (Deryugina and Quigley 2015). However, what is interesting is that the neutrophils-derived MMP-9 can induce angiogenesis in the absence of VEGF-A, as it was described in pancreatic ductal adenocarcinoma (Bausch et al. 2011).

5.5.3 Oncostatin M (OSM)

Oncostatin M is a cytokine belonging to the IL-6 family of cytokines. It is produced by macrophages, monocytes, T cells, neutrophils, mast cells, and dendritic cells (DCs). Neutrophils treated with GM-CSF express and release high level of this cytokine. OSM effect in TME is connected with, e.g., stimulation of tumor cells' proliferation, stimulation of angiogenesis, and induction of mesenchymal phenotype of cancer cells (Elbjeirami et al. 2011; Richards 2013; Junk et al. 2017; West et al.

2018). The involvement of neutrophil-derived OSM in the tumor progression was well described in breast cancer model. The studies by Queen et al. (2005) have evidenced that human breast cancer cell lines through GM-CSF stimulate neutrophils to the secretion of oncostatin M, which in turn enhances VEGF production in cancer cells and increases their invasive capacity. More recently, Li et al. (2015) have described that TANs isolated from human hepatocellular carcinoma tissue exhibit high rate of autophagy, which correlates with elevated secretion of OSM and finally with a disease progression in HCC patients.

5.5.4 Cathepsin G (CG)

Cathepsin G is a serine protease stored in azurophilic granules of neutrophils and exhibits chymotrypsin-like and trypsin-like substrate specificity. This enzyme is known to participate in the destruction of extracellular pathogens and in the modification of chemokines and cytokines activity (proforms are cleaved into active forms). It also increases the permeability of endothelium (Pham 2008; Meyer-Hoffert and Wiedow 2011). The role of CG in tumor progression is rather barely known, but some studies have indicated on its participation in tumor cell invasion. As it was described, CG induces, *in vitro*, aggregation, formation of multicellular spheroids, and migration of human MCF-7 cells (breast cancer cells line) (Yui et al. 2005, 2014; Morimoto-Kamata et al. 2020). Other study, in the murine breast adenocarcinoma model, has demonstrated that CG enhances the tumor cells induced by osteoclastogenesis and subsequent osteolysis. It turn, the mammary tumor cells were responsible for elevated secretion of cathepsin G (Wilson et al. 2008). Similarly to NE, CG released from neutrophils can enter into endosomes of tumor cells (human lung adenocarcinoma cell lines) in the clathrin-dependent manner. However, in contrast to NE, cathepsin G is unable to degrade IRS-1, and its influence on intracellular signaling pathways is unknown (Gregory and Houghton 2011).

5.5.5 Arginase 1 (ARG-1)

The N2 TANs characterize with the expression of ARG-1, which is located in gelatinase granules. The neutrophils can secret ARG-1 after stimulation with IL-8 or TNF-α, which are products of tumor cells as was shown in the model of nonsmall cell lung cancer cell lines. The effect of ARG-1 on TME is connected with its strong immunosuppressive action. It was described that ARG-1-positive TANs positively correlate with suppressed functions of T cells (Rodriguez et al. 2009; Rotondo et al. 2009; Grzywa et al. 2020). ARG-1 catalyzes degradation of L-arginine to ornithine and urea resulting in the depletion of arginine in the extracellular milieu. Lack of L-arginine downregulates the expression of CD3ζ chain, a critical element of the CD3/TCR complex. This leads to an impairment of T-cell functions which, although

alive, do not proliferate and do not produce cytokines and chemokines. Moreover, the absence of L-arginine affects NK cells, e.g., the expression of NKp46 and NKp30 activating receptors and IFN-γ secretion are diminished as well as the proliferation of these cells is lowered (Grzywa et al. 2020; Oberlies et al. 2009; Munder 2009). The clinical significance of neutrophils' ARG-1 was described by Sippel et al. (2011). The authors have showed that immunosuppression in patients with glioblastoma is related to degranulation of neutrophils and release of ARG-1. Another study has evidenced that neutrophils isolated from blood and tumor tissue of glioma patients characterize with high expression of ARG-1 and have potent immunosuppressive effect on T cells (Gielen et al. 2016). It was also described that $CD15^+$ $ARG\text{-}1^+$ cells with neutrophil's morphology were frequently identified in tumor tissue of gastric, colorectal, and prostate carcinomas, while in adenomas, their expression was very low or undetectable (Jang et al. 2018).

5.5.6 PD-L1

The neutrophils of cancer patients can express an immune checkpoint molecule, a ligand for programmed cell death protein 1 (PD-L1). The PD-L1-positive neutrophils were found in tumor tissue of, e.g., hepatocellular carcinoma (He et al. 2015) and gastric cancer (Wang et al. 2017). Moreover, higher number of PD-L1-positive neutrophils in tumor tissue of gastric cancer has correlated with disease progression and poor survival of patients (Wang et al. 2017). The expression of PD-L1 can also be found on macrophages, some activated T cells and B cells, DCs, and primarily on tumor cells (Han et al. 2020). In human cancers, the expression of programmed cell death protein 1 (PD-1) is observed on T cells, B cells, NK cells, macrophages, and DCs. The PD-1/PD-L1 axis is responsible for cancer immune escape. For example, the interaction of PD-L1 (present on cancer or immune cells) with PD-1 expressed on T cells induces the immunosuppressive signal, leading to the impairment of T-cell effector function (e.g., proliferation, cytokine secretion) (Han et al. 2020; Sun et al. 2018b). The involvement of PD-L1-positive neutrophils in diminishing the activity of T cells was evidenced by Wang et al. (2017). The authors have clearly demonstrated that $PD\text{-}L1^+$ neutrophils from gastric cancer tissue, activated by GM-CSF, suppress the proliferation of autologous T cells, *in vitro.* Apart from T cells, the NK cells can also interact with neutrophils through PD-1/PD-L1 axis. In a mouse model of colon cancer, an inhibitory effect of neutrophils on NK-cell cytotoxicity in the PD-1/PD-L1-dependent manner was observed (Sun et al. 2020). Another study showed that checkpoint molecules play an important role in the direct interaction of neutrophils with tumor cells. Gershkovitz et al. (2020) described that PD-L1-negative neutrophils exert higher cytotoxic activity against breast cancer cell lines then their PD-L1-positive counterparts.

5.5.7 *Neutrophils Extracellular Traps (NETs)*

NETs were discovered in the year 2004 by Brinkmann et al. (2004) as an antimicrobial mechanism degrading viruses and bacteria. The formation of NET is called netosis, and this process is connected with changes in neutrophils' morphology and function and is considered as a unique form of cell death. NETs consist of the chromatin DNA filaments and variety of proteins from granules, cytoplasm, and cytoskeleton. Induction of netosis requires stimulation of neutrophils and is directly connected with an activation of NADPH oxidase and ROS production. ROS in turn activates protein-arginine deiminase type 4 (PAD4), which catalyzes hypercitrullination of histones responsible for decondensation of chromatin. However, ROS-and PAD4-independent way of NETs release was also noted. Among the best-known stimulators of netosis are bacterial products (e.g., LPS, PMA), cytokines, chemokines (e.g., IL-8, TNF-α, G-CSF), and drugs (e.g., statins). Apart from its participation in the immune defense, NETs play a role in some pathological conditions like arteriosclerosis, autoimmunity, diabetes, and malignant disease (Kaplan and Radic 2012; Papayannopoulos 2018; Liu and Liu 2019).

TME is rich in cytokines/chemokines (e.g., TNF-α, G-CSF, IL-8) and proinflammatory factors (e.g., leukotriene B4) that can easily activate neutrophils and potentially induce netosis. Although current knowledge regarding NETs and tumors is still at the beginning of elucidation, generally it is though that the generation of NETs favors the tumor progression and formation of metastasis (Masucci et al. 2020). The presence of NETs in human TME was not extensively studied; however, their expression was found in patients' tumor tissue of Ewing sarcoma (Berger-Achituv et al. 2013), lung cancer (Li et al. 2019), and triple-negative human breast cancer (Park et al. 2016). The involvement of NETs in the development of metastasis to distinct anatomical sites was proofed in mouse models of lung carcinoma (which form metastasis to liver) (Cools-Lartigue et al. 2013), in mouse model of ovarian cancer (which metastasized to omentum) (Lee et al. 2018) as well as in metastatic lung lesion of breast cancer patients (Park et al. 2016) and in liver metastasis of colorectal cancer (Tohme et al. 2016). The direct involvement of tumor cells in the formation of NETs was recently a hot topic in a research area. The *in vitro* studies on pancreatic cancer cells (Jung et al. 2019) or triple-negative breast cancer cells (Park et al. 2016) have clearly demonstrated that tumor cells can successfully induce NETs' formation. What is more, studies with breast cancer cells evidence that secreted G-CSF is responsible for human neutrophils activation and NETs' formation (Park et al. 2016; Arpinati et al. 2020).

The effect of NETs on tumor progression is related to the activity of NE, CG, and MMP-9, which functions in TME were described above. Moreover, NETs can also trap tumor cells circulating in blood vessels and arrest them into metastatic site as was proofed in the mouse model of various cancers and on clinical samples of triple-negative breast cancer patients (Uribe-Querol and Rosales 2015; Park et al. 2016; Cools-Lartigue et al. 2013, 2014). Another mechanism of tumor promotion through NETs includes stimulation of cancer cell proliferation and migration. Yang et al.

(2020) showed that DNA of NETs binds to the CCDC25 receptor present on cell surface of human breast cancer cell lines and on patients' primary breast cancer cells, enabling tumor cells' adhesive properties, invasive potential, and proliferation. Very interesting mechanism of NETs' involvement in tumor immune escape has been recently proposed by Teijeira et al. (2020). They have shown that chemokines (targeted CXCR1 and CXCR2 on neutrophils), secreted by human colorectal adenocarcinoma cell line, effectively induce neutrophils' netosis. In turn, NETs wrap and coat colorectal cancer cells protecting them from the cytotoxicity of effector $CD8^+$ T cells. Another protumoral capability of NETs was described by Martins-Cardoso et al. (2020). NETs isolated from supernatant of PMA activated blood neutrophils to induce the prometastatic phenotype of human breast cancer cells (MCF7 cell line) through the promotion of EMT.

5.5.8 Tumor Cell Extravasation

The first step of metastasis process is a detachment of neoplastic cells from primary tumor, breaking down the basement membrane of tumor blood vessels and then intravasation of cells into circulation. The circulatory tumor cells (CTCs) must survive blood flow shear forces and immune system challenges, and thus, shortly after entering into capillaries, they pass through the endothelial vessel wall (extravasation) into surrounding areas or distant sites from primary tumor. The CTCs' extravasation is multistep process and requires: (1) initial attachment and next firm adhesion of tumor cells to endothelium; (2) modulation of endothelial barrier; and (3) transmigration through endothelium into the tissue (Madsen and Sahai 2010; Strilic and Offermanns 2017; Sökeland and Schumacher 2019).

Several reports have indicated that neutrophils facilitate and enhance tumor cells' extravasation process. Wu et al. (2001) 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 cells' transendothelial migration. Importantly, MDA-MB-231 cells alone do not transmigrate. Slattery and Dong (2003) have reported that neutrophils enhance the migration of human melanoma cells (C8161) under flow conditions and improve C8161 cells' adhesion to fibroblast L-cells. In later studies, Dong et al. (2005) concluded that neutrophils facilitate melanoma cells' tight adhesion on the endothelium and their subsequent transendothelial migration. The direct evidence for the neutrophils' involvement in the metastasis process of tumor cells was provided by Spicer et al. (2012) and McDonalds et al. (2009). Using *in vivo* models of metastasis and intravital microscopy, they have shown that neutrophils promote cancer cells' adhesion within liver sinusoids, and neutrophils may act as a bridge to facilitate interactions between cancer cells and the liver parenchyma.

The mechanism of 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 CTCs first interact with circulating neutrophils to form "heterotypic aggregates" and subsequently bind to the endothelium through neutrophils (Piccard et al. 2012; Liang et al. 2005, 2008; Fu et al. 2011). The contribution of neutrophils in extravasation process is mediated by the direct contact between neutrophils, CTCs, and endothelial cells. This three-way interaction occurs due to the expression of CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1, β2 integrins) on the neutrophil surface; the presence of E-selectin and ICAM-1 on endothelial cells; and the expression of ICAM-1 and Sialyl-Lewis X (sLeX) on tumor cells (Sionov et al. 2015; Piccard et al. 2012; Slattery and Dong 2003; Fu et al. 2011; Wu et al. 2020). Although several cytokines and chemokines can be implicated in the adhesive activity of neutrophils and tumor cells, IL-8 is particularly important. This cytokine (released by neutrophils and tumor cells) enhances Mac-1 and LFA-1 expression. In addition, IL-8 activates endothelial cells and promotes angiogenesis (Dong et al. 2005; Waugh and Wilson 2008). 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. (Huh et al. 2010). They have demonstrated that the reduction of IL-8 expression in melanoma cells (WM35 cell line) using small interfering RNA (siRNA) decreases their interaction with neutrophils and diminishes the melanoma cells tethering on endothelium and across endothelial cell layer (Peng et al. 2007). The summary of neutrophils' prometastatic role has been expressly presented by Liang et al. (2009). The authors have described that neutrophils facilitate the adhesion of melanoma cell lines (C8161.c9; WM9) to the endothelial cells allowing tumor cells to extravasation. Moreover, the authors proved that ICAM-1 on melanoma cells, as well as LFA-1 and Mac-1 on neutrophils significantly participate in above processes, while IL-8 regulates β2 integrin expression.

5.6 Conclusion

In TME, neutrophils are called tumor-associated neutrophils and represent highly heterogenic population of cells, which display both positive (antitumoral) and negative (protumoral) role. The first role is mainly related to the cytotoxic action of neutrophils and relay on the production of reactive oxygen species and expression of death receptors. The protumoral properties of neutrophils are more frequently observed in several malignant diseases and are primarily connected with release of various products of granules displaying prometastatic, proangiogenic, and immunosuppressive activities. No less important protumoral activity of neutrophils is connected with their ability to directly help tumor cells in extravasation process. Thus, neutrophils, although rather short-living cells, are one of the key players in the functions of whole TME.

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Chapter 6
Role of NK Cells in Tumor Progression

Iñigo Terrén and Francisco Borrego

Contents

Abstract Natural Killer (NK) cells are effector lymphocytes with the ability to generate an antitumor response. NK cells encompass a diverse group of subsets with different properties and have the capacity to kill cancer cells by different means. However, tumor cells have developed several mechanisms to evade NK cell-mediated killing. In this chapter, we summarize some aspects of NK cell biology with the aim to understand the competence of these cells and explore some of the challenges that NK cells have to face in different malignancies. Moreover, we will review the current knowledge about the role of NK cells in tumor progression and describe their phenotype and effector functions in tumor tissues and peripheral blood from cancer patients. Finally, we will recapitulate several findings from different studies focused on determining the prognostic value of NK cells in distinct cancers.

Keywords NK cells · Cancer · TME · Tumor microenvironment · Tumor evasion · Solid tumors · Tumor-infiltrating lymphocytes · TINK cells · Prognosis · CD56

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Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
CCL3	C-C Motif Chemokine Ligand 3
CIC	Cancer-initiating cell
CSC	Cancer stem cell
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
Eomes	Eomesodermin
FcγRIII	Fc gamma receptor 3
GM-CSF	Granulocyte-macrophage colony–stimulating factor
HCC	Hepatocellular carcinoma
HIF-1α	Hypoxia-inducible factor 1 alpha
HLA	Human leukocyte antigen
HNC	Head and neck cancer
IFNγ	Interferon gamma
IL	Interleukin
ILC	Innate lymphoid cell
KIR	Killer-cell immunoglobulin-like receptor
MHC	Major histocompatibility complex
MICA	MHC class I chain–related protein A
NCAM	Neural cell adhesion molecule
NK	Natural Killer
NSCLC	Non–small cell lung cancer
scRNA-seq	Single-cell RNA sequencing
TGF-β	Transforming growth factor beta
TINK	Tumor-infiltrating NK cell
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand

6.1 Introduction

Natural killer (NK) cells are effector lymphocytes that belong to the innate lymphoid cells (ILCs) family. They constitute around 5–15% of lymphocytes in the blood, and different subsets of NK cells can be also found in multiple tissues and organs (Freud et al. 2017). In contrast to cytotoxic T lymphocytes (CTLs), NK cells do not require prior sensitization to exert their cytotoxic activity. Moreover, NK cells show limited reactivity against healthy cells, which, when combined with their ability of recognizing and killing tumor cells, make them key players in the defense against several malignancies. Furthermore, NK cells have the ability to produce and secrete a number of cytokines and chemokines that can orchestrate innate and adaptive

immune responses, further contributing to tumor surveillance. In this chapter, we will summarize the current knowledge on NK cell biology, focusing on their phenotype and effector functions, and review some mechanisms that cancer cells have developed to escape from NK cell cytotoxic activity. Finally, we will explore the role of NK cells in tumor progression, describing the characteristics of NK cells in cancer patients and their value as prognostic markers.

6.2 NK Cell Biology

6.2.1 NK Cell Diversity

NK cells were firstly described in 1970s as non–thymus-derived non-Ig-bearing lymphoid cells with the ability to kill target cells via antibody-dependent cell-mediated cytotoxicity (ADCC) and other cell contact–dependent mechanisms (Greenberg et al. 1973; Kiessling et al. 1975a; Kiessling et al. 1975b). These initial studies identified NK cells by excluding other lymphocytes but failed to find a specific NK cell marker. Almost 50 years later, understanding of NK cell biology has greatly improved, but immunologists have not yet found a specific marker for NK cells. Currently, human NK cells can be phenotypically identified by the lack of expression of specific markers of other leukocytes, including markers of T cells (e.g., CD3), B cells (e.g., CD19), and myeloid cells (e.g., CD14), and by the expression of neural cell adhesion molecule (NCAM, also known as CD56) and Fc gamma receptor 3 (FcγRIII, also known as CD16). Two major NK cell subsets can be distinguished based on CD56 and CD16 expression: $CD56^{bright}CD16^{low/-}$, and $CD56^{dim}CD16^{+}$ NK cells. Together, these subsets constitute the majority of NK cells in peripheral blood, but the lack of specific NK cell markers makes the identification of other NK cell subset that do not express CD56 very challenging. For instance, it has been reported that $CD56^{neg}$ NK cells, which exhibit a phenotype similar to the $CD56^{dim}$ subset (Voigt et al. 2018), are expanded under certain pathologies, such as patients infected with human immunodeficiency virus or hepatitis C virus, patients coinfected with cytomegalovirus and Epstein–Barr virus, and patients with multiple myeloma (Mavilio et al. 2005; Alter et al. 2011; Müller-Durovic et al. 2019; Vitallé et al. 2019; Orrantia et al. 2020a). Furthermore, CD16 expression can be downmodulated due to activation or cryopreservation (Peruzzi et al. 2013; Oliviero et al. 2017; Tang et al. 2015; Zhou et al. 2013; Romee et al. 2013; Lugthart et al. 2015) and could hamper the identification of $CD56^{neg}$ NK cells, which, on the other hand, share phenotypic similarities with other ILCs (Vivier et al. 2018), so the proper classification and identification of these cells may be challenging. To this end, alternative NK cell identification strategies have been proposed based on the expression of NKp80 and/or the transcription factor eomesodermin (Eomes) (Orrantia et al. 2020a; Vivier et al. 2018; Vitale et al. 2001; Freud et al. 2016; Verma et al. 2020; Orrantia et al. 2020b).

In addition to CD56 and CD16, human NK cells express a vast repertoire of surface molecules that have been used to characterize multiple subsets in peripheral blood and different tissues (Freud et al. 2017). Excitingly, this field is constantly evolving and new NK cell subsets are being revealed and characterized. The immunophenotypic profiling of NK cells (and other cells) requires simultaneously analyzing multiple markers and has been always limited by existing technologies. In this way, improvements in flow cytometry technology have allowed to understand how diverse the NK cell repertoire is. Furthermore, emerging technologies such as mass cytometry and single-cell RNA sequencing (scRNA-seq) are providing a more insightful understanding of NK cell biology and the number of different subsets in health and disease. For instance, by using mass cytometry, it has been estimated that there could be 6000–30,000 phenotypic NK cell subpopulations in any human being (Horowitz et al. 2013). Transcriptomic analysis based on scRNA-seq has also confirmed the heterogeneity of these lymphocytes and has been useful to reveal organ-specific signatures (Crinier et al. 2018; Yang et al. 2019; Smith et al. 2020). Interestingly, whether surface markers can effectively identify and classify NK cell subsets is still a matter of debate. For instance, innate lymphoid cell (ILC) 1 and NK cells can be defined by their phenotype at steady state, but the analysis becomes more complex when cells become activated (Seillet et al. 2021). Moreover, in the context of tumor microenvironment (TME), it has been described that murine NK cells can be converted to ILC1-like cells by the effect of transforming growth factor beta (TGF-β) (Gao et al. 2017). The fact that a specific phenotype is linked to determined effector functions has also been discussed by recent data. It has been traditionally accepted that among the two major subsets of NK cells, $CD56^{bright}$ cells play an immunomodulatory role, while $CD56^{dim}$ cells are specialized in target killing (Freud et al. 2017). However, it has been described that $CD56^{bright}$ NK cells can perform potent cytotoxic activity following priming with interleukin (IL)-15 or feeder cell–based expansion protocols (Wagner et al. 2017; Poznanski et al. 2018). In light of these findings, some authors have proposed that NK cells could be also classified depending on their metabolism, which is intricately linked to their maturation status or functional state (Poznanski and Ashkar 2019; O'Brien and Finlay 2019; Terrén et al. 2019; Marçais et al. 2014). Nonetheless, using phenotypic and/or metabolic features to characterize NK cells leads to the conclusion that this lymphocyte subset constitutes a complex and diverse group of cells.

6.2.2 NK Cell Effector Functions

The relevance of NK cells in tumor surveillance is evidenced by their capacity of killing malignant cells while avoiding any damage to healthy cells. This ability is the result of a balance between signals from germline-encoded activating and inhibitory receptors, which will determine if NK cells become activated. NK cells go through an education process through which they gain reactivity and the capability of being inhibited by major histocompatibility complex (MHC) class I proteins, termed

human leukocyte antigens (HLAs) class I in humans (Boudreau and Hsu 2018). MHC class I molecules expressed in healthy cells are recognized by a variety of MHC-specific inhibitory receptors expressed in NK cells, including the polygenic and polymorphic family of killer-cell immunoglobulin-like receptors (KIRs) and the heterodimeric receptor CD94/NKG2A. Missing-self hypothesis explains how MHC class I recognition by inhibitory receptors prevents NK cell activation and thus protects healthy cells. Contrarily, malignant cells downmodulate the expression of MHC class I molecules and/or increase the expression of stress ligands that can bind to numerous activating receptors expressed on NK cells, such as NKG2D, natural cytotoxicity receptors (including NKp30, NKp44 and NKp46), CD94/NKG2C, DNAM1, or 2B4, among others. Additionally, NK cells can be activated through CD16, which can bind to opsonized target cells and then induce ADCC (Terrén et al. 2020). Therefore, when encountering tumor cells with the above-mentioned features, the balance between inhibitory and activating signals is tilted toward the latter, and NK cells will become activated.

NK cells can directly and indirectly kill cancer cells through different mechanisms (Fig. 6.1). Upon target cell recognition, NK cells can exert direct cytotoxicity or ADCC by releasing granules containing cytotoxic molecules, including perforin and granzymes. Perforin molecules are released as monomers that aggregate and form pores in the target cell membrane, thereby allowing the internalization of granzymes and inducing an osmotic imbalance (Prager and Watzl 2019). Of note, it has been also proposed that granzyme B can enter into target cells independent of perforin via endocytosis (Veugelers et al. 2004). Once internalized, granzymes can induce caspase activation, mitochondrial dysfunction, and other caspase-independent mechanisms that will result in target cell apoptosis (Prager and Watzl 2019). Alternatively, NK cells can kill target cells by inducing death receptor–dependent apoptosis. This mechanism is dependent on the binding of ligands from the tumor necrosis factor (TNF) superfamily expressed by NK cells, including FasL (also known as CD95L), TRAIL (TNF-related apoptosis-inducing ligand) and TNF, to their respective (death) receptors expressed in target cells. Upon engagement, death receptors initiate a signaling cascade that, similar to granzymes, leads to the activation of caspases and subsequent mitochondrial damage, thereby inducing apoptosis of target cells. Intriguingly, during serial killing, NK cells preferentially use granule-mediated mechanisms (i.e., direct cytotoxicity and ADCC) for their first encounters, and then switch to death receptor–related mechanisms for the subsequent target cell encounters (Prager et al. 2019). Furthermore, activated NK cells can modulate immune response by producing and secreting cytokines, chemokines, and growth factors, including TNF, IFNγ (interferon gamma), IL-5, IL-10, IL-13, CCL3 (C-C Motif Chemokine Ligand 3), CCL4, CCL5, GM-CSF (granulocyte-macrophage colony–stimulating factor), and others (Bald et al. 2020; Caligiuri and a. 2008; Morvan and Lanier 2016). Through these molecules, NK cells provide an additional mechanism to control tumor growth. For instance, it has been described that NK cells can recruit conventional type 1 dendritic cells (DCs) into the TME via CCL5 and CXCL1 chemokines (Böttcher et al. 2018). DCs, among other functions, can then recruit effector CD8+ T cells into TME and also activate naïve CD8+ T cells in the

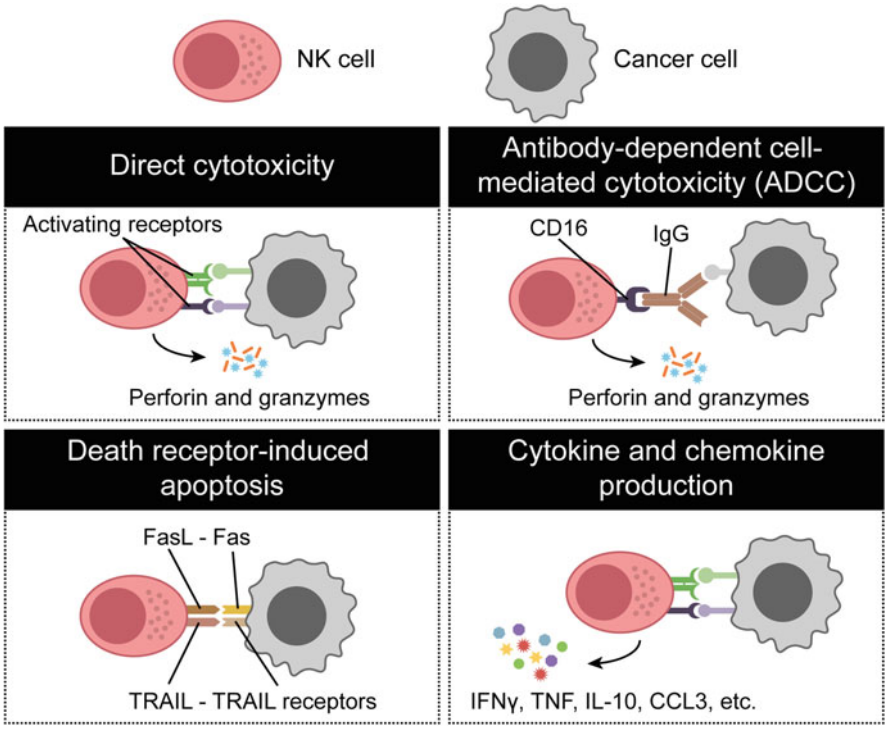

Fig. 6.1 Killing mechanisms mediated by NK cells. NK cells can kill tumor cells by releasing perforin- and granzyme-containing granules following activation through activating receptors (direct cytotoxicity) or through CD16 (ADCC). Alternatively, NK cells can induce apoptosis of tumor cells by binding FasL or TRAIL to their respective receptors expressed in cancer cells (death receptor–dependent apoptosis). Moreover, activated NK cells can secrete different cytokines and chemokines that modulate the innate and adaptive immune responses

lymph nodes, thus serving as a bridge between NK cells and adaptive immune responses (Peterson and Barry 2021). Altogether, these functions highlight the versatility of NK cells and their capacity to eliminate target cells through different mechanisms.

6.3 Mechanisms of Tumor Resistance to NK Cell–Mediated Killing

Unfortunately, cancer cells have developed a wide variety of mechanisms to escape from NK cell–mediated killing. Besides tumor cells, many tumor-associated cells can be found in the TME, such as myeloid-derived suppressor cells, regulatory T cells, or tumor-associated fibroblasts and macrophages (Vitale et al. 2014). Together, tumor and tumor-associated cells generate an immunosuppressive

microenvironment that blunts NK cell effector functions. These cells create a nutrient-depleted TME that induce a metabolic restriction on tumor-infiltrating NK (TINK) cells, thus limiting their effector functions (Terrén et al. 2019). Particularly, hypoxia is commonly found in solid tumors due to disorganized vascularization. Hypoxia has been described to reduce cytokine and chemokine secretion, and cytotoxicity of NK cells, as well as the expression of activating receptors and cytotoxic molecules (Parodi et al. 2018; Balsamo et al. 2013; Sarkar et al. 2013; Solocinski et al. 2020; Guan et al. 2020). Cells adapt to hypoxia by stabilizing the transcription factor HIF-1α (hypoxia inducible factor 1 alpha) and therefore increasing its expression. Consequently, TINK cells showed higher expression of HIF-1α and upregulation of the transcription of HIF-1α target genes (Guan et al. 2020). Inconveniently, the increased HIF-1α expression may be detrimental to NK cell functions. A recent report revealed that the expression of HIF-1α in TINK cells negatively associates with their antitumor potential in both mouse and humans (Ni et al. 2020). Thus, hypoxia represents one of the major obstacles for NK cell functions in the TME.

Hypoxia can further contribute to tumor escape by downmodulating the expression on malignant cells of MICA (MHC class I chain-related protein A) and MICB, which are ligands of the activating receptor NKG2D (Barsoum et al. 2011; Yamada et al. 2012; Schilling et al. 2015; Siemens et al. 2008; Lu et al. 2015b). The reduction in the surface expression of these ligands is mediated by the hypoxia-induced upregulation of the metalloproteinase ADAM10 (Barsoum et al. 2011; Ou et al. 2019). In some malignancies, shedding of surface MICA and MICB is paralleled with an increment in soluble MICA and MICB (Lu et al. 2015b; Ou et al. 2019; Basher et al. 2020), although it has been reported some exceptions in which soluble MICs are not increased (Yamada et al. 2012). Soluble NKG2D ligands have been described to induce the internalization of NKG2D and thus downmodulate the expression of this receptor in NK cells (Doubrovina et al. 2003; Song et al. 2006). Accordingly, elevated soluble MICA levels found in serum of patients with prostate cancer or hepatocellular carcinoma (HCC) inversely correlated with surface NKG2D expression in NK cells (Wu et al. 2004; Jinushi et al. 2005). Moreover, serum levels of soluble MICB inversely correlated with the frequency of circulating NK cells in patients with metastatic prostate cancer (Liu et al. 2013). A recent report thoroughly analyzed this effect and found that NK cells cultured with soluble MICB downmodulated genes regulating cell proliferation and survival, and increased proapoptotic genes and genes that are inhibitors of the cell cycle (Basher et al. 2020). Interestingly, it has been proven that soluble MIC-neutralizing antibodies can restore NK cell homeostasis and function against MIC+ tumor cells (Basher et al. 2020; Lu et al. 2015a; Basher et al. 2016). In conclusion, shedding of surface NKG2D ligands by cancer cells may impair NK cell antitumor activity at different levels and therefore, targeting soluble NKG2D ligands represents a therapeutic approach aimed to increase NK cell activity.

Elevated TGF-β levels are commonly found in the TME and serum of many cancer patients (Ma et al. 2020; Lee et al. 2004; Zecca et al. 2020). TGF-β has been described to antagonize the induction of the transcription factor T-bet, a positive

regulator of IFNγ production, in NK cells following stimulation with IL-12, IL-15, and IL-18 (Yu et al. 2006). Accordingly, TGF-β inhibits NK cell production of IFNγ in response to IL-12 and IL-18 stimulation, and following CD16 activation (Laouar et al. 2005; Trotta et al. 2008). TGF-β can also modulate NK cell phenotype. The expression of CXCR3, CXCR4, and CX_3CR1 chemokine receptors is greatly affected by TGF-β (Castriconi et al. 2013). Several authors have reported that TGF-β downmodulates the expression of NKp30 and NKG2D receptors in NK cells (Lee et al. 2004; Castriconi et al. 2013; Castriconi et al. 2003; Fujii et al. 2018; Wilson et al. 2011; Han et al. 2018; Zenarruzabeitia et al. 2017; Tran et al. 2017). Intriguingly, CD16 expression is not altered when NK cells were cultured overnight with TGF-β (Trotta et al. 2008), while longer cultures (15 days) induced the downregulation of this receptor (Allan et al. 2010; Keskin et al. 2007). Loss of CD16 expression in NK cells cultured with TGF-β was also paralleled with an increased expression of CD103 and CD9, characteristic of decidual NK cells (Freud et al. 2017; Keskin et al. 2007). The upregulation of CD103 and CD9 in response to TGF-β was also reported by other authors (Cerdeira et al. 2013; Montaldo et al. 2016; Hawke et al. 2020a; Hawke et al. 2020b). However, Hawke et al. suggested that, similar to what has been demonstrated in mouse NK cells (Gao et al. 2017), human NK cells acquire an ILC1-like phenotype, instead of decidual-like phenotype upon TGF-β exposure (Hawke et al. 2020a; Hawke et al. 2020b). Besides its effect over NK cell functions and phenotype, TGF-β is able to inhibit metabolic activity of NK cells (Viel et al. 2016; Zaiatz-Bittencourt et al. 2018). This effect has been shown to be particularly relevant in patients with metastatic breast cancer, in which NK cells exhibited metabolic and functional defects. Remarkably, authors found that neutralizing TGF-β restored the metabolic activity and IFNγ production of the patient NK cells (Slattery et al. 2021). Similarly, other authors reported that plasma from HCC patients contained elevated levels of TGF-β, and that exposing NK cells from healthy donors to plasma from HCC patients induced metabolic and functional defects. These defects were also restored when anti-TGF-β antibodies were added (Zecca et al. 2020). Therefore, in the TME, TGF-β plays a major role by modulating NK cell effector functions, phenotype, and metabolism.

In the TME, numerous soluble factors can be found, such as prostaglandin E2 and L-kynurenine, that also have an immunosuppressive effect on NK cell functions by reducing the expression of activating receptors and cytotoxic activity (Chiesa et al. 2006; Li et al. 2012; Park et al. 2018; Pietra et al. 2012). These molecules derived from tumor and tumor-associated cells modulate NK cell phenotype and functions, so cancer cells can resist against functionally suppressed NK cells. Also, certain tumor cells have the ability to resist the lytic activity of fully competent NK cells. It has been described a large variety of mutations that allow cancer cells to resist NK cell cytotoxicity, such as gene mutations that interfere with the activity of death receptors or caspases (Sordo-Bahamonde et al. 2020). Knowledge about NK cell killing-resistance mechanisms is crucial to predict the role that NK cells can play during the progression of the disease. Equally, a thorough characterization of TINK cells and the TME in which they are located is necessary to recognize if NK cells are relevant in the outcome of different types of cancer.

6.4 NK Cells in Tumor Progression

In certain tumors, HLA class I expression is low or absent. This feature confers cancer cells protection against the CTL-mediated killing, but not against the NK cell–mediated cytotoxic activity. Reduced expression of HLA class I was also found in cancer stem cells (CSCs) or cancer-initiating cells (CICs), a rare subpopulation within a tumor that are resistant to chemotherapy and radiotherapy (Ravindran et al. 2019). Notably, multiple reports have indicated that NK cells can recognize and eliminate CSCs and CICs (Tallerico et al. 2013; Ames et al. 2015; Pietra et al. 2009; Tallerico et al. 2017; Ferreira-Teixeira et al. 2016; Castriconi et al. 2009; Close et al. 2020; Cristiani et al. 2019). Therefore, NK cells have the potential of eradicating tumor and tumor-initiating cells, which could be crucial to prevent tumor progression and metastases (López-Soto et al. 2017). However, NK cells have a poor infiltration in solid tumors, and those NK cells that reached the TME show an altered phenotype and effector functions because of the immunosuppressive TME. In this section, we will discuss these points and the current knowledge about the role of NK cells in tumor progression.

6.4.1 NK Cell Infiltration in the TME

NK cells play a key role in initiating and promoting the antitumor response. This process involves several steps, including the recruitment of NK cells to the TME, recognition of tumor cells and activation, killing of the target cells, and orchestrating innate and adaptive immunity (Bald et al. 2020). The first step depends, at least in part, on the expression of several homing receptors by NK cells and the presence of their respective soluble chemokine ligands in the TME (Bald et al. 2020; Yao and Matosevic 2021). It has been proposed that tumor cells can modulate the expression of chemokines in the TME to preferentially attract less cytotoxic NK cells. Reduced expression of CXCL12, CX_3CL1, CXCL1, and CXCL8 could hinder the recruitment of $CD56^{dim}$ NK cells, while increased expression of CXCL2, CXCL9, CXCL10, CCL5, and CCL19 could promote the migration of $CD56^{bright}$ NK cells (Bald et al. 2020; Castriconi et al. 2018). Moreover, TGF-β can modulate the expression of chemokine receptors in NK cells, thereby representing another mechanism through which NK cell recruitment to the TME can be hampered by cancer cells (Castriconi et al. 2013).

As previously mentioned, it could be challenging to properly identify NK cells due to the lack of specific markers. Initial studies used CD57 to identify NK cells, although this is a marker of a subset of $CD56^{dim}$ NK cells and can also be expressed by CD8+ T cells (Nielsen et al. 2013; Russick et al. 2020). Some authors used CD56 as a marker for NK cells. However, CD56 is also expressed by ILC3 and intraepithelial ILC1, and by some T cell subsets (Simoni and Newell 2018; Kovalenko et al. 2021). Recent publications suggest that NKp46 could be more

accurate to identify NK cells in the TME (Cózar et al. 2021), although this receptor is also shared with other ILCs (Seillet et al. 2021; Simoni and Newell 2018). Thus, knowledge about NK cell infiltration in solid tumors and their prognostic value can be different depending on the strategy used to identify NK cells. Nonetheless, multiple studies using either CD57, CD56 or NKp46 markers have confirmed the presence of NK cells in a wide variety of solid tumors (Russick et al. 2020; Cózar et al. 2021; Nersesian et al. 2021).

6.4.2 Phenotype of TINK Cells

TME can selectively recruit certain NK cell subsets and modulate their phenotype. Consequently, TINK cells and circulating NK cells have differences in their phenotype and transcriptional programs (Guan et al. 2020; de Andrade et al. 2019). It is interesting to note that some of these phenotypic changes include a differential expression of immune checkpoints, such as PD-1 or TIM-3. Higher frequency of PD-1+ NK cells has been reported in the peritoneal fluid of patients with ovarian carcinoma, compared to peripheral blood NK cells from both patients and healthy donors (Pesce et al. 2017). Similar results have been recently reported in TINK cells from patients with non–small cell lung cancer (NSCLC) and head and neck cancer (HNC) (Trefny et al. 2020; Concha-Benavente et al. 2018). Interestingly, it has been found that PD-1 expression could be higher in circulating NK cells from patients with certain cancers, such as Kaposi sarcoma and HNC, compared to healthy donors (Concha-Benavente et al. 2018; Beldi-Ferchiou et al. 2016). Another study in HCC patients revealed that there is an accumulation of tissue-resident CD49a+ NK cells in the TME, and that this subset also expressed higher levels of PD-1, TIGIT, and CD96 than the intratumoral CD49a- NK cell subset (Sun et al. 2019). PD-1 upregulation in TINK cells can be a consequence of tumor-derived cytokines, or the increased glucocorticoids levels found in the plasma of cancer patients, or even the chemotherapeutic agents used for the treatment (Park et al. 2017; Quatrini et al. 2021; Makowska et al. 2020). Higher TIM-3 expression has been reported in NK cells from sarcoma and breast tumor resections, and in HCC tumor tissues (Neo et al. 2020; Tan et al. 2020). In contrast, other studies reported no differences in the expression of TIM-3 between circulating NK and TINK cells from HNC and NSCLC patients (Trefny et al. 2020; Concha-Benavente et al. 2018). Considering the efficacy of antitumor therapies targeting immune checkpoints, it is of utmost relevance to better understand the regulation of these receptors in TINK cells and their functional consequences.

6.4.3 *Prognostic Value of NK Cells*

Compared to T and B cells, NK cells represent a minor fraction of tumor-infiltrating lymphocytes (López-Soto et al. 2017; Cózar et al. 2021). Furthermore, those NK cells that migrate to the TME showed altered phenotype and functions. So, could NK cells have a relevant impact in tumor progression? Despite representing a small proportion of lymphocytes in the TME, multiple studies have reported the presence of TINK cells in various cancers, although their presence is variable in different malignancies (Russick et al. 2020; Cózar et al. 2021). A recent meta-analysis evaluated the prognostic value of NK cells in 53 studies of distinct cancers, including HNC, breast, colorectal, gastric, lung, liver, ovarian, endometrial, vulvar, kidney, sarcoma, melanoma, periampullary adenocarcinoma, gallbladder, and glioblastoma. Authors concluded that NK cell infiltration in solid tumors is associated with a decreased risk of death (Nersesian et al. 2021). Similar conclusions were reported by other authors who concluded that the abundance of TINK cells is associated with increased overall survival and improved prognosis also in other malignancies, including HCC (Wu et al. 2020), NSCLC (Soo et al. 2018), and renal cell carcinoma (Remark et al. 2013). Remarkably, many studies reported no impact of NK cells in overall survival (Nersesian et al. 2021). These contradictory results may be due to the methods used to identify NK cells (i.e., strategies based on CD57, CD56, or NKp46 markers). Hence, to obtain more robust conclusions, it is crucial to develop and apply a precise identification strategy that discriminates between NK cells and other cells, such as ILCs and subsets of T cells. Moreover, it would be very useful to differentiate between NK cell subsets, since some of them could have opposite roles in tumor progression. For instance, CD11b-CD27- NK cell subset has been found to accumulate in the TME of patients with HCC and NSCLC. This subset showed immature phenotype and poor cytotoxic capacity, and its presence in the TME is associated with tumor progression (Jin et al. 2013; Zhang et al. 2017). A subpopulation of CD49a+ NK cells can be also found in the intratumoral tissues of HCC patients, and the accumulation of this subset is associated with poor clinical outcome (Sun et al. 2019). Besides TINK cells, the role of circulating NK cells in tumor progression should be also considered. Decreased proportions of peripheral blood NK cells expressing NKp30, NKp46, NKG2D, and DNAM-1 correlated with tumor progression in gastric cancer patients (Han et al. 2018). Frequency of CCR7+CD56^{bright} NK cells marks disease evolution in patients with melanoma (Cristiani et al. 2019). Excitingly, peripheral blood NK cells can serve as a prognostic indicator in certain malignancies. Higher frequency of circulating PD-1+ NK cells has been associated with increased survival in HNC patients (Concha-Benavente et al. 2018). It has been reported that levels of circulating NKp46+CD56^{dim}CD16+ NK cells influence survival of NSCLC patients (Picard et al. 2019). Another report also found that NSCLC patients with high NK cell count showed increased overall survival and progression-free survival (Mazzaschi et al. 2019). Therefore, current knowledge suggests that NK cells play a key role in tumor progression and that they could be used as a prognostic factor in a number of

malignancies. A deeper analysis of the phenotype and functionality of distinct NK cell subsets would contribute to understanding of their exact role in different cancers.

6.5 Concluding Remarks

Considering the antitumor activity of NK cells, many studies have focused on them and tried to understand if these cells are relevant in a number of malignancies. However, contradictory conclusions have been published, although this is not unexpected. The strategies used to identify NK cells in these studies have been updated as our knowledge about NK cell biology improved rapidly. Moreover, NK cells are a diverse group of cells and distinct subsets may perform opposite functions during the disease. Thus, thorough analyses of NK cell subpopulations in cancer patients will be helpful to better understand their role. It is equally important to examine NK cells in their specific context. Tumor cells can develop resistance to NK cell–mediated killing through different mechanisms. It is critical to understand if NK cells become dysfunctional in certain cancers due to the specific conditions of the TME, or as a consequence of tumor-derived suppressive molecules, such as soluble MIC ligands or TGF-β. Identifying these mechanisms will provide with new targets that could be exploited in cancer immunotherapy and improve the clinical outcome of many patients. Further studies are needed to completely understand the role of NK cells in tumor progression, and undoubtedly, new findings on NK cell biology and the characteristics of different cancers will unveil the contribution of these cells during the disease. For now, current data indicates that NK cells are crucial elements of the antitumor response. They have the ability to mount an early response during the first steps of cancer development. Moreover, due to their capacity of recognizing and killing CSCs, NK cells could be extremely relevant to control metastases. Future studies will elucidate if NK cells play a relevant role during other stages of tumor progression.

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Chapter 7
The Role of Myeloid-Derived Suppressor Cells in Tumor Growth and Metastasis

Defne Bayik, Juyeun Lee, and Justin D. Lathia

Contents

Abstract Myeloid-derived suppressor cells (MDSCs) are immature bone marrow–derived suppressive cells that are an important component of the pathological immune response associated with cancer. Expansion of MDSCs has been linked to poor disease outcome and therapeutic resistance in patients with various malignancies, making these cells potential targets for next-generation treatment strategies. MDSCs are classified into monocytic (M-MDSC) and polymorphonuclear/granulocytic (PMN-MDSC) subtypes that undertake distinct and numerous roles in the tumor microenvironment or systemically to drive disease progression. In this chapter, we will discuss how MDSC subsets contribute to the growth of primary tumors and induce metastatic spread by suppressing the antitumor immune response, supporting cancer stem cell (CSC)/epithelial-to-mesenchymal transition (EMT) phenotypes and promoting angiogenesis. We will also summarize the signaling networks involved in the crosstalk between cancer cells and MDSCs that could represent putative immunotherapy targets.

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Abbreviations

Arg1	arginase 1
CCA	cholangiocarcinoma
CMP	common myeloid progenitor
COX2	cyclooxygenase 2
CSC	cancer stem cell
CTC	circulating tumor cell
DC	dendritic cell
EMT	epithelial-to-mesenchymal transition
ER	endoplasmic reticulum
FAO	fatty acid oxidation
GBM	glioblastoma
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony–stimulating factor
GMP	granulocyte-macrophage progenitor
GP	granulocyte progenitor
HCC	hepatocellular carcinoma
HIF-1a	hypoxia-inducible factor 1α
HNSCC	head and neck squamous cell carcinoma
HSC	hematopoietic stem cell
IDO	indole amine 2,3 dioxygenase
IFN	interferon
IL	interleukin
IRF8	interferon regulatory factor-8
L-Arg	L-arginine
M-CSF	macrophage colony–stimulating factor
MDP	macrophage and DC progenitor
MDSC	myeloid-derived suppressor cell
MIF	macrophage migration–inhibitory factor
MLPG	monocyte-like precursor of granulocyte
M-MDSC	monocytic MDSC
MMP	matrix metalloproteinase
MPO	myeloperoxidase
NET	neuroendocrine tumor
NF-κB	nuclear factor kappa B
NO	nitric oxide
NOS	NO synthase
PD-L1	programmed death-ligand 1

PGE_2	prostaglandin E2
PMN-MDSC	polymorphonuclear MDSC
PNT	peroxynitrite
RCC	renal cell carcinoma
ROS	reactive oxygen species
STAT	signal transducer and activator protein
TAM	tumor-associated macrophage
TGF	transforming growth factor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	TNF receptor
VEGF	vascular endothelial growth factor

7.1 Introduction

7.1.1 MDSC Subsets

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells associated with numerous pathological conditions including cancer, inflammation, and infection (Gabrilovich and Nagaraj 2009). MDSCs exert potent immunosuppression, particularly on T cells, thereby abrogating adaptive immune responses. Based on phenotypic, molecular, and functional differences, MDSCs are categorized into two subsets: monocytic (M-MDSCs) and polymorphonuclear/granulocytic (PMN-MDSCs or G-MDSCs)(Movahedi et al. 2008). These MDSC subsets share phenotypic characteristics with inflammatory myeloid cells, and, as such, many efforts have been made to discriminate between these cell subsets (Fig. 7.1). In mice, MDSCs were first defined as $CD11b^+Gr1^+$ cells, and additional phenotypic markers have been found to identify each subset. M-MDSCs are defined as $CD11b^+Ly6C^{high}Ly6G^-$ cells, lacking expression of major histocompatibility complex (MHC) class II and the macrophage and dendritic cell markers CD68 and CD11c on the cell surface. PMN-MDSCs are characterized as $CD11b^+Ly6C^{low}Ly6G^+$, which also mark neutrophils (Bronte et al. 2016). Although elevated expression of surface markers such as CD115 and CD244 on PMN-MDSCs compared to neutrophils has been reported (Youn et al. 2012), the use of these markers is limited due to the high heterogeneity of PMN-MDSCs (Veglia et al. 2021).

In humans, the cell surface immunophenotyping of MDSCs is confounded by the lack of Gr-1 expression on human leukocytes. M-MDSCs are defined as $CD11b^+CD33^+CD14^+CD15^-CD66b^-$ and express a very low level of MHC class II molecules, making them distinct from monocytes, which express MHC class II. Similar to murine cells, human PMN-MDSCs and neutrophils share the phenotype characterized as $CD11b^+CD33^+CD14^-CD15^+CD66b^+$(Bronte et al. 2016).

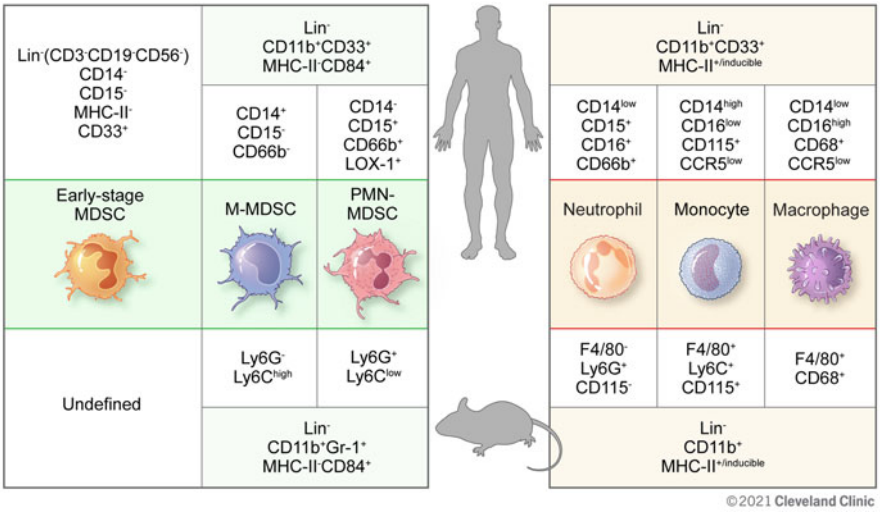

Fig. 7.1 Phenotypic markers to define MDSCs in humans and mice. Characterization of MDSCs and inflammatory myeloid cells based on surface markers

Traditionally, density-gradient centrifugation has been used to separate PMN-MDSCs and neutrophils. After centrifugation, PMN-MDSCs are enriched in the low-density fraction, while neutrophils are isolated from the high-density fraction of the peripheral blood mononuclear cells (PBMCs)(Dumitru et al. 2012). However, this method does not ensure clear separation of two cell types as activated neutrophils can also be found in the low-density fraction. Recently, lectin-type oxidized LDL receptor 1 (LOX-1) was identified as a PMN-MDSC-specific marker that provides better distinction without gradient centrifugation and functional assays (Condamine et al. 2016). Additionally, a third subset of MDSC has been identified in humans, known as an early-stage MDSC (eMDSC)(Bronte et al. 2016; Solito et al. 2011; Diaz-Montero et al. 2009; Kumar et al. 2016b). This population consists of progenitor cells that exhibit an immature phenotype and lack both monocytic ($CD14^{-}$) and granulocytic ($CD15^{-}$) markers. The equivalent murine cell type is yet to be defined.

Recently, CD84 has been proposed as a novel marker for both M- and PMN-MDSC subsets (Alshetaiwi et al. 2020). Using single-cell RNA sequencing of MDSCs isolated from the MMTV-PyMT (mouse mammary tumor virus–polyomavirus middle T antigen) mouse model of breast cancer, Alshetaiwi et al. identified several surface markers, including CD84, that were MDSC specific in both mouse and human (Alshetaiwi et al. 2020). These authors further confirmed that $CD11b^{+}Gr1^{+}CD84^{+}$ cells exhibit immunosuppressive function and high reactive oxygen species (ROS) production (Alshetaiwi et al. 2020). The current gold standard for defining MDSCs is evaluating suppressive function by inhibition of T cells in conjunction with the phenotypic criteria described above. However, suppression assays present technical challenges in the clinical setting, as the number of isolated

cells can be limited. A more definitive panel of surface markers for MDSCs would remove the requirement for such assays in the future.

7.1.2 Lineage Relationship of MDSCs

The development of MDSCs requires certain cues to be initiated. In healthy individuals or naïve mice, MDSCs are undetectable or rarely present in the circulation. In the context of pathophysiological conditions, a number of factors interfere with the maturation of myeloid cells, leading to the accumulation of MDSCs. MDSCs are a functional state, as cells isolated from healthy mice lacked suppressive activity compared to cells isolated from a tumor-bearing host (Youn et al. 2008; Kusmartsev et al. 2004). In the process of conventional myelopoiesis, hematopoietic stem cells (HSCs) differentiate into common myeloid progenitors (CMPs), followed by granulocyte-macrophage progenitors (GMPs) and further differentiation into granulocyte progenitors (GPs) and macrophage and dendritic cell (DC) progenitors (MDPs). This process is tightly regulated by the orchestration of multipsle cytokines and transcriptional factors. However, in the presence of a tumor or other chronic inflammatory disease, myelopoiesis becomes defective and myeloid precursor cells including GPs and MDPs differentiate into immature MDSCs (Fig. 7.2). Recently, Mastio et al. reported a novel MDSC progenitor population, termed monocyte-like precursors of granulocytes (MLPGs), and described these cells as monocytic precursors that can be differentiated into PMN-MDSCs specifically in the presence of a tumor (Mastio et al. 2019). Studies have shown that a larger proportion of the PMN-MDSC pool is derived from MLPGs than from their GP counterparts.

The generation of MDSCs begins via expansion of immature myeloid cells in the bone marrow induced by highly produced growth factors such as granulocyte-macrophage colony–stimulating factor (GM-CSF), granulocyte colony–stimulating factor (G-CSF), macrophage colony–stimulating factor (M-CSF), and vascular endothelial growth factor (VEGF)(Morales et al. 2010; Waight et al. 2011; Lechner et al. 2010). Each growth factor appears to stimulate production of a particular MDSC subset, as GM-CSF induces preferential expansion of M-MDSCs, whereas G-CSF is crucial for PMN-MDSC expansion (Dolcetti et al. 2010). Tumor-derived VEGF, GM-CSF, interleukin (IL) 6, M-CSF, and S100A9 further halt the differentiation of myeloid cells into DCs, resulting in increased accumulation of MDSCs (Dolcetti et al. 2010; Cheng et al. 2008; Gabrilovich et al. 1998; Menetrier-Caux et al. 1998). This effect relies on activation of key transcription factors. Binding of each growth factor to its receptor activates transcriptional regulators including signal transducer and activator protein (STAT) 3, interferon regulatory factor-8 (IRF8), and CCAAT/enhancer-binding protein beta (C/EBP-β), subsequently skewing cell fate toward MDSCs. In addition, epigenetic silencing of retinoblastoma has been linked to the development of PMN-MDSCs (Youn et al. 2013). In tumor-bearing mice, MDSCs exhibit elevated phosphorylated STAT3 expression. Cheng et al. showed that increased phospho-STAT3 upregulated transcription of the myeloid-related

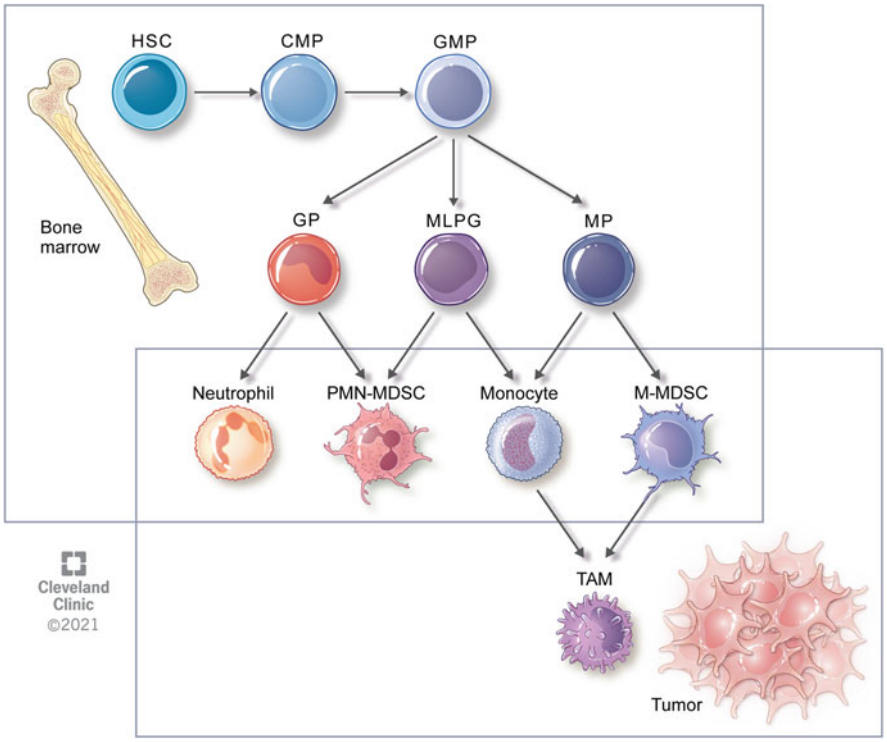

Fig. 7.2 Hematopoietic lineage of MDSCs. MDSCs are derived from HSCs in bone marrow and differentiate into subsets during tumor progression

proteins S100A8 and S100A9, which in turn increases ROS and directs MDSCs toward differentiation (Cheng et al. 2008). Abrogation of STAT3 signaling reduced expansion of MDSCs in vivo (Kortylewski et al. 2005; Nefedova et al. 2005) and inhibited the induction of immunosuppressive function in tumor-infiltrating MDSCs in vitro (Al-Khami et al. 2017). Another transcription factor critical for MDSC expansion is IRF8. IRF8 positively regulates the development of monocytes and DCs while acting as a negative regulator for neutrophils and MDSCs (Becker et al. 2012). IRF8-deficient mice showed an increase in the number of neutrophils, which display MDSC-like characteristics (Becker et al. 2012; Waight et al. 2013). Further study revealed that downregulation of IRF8 in MDSCs is regulated by the STAT3 and STAT5 pathways (Waight et al. 2013). C/EBP-β has also been suggested to function as a "master" transcription factor for MDSC development, as C/EBP-β directly binds to the promoter regions and enhances the transcription of genes related to MDSC expansion, as well as those that regulate immunosuppressive functions, such as arginase 1 (Arg1), inducible nitric oxide synthase (iNOS or NOS2), and cyclooxygenase 2 (COX2) (Fultang et al. 2020). Phospho-STAT3 is known to upregulate C/EBP-β expression, and a recent study by Li et al. suggested c-Rel as a novel upstream regulator of C/EBP-β (Li et al. 2020). Deletion of c-Rel in myeloid

cells significantly reduced tumor growth and altered the immunosuppressive machinery of MDSCs. c-Rel also directly regulates the transcription of MDSC signature genes by forming a transcriptional complex with phospho-STAT3, C/EBP-β, and p65.

Accumulated MDSCs further undergo functional changes regulated by multiple inflammatory cytokines including interferon (IFN) γ, IL-1β, IL-6, and tumor necrosis factor (TNF) α, which mainly signals through nuclear factor kappa B (NF-κB), STAT1, or STAT6(Condamine et al. 2015). The IFNγ-STAT1 axis is likely to be crucial for the suppressive function of M-MDSCs, potentially through activation of a negative feedback loop (Schouppe et al. 2013), whereas activation of STAT1 by IFNγ led to decreased survival and functionality of PMN-MDSCs (Medina-Echeverz et al. 2014). In addition, Toll-like receptor (TLR) signaling induces MDSC accumulation and enhances immunosuppressive function, resulting in tumor progression. In tumor-bearing mice, adjuvant therapy with TLR2 ligand increased expansion and suppressive function of M-MDSCs, which was further enhanced by IFNγ secreted from T cells (Shime et al. 2017). TLR4 also positively regulates MDSCs, as administration of lipopolysaccharide and IFNγ into naïve mice resulted in expansion of MDSC subsets and impaired induction of DCs in the spleen (Greifenberg et al. 2009). Upregulation of COX2 through prostaglandin E2 (PGE_2) has also been suggested to be a key factor that induces suppressive MDSCs, in accordance with elevated COX2+ MDSCs and circulating PGE2 detected in blood from cancer patients (Obermajer et al. 2011a). In addition to aberrant myelopoiesis, the conversion of mature myeloid cells such as neutrophils or monocytes into MDSCs has also been reported. Several studies have reported that after exposure to tumor cells, $CD14^+$ monocytes are able to acquire an M-MDSC phenotype through PGE_2(Mao et al. 2013) or an IL-10-dependent mechanism (Rodrigues et al. 2010). Additionally, in humans, endoplasmic reticulum (ER) stress can induce the conversion of neutrophils into PMN-MDSCs that express LOX-1(Condamine et al. 2016). After migrating to tumors, M-MDSCs further differentiate into immunosuppressive tumor-associated macrophages (TAMs). Growth factors in the tumor microenvironment play a crucial role in this process. Blockade of the GM-CSF and M-CSF pathways significantly reduced the tumor infiltration of MDSCs and impaired their differentiation into TAMs with immunosuppressive properties (Zhu et al. 2014; Van Overmeire et al. 2016). Hypoxia in the tumor site is another critical factor that regulates the conversion of MDSCs into TAMs. Adoptively transferred M-MDSCs become immunosuppressive TAMs in a hypoxia-inducible factor 1α (HIF-1α)-dependent mechanism (Corzo et al. 2010). Another study showed that hypoxic conditions induce upregulation of CD45 phosphatase, leading to decreased STAT3 phosphorylation (Kumar et al. 2016a). These findings further emphasize the importance of STAT3 signaling in the development and maintenance of MDSCs. In addition to TAMs, the differentiation of MDSCs into regulatory DCs (Zhong et al. 2014) or fibrocytes (Niedermeier et al. 2009; Zoso et al. 2014) has been reported in various tumor models as well.

7.1.3 MDSCs in Cancer

Earlier studies focusing on immunosuppressive mechanisms in cancer identified alterations in hematopoietic lineage commitment characterized by an accumulation of immature myeloid cells. Later studies identified these cells as MDSCs and reported that the frequency of this heterogeneous cell population increases in a variety of malignancies (Table 7.1). While some tumors, such as renal cell carcinoma and pancreatic cancer, are characterized by expansion of PMN-MDSCs, M-MDSCs are reported to be the dominant population in other malignancies, such as liver cancer. These differences are in part driven by the distinct chemokine

Table 7.1 MDSCs expand in the peripheral circulation and infiltrate tumors of patients with malignancies

Subtype	Cancer	Anatomical Location	Reference
MDSC/ PMN-MDSC/M-MDSC	Breast	Peripheral blood, tumor	(Wang and Yang 2016; Yu et al. 2013; Peng et al. 2016; Almand et al. 2000; Cassetta et al. 2020)
M-MDSC	CCA	Peripheral blood	(Xu et al. 2016)
M-MDSC/ PMN-MDSC	Colorectal	Peripheral blood, tumor	(Bayik et al. 2020a; Wu et al. 2014; Cassetta et al. 2020)
M-MDSC	Esophageal	Peripheral blood	(Chen et al. 2014)
M-MDSC/ PMN-MDSC	Gastrointestinal	Peripheral blood	(Mundy-Bosse et al. 2011)
M-MDSC/ PMN-MDSC	Glioblastoma	Peripheral blood, tumor	(Alban et al. 2018; Bayik et al. 2020b; Cassetta et al. 2020; Raychaudhuri et al. 2011, 2015; Chai et al. 2019)
MDSC/M-MDSC	HCC	Peripheral blood	(Bayik et al. 2020a; Hoechst et al. 2008; Arihara et al. 2013; Shen et al. 2014)
MDSC/M-MDSC/ PMN-MDSC	HNSCC	Peripheral blood	(Lang et al. 2018; Almand et al. 2000, 2001; Cassetta et al. 2020; Young et al. 1997; Zhong et al. 2019)
MDSC/M-MDSC/ PMN-MDSC	Lung	Peripheral blood, tumor	(Yamauchi et al. 2018; Almand et al. 2000, 2001; Liu et al. 2010)
M-MDSC	Melanoma	Peripheral blood	(Meyer et al. 2014; Lesokhin et al. 2012; Filipazzi et al. 2007)
M-MDSC	NET	Peripheral blood	(Bayik et al. 2020a)
PMN-MDSC	Pancreas	Peripheral blood, tumor, bone marrow	(Khaled et al. 2014; Porembka et al. 2012)
M-MDSC	Prostate	Peripheral blood	(Vuk-Pavlovic et al. 2010)
PMN-MDSC	RCC	Peripheral blood	(Najjar et al. 2017)
PMN-MDSC	Ovarian	Peripheral blood	(Cassetta et al. 2020)
PMN-MDSC	Urothelial cancer	Peripheral blood, tumor	(Sheng et al. 2020; Eruslanov et al. 2012)

expression profiles of tumor cells but can also be biased by differences in analysis methods. Notably, PMN-MDSCs do not recover from cryopreservation: fresh samples must be used to achieve an accurate profiling of this subset (Kotsakis et al. 2012). Despite the confounding factors, which include storage conditions, time of analysis, and method of leukocyte isolation (Florcken et al. 2015), enhanced MDSC frequency is linked to higher-grade disease and poor prognosis of patients with a variety of solid tumors and blood cancers, including breast cancer, ovarian cancer, liver cancer, melanoma, non–small cell lung carcinoma, head and neck cancer, esophageal squamous cell carcinoma, Hodgkin lymphoma, and glioblastoma (GBM)(Diaz-Montero et al. 2009; Cui et al. 2013; Alban et al. 2018; Bayik et al. 2020a; Mizukoshi et al. 2016; Weide et al. 2014; Lang et al. 2018; Vetsika et al. 2014; Wang and Yang 2016; Chen et al. 2014; Romano et al. 2015). Furthermore, high MDSC levels inversely correlate with response to cystectomy in urothelial cancer, chemotherapy in liver and colorectal cancers, and immunotherapy in melanoma (Mizukoshi et al. 2016; Meyer et al. 2014; Ornstein et al. 2018; Limagne et al. 2016). These findings make MDSCs a promising target for cancer immunotherapy and warrant further investigation of the signaling networks that drive MDSC accumulation and function.

7.2 Signaling Networks Driving MDSCs in Cancer

MDSC subset recruitment, maintenance, and function are informed by multiple factors that collectively contribute to tumor progression through immunomodulation.

7.2.1 Recruitment

Tumor cells secrete a number of chemokines to drive accumulation of MDSCs in the tumor microenvironment (Fig. 7.3). For PMN-MDSCs, CXCR2 serves as the main chemoattractant receptor, and tumor cells abundantly express the CXCR2 ligands CXCL1, CXCL2, and CXCL5(Clavijo et al. 2017; Wang et al. 2016). In addition to CXCR2, PMN-MDSC expression of CXCR4 also drives their trafficking to the liver premetastatic niche in response to CXCL1 and CXCL12(Wang et al. 2017a; Seubert et al. 2015). In patients with renal cell carcinoma, in addition to CXCL5, CCL3, IL-8, and IL-1β expression correlates with PMN-MDSC levels, pointing to the presence of additional mediators (Najjar et al. 2017). IL-1β-overexpressing fibrosarcoma and mammary carcinoma are characterized by more tumor-infiltrating, circulating, and splenic MDSCs with increased suppressive function (Bunt et al. 2006; Song et al. 2005; Bunt et al. 2007). Follow-up mechanistic studies demonstrated that downstream IL-6 signaling was partially responsible for MDSC accumulation in breast cancers (Bunt et al. 2007), while colorectal cancer cells engineered to

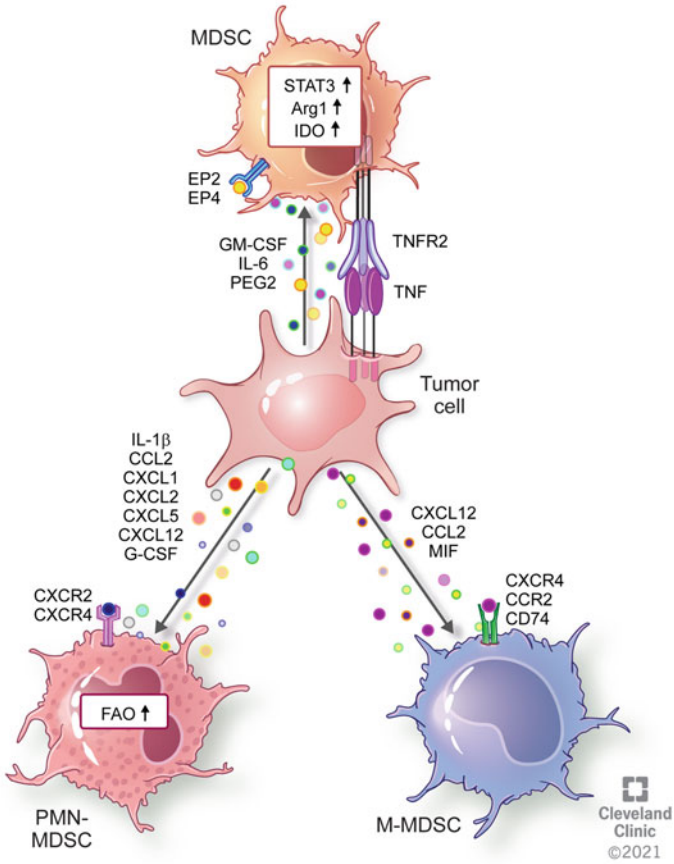

Fig. 7.3 Tumor-derived factors regulate MDSC subset recruitment and function. Tumor cells secrete a multitude of cytokines and chemokines that collectively facilitate MDSC accumulation, metabolic reprogramming, and activation of immunosuppressive pathways. Earlier studies focusing of MDSC subsets as a single population identified conserved informative signals (IL-6, GM-CSF, PGE_2 and TNFR2), while later studies defined an array of subset-specific factors. G-CSF, IL-1β, CXCL1, CXCL2, CXCL5, and CXCL2 primarily act on PMN-MDSCs to drive trafficking through CXCR2 and CXCR4. In contrast, CCL2 and MIF selectively recruit M-MDSCs in a CCR2- and CD74-dependent manner

overexpress IL-1β had higher CXCL1, CXCL2, and CXCL5 levels and enhanced PMN-MDSC infiltration (Tannenbaum et al. 2019). Tumor-derived G-CSF also mobilizes PMN-MDSCs in breast cancer models to form a protumorigenic microenvironment in the lungs (Kowanetz et al. 2010). However, this could be disease/model specific, as in colorectal cancer, the effect of G-CSF was limited to expansion and did not induce recruitment (Tannenbaum et al. 2019). Redundancy in chemokines and differences across models point to tumor-type-, stage-, and localization-dependent regulation of MDSC infiltration. Furthermore, bidirectional

communication between MDSCs and tumor cells can activate a positive feedback loop that augments MDSCs infiltration and accelerates tumor growth. S100A8/S100A9 produced by bone marrow–derived cells activate mitogen-activated protein kinases and NF-κB in colon cancers to increase CXCL1 production and subsequent MDSC infiltration (Ichikawa et al. 2011). Some of these signaling axes can also be activated in response to treatment as a compensatory mechanism. In mouse models, depletion of TAMs resulted in elevated CXCL2 expression from tumors and PMN-MDSC infiltration into cholangiocarcinoma (CCA) tumors (Loeuillard et al. 2020) (Fig. 7.3).

Coculture studies using human tumors and preclinical animal studies identified CCL2 as a key cytokine mediating the migration of CCR2-expressing MDSCs (Huang et al. 2007; Chang et al. 2016; Lesokhin et al. 2012). However, CCL2 signaling also enhances the T cell suppressive activity of PMN-MDSCs in colon cancer, suggesting that it can affect both subsets of MDSCs (Chun et al. 2015). Circulating M-MDSCs in patients with non–small cell lung carcinoma also express high levels of CCR5, suggesting that additional CCR2 ligands can inform recruitment of these cells (Yamauchi et al. 2018). PGE_2-dependent CXCR4 expression is also implicated in CXCL12 response and M-MDSC migration toward ovarian cancer ascites (Obermajer et al. 2011b). Several additional proinflammatory mediators are linked to MDSC accumulation in tumor-bearing mice. This could be a consequence of activation of the resolution pathway as a negative feedback loop to restrain inflammatory damage. Macrophage migration inhibitory factor (MIF) is one such inflammatory cytokine associated with MDSC accumulation in GBM (Otvos et al. 2016). Mechanistic studies have revealed that MIF, which is enriched in cancer stem cells (CSCs) over the non-CSC fraction, acts on surface CD74 to recruit M-MDSCs into the tumor microenvironment (Otvos et al. 2016; Alban et al. 2020). Notably, TNF receptor 2 knockout ($Tnfr2^{-/-}$) mice were characterized by impaired MDSC expansion upon tumor implantation (Zhao et al. 2012). Transmembrane but not soluble TNFα served as a ligand for TNFR2 to drive the MDSC phenotype, and neutralization of TNFα prevented accumulation of these cells in preclinical tumor models (Zhao et al. 2012; Hu et al. 2014). In vitro coculture assays suggested that specific polarization of M-MDSCs, but not PMN-MDSCs from bone marrow precursors, in response to GM-CSF, was blocked in $Tnfr2^{-/-}$ mice (Polz et al. 2014). This is consistent with the studies demonstrating that GM-CSF is a stronger inducer of M-MDSCs than of PMN-MDSCs (Tannenbaum et al. 2019; Lesokhin et al. 2012). However, $Tnfr2^{-/-}$ mice had lower metastatic burden in the liver, a process primarily regulated by the PMN-MDSC subset, along with reduced $CD11b^{+}Gr\text{-}1^{+}$ cell frequency, suggesting that TNF signaling likely also plays a role in the maintenance and/or recruitment of PMN-MDSCs (Ham et al. 2015) (Fig. 7.3).

In addition to tumor-derived factors, recruitment of MDSCs can be facilitated by stromal cells present in the tumor microenvironment, informed by host factors, and impacted by treatment strategies. In CCA, a leaky gut barrier results in the activation of the TLR4 signaling axis by commensal bacteria and subsequent CXCL1 production from hepatocytes to recruit PMN-MDSCs (Zhang et al. 2021). IL-8 and GM-CSF secretion by an IL-17-producing γδ T cell subset (γδT17) sorted from

human colorectal tumors stimulated transwell migration of PMN-MDSCs (Wu et al. 2014). In melanoma, indole amine 2,3 dioxygenase (IDO) expression promotes systemic expansion and tumor recruitment of MDSCs in a regulatory T cell (Treg)-dependent manner (Holmgaard et al. 2015). Treatment with cytokine-induced killer cells also promotes accumulation of PMN-MDSCs and M-MDSCs in hepatocellular carcinoma models partially by upregulating IL-3 and CX3CL1 secretion from tumor cells (Yu et al. 2019). In contrast, macrophages and microglia constitute the main source of CCL2 in the human GBM microenvironment, indicating that they contribute to the migration of M-MDSCs (Chang et al. 2016). Communication between macrophages and MDSCs is not unique to GBM. Bidirectional crosstalk between these two cell populations in breast cancer collectively drives immunosuppression and is facilitated by IL-10 secretion from MDSCs and IL-6 production from peritoneal macrophages (Beury et al. 2014; Sinha et al. 2007b). These observations suggest that the predominant cell type interacting with MDSCs can be organ or tumor specific.

7.2.2 Maintenance and Function

The maintenance and function of MDSCs are defined by a multitude of soluble ligands that activate conserved intracellular signaling networks and regulate cellular metabolism. Tumor cells can stimulate the expression of immunomodulatory factors by MDSCs, and production of both IL-6 and NO increases when MDSCs are cocultured with mouse breast cancer cells (Beury et al. 2014). Lung tumor cells can also induce Arg1 expression through PGE_2 signaling through the E-prostanoid (EP)2/EP4 receptor (Rodriguez et al. 2005; Sinha et al. 2007a). In human M-MDSCs, PGE_2 treatment is accompanied by upregulation of functional MDSC markers such as Arg1, COX2, IL-10, and IDO1(Obermajer et al. 2011a). MDSC chemoattractants are in part responsible for downstream functional effects, as well. MIF drives expression of Arg1 in MDSCs, while GM-CSF, CCL2, and IL-6 are among the factors that activate the STAT3 pathway, which is integral for MDSC behavior (Al-Khami et al. 2017; Chun et al. 2015; Otvos et al. 2016; Panni et al. 2014; Yu et al. 2013; Jiang et al. 2017). STAT3 phosphorylation subsequently drives expression of immunosuppressive mediators such as IDO1(Yu et al. 2013). Both M-MDSCs and PMN-MDSCs isolated from IL-1β-overexpressing colon tumors are characterized by higher expression of immunosuppressive markers, including Arg1, iNOS, transforming growth factor (TGF) β, matrix metalloproteinase (MMP) 9, and S100A9(Tannenbaum et al. 2019). Similarly, inhibition of IDO reverses the inhibitory effect of MDSCs on T cell proliferation (Holmgaard et al. 2015). Transmembrane TNF signaling through TNFR2 promotes the survival and suppressive function of MDSCs through downstream p38 and NF-κB signaling, and TNFR2-deficient M-MDSCs had impaired suppressive activity and reduced production of immunosuppressive mediators (Hu et al. 2014; Polz et al. 2014).

The cellular metabolism of MDSCs is closely linked to their functionality, particularly within the tumor microenvironment. Compared to monocytes, human M-MDSCs infiltrating hepatocellular carcinoma were characterized by a low rate of glycolysis and reduced expression of the glucose-uptake receptor Glut-1(Baumann et al. 2020). This metabolic state was linked to the generation of dicarbonyl methylglyoxal, a metabolite that was also important for the suppressive capacity of M-MDSCs (Baumann et al. 2020). Preclinical studies suggested that $CD11b^{+}Gr1^{+}$ MDSCs can undergo metabolic reprogramming in the tumor microenvironment that results in enhanced fatty acid oxidation (FAO) over glycolysis (Al-Khami et al. 2017). While, in peripheral organs, both M-MDSC and PMN-MDSC expansion relied on glycolysis, in tumors, MDSC suppressive function depended on FAO (Al-Khami et al. 2017; Hossain et al. 2015; Jian et al. 2017). This metabolic alteration was in part driven by STAT3/STAT5-dependent upregulation of lipid uptake receptors in response to tumor-derived G-CSF, GM-CSF, and IL-6 (Al-Khami et al. 2017). GM-CSF-induced STAT5 phosphorylation was also required for enhanced expression of fatty acid transporter protein 2 and production of PGE_2 in PMN-MDSCs (Veglia et al. 2019). Importantly, MDSC metabolism and fate can also be informed by the tumor microenvironment. In addition to promoting MDSC differentiation, hypoxia can also reprogram MDSCs to increase programmed death-ligand 1 (PD-L1) expression level in a HIF-1α-dependent manner (Noman et al. 2014).

7.3 Differential Roles of MDSC Subsets in Cancer

PMN-MDSCs and M-MDSCs can undertake specialized roles in the tumor microenvironment (Fig. 7.4). Earlier reports studying MDSC subtypes as a single population demonstrated that these cells induce Treg development by secreting IL-10 and TGFβ (Huang et al. 2006) and suppress proliferation of T cells partially through Arg 1-dependent depletion of extracellular L-arginine (L-Arg)(Rodriguez et al. 2005; Rodriguez et al. 2004). L-Arg is essential for T cell activation, as its deprivation downregulates expression of the CD3ζ chain and leads to arrest of T cell proliferation (Taheri et al. 2001; Raber et al. 2012). While enzyme-mediated L-Arg depletion is the dominant pathway and is primarily regulated by Arg1, both MDSC subsets were also reported to express cationic amino acid transporter 2 (CAT2) to take up L-Arg from the tumor microenvironment to locally suppress T cell activity (Cimen Bozkus et al. 2015). This approach is not limited to regulation of L-Arg levels; sequestration of extracellular cystine by MDSCs through import receptors was shown to contribute to T cell suppression through deprivation of the amino acid cysteine (Srivastava et al. 2010) (Fig. 7.4a). While the role of individual MDSC subsets in cysteine regulation remains to be investigated, later studies focusing on the distinct functions of M-MDSCs and PMN-MDSCs identified divergent pathways that modulate L-Arg metabolism. Although both MDSC subsets were shown to express Arg1, this enzyme is more central to PMN-MDSC function, and human

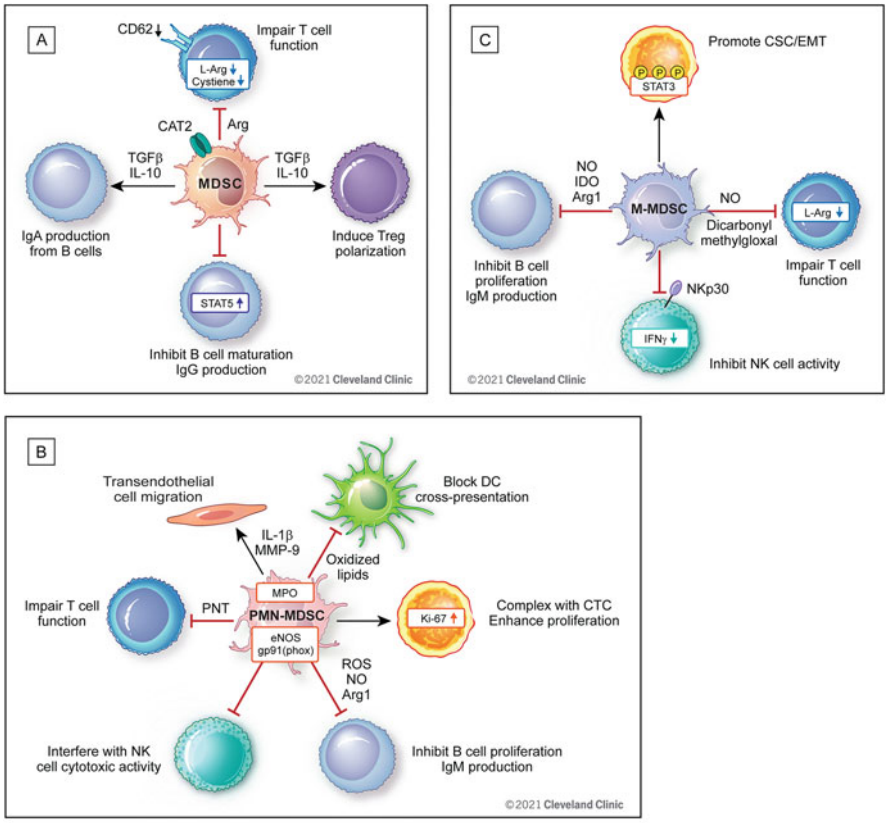

Fig. 7.4 MDSC subsets suppress the function of antitumoral immune cells and interact with tumor cells. MDSC subsets perform differential roles systemically and in the tumor microenvironment. (**a**) As a bulk population, MDSCs induce Treg polarization, suppress cytotoxic T cell activity, and regulate B cell maturation. (**b**) PMN-MDSCs drive metastatic spread by complexing with circulating tumor cells and acting on endothelial cells. They suppress T cell, dendritic cell, NK cell, and B cell function primarily by producing ROS and PNT. (**c**) NO production is the main B cell and T cell suppressive pathway for M-MDSCs. These cells also activate STAT3 signaling in tumor cells to promote a cancer stem cell phenotype

PMN-MDSCs release Arg1 into their environment (Raber et al. 2012; Rodriguez et al. 2009). PMN-MDSCs also produce peroxynitrite (PNT) in a gp91 (phox)- and endothelial NOS (eNOS)-dependent manner and ROS to suppress T cell activity (Youn et al. 2008; Raber et al. 2014) (Fig. 7.4b). Importantly, IDO1 expression prevents differentiation of MDSCs into proinflammatory neutrophils by promoting ROS scavenging in the context of graft-versus-host disease (Ju et al. 2021). As catabolism of tryptophan by IDO interferes with T cell proliferation and inhibition of IDO reverses the suppressive activity of MDSCs, tryptophan regulation also emerges as a potential pathway (Holmgaard et al. 2015; Yu et al. 2013; Lee et al. 2002). In contrast, the main pathway by which murine M-MDSCs impair T cell

function is through nitric oxide (NO) production by iNOS (Youn et al. 2008; Lesokhin et al. 2012; Huang et al. 2006; Raber et al. 2014). Human M-MDSCs were also shown to deplete intracellular L-Arg in T cells via cellular transfer of dicarbonyl methylglyoxal (Baumann et al. 2020) and suppress T cell proliferation by releasing TGFβ (Filipazzi et al. 2007), suggesting that MDSCs employ multiple complementary mechanisms to block T cell activation (Fig. 7.4c). In addition to soluble mediators, induction of Tregs and suppression of T cell proliferation were in part dependent on CD40 and integrin expression on MDSCs, suggesting that cell–cell contacts are important for the suppressive function of MDSCs (Kusmartsev et al. 2004; Pan et al. 2010). One notable mechanism of action is downregulation of the homing-receptor CD62 (L-selectin) by MDSCs, possibly through plasma membrane expression of ADAM17(Schouppe et al. 2013; Hanson et al. 2009) (Fig. 7.4a). M-MDSCs also reduce CD44 and CD25 levels, suggesting that they can interfere with T cell homing and IL-2 response (Schouppe et al. 2013).

The inhibitory activity of MDSC subsets extends beyond T cells. M-MDSCs suppress natural killer (NK) cell IFNγ production and NKG2D expression through direct cell–cell contact and via membrane-bound TGFβ (Hoechst et al. 2009; Li et al. 2009) (Fig. 7.4c). An MDSC-dependent reduced cytotoxicity of hepatic and splenic NK cells was consistent across melanoma, lung, liver, and lymphoma models, indicating that tumor-associated MDSCs have a global effect on immune activation (Li et al. 2009). At the premetastatic site, PMN-MDSCs can create a permissive environment by interfering with the cytotoxic function of NK cells (Sceneay et al. 2012). PMN-MDSCs can also interfere with antigen cross-presentation by DCs. This effect was dependent on MDSC myeloperoxidase (MPO) expression and mediated by transfer of oxidized lipids to DCs (Ugolini et al. 2020) (Fig. 7.4b). Furthermore, MDSCs can inform immune response at distant organs, although there are significant differences in the expression profile, metabolic activity, and suppressive capacity of tumor-infiltrating versus peripheral MDSCs. Notably, peripheral MDSCs are less effective at suppressing T cell activity compared to those isolated from tumor beds (Haverkamp et al. 2011; Maenhout et al. 2014). As such, PMN-MDSCs localized in human lung tumors or colorectal carcinoma have higher levels of PD-L1 or expression of CD73 and the ectonucleotidase CD39 compared to their counterparts in the peripheral blood (Limagne et al. 2016; Yamauchi et al. 2018). Accumulation of $CD11b^{+}Gr1^{+}$ MDSCs in spleens and bone marrow of tumor-bearing mice impaired B cell development and immunoglobulin (Ig) G production through down-modulation of STAT5 signaling (Wang et al. 2018). Another study in mouse fibrosarcoma models demonstrated that splenic MDSCs can induce IgA production from B cells by secreting IL-10 and TGFβ1(Xu et al. 2017). While the differential roles of MDSC subsets have not yet been fully investigated, both M-MDSCs and PMN-MDSCs isolated from healthy donors were capable of suppressing B cell proliferation and IgM secretion in vitro through a combination of secreted factors and contact-dependent mechanisms (Lelis et al. 2017; Jaufmann et al. 2020) (Fig. 7.4a). Collectively, these observations support the notion that MDSCs can potentially impact antibody response in cancer.

Beyond suppressing the antitumor immune response, MDSC subsets can also directly interact with tumor cells to promote CSC maintenance and cell migration, remodel the extracellular matrix, and drive angiogenesis. Earlier studies showed that splenic- and tumor-infiltrating $CD11b^{+}Gr1^{+}$ cells from melanoma, colorectal or lung models secrete proangiogenic factors including VEGF, fibroblast growth factor, or MMP-9 to promote endothelial cell differentiation and function in vitro or in vivo (Kujawski et al. 2008; Yang et al. 2004). This effect is attributed to PMN-MDSCs, as later studies demonstrated that Ly6G+ cells secrete multiple proangiogenic factors, activate endothelial cells, and promote transendothelial migration of tumor cells (Spiegel et al. 2016; Binsfeld et al. 2016) (Fig. 7.4b). Several preclinical studies indicated that this function of PMN-MDSCs assists metastatic spread. In a breast cancer model, $CD11b^{+}Gr1+$ cells promoted leaky and aberrant vasculature via MMP-9 secretion to drive lung metastasis (Yan et al. 2010). PMN-MDSC accumulation in livers contributed to premetastatic niche formation in colorectal cancer models, whereas pulmonary metastasis of breast cancers was in part driven by PMN-MDSC-induced tumor cell proliferation and mesenchymal-to-epithelial reversion (Wang et al. 2017a; Seubert et al. 2015; Ouzounova et al. 2017). Neutrophils with an expression profile consistent with that of PMN-MDSCs were also shown to pair with circulating tumor cells (CTCs) in breast cancer patients and mouse models. This clustering enhanced metastatic potential of CTCs by conferring a proliferative advantage (Szczerba et al. 2019) (Fig. 7.4b). Compared to PMN-MDSCs, a protumorigenic role for M-MDSCs is not well studied. The main function associated with M-MDSCs is their ability to promote epithelial-to-mesenchymal transition (EMT)/the CSC phenotype through STAT3 phosphorylation (Panni et al. 2014; Ouzounova et al. 2017; Peng et al. 2016). In coculture experiments with human breast cancer cell lines, this effect was dependent on production of IL-6 and NO (Peng et al. 2016). Similarly, M-MDSCs localizing at the invasive front of breast cancer models were shown to induce EMT via NO (Ouzounova et al. 2017) (Fig. 7.4c). Given that M-MDSCs localize adjacent to CSCs in GBM (Otvos et al. 2016), it is possible that spatial organization of these cells can determine their function. However, this CSC-promoting phenotype is not unique to M-MDSCs. PMN-MDSCs can enhance the stemness of tumor cells by upregulating STAT3 phosphorylation and DNA methyltransferase 3 beta activation, partially via exosomal S100A9(Ai et al. 2019; Wang et al. 2019).

7.4 Conclusion

Targeting MDSCs in cancer comprises a therapeutic opportunity to prime immunotherapy response. In preclinical models, inhibition of MDSCs primed the response to other immunotherapies, including checkpoint inhibitors and tumor vaccines (Clavijo et al. 2017; Davis et al. 2017; Highfill et al. 2014; Kamran et al. 2017). The therapeutic strategies to control MDSCs can be categorized as follow: (1) blocking development, recruitment, and/or immunosuppressive function and

(2) reprogramming MDSCs into mature antitumoral cells. Chemotherapies, including 5-fluorouracil, fludarabine, gemcitabine, and sunitinib, have been used to nonspecifically deplete MDSCs (Otvos et al. 2016; Bayik et al. 2020b; Le et al. 2009; Peereboom et al. 2019; Vincent et al. 2010; Wang et al. 2017b; Ko et al. 2009). In addition, key molecular pathways that are required for MDSC survival and distinguish these cells from monocytes and granulocytes comprise therapeutic targets. Recently, activation of the liver-X nuclear receptor (LXR)/apolipoprotein E (ApoE) axis was shown to reduce the number of MDSCs of both subsets through apoptosis (Tavazoie et al. 2018). The unfolded protein and ER stress responses are also linked to MDSC physiology (Condamine et al. 2014; Mohamed et al. 2020; Thevenot et al. 2014). Consistently, blockade of ER stress and linked TNF-related apoptosis-inducing ligand receptor signaling has been shown to induce MDSC-specific depletion (Condamine et al. 2014). Blockade of mitochondrial complex I activity with dimethylbiguanide (Baumann et al. 2020) and inhibition FAO (Hossain et al. 2015) can also abolish MDSC suppressive activity through metabolic reprogramming. Inhibition of IL-1β has been effective in renal cell carcinoma, breast cancer, and GBM models by consistently reducing the frequency of circulating PMN-MDSCs, while CXCR2 blockade interferes with trafficking of these cells in sarcoma and head and neck cancer models (Najjar et al. 2017; Bunt et al. 2007; Highfill et al. 2014; Bayik et al. 2020b; Greene et al. 2020). Finally, targeting of COX2/PGE_2 or PDE5 via tadalafil also interferes with MDSC expansion and function (Obermajer et al. 2011a; Yu et al. 2019; Fujita et al. 2011; Veltman et al. 2010). Earlier studies focusing on MDSC maturation pathways established that all-trans retinoic acid can induce their differentiation into DCs (Almand et al. 2001). Metabolic reprogramming of MDSCs by targeting protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) signaling or by promoting glycolysis also induces the maturation of M-MDSCs into macrophages with antitumoral activity (Mohamed et al. 2020; Liu et al. 2014). This type of differentiation into inflammatory macrophages can also be achieved by stimulation with TLR7/8, TLR9 agonists or inflammatory cytokines (Bayik et al. 2018; Shirota et al. 2012; Wang et al. 2015). Collectively, these preclinical findings resulted in clinical translation of several MDSC-targeting approaches to treat advanced malignancies (Table 7.2). While these studies are underway, broadening the understanding of the heterogeneity and molecular programming of MDSC subsets is poised to identify additional therapeutic opportunities with higher specificity.

Table 7.2 Clinical trials targeting MDSCs

Strategy	Target	Drug (Intervention)	Combo Partner	Tumor	Clinical Trial identifier	Status (phase)
Inhibiting suppressive function	COX-2	Celecoxib	Nivolumab/ ipilimumab	Colon carcinoma	NCT03026140	Recruiting (phase II)
	PDE5	Tadalafil	Anti-MUC1 vaccine	Head and neck cancer	NCT02544880	Active, not recruiting (phase I)
	Arg1	Arg1 peptide	PD-L1 peptide	Myeloproliferative neoplasms	NCT04051307	Recruiting (phase I/II)
	IDO	BMS-986205	Nivolumab/ temozolomide	Glioblastoma	NCT04047706	Recruiting (phase I)
	PI3K	IPI-549	Nivolumab	Advanced solid tumors	NCT02637531	Active, not recruiting (phase I)
Inhibiting differentiation & infiltration	CSF-1R	Cabiralizumab	Nivolumab	Advanced solid tumors	NCT02526017	Completed (phase I)
	VEGF	Bevacizumab	Atezolizumab	Metastatic cancer/renal cancer	NCT03024437	Recruiting (phase I/II)
	VEGFR	Regorafenib	Nivolumab	Hepatocellular carcinoma	NCT04170556	Recruiting (phase I/II)
	CXCR1/ 2	Navarixin	Pembrolizumab	Advanced solid tumors	NCT03473925	Active, not recruiting (phase II)
	CCR5	Vicriviroc	Pembrolizumab	Colorectal cancer	NCT03631407	Active, not recruiting (phase II)
Depletion	LXR	RGX-104	Nivolumab/ pembrolizumab	Advanced solid tumors/ lymphoma	NCT02922764	Recruiting (phase I)
	DNA	Gemcitabine	Nivolumab	Non–small cell lung cancer	NCT04331626	Not yet recruiting (phase IV)
Promoting differentiation	ARTA	RAR/RXR	Pembrolizumab	Advanced melanoma	NCT03200847	Active, not recruiting (phase I/II)
	TLR9	CMP-001	Nivolumab	Melanoma/lymph node cancer	NCT03618641	Active, not recruiting (phase II)

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Chapter 8
Cancer Stem Cells: An Ever-Hiding Foe

Jacek R. Wilczyński

Contents

Abstract Cancer stem cells are a population of cells enable to reproduce the original phenotype of the tumor and capable to self-renewal, which is crucial for tumor proliferation, differentiation, recurrence, and metastasis, as well as chemoresistance. Therefore, the cancer stem cells (CSCs) have become one of the main targets for anticancer therapy and many ongoing clinical trials test anti-CSCs efficacy of plenty of drugs. This chapter describes CSCs starting from general description of this cell population, through CSCs markers, signaling pathways, genetic and epigenetic regulation, role of epithelial-mesenchymal transition (EMT) transition and autophagy, cooperation with microenvironment (CSCs niche), and finally role of CSCs in escaping host immunosurveillance against cancer.

Keywords Cancer stem cells · Metastasis · Chemoresistance · Epithelial-mesenchymal transition · Niche

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Abbreviations

AKT	protein kinase B
ALDH1	aldehyde dehydrogenase-1
CAFs	cancer-associated fibroblasts
CSCs	cancer stem cells
CTCs	circulating tumor cells
CXCR	C-X-C motif chemokine receptor
DKK1	Dickkopf-related protein 1
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial–mesenchymal transition
EpCAM	epithelial cell adhesion molecule
ERK	extracellular-signal-regulated kinase
FAK	focal adhesion kinase
HDAC	histone deacetylase
HGF	hepatocyte growth factor
Hh	hedgehog signaling
HIF-1α	hypoxia-inducible factor-1α
IL	interleukin
JAK	Janus kinase
Klf4	Krüppel-like factor-4 transcription factor
LIF	leukemia-inhibiting factor
MAPK	mitogen-activated protein kinases
MDSCs	myeloid-derived suppressor cells
MET	mesenchymal-to-epithelial transition
MMPs	metalloproteinases
MSCs	mesenchymal stem cells
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cells
NKG2D	activating receptor of NK cells
NOTCH	neurogenic locus notch homolog protein
NRF2	nuclear factor erythroid-2-related factor-2
NUMB	protein numb homolog
Oct4	octamer-binding transcription factor-4
OXPHOS	oxidative phosphorylation
PD-L1	programmed death ligand 1 (also called B7-H1)
PGE_2	prostaglandin E_2
PI3K	phosphatidylinositol 3-kinase/phosphatase
PTEN	phosphatase and tensin homolog
ROS	reactive oxygen species
Sox2	sex-determining region-Y box-2 transcription factor
STAT	signal transducer and activator of transcription

TAMs	tumor-associated macrophages
TAZ	transcriptional coactivator with PDZ-binding motif
TGF-β	transforming growth factor-β
TLR	toll-like receptor
TNF-α	tumor necrosis factor-α
Tregs	T regulatory cells
VEGF	vascular-endothelial growth factor
YAP	Yes-associated protein
ZEB1	zinc finger E-box-binding homeobox-1

8.1 Introduction: Cancer Stem Cells: Definition and General Description

Cancer stem cells (CSCs), alternatively known as tumor-initiating or tumor-propagating cells (TICs, TPCs), are a population of cells enable to reproduce the original phenotype of the tumor, but more importantly capable to self-renewal, which is crucial to tumor proliferation, differentiation, recurrence, and metastasis, as well as chemoresistance (Irani 2019; Irani and Dehghan 2017, 2018; Irani and Jafari 2018; Wang 2019). Tumor cells are considered to be CSCs while possessing simultaneously all following features: have specific surface markers, are able to form floating spheres in serum-free medium, and form tumors when transplanted into laboratory animals (Choudhury et al. 2019). From mechanical perspective, CSCs are softer and more deformable cells than both nonmalignant and normal malignant cells (Vander Linden and Corbet 2019; Helmlinger et al. 1997, 2002; Vaupel et al. 1981). CSCs were first identified in 1997 in acute myeloid leukemia (Bonnet and Dick 1997) followed by identification in many solid tumors including prostate, ovarian, breast, pancreatic, colon, head and neck, lung, liver cancer, and glioblastoma (reviewed in: Nazio et al. 2019). Population of CSCs may be divided based on their cell cycle behavior and chemoresistance into two subpopulations: proliferating and quiescent. These two subpopulations occupy different niches inside tumor and exclusively quiescent CSCs are characterized by autophagic state (Marcucci et al. 2017, 2019; Liu et al. 2013). The proliferative CSCs possess acquired chemoresistance in response to treatment, as well as intrinsic chemoresistance to some drugs that have not been used before. The proliferative CSCs could be killed by chemotherapeutics; however, the demanded dose of antimitotic drug is higher compared to normal tumor cells. Otherwise, quiescent CSCs are capable to survive even high doses of antimitotic drugs, thus promoting tumor relapse (Wang 2019; Lee et al. 2019; Batlle and Clevers 2017; Schmidt and Efferth 2016; Naik et al. 2016).

Regarding the origin of CSCs there are two possible mechanisms—differentiation from progenitor or normal stem cells or from normal cancer cells, which acquire stemness characterization via epithelial-mesenchymal transition process (EMT) (Marcucci et al. 2019; Mani et al. 2008). Nowadays, an EMT process is not viewed

as a "switch" from epithelial to mesenchymal state of the cell. Instead, it is perceived as a continuum of states from a fully epithelial/proliferative to fully mesenchymal/invasive phenotype comprising a spectrum of intermediate hybrid states. CSCs could represent any of these final or intermediate phenotypic states (Tam and Weinberg 2013). According to the hierarchical model of tumor growth, only CSCs exhibit self-renewal capacity, while other tumor cells possess only limited proliferative potential. Alternatively, stochastic tumor growth model points out that all cancer cells are capable to undergo either self-renewal as CSCs or differentiation into nonproliferating cancer cells depending on genetic and environmental signals (Wang 2019). In different tumors, CSCs indicate astonishing and diversified plasticity allowing to conclude that CSCs hierarchy is not a rigid phenomenon, and non-CSCs cells could be reprogrammed to functional CSCs by various environmental and epigenetic stimuli. A situation met in real tumors seems to be rather a mixture of what is described by pure hierarchical and stochastic models (Chen et al. 2012; Suva et al. 2014). This fact has a profound influence on the anti-CSCs treatment efficacy. If CSCs were strictly defined (as does the hierarchical model) it would be relatively easy to eliminate them. But if stemness were a stochastic and transient feature of competing cancer cells, therapeutic targeting against CSCs would be of a great challenge (Wang 2019; Vlashi and Pajonk 2015).

The capacity of CSCs and non-CSCs populations to interconvert is a unique feature of CSCs, which distinguishes them from normal stem cells. Another difference is based on observation that CSCs are able to form tumors when transplanted into laboratory animals, while normal stem cells cannot do this (Wang 2019). The third main difference involves stem cell niche composition. While normal stem cell niche is tumor suppressive and produces signals arresting cell growth, the CSCs niche produces signals supporting CSCs growth and activation of survival pathways (Khan et al. 2019; Asadzadeh et al. 2019; Batlle and Clevers 2017; Lopez-Lazaro 2015).

Tumor-initiating potential of CSCs could be obtained due to different events occurring in their environment, mainly stressors (hypoxia, pH, drugs, mechanical stress, immunological response), stressor-promoted epigenetic changes (i.e., histone and noncoding RNA modifications), and finally activation of "stemness" signaling pathways (i.e., wingless-related integration site—Wnt, Hedgehog, neurogenic locus notch homolog protein—NOTCH). As the action of these factors could vary between different tumors, and even in different areas of the same tumor, the functions and, to some extent, a phenotype of CSCs could differ spatially and temporally (Takebe et al. 2015; Berabez et al. 2018; Marcucci et al. 2014; Dumont et al. 2008; Wallin et al. 2012; Visvader and Lindeman 2008; Vermeulen et al. 2012; Taniguchi et al. 2019). The CSCs abundance inside tumors could vary from 0,0001–0,1% to as many as 25% of tumor mass depending on method of their identification, and even more on the environment they used to exist in (Capp 2019; Quintana et al. 2008; Rosen and Jordan 2009). According to that functional and phenotypic diversity of CSCs it might be stated that CSCs are a population of cells with both increased gene expression variability and epigenetic plasticity followed by a disturbed interactions with other cells, which disables existence of normal

intercellular interaction network (Capp 2019). From evolutionary perspective, CSCs are a result of an adaptive tumor response sustaining malignant progression shaped by genetic alterations and selective environment (Vander Linden and Corbet 2019).

8.2 Cancer Stem Cells Markers

The CSCs surface markers are not specific for CSCs, but are also expressed on normal stem cells. Moreover, the presence of some surface molecules is not enough to recognize CSCs. They have to indicate precisely defined behavior in in vitro spheroid formation or aldefluor assays to be properly recognized. In vivo limiting dilutions assays and formation of tumors after transplantation to laboratory animals remain the gold standard for CSCs identification. Despite this, several markers have been suggested to identify CSCs, but their precise clinical significance is incomplete as they are applied only as surrogate markers for CSCs identification. The composition of CSCs' surface markers may vary between tumors originating from different tissues. However, there is a group of markers most frequently and reproducibly describing CSCs. Among them CD133, CD44, ALDH1, and CD24 are the most universal and have been most widely studied (Irani 2019). Elevated levels of CD133, glycoprotein known as prominin-1, were noticed in metastatic tumors, correlating to migration, stemness, and tumorigenicity resulting from EMT. Expression of CD133 enhances invasive abilities and chemoresistance of tumor cells. In ovarian cancer, CD133 augmented the adhesion of cancer cells to peritoneal mesothelium, promoting formation of peritoneal implants (Motohara and Katabuchi 2019; Roy et al. 2018). CD44 is a cell surface antigen engaged in cell–cell interactions, migration, and adhesion. Its expression regulates lymphocyte activation and hyaluronic metabolism. It is also responsible for metastatic properties and invasiveness of cancer cells both by regulation of EMT and interaction with hyaluronan acid in extracellular matrix (Irani 2019). In ovarian cancer, peritoneal disseminated implants are enriched in CD44 expression compared to primary tumors, indicating growing aggressiveness (Miranda et al. 2016). CD44 is involved in activation of a variety of receptor tyrosine kinase–induced pathways including hepatocyte growth factor receptor (HGF/c-Met), Src and focal adhesion kinase (Src/FAK), and phosphatidylinositol 3-kinase/phosphatase/protein kinase B (PI3K/AKT), which increase proliferation and survival of cells (Chen and Wang 2019**;** Marjanovic et al. 2013; Matzke et al. 2007; Skupien et al. 2014). Aldehyde dehydrogenase-1 (ALDH1) is a member of protein enzymes involved in cell differentiation, metastasis, detoxification, and drug resistance through the oxidation of intracellular aldehydes (Rodriguez-Torres and Allan 2016). Expression of ALDH1 correlates with migration of cancer cells and unfavorable prognosis for cancer patients (Irani 2019). CD24 is a protein known as heat-stable antigen CD24 engaged in cell adhesion. Lack of or low CD24 expression on CSCs is probably responsible for their increased invasive and metastatic potential and is responsible for worse clinical prognosis (Jaggupilli and Elkord 2012; Taniuchi et al. 2011). In breast cancer, CD44+/CD24−/low phenotype characterizes

mesenchymal and quiescent, while ALDH1+ cells characterize epithelial and proliferative CSCs, respectively (Zhou et al. 2019). The other cancer-specific CSCs markers are CD26, CD29, CD49f, CD117, CD166, EpCAM, CK17, CXCR4 (Organista-Nava et al. 2019; Motohara and Katabuchi 2019). It is noteworthy that at the beginning of cervical cancerogenesis, oncogenic human papilloma virus targets exclusively CD133 + CD44+ CD49f + CD17+ cells considered to be stem cells for cervical epithelium. Through the action of viral E6 and E7 proteins, these cells acquire stemness features of CSCs (Organista-Nava et al. 2016, 2019; Hou et al. 2015). In ovarian cancer, EpCAM/Bcl-2 signaling pathway prevents platinum-dependent apoptosis of cancer cells, resulting in chemoresistance. EpCAM expression is increased in tumors of chemo-resistant patients and correlates with unfavorable outcome. Tyrosine kinase receptor CD117 is responsible for tumor formation, chemoresistance, and poor prognosis in ovarian cancer patients (Motohara and Katabuchi 2019).

Apart from surface markers, there is a group of transcription factors, which by altered expression could characterize CSCs cells. Among them, Oct4, Sox2, Klf4, c-Myc (so-called Yamanaka factors), and Nanog are the best described intracellular CSCs markers (Vlashi and Pajonk 2015; Yamanaka and Blau 2010). Octamer-binding transcription factor-4 (Oct4) is involved in embryonic development and cellular pluripotency. Its function is to stabilize the higher-order structure of chromatin in the Nanog locus (Levasseur et al. 2008). Cytoplasmic expression of Oct4 regulates EMT transformation and is recognized predictor of adverse clinical outcome in cancer. Sex-determining region-Y box-2 transcription factor (Sox2) makes complex with Oct4 and is essential for embryonic and acquired pluripotency and self-renewal of cells. Deregulated Sox2 expression was noticed in several malignant tumors and linked to risk of cancer recurrence and poor prognosis (Takahashi and Yamanaka 2006; Vlashi and Pajonk 2015). Krüppel-like factor-4 transcription factor (Klf4) targets genes involved in cell cycle control and inhibits proliferation by maintaining cell arrest in the G1/S and G2/M checkpoints. In most circumstances, Klf4 acts as cancer suppressor (Chen et al. 2003). Another transcription factor with changed expression in CSCs is c-Myc belonging to Myc regulatory gene and proto-oncogene family. c-Myc is a downstream target for leukemia inhibitory factor/signal transducer and activator of transcription (LIF/STAT3) signaling pathway and amplifies expression of other Yamanaka factors to induce cellular pluripotency (Takahashi and Yamanaka 2006). Nanog is a homeobox protein family transcription factor engaged in upregulation of embryonic stem cells pluripotency and co-operating with Oct4 and Sox2. Nanog is highly expressed in CSCs and its expression correlates negatively with patient's outcome (Chen and Wang 2019; Chiou et al. 2008; Habu et al. 2015). All mentioned above transcription factors augment CSCs maintenance and self-renewal, tumor formation, and chemoresistance. Exclusive increased expression of these transcription factors in CSCs is determined by the fact that they are all substrates for 26S proteasome activity but are spared from degradation as proteasome activity is not present in CSCs cells (Vlashi and Pajonk 2015).

8.3 Cancer Stem Cells Signaling Pathways

CSCs survival depends on the activation of intracellular signaling pathways responsible for stemness. The most important pathways engaged in CSCs function are Wnt/β-catenin, Hedgehog, Hippo/Yes-associated protein (YAP), NOTCH, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and hypoxia-inducible factor-1α (HIF-1α). Wnt/β-catenin is canonical and conservative signal pathway necessary for initiation and regulation of cell self-renewal, growth, migration, survival, and participation in organogenesis. Disturbed Wnt/β-catenin signaling was observed in many malignancies and is indispensable feature of CSCs stemness. In breast cancer, Wnt/β-catenin signaling is associated with epithelial ALDH1+ CSCs populations and their expansion. Inhibition of Wnt/β-catenin signals in CSCs enables entering by them quiescence state and inhibits CSCs metastatic potential. Wnt/β-catenin pathway enhances chemoresistance of breast cancer cells and correlates with poor clinical outcome (Sulaiman et al. 2018, 2019; Forget et al. 2007; Dey et al. 2013; Tzeng et al. 2015; Pohl et al. 2017; Jang et al. 2015; Yang et al. 2016; Khan et al. 2019). In ovarian cancer, CD117 overexpression upregulates ATP-binding cassette G2 (ABCG2) drug resistance system through the Wnt/β-catenin pathway, thereby increasing chemoresistance of ovarian tumor in a hypoxic microenvironment (Chau et al. 2013).

The Hedgehog (Hh) signaling is extremely important for interactions between CSCs and cancer-associated fibroblasts (CAFs) being a key component of tumor CSCs niche. It was observed in breast cancer, that CSCs secrete sonic hedgehog homolog (Shh), a ligand for Hh, which in turn activates CAFs to secrete factors for self-renewal and expansion of CSCs. Hh-signaling takes also part in EMT transition of CSCs and formation of cell-signaling surface structures called primary cilia (De Angelis et al. 2019; Guen et al. 2017). In ovarian cancer, activation of Hh-signaling pathway is connected to formation of CSCs spheroids and chemoresistance (Ray et al. 2011; Song et al. 2018; Park et al. 2010).

The Hippo/YAP pathway is essential signaling component in regulation of tissue growth and organ size as well as stemness maintenance. Trigger regulatory signals are very interesting as they consist of cell density, stiffness of extracellular matrix, shear stress, and nutrient abundance. YAP overexpression promotes cell proliferation, metastasis, and chemoresistance in breast and ovarian cancers. It also enhances CSCs through upregulation of stemness regulatory genes via YAP/transcriptional coactivator with PDZ-binding motif (TAZ)/interleukin (IL)-6/SRF pathway (Kim et al. 2015; Halder and Johnson 2011). YAP/TAZ (transcriptional coactivator with PDZ-binding motif) activity correlates with poor prognosis in breast cancer (Zanconato et al. 2019).

NOTCH signaling is a conservative cell-to-cell communication pathway responsible for cell proliferation, differentiation, and tissue angiogenesis. Deregulated NOTCH signaling is critically involved in maintenance of cellular stemness and migration of CSCs and inside hypoxic niche conditions acts together with HIF-1α pathway. Its function in CSCs dormancy was also reported (Capulli et al. 2019;

Venkatesh et al. 2018; Khan et al. 2019). NOTCH signaling enables increased expression of intracellular CSCs markers including Oct4, Nanog and Klf4. NOTCH pathway is activated in recurrent ovarian cancer and correlates with poor survival (Park et al. 2010).

NF-κB signaling takes place in multiple processes including proliferation, inflammation, angiogenesis, and migration. NF-κB pathway is a common target for different signals like cytokines, infective agents, DNA damage, stress, and hypoxia. NF-κB pathway co-operates with other signaling pathways like Wnt/β-catenin, PI3K, and Janus kinase (JAK)/STAT3 especially in promotion of pro-inflammatory tumor environment and chemoresistance. Breast cancer CSCs showed increased expression of NF-κB (Xia et al. 2014; Gallo et al. 2018; Yamamoto et al. 2013).

HIF-1α transcription factor signaling is one of the key pathways engaged in promotion of CSCs. The areas of hypoxic tumor and hypoxic niche for CSCs make this pathway one of the most important ways to perform cancer cells proliferation, dormancy, and chemoresistance. Through HIF-1α, hypoxia regulates EMT transition and promotes epithelial CSCs (Xia et al. 2014; Wong et al. 2011; Shiraishi et al. 2017).

Function of several other signaling pathways in maintenance of CSCs has been described, including JAK/STAT pathway, TGF-β-signaling pathway or PI3K/phosphatase, and tensin homolog (PTEN) pathway (Roca et al. 2019).

8.4 Genetic and Epigenetic Regulation of Cancer Stem Cells

Cancer stem cells have increased ability to DNA repair what helps them to resist hypoxic conditions and drug toxicity produced by hostile environment or cancer treatment, respectively. Therefore, mutations and epigenetic changes of *BRCA* genes play important role in maintenance of CSCs population. *BRCA1* expression was found to be enhanced in CD133+ lung cancer CSCs and highly aggressive pancreatic cancer cells (Desai et al. 2014; Mathews et al. 2011). BRCA proteins activate JAK/STAT and NOTCH pathways, as well as regulate Hh-signaling, while loss of *BRCA1* expression activates the PI3K-signaling pathway in CSCs cells, respectively (Gorodetska et al. 2019). Defective genes (i.e., *CTNNB1, PTC, SMO, NOTCH, k-Ras, b-Raf, MEK*) cause improper function of Wnt/β-catenin, Hedgehog, NOTCH, RAS/MEK, or PI3K signaling pathways in ovarian cancer CSCs (Suster and Virant-Klun 2019; Testa et al. 2018). Additionally, genes responsible for cell-cycle regulation and activation of apoptosis are also frequently mutated in CSCs cells (Lee et al. 2019; Karimi-Busheri et al. 2010). Studies devoted to ovarian cancer indicated that besides changes of *BRCA* and *TP53* gene expression, ovarian cancer CSCs showed deregulation of genes responsible for function of centrosome, cell membrane receptors, and cell cycle, like *NAB1, PROS1, GREB1, KLF9* (Suster and Virant-Klun 2019; Huang et al. 2014). Another group of genes involved in CSCs maintenance are *HOX* genes, which in physiological conditions regulate

morphogenesis and organogenesis of the embryo through changes of cell proliferation, differentiation, migration and apoptosis (Smith et al. 2019; Hombria and Lovegrove 2003). In cancer, *HOX* genes could play a role of both stimulators and suppressors of oncogenesis. Aberrant *HOX* function in cancer may cause dedifferentiation of cells and increase of their plasticity inducing the population of CSCs cells (Bhatlekar et al. 2018; Ben Khadra et al. 2014). Epigenetic deregulation of *HOX* genes can support CSCs self-renewal, death evasion, metastasis potential, EMT transition, and chemoresistance (Bhatlekar et al. 2018; Haria and Naora 2013; Jin et al. 2012).

Epigenetic changes of gene expression are the most important genetic factor shaping CSCs' behavior and responsible for CSCs plasticity. Accumulative evidence shows that noncoding RNAs play a key role in these mechanisms through modification of target genes locally and in distant places (i.e., premetastatic or metastatic niche) when being transported to them via exosomes (Irani 2019).

Small single-strand noncoding regulatory micro RNAs (miRNAs) by changing the expression of target genes are capable to act both as stimulators and suppressors of CSCs stemness, self-renewal, proliferation, migration, and chemo- and radioresistance. The main way to perform biological functions by miRNAs is epigenetic modification of signaling pathways in CSCs. miRNAs could modulate the DNA repair genes, like *RAD51*, apoptosis regulator *MCL1*, F2R like thrombin or trypsin receptor 3 (*F2RL3*), and Poly(ADP-Ribose) polymerase 1 (*PARP1*) (Schulz et al. 2019; Gong et al. 2015). Function of CSCs could be suppressed by orchestrated influence of many miRNAs affecting transduction of cellular signals: miR-200c and miR-145 (protein containing a disintegrin and metalloprotease (ADAM) pathway), miR-494 (polycomb complex protein BMI-1 pathway), miR-195-5p and miR-34 (NOTCH pathway), miR-99a (mammalian target of rapamycin (mTOR) pathway), miR-519d and miR-128 (caspases). Conversely CSCs' functions are stimulated by other miRNAs: miR-19 and miR-501-5p (via Wnt/β-catenin pathway), miR-21 and miR221/222 (PTEN pathway), miR-483-5p (cyclin D1 pathway), miR-196b-5p (STAT3 pathway), and miR-494-3p (NOTCH1 pathway) (reviewed in Khan et al. 2019). Some miRNAs have ability to regulate function of many different signaling pathways or target genes, while other miRNAs are able to regulate only one pathway or gene, respectively. For instance, miR-372/373 studied in colorectal cancer CSCs is capable to modulate as many as eight pathways including Nanog, Hedgehog, NF-κB, mitogen-activated protein kinase (MAPK), vitamin-D receptor (VDR), JAK/STAT, TGF-β, PI3K/Akt (Khan et al. 2019; Wang et al. 2018a, b, c; Xu et al. 2018). Similarly miR-128 studied in lung cancer CSCs regulates AKT/extracellular-signal-regulated kinase (ERK), p38, PI3K/Akt, vascular-endothelial growth-factor (VEGF), IL-6/JAK/STAT signal pathways (Kwon et al. 2018; Yang et al. 2017; Jiang et al. 2016; Hu et al. 2014). In prostate cancer, miR-302/367 targets four genes (encoding Oct4, Sox2, Nanog, Klf4) and two signaling pathways (BMI-1, large tumor suppressor kinase-2 (LATS2) /YAP) (Guo et al. 2017a, b). On the contrary, some miRNAs are specific regulators of one pathway in CSCs, like miR-138 in lung cancer, which regulates TGF-β pathway, or miR-92a in ovarian cancer regulating Wnt/β-catenin pathway (Zhang et al. 2018;

Chen et al. 2017). There are miRNAs, which function as CSCs modulators and are represented in many cancers (like miR-200c or miR-21), as well as miRNAs, which have been described exclusively in one type of cancer (reviewed in Khan et al. 2019). The most critical miRNAs for acquisition of stemness properties by cancer cells in most circumstances differ between different cancers. miR-21 is of greatest importance for induction of stemness in colorectal and head/neck cancer (Ju 2011; Yu et al. 2013), miR-218 in lung cancer (Yang et al. 2017), miR-221/222 in breast cancer (Li et al. 2017), miR-383 in prostate cancer (Guo et al. 2017a, b), and finally miR-744 in pancreatic cancer (Zhou et al. 2015).

Circular RNAs (circRNAs) are noncoding stable RNAs that act as "sponges" to bind and regulate function of miRNAs, and could be found both intracellularly and inside exosomes. CircGprc5a and circ-ITCH are examples of circRNAs capable of stimulation of self-renewal of CSCs. CircGprc5a modifies function of retinoic acid–induced protein-3 gene (*GPRC5A*) enhancing stemness of CSCs in bladder cancer, while circ-ITCH functions as a "sponge" for miR-214, which modulates stemness by Wnt/β-catenin signaling pathway (Feng et al. 2019; Gu et al. 2018; Qi et al. 2015). Hsa_circ_0020397 through binding with miR-138 regulates proliferation functions of telomerase reverse transcriptase in CSCs. Another circRNA, hsa_circ_0005075 produces "sponge" for miR-93 followed by mesenchymal-to-epithelial transition (MET) and inhibition of CSCs differentiation. CircUBAP2 enhances the expression of antiapoptotic Bcl-2 in CSCs by formation of "sponge" with miR-143. In laryngeal cancer migration of CD133 + CD44+ CSCs could be induced via EMT caused by upregulation of STAT signaling pathway by hg19_circ_0005033 circRNA (Zhang et al. 2017; Shang et al. 2016; Vadde et al. 2015; Wu et al. 2018). CircRNAs could also influence interactions between CSCs and microenvironment by causing anoikis of CSCs deprived of attachment to components of extracellular matrix (ECM) (Agliano et al. 2017).

The function of CSCs could be also regulated by long noncoding RNAs (lncRNAs) defined as RNA transcripts exceeding 200 nucleotides but not translated to proteins. They participate in the regulation of gene transcription, as well as in posttranslational and epigenetic regulation. Epigenetic regulation by expression of *HOX*-derived lncRNAs can influence CSCs function. The *HOTAIR* gene encodes lncRNA that supports CSCs phenotype and EMT transition in breast and colon cancer CSCs. HOTTIP, another lncRNA originating from HOX cluster, stimulates pancreatic CSCs functions by regulation of Wnt signaling (Padua Alves et al. 2013; Zhang et al. 2014; Fu et al. 2017). Highly upregulated lncRNA of transcription factor 7 seen in liver cancer CSCs activates Wnt/β-catenin signaling and leads to tumor propagation (Toh et al. 2017; Wang et al. 2015).

Another mechanism of epigenetic regulation in CSCs is dependent on methylation of both histones and non-histone proteins. Methylation is associated with either activation or repression of regulated gene. Methylation of histone H3 lysine 4 (H3K4), H3K36, and H3K79 results in gene activation, whereas methylation of H3K9, H3K27, and H4K20 produces a gene repression. Methylation concerns also DNA, when methyl groups are transferred from S-adenosyl methionine (SAM) to CpG groups of gene promoters and regulatory regions. Hypermethylation of DNA in

cancer results in silencing of tumor suppressor or differentiation genes and may contribute to formation of CSCs (Kouzarides 2007; Esteller 2007). Aberrant Wnt/β-catenin activation in CSCs could result from methylation of promoters for Wnt inhibitors and negative regulators, namely, secreted frizzled-related protein 1 (SFRP-1), and Dickkopf-related protein 1 (DKK1), as was found in breast and colon cancers (Klarmann et al. 2008; Suzuki et al. 2004; Koinuma et al. 2006). Disturbed histone H3K16 and H3K27 modifications could also inhibit the expression of Wnt antagonists (Hussain et al. 2009). Disturbed methylation of *Shh* gene promoter results in upregulation of Hh-signaling pathway in breast and gastric cancers (Cui et al. 2010; Wang et al. 2006). Methylation of H3K27 histone causes silencing of miR-200c and miR-205 expression, thus activating EMT transition and CSCs phenotype (Tellez et al. 2011). Histone methylation is also responsible for increased expression of ATP-binding cassette (ABC) family of transmembrane transporters responsible for chemoresistance of CSCs (To et al. 2008). Dysregulated function of histone acetyltransferases (HAT) and deacetylases (HDAC) is also connected to cancer progression. HDAC1 and HDAC7 enzymes promote stemness in CSCs of breast and ovarian cancer. Knockdown of HDAC function resulted in arrest of growth and entering apoptosis in many cancers (Roca et al. 2019; West and Johnstone 2014; Cai et al. 2018). Enhanced histone acetylation of jagged canonical NOTCH ligand-2 gene (*JAG2*) promoter in multiple myeloma affects NOTCH pathway activity in CSCs (Ghoshal et al. 2009).

8.5 Cancer Stem Cells and EMT Transition

Epithelial-to-mesenchymal transition is a process, which occurs in three different types: type-1 EMT during embryogenesis, type-2 EMT during wound healing and regeneration, and type-3 EMT in cancer (Hass et al. 2019; Kalluri and Weinberg 2009). Type-3 EMT facilitates cells' metastasize potential and promotes CSCs motility and invasion. EMT changes cell apico-basal polarity, cytoskeleton remodeling, cell morphology, cell–matrix interaction, attenuates cell–cell adhesion, and facilitates cell migration (Jolly and Celià-Terrassa 2019; Savagner 2015). Acquisition of mesenchymal phenotype by CSCs enables them to migrate into surrounding tissues ("invasive front"), microvasculature (lymphatic and blood microvessels), and to distant localizations. Moreover, this enhances their survival and chemoresistance, thus promoting tumor recurrence. In the target organs (metastatic niches), CSCs go through MET transition gaining again epithelial phenotype. MET transition augments intercellular contact, proliferation, and differentiation of metastatic tumors (Ishiwata 2016). "Invasive front" of the tumor is defined as an interface between growing tumor and surrounding stroma. The components of the "invasive front" are extracellular matrix, cells (including lymphocytes, tumor-associated macrophages—TAMs, fibroblasts, myeloid progenitor cells) and blood and lymphatic vessels. At the "invasive front" TAMs initiate EMT and promote CSCs via activation of TGF-β, Wnt/β-catenin, and RAS/ERK signaling pathways (Clark

and Vignjevic 2015, Shiga et al. 2015, Lee et al. 2018). In breast cancer epithelial state, CSCs are proliferative, localized inside the tumor, and marked as ALDH1+ E-cadherin ^{high} vimentin ^{low} zinc finger E-box-binding homeobox-1 (ZEB1) ^{low}. Mesenchymal-state CSCs are quiescent, localized at the "invasive front" and marked as CD44 + CD24- E-cadherin ^{low} vimentin ^{high} ZEB1 ^{high} (De Angelis et al. 2019; Liu et al. 2014). In the primary tumor, the cells undergoing EMT adopt mesenchymal phenotype, then migrate to distant organs where they produce metastases, and finally revert into epithelial phenotype.

EMT transition is also a way by which differentiated cancer cells could possess stemness and become CSCs (Brabletz et al. 2005). The CSCs arisen in this process could have a phenotypic heterogeneity. They either show "pure" epithelial (E) or mesenchymal (M) phenotypes, or alternatively they show hybrid E/M phenotype combining both epithelial and mesenchymal features in different proportions. These hybrid E/M state cells are highly tumorigenic, and display stemness features like self-renewal and plasticity (Suster and Virant-Klun 2019). The epithelial, hybrid, and mesenchymal states are interchangeable in response to signals coming from intrinsic (i.e., tumor niche) and extrinsic (i.e., chemotherapy) sources. Their response depends also on the history of previous signals—"cellular memory" (Elowitz et al. 2002; Chang et al. 2006). Hybrid E/M phenotypes are sustained by "stability factors," like protein numb homolog (NUMB), transcription factor Ovo-like-2 (OVOL2), grainyhead-like protein-2 homolog (GRHL2), and nuclear factor erythroid-2-related factor-2 (NRF2), as well as by TGFβ- and NOTCH signaling (Bocci et al. 2019; Matsumura et al. 2019; Boareto et al. 2016). Hybrid E/M cells behave as aggressive CSCs, and their function is regulated between different interim E/M phenotypes by Wnt, NOTCH, and NF-κB signaling (Colacino et al. 2018; Kroger et al. 2019). Subset of CSCs of intermediate E/M phenotype shows probably the highest level of adaptation to secondary localizations and sometimes are called circulating CSCs (CTCs) (Agnoletto et al. 2019; Tam and Weinberg 2013). They have been identified in several metastatic cancers, including breast, lung, gastric, colon, and hepatocellular cancer (Vishnoi et al. 2015; Koren et al. 2016; Nel et al. 2014; Katoh et al. 2015; Li et al. 2014). CTCs are also described by increased expression of ALDH1, and associated with high tumor grade, poor outcome, and high level of expression of multi-drug resistance proteins (Aktas et al. 2009; Ginestier et al. 2007; Gradilone et al. 2011). Transcriptional and epigenetic regulation of EMT involves *CDH1* (for E-cadherin) gene promoter and downstream NF-κB pathway targets (Markopoulos et al. 2019; Jing et al. 2011). Epigenetic regulation of EMT embraces H3K27me3 histone methylation and changes in miR-200 and miR-34 expression (zinc finger transcription factors ZEB/miR-200 and SNAIL/miR-34 regulatory loops), which additionally governs the EMT-dependent activation of CSCs (Polyak and Weinberg 2009; Brabletz and Brabletz 2010). The presence of balanced interactions between feedback regulatory loops of p53- and NF-κB-dependent miRNA regulations is crucial for EMT and CSCs behavior (Markopoulos et al. 2018). Inflammatory environment in tumors created by TAMs, CAFs, MDSCs, cytokines (IL-1, IL-6, TNFα, TGFβ), and chemokines (IL8) participates in EMT and promotion of CSCs and depends on

TGF-β and NF-κB signaling. Besides those two pathways, Hedgehog, Wnt/β-catenin, and NOTCH pathways also regulate EMT (Iliopoulos et al. 2009; Hass et al. 2019).

8.6 Cancer Stem Cells and Tumor Microenvironment Niche

CSCs niche is a specialized tumor microenvironment taking part in origination and regulation of CSCs. Components of CSCs niche provide both nutrients and signals needed for the effective function of CSCs. In cancer, functionally understood niche is composed of CAFs, mesenchymal stem cells (MSCs), immune cells including tumor-associated macrophages (TAMs), non-CSCs cancer cells, adipocytes, components of extracellular matrix, blood and lymphatic vessels, cytokines, chemokines, and growth factors. CSCs niche enhances cell differentiation, accumulation of genetic mutations and epigenetic signals, resistance to apoptosis and toxic agents. The proper function of CSCs niche demands interchange of signals between CSCs and niche microenvironment (Kubo et al. 2016; Quante et al. 2011).

One of the most important cellular components of CSCs niche is CAFs, which regulate EMT transition, secrete proangiogenic factors, produce cytokines (IL-6, LIF, TGF-β), chemokines (IL-8, CXCL12, CXCL1), prostaglandins (PGE), and growth factors (HGF, VEGF). CAFs are situated mainly at the tumor "invasive front" (Zhang and Peng 2018; Guo et al. 2017a, b). Observation in breast cancer prooved that CSCs stemness and EMT transition was regulated by CAFs-derived exosomes containing regulatory molecules like miR-21, miR-378e, miR-143 and lnc RNA h19 (Huang et al. 2019; Ren et al. 2018; Donnarumma et al. 2017). They also activate the NF-κB, STAT, and NOTCH pathways in CSCs, thus supporting their drug resistance (Lee et al. 2019; Boelens et al. 2014). Exosomes containing miR-105 derived from cancer cells are a signal that force CAFs to reciprocally support CSCs. Cancer-associated fibroblasts have been classified functionally into different subpopulations. Inflammation in cancer niche is very important phenomenon and is dependent on the "inflammatory" iCAFs function. iCAFs inflammasome pathway is regulated by NOD-LRR-and pyrin domain-containing protein-3 (NLRP3), IL-6/STAT3/PTEN/NF-κB, TGF-β/SMAD and IL-1 mediated signals, and supports tumor progression by creation of immune-suppressive environment (Ershaid et al. 2019; Yan et al. 2018; Iliopoulos et al. 2011). In breast cancer, IL-6 secreted by CAFs regulates stemness mainly in CSCs of mesenchymal phenotype, while IL-8 stimulates mainly epithelial ALDH1+ CSCs (Chan et al. 2019; Chang et al. 2014; Ginestier et al. 2010). CAFs are even able to travel with CSCs to distant localizations to produce metastases. During chemo- or radiotherapy, cancer cell niche is enriched in CAFs by action of IL-8-CXCL1-pathway. Chemotherapy-recruited CAFs produce several CXCL chemokines, which further stimulate expansion of CSCs (Duda et al. 2010; Chan et al. 2016; Ginestier et al. 2010). In breast cancer, they are CXCL12 (stromal-cell-derived factor-1—SDF-1) and CCL2 (monocyte chemoattractant protein-1), which act on cancer cells, and activate prostemness

pathways, mainly Wnt/β-catenin, PI3K/AKT and NOTCH. Also in breast cancer, it was discovered that high mobility group box-1 (HMGB1) protein secreted by autophagic CAFs enhances stemness of breast CSCs via toll-like receptor-4 (TLR-4) (Tsuyada et al. 2012; Todaro et al. 2014; Zhao et al. 2017). The direct cell-cell contact between CAFs and CSCs is also an indispensable component of niche properties. CD44 and CD10/GPR77 membrane molecules are both engaged in this kind of interaction (Su et al. 2018).

Mesenchymal stem cells are a population of multipotent mesenchymal stromal cells capable to generate different cell types. They are described functionally by ability to migration into sites of inflammation, tissue injury, and cancer where they suppress immune response. MSCs are recruited into the tumors via TGF-β- and CXCL12-dependent ways (Quante et al. 2011). Inside tumors MSCs participate in regulation of EMT phenomenon, angiogenesis, and chemoresistance, as well as are able to differentiate into CAFs (Ma et al. 2014; Ishihara et al. 2017; Chang et al. 2015). They activate stemness in CSCs by secretion of IL-6, IL-8, CCL2, CCL5, PGE-2, metalloproteinase inhibitor-2 (TIMP-2), VEGF, fibroblast growth factor (FGF), and JAG1. Similar to CAFs, MSCs are multiplied in radio- or chemotherapy-treated tumors and through secretion of CXCL12 chemokine and activation of STAT3 signaling, they augment CSCs stemness and resistance to therapy. In breast and ovarian cancer, interaction with MSCs upregulated the PI3K/AKT pathway and MDR proteins in CSCs, resulting in resistance to trastuzumab and paclitaxel/carboplatin, respectively (Lee et al. 2019; Kalluri and Zeisberg 2006; Rafii et al. 2008; Chan et al. 2016; Park et al. 2009; Wang et al. 2018a, b, c). MSCs cell possess a unique possibility to fuse with cancer cells to form so-called hybrid cancer cells. This cell population although not very numerous has been identified in several cancers and contributes to cancer plasticity, genetic variability, and metastases (Melzer et al. 2018; Pawelek and Chakraborty 2008).

Provascular signals from tumor niche trigger neovascularization. CSCs participate in this phenomenon by "vasculogenic mimicry" where CSCs and cancer cells form vascular-like channels to supply nutrition during prevascular phase of tumor growth. Later on CSCs could differentiate to epithelial and vascular smooth muscle–like cells, creating the "mosaic pattern" of vascularization. CSCs are also able to secrete HIF-1α and VEGF in response to exogenic expression of these factors (Maniotis et al. 1999; Ping and Bian 2011). Epithelial cells of the niche vessels secrete many factors maintaining CSCs stemness phenotype, including IL-1, IL-3, IL-6, granulocyte colony–stimulating factor (G-CSF), and granulocyte-macrophage colony–stimulating factor (GM-CSF) (Barbato et al. 2019; Pirtskhalaishvili and Nelson 2000; Butler et al. 2010). Secretion of TNF-α by endothelium upregulates NF-κB signaling and stimulates chemoresistance of CSCs (Tang 2012).

Inflammation inside tumor is directly connected to EMT transition, thus influencing cancer progression and metastatic potential. It also upregulates significantly resistance of CSCs against host immune surveillance. Several proinflammatory cytokines/chemokines, including TGF-β, TNF-α, IL-1, IL-6, and IL-8, are secreted by the cells occupying CSCs niche and are potent EMT-inducers. Cytokine-triggered signaling pathways activate transcription factors and epigenetic regulation in

CSCs. One of the key regulators of EMT is TGF-β, which has a dual role for cancer progression—cancer suppressor in early cancer and tumor promoter in advanced cancer. Switch to promoter function is connected to initiation of EMT transition and depends on Smad3/Smad4 transcription factors, RAS/RAF/MAPK, and NF-κB signaling (Tian et al. 2013, Balkwill 2009, reviewed in Markopoulos et al. 2019). TGF-β-induced Smad expression is crucial for EMT as it upregulates ZEB and SNAIL zinc finger transcription factors followed by downregulation of miR-200 and miR-34. Complex functionality of TGF-β-induced regulatory loops consists of SNAIL/miR-34- and ZEB/miR-200-dependent switch between EMT and MET CSCs phenotypes, respectively. NF-κB signaling triggered by TGF-β promotes EMT and consequently CSCs motility, stemness, metastasis, and drug resistance (Tian et al. 2013, Markopoulos et al. 2018, reviewed in: Markopoulos et al. 2019). Prolonged stimulation of breast epithelial cells by TGF-β stimulated EMT and caused increase of CSCs-phenotype CD44 + CD24- cells (Bhat et al. 2019; Katsuno et al. 2019). In gastric cancer, *Helicobacter* infection stimulates TGF-β secretion followed by EMT activation, IL-17 secretion by Th17 cells, neutrophil recruitment, and creation of chronic inflammatory reaction sustaining CSCs (Rezalotfi et al. 2019; Lina 2014; Choi et al. 2015). TNF-α is a main proinflammatory cytokine engaged in regulation of differentiation and apoptosis in cancer. Its downward signaling activates NF-κB, caspase, p38, c-Jun N-terminal kinases (JNK), and ERK pathways. Through NF-κB pathway, TNF-α stimulates cytokine and chemokine effectors [IL-6, IL-8, IL-18, inducible nitric synthase (iNOS), cyclo-oxygenase (COX)-2, and lipoxygenase (LOX)], which link inflammation to cancer progression. TNF-α could negatively affect growth of early tumors; however, it promotes survival, angiogenesis, and EMT in advanced tumors. TNF-α co-operates strongly with TGF-β to accelerate the process of EMT (Balkwill 2009; Aggarwal et al. 2012; Brenner et al. 2015; Onder et al. 2008; Bates and Mercurio 2003). Breast cancer cells exposed to TNF-α have been enriched by CSCs CD44 + CD29+ cells (Weitzenfeld et al. 2016). IL-1 is another proinflammatory cytokine exerting effects on CSCs residing in niche. IL-1-dependent NF-κB signaling upregulates stemness-promoting genes, like proto-oncogene polycomb ring finger gene (*BMI1*) and nestin gene (*NES*). In head and neck cancer, CSCs IL-1 activates EMT by downregulation of E-cadherin gene (*CDH1*) expression, while in breast cancer, CSCs activate EMT by IL-1/IL-1R/β-catenin pathway, which additionally leads to estrogen receptor *ESR1* gene silencing and tamoxifen resistance (Mantovani et al. 2018; Soria et al. 2011; Li et al. 2012; Charuworn et al. 2006; Jiménez-Garduño et al. 2017). IL-6 is another proinflammatory cytokine activated in tumor microenvironment by TGF-β, TNF-α and IL-1, NF-κB and STAT3 transcription factors, and RAS/RAF/MEK and PI3K signaling pathways. Through the upregulation of NF-κB and STAT3 transcription factors, IL-6 increases expression of miR-21, miR-181b-1, and Let-7; enhances cancer-associated inflammation; and activates EMT. IL-6-induced EMT induces invasion and migration of cancer cells via activation of metalloproteinases (Chang et al. 2014; Ancrile et al. 2007; Chou et al. 2005). In breast cancer, IL-6 was shown to stimulate CSCs stemness by increase of CD44 and Oct4 expression. It is also capable of autocrine augmentation of self-secretion in CSCs via JAG1/NOTCH3

signaling, thus stimulating self-renewal and proliferation of CSCs (Kim et al. 2013; Sansone et al. 2007; Al-Hajj et al. 2003). Breast cancer ALDH1+ CSCs were shown to have higher expression of IL-8 receptor and alpha-chemokine receptor *CXCR* gene. IL-8 signaling was connected to increased CSCs activity both in HER2-positive and triple negative breast cancers (Dominguez et al. 2017; Singh et al. 2013; Charafe-Jauffret et al. 2009).

Metabolic reprogramming of CSCs is one of the key factors influencing their stemness, migratory potential, and chemoresistance. CSCs show unique adaptation to variable levels of tissue oxygenation found inside the tumors and are capable of functioning using both aerobic glycolysis and oxidative phosphorylation (OXPHOS) (Nazio et al. 2019; Peixoto and Lima 2018; Menendez et al. 2013; Pacini and Borziani 2014). Generally, in normoxic and most hypoxic conditions, CSCs rely on OXPHOS, which is more energetically efficient process. In this situation, the maintenance of CSCs stemness depends on increase of antioxidant defense against reactive oxygen species (ROS) derived by enhancement of OXPHOS rate and mitophagy, which through degradation of defective mitochondria prevents apoptosis of CSCs (Nazio et al. 2019, Held and Houtkooper 2015, Peiris-Pagès et al. 2016, Snyder et al. 2018, reviewed in Jagust et al. 2019). ROS balance and resistance to ROS inducers (like chemo- and radiotherapy) are regulated in CSCs by c-Myc, p53, HIF-1α, NF-κB, and NRF2 pathways. HIF-1α via signaling pathway reduces ROS production and protects CSCs from their adverse effects. ALDH1, the CSCs marker, directly reduces ROS and produces antioxidants, as well as facilitates resistance to paclitaxel (Takahashi and Yamanaka 2006). In hypoxic conditions of the niche, CSCs can switch from OXPHOS to aerobic glycolysis. Although it is usually less efficient in production of energy, in cancer cells, it could achieve levels of energy comparable to OXPHOS. Besides this, it was found that even in hypoxic environment, cancer cells use simultaneously OXPHOS and glycolytic metabolic pathways (reviewed in Jagust et al. 2019). Hypoxia-activated cascade of cellular pathways dependent on HIF-1α helps to endure hostile conditions by reprogramming CSCs, which can finally enter the state of quiescence. Genes and transcription factors responsible for CSCs pluripotency were demonstrated to be engaged in switch from OXPHOS to glucose-dependent metabolism (reviewed in Jagust et al. 2019). CAFs and other cells of CSCs niche support CSCs metabolic reprogramming and help to remove lactates in so-called reverse Warburg effect. (Nazio et al. 2019, Yoshida 2017, reviewed in Jagust et al. 2019). CSCs are generally situated inside or close to hypoxic areas inside tumors; however, in some brain tumors, CSCs reside in well-oxygenated perivascular niches (Gilbertson and Rich 2007). Tumors possessing high expression of HIF-1α have been associated with higher mortality and resistance to chemotherapeutics. In breast cancer, HIF-1α was correlated to MDR proteins expression (Semenza 2014; Cao et al. 2013). The presence of HIF-1α expression enhances activation of EMT and stemness activators like Wnt/β-catenin, Hedgehog, NOTCH pathways and CD133, Nanog and Sox2 in CSCs markers (Liu et al. 2014; Majmundar et al. 2010). Tumor environment is described by acidosis, resulting from glycolytic activity and mitochondrial respiration–derived carbon dioxide hydration. Acidosis seems to be a triggering

and a maintenance factor for CSCs stemness. Acidic conditions stabilize HIF-1α, change histone epigenetic regulation, and downregulate von Hippel-Lindau (VHL) tumor suppressor molecule. They also stimulate MSCs, increase expression of transcription factors Oct4 and Nanog in CSCs, and secretion of VEGF and IL-8 in CSCs niche (Vander Linden and Corbet 2019; Schornack and Gillies 2003; Corbet and Feron 2017; Hjelmeland et al. 2011; Mekhail et al. 2004). Acidosis drives energy gain into OXPHOS mechanism and changes lipid metabolism. It augments drug resistance by direct influence on cell membrane integrity, efficacy of membrane transporters, cancer cell dormancy, and autophagy (reviewed in Vander Linden and Corbet 2019).

Dysregulation of lipid metabolism is observed in the most aggressive tumors. Lipid desaturation plays important role in self-renewal and tumorigenicity of CSCs through the changes of lipid composition of cell membrane and Wnt/β-catenin signaling. Monounsaturated fatty acids/Stearoyl-CoA desaturase-1 (SCD-1) converses fatty acids into monosaturated fatty acids. Upregulation of monounsaturated fatty acids/Stearoyl-CoA desaturase-1 (SCD-1) enhances tumor proliferation, while inhibition of SCD-1 results in decrease of ALDH1, Nanog and Oct4 activity, and restores chemoresistance in lung CSCs (Begicevic et al. 2019; Kim and Ntambi 1999; Colacino et al. 2016; Noto et al. 2013). Lipids can also function as second messengers of signal transduction in CSCs via NOTCH, AKT, and NF-κB pathways (reviewed in: Jagust et al. 2019). Lipids are also an important substrate for energy supply; therefore, blockade of fatty acid synthase (FASN) inhibits CSCs growth (Wang et al. 2013). In breast cancer, JAK/STAT3 pathway was found to regulate lipid metabolism in CSCs, thus stimulating their stemness.

Adipocytes from cancer microenvironment (cancer-associated adipocytes—CAAs) are capable to provide lipids for CSCs. Increased lipid uptake results in lipid droplet accumulation inside CSCs. High concentration of lipid droplets is correlated with tumor aggressiveness and poor survival. Fatty acids stored up in CSCs serve as energetic reserve for the cells during the periods of metabolic restrictions and are then mobilized during a lipophagy process (Lue et al. 2017). Breast adipocytes via secretion of leptin and IL-8 could participate in lipid metabolism of CSCs using the same STAT3 signaling pathway. Adipocytes and adipose progenitor cells being component of breast cancer niche and secreting GM-CSF and metalloproteinase 9 are also capable of stimulating breast cancer CSCs (Reggiani et al. 2017; Wang et al. 2018a, b, c; Al-Khalaf et al. 2019). In ovarian cancer, omental implants are an example of another niche, in which adipocytes play important role in nesting and proliferation of CSCs (Nieman et al. 2011).

In ovarian cancer, ascites represents a unique microenvironment for CSCs and accounts for transcoelomic spread of metastases/implants. During this process, cancer cells go through EMT and, in the form of single cells or cell spheroids containing a lot of CSCs, are transported passively over peritoneal cavity, then homing mesothelium, going through MET, and starting to grow extensively (Bregenzer et al. 2019; Yeung et al. 2015). Ascites also facilitates entry of cancer cells into lymphatic vessels. Pro-inflammatory IL-6 from ascites stimulates stemness in CSCs via JAK/STAT3 and Wnt/β-catenin signaling (Abubaker et al. 2014).

VEGF is also a regulator of peritoneal carcinomatosis, and IL-8 recruits cancer cells into the surface of omentum due to the tropism between CSCs and adipocytes (Winiarski et al. 2013; Nieman et al. 2011). Extracellular vesicles play important role in regulatory network inside ascitic fluid. They are able to transport miRNAs, lipids, cytokines and growth factors, as well as CSCs markers like CD44 or EpCAM molecules (Zong and Nephew 2019; Runz et al. 2007; Gutwein et al. 2005). One of the astonishing mechanisms resulting in enrichment of CSCs in peritoneal implants is response of cancer cells to mechanic stimuli and mechanic stress produced by peritoneal extension due to ascitic fluid. Mechanic stimuli cause activation of mechanotransduction signals involving mainly YAP/TAZ signaling pathway, and accessory NF-κB, ERK, FAK, and Rho/Rho-associated protein kinase (Rho/ROCK) pathways. There a plenty of mechanical stressors that influence behavior of CSCs in ovarian cancer. The first of them are shear and compression produced mainly by ascites build-up and movement, then tension and compression caused by tumor growth against surrounding tissue, and finally stiffness resulting from ECM remodeling and desmoplastic response (reviewed in: Bregenzer et al. 2019). Activation of mechanotransduction signals regulates EMT/MET transition, changes cancer cell shape and morphology, enhances CD133 + CD44 + Oct4+ CSCs population, increases CSCs chemoresistance through upregulation of ABCG2 and P-gp membrane transporting systems, increases angiogenesis via VEGF secretion, and regulates interaction with ECM (reviewed in Bregenzer et al. 2019). Response of CSCs to mechanic stressors in metastatic locations augments ovarian cancer invasiveness, chemoresistance, and stemness of cancer cells.

ECM composition is altered inside tumor niche due to the activity of CAFs and cancer cells themselves. The changes of metalloproteinase activity and VEGF in tumor environment influence ECM behavior and are the source of different changes including desmoplastic reaction. Disturbed ECM and aberrant tumor vasculature results in fluctuations of tumor interstitial fluid pressure that would further influence pathways regulating EMT transition, hypoxia, and chemoresistance. Components of ECM could co-operate with CSCs in different ways. CD44, a marker of CSCs, is a receptor of hyaluronic acid and versican, constituents of ECM. CD133, whose expression is connected to CSCs stemness, could be activated by type I collagen. Both CD44 and CD133 promote attachment of cancer cells to mesothelium on the surface of peritoneum. Mechanic signals are also transmitted to CSCs by syndecan-1 (CD138) and discoidin domain receptor-1 (DDR1) activated by fibronectin and collagen, respectively (reviewed in Choudhury et al. 2019). In breast cancer, tenascin C expressed in ECM supports WnT/β-catenin and NOTCH signaling, thus stabilizing CSCs functions (Oskarsson et al. 2011).

8.7 Cancer Stem Cells and Autophagy

Autophagy is defined as a self-digestion inside auto-phagosomes of proteins, lipids, and damaged cellular organelles followed by recycling of digestion products. In normal conditions, autophagy is a mechanism of controlling cell homeostasis, but

during stress produced by hypoxia, starvation or toxic drugs autophagy is a mode of cell survival. The role of autophagy is ambivalent—it could act as antitumor mechanism, or it could promote tumorigenesis. Protection against tumor initiation depends on ability to control cell homeostasis in chronically inflamed or mutagenic environment. In cancer, autophagy helps to maintain tumor survival and progression despite hostile conditions. Forkhead box family transcription factor-3 (FOXO3) signaling pathway mediates transcription of autophagy-regulating genes including autophagy-related genes (*ATG*), beclin-1 gene (*BECN1*), Unc-51-like autophagy-activating kinase-1 gene (*ULK1*), and gamma-aminobutyric acid receptor–associated protein-like-1 gene (*GABARAPL1*) (Nazio et al. 2019; Van Der Vos and Coffer 2008). Autophagy is linked to EMT and present in cells with mesothelial phenotype. It is also connected to chemoresistance of CSCs. Autophagy protects cancer cells from proapoptotic stimuli and genome instability. It is also capable to modify antitumor immune responses and maturation of some immune cells. During premetastatic latency breast cancer, CSCs indicate dormancy phenotypes supported by several mechanisms including autophagy. Autophagy in dormant CSCs could be regulated by activation of SRC-mediated TNF-related apoptosis-inducing ligand (TRAIL) resistance (in bone metastases), effective DNA repair and p53 function sustained by expression of *ATG7* gene, and by decrease of 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase-3 (PFKFB3) concentration in the cells (Zhang et al. 2009; Lee et al. 2012; Shinde et al. 2019; Janji et al. 2016).

8.8 Cancer Stem Cells and Immunosurveillance

Cancer CSCs would not be so dangerous if they were not able to escape from immune surveillance. This property is known as immune CSCs resistance, and is based on lower CSCs immunogenicity and ability to manipulate immune system through secretion of suppressor molecules, recruitment of immune-regulatory cells, and decreased expression of cell antigens. CSCs are capable to mimic the function of antigen-presenting cells, however, in altered way as they show an eleveted expression of check point programmed death ligand-1 (PD-L1) and decreased expression of MHC molecules. As a result of defective antigen presentation, inhibition of T cell effectors, stimulation of Tregs, and promotion of tumor tolerance occur. Glioblastoma CSCs have altered expression of PD-L1, galectin-3, and macrophage-inhibitory cytokine-1, thus being able to avert cytotoxic T reactions and phagocytosis (Kim et al. 2016; Downs-Canner et al. 2017). Moreover, upregulated expression of HLA-E class II molecule and simultaneous low expression of MHC class I and NKG2D molecules on glioblastoma CSCs were shown to inhibit cytotoxic T cell and NK effectors (Sultan et al. 2017; Du et al. 2014). In cancer breast cells, PD-L1 overexpression was connected with increased function of Oct4 and Nanog transcription factors promoting stemness (Zhao et al. 2009). Breast CSCs also indicate downregulation of MICA and MICB ligands for NK cell receptor NKG2D that makes them resistant against NK cell–mediated cytotoxicity (Gagliani et al. 2015).

CD95 molecule is a death-promoting factor for regulation of activation-induced death of T lymphocytes and many other types of cells. In gastric cancer, CD95/CD95-ligand signaling promotes EMT and supports maintenance of CSCs population (Badrinath and Yoo 2019; Ceppi et al. 2014).

Immune cells present in CSCs niche comprise mainly of TAMs, MSCs, and MDSCs, which through TGF-β signals stimulate tumor EMT, progression, and metastatic potential. All three cell populations contribute to immunosuppressive environment in tumor and CSCs niche. Through secretion of macrophage inflammatory proteins (MIP1 and MIP2) and PGE, MSCs recruit suppressor M2 macrophages into the tumor (Vasandan et al. 2016). Moreover, TAMs and MSCs stimulate the T regulatory CD4 + CD25 + FoxP3 cells, while MDSCs recruit the T helper IL-17-secreting suppressors (Barbato et al. 2019; Kalluri and Weinberg 2009; Kitamura et al. 2015). In ovarian cancer, IL-17 activates NF-κB and p38-mitogen-activated protein kinase pathways, which increase stemness of cancer cells (Xiang et al. 2015) R. In colon cancer, regulatory T FoxP3 + IL-17+ cells promote expansion of CSCs in hypoxic environment (Sultan et al. 2017; Silver et al. 2016). CSCs cells in glioblastoma and colon cancer were shown to secrete increased levels of immunosuppressive TGF-β and IL-4, respectively (Codony-Servat and Rosell 2015; Viry et al. 2014; Lorin et al. 2013). They could downregulate the intensity of host immune antitumor response. Acidic conditions in CSCs microenvironment and premetastatic niche also decrease antitumor efficacy of T lymphocytes and NK cells, as well as the secretion of IL-2, interferon (IFN) γ, perforin, and granzyme B. Acidosis inhibits also maturation of dendritic cells. Accumulation of H^+ ions and lactate inhibits glycolytic processes in T cells and expression of nuclear factor of activated T cells (NFAT). Acidic conditions help also to deviate TAMs activity into M2 tumorigenic phenotype (Fischer et al. 2007; Gottfried et al. 2006; Dietl et al. 2010; Brand et al. 2016). TAMs present in cancer cells niche produce TNF-α and TGF-β for maintaining CSCs. In breast cancer, TAMs promote CSCs via EGFR/STAT3/Sox2 signaling pathway. The function of FoxP3+ Tregs and PD-1/PD-L1 pathway also support the CSCs population (Zhou et al. 2019; Plaks et al. 2015; Yang et al. 2013; Seo et al. 2013; Malta et al. 2018). Metastatic aggressive foci characterized by high level of autophagy are poorly infiltrated by tumor-infiltrating lymphocytes (TILs) (Zarogoulidis et al. 2016).

8.9 Conclusion

Cancer stem cells have become one of the main targets for anticancer therapy and many ongoing clinical trials test anti-CSCs drugs. However, high plasticity of CSCs gives rise to many doubts concerning efficacy of these drugs. Some treatment options propose multidirectional inhibition of CSCs by simultaneous use of several drugs having different points of action, but results of such trials are still inconclusive. Only time will tell if we can tame and neutralize CSCs successfully, but even today, many are skeptical. And this will not change in the nearest future, I am afraid.

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

Raphaëlle Toledano Zur, Galit Adler, Katerina Shamalov, Yair Tal, Chen Ankri, and Cyrille J. Cohen

Contents

Abstract As an important part of the immune system, T lymphocytes exhibit undoubtedly an important role in targeting and eradicating cancer. However, despite these characteristics, their natural antitumor response may be insufficient. Numerous clinical trials in terminally ill cancer patients testing the design of novel and efficient immunotherapeutic approaches based on the adoptive transfer of autologous tumor-specific T lymphocytes have shown encouraging results. Moreover, this also led to the approval of engineered T-cell therapies in patients. 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.

Keywords Adoptive Cell Therapy · Cancer · CAR-T-cells · T-cells engineering · T-cell Receptor · Tumor-Infiltrating Lymphocytes

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Abbreviations

ACT	Adoptive Cell Therapy
ALL	Acute Lymphoblastic Leukemia
ATP	Adenosine Tri-Phosphate
BCMA	B Cell Maturation Antigen
CAR	Chimeric Antigen Receptor
CD19	Cluster of Differentiation 19
CEA	Carcinoembryonic Antigen
CLL	Chronic Lymphoblastic Leukemia
CR	Complete Response
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
HER2	Human Epidermal Growth Factor Receptor 2
HLA	Human Leukocyte Antigen
HPV	Human Papillomavirus
IL-10	Interleukin 10
MART1	Melanoma Antigen Recognized by T cells 1
MHC	Major Histocompatibility Complex
MM	Multiple Myeloma
NCR	Natural Cytotoxicity Receptor
NK	Natural Killer Cells
NY-ESO	New York Esophageal Squamous Cell Carcinoma Antigen
OR	Overall Response
ORR	Objective Response Rate
PD1	Programmed Cell Death Protein 1
PDL-1	Programmed Cell Death Ligand Protein 1
PMEL	Premelanosome Protein
RCC	Renal Cell Carcinoma
TAA	Tumor-Associated Antigen
TCR	T cell Receptor
TGF	Transforming Growth Factor
Th2	T helper type 2 lymphocytes
TIL	Tumor-Infiltrating Lymphocytes
Tregs	Regulatory T cells

9.1 Introduction

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 (Eisenberg et al. 2019). An important milestone in the development of immunotherapy occurred in 1987 when tumor-infiltrating lymphocytes from patients with metastatic malignant melanoma were successfully cultured and expanded using

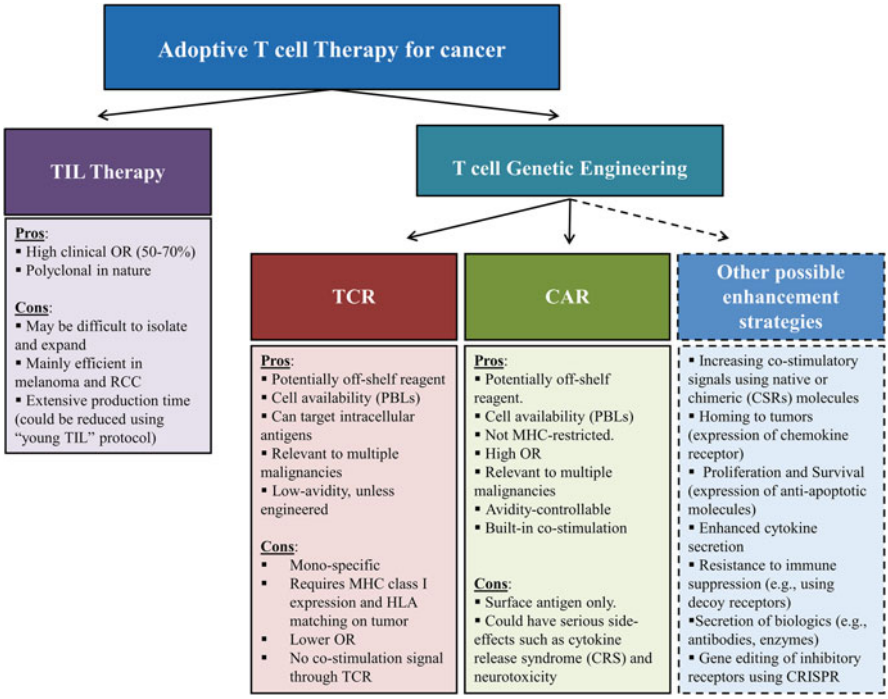

Fig. 9.1 A summary of different adoptive T-cell therapy approaches. *CAR* chimeric antigen receptor, *CRS* cytokine release syndrome, *CSRs* chimeric switch receptors, *OR* objective response, *PBLs* peripheral blood lymphocytes, *RCC* renal cell carcinoma, *TCR* T-cell receptor, *TILs* tumor-infiltrating lymphocytes

the T-cell growth factor interleukin 2 (IL-2) (Muul et al. 1987). The main idea behind immunotherapy is to exploit the potential of the host immune system and to utilize it to fight cancer cells and to enhance resistance to tumor-induced immunosuppression. It was shown that the immune system eliminates arising cancer cells constantly and is also reactive in the microenvironment of the tumor during tumor development (Gonzalez et al. 2018). While also part of the targeted therapy arsenal, monoclonal antibodies that target a specific antigen present on cancer cells are often considered a form of immunotherapy. They can be conjugated with therapeutic drugs that would produce a cytotoxic effect on cancer cells. A main development in antibody-based immunotherapy was the advent of immune checkpoint inhibitors—these monoclonal antibodies that can either costimulate T-cells or block the coinhibitory pathways to allow T-cells to be activated and clear tumor cells. Another immunotherapeutic strategy is related to cancer vaccines, by which a whole or fragments of cancer cells, antigens, or mRNA-encoding antigens are designed to stimulate an immune response. A rapidly evolving strategy is represented by autologous adoptive cell therapy (ACT) in which immune cells (usually T-cells or natural killer (NK) cells) are isolated from the patient's blood or tumor tissue, expanded and/or modified

ex vivo (Ott et al. 2017), and then reinfused back to the patient to fight cancer. T-cells used in ACT can be tumor-infiltrating lymphocytes (TILs) that express natural T-cell receptor (TCR), or genetically engineered T-cells that express an exogenous cancer-specific TCR or chimeric antigen receptor (CAR) (Mondino et al. 2017) (Fig. 9.2).

9.2 Tumor Antigens and Microenvironment: Opportunities and Hurdles

To design novel and promising immunotherapies, one must better understand the determinants and challenges present in the tumor and its environment.

9.2.1 Tumor Antigens: The Target of the Immune System

T-cells play a central role in the immune response against cancer. Their activation is initiated by the interaction of TCR with its associated major histocompatibility complex (MHC)/peptide complex presented on the surface of the target cell, which activates them specifically (He et al. 2019). Whether T-cells could recognize endogenous (tumor) tissues was a matter of debate for 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 (Coulie et al. 2014). Accordingly, tumor-specific T-cells have been shown to be activated through the binding of their TCR to specific epitopes derived from tumor antigens (TAs) presented by an MHC molecule. TAs can be termed tumor-specific antigens (TSAs) if present only on tumor cells, while if they are also expressed by normal tissues, they will be considered tumor-associated antigens (TAAs). They can be classified into several categories; this division pertains to the pattern of expression of these antigens (e.g., overexpressed, onco-fetal, etc.) and whether these antigens are "self" or mutated (Vigneron 2015). Several sources indicate different classifications, but five known classes of TA can be broadly described:

- *Mutated self-proteins/NeoAntigens*—Usually when mutations occur in the initial cancerous cell (or one of its early daughter cells), this class of tumor antigens can provide excellent targets for T-cell-based immunotherapy of cancer as they are to be expressed in most of the tumor tissues but not in healthy ones (Yamamoto et al. 2019).
- *Cancer/testis antigens (C/T)*—they are expressed not only in various human cancers, but also in normal testis tissues (e.g., NYESO1). Some evidences suggest that there might be a certain level of T-cell tolerance toward these antigens (Pearson et al. 2017; Simpson et al. 2005).

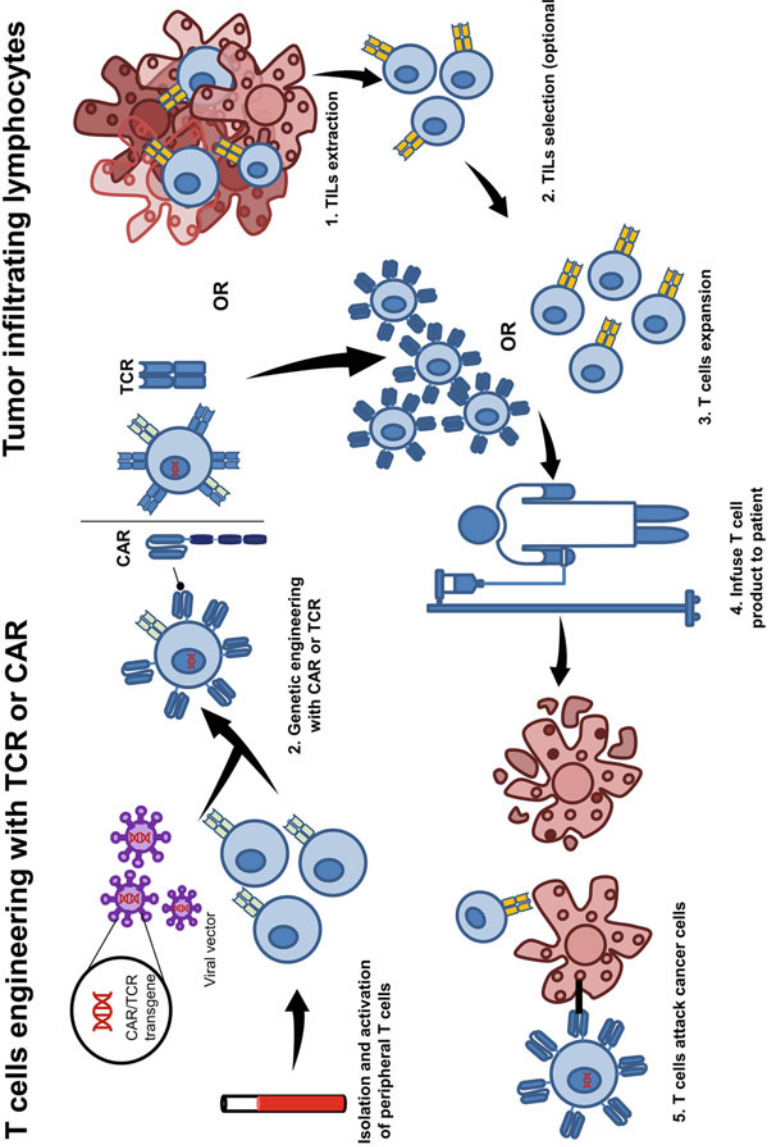

Fig. 9.2 Schematic representation of the different adoptive T-cell therapy processes. *CAR* chimeric antigen receptor, *TCR* T-cell receptor, *TILs* tumor-infiltrating lymphocytes

- *Tissue-specific differentiation antigens*—these antigens are only expressed 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 (Lai et al. 2018; Joyce III 1988). These antigens have emerged as potentially promising target antigens for T-cell-based adoptive immunotherapy, but their presence on normal tissues can be the source of autoimmune manifestations.
- *Overexpressed antigens*—this type of antigens also constitutes an important TA class, which is relevant in both T-cell therapy and antibody-based treatments. Overexpressed tumor-self antigens can also serve new vaccine targets (Bright et al. 2014). Based on clinical data, it seems that the combination of their overexpression in several tumor tissues (e.g., Her2) and their reduced levels in healthy cells may limit the potential for deleterious autoimmune side effects (Linnemann et al. 2011). Even when they are express at high levels in normal tissues, they can also be valuable targets if they are normally expressed in nonessential tissues such as in the case of CD19 or BCMA, which are antigens for hematological malignancies.
- *Viral antigens*—as it is believed that around 20% of all cancer cases are linked to infectious agents (Zur 2009; de Martel et al. 2020), antigens derived from oncogenic viruses would provide a source of "nonself" targets, which would be recognized more efficiently than TAA due to a potential lack of tolerance against the viral epitopes (De Re et al. 2020).

9.2.2 Tumor Microenvironment (TME): Halting the Immune Response

Solid tumors contain many other cell types including cells derived from the innate and adaptive immune system, stromal cells, and myeloid-derived suppressor cells (MDSCs) (Schouppe et al. 2012; Haas and Obenauf 2019). 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 tumor-supportive environment they promote, might represent an effective approach to enhance the destruction of cancer cells, leading to tumor elimination (Baghban et al. 2020). In parallel, several tumors exhibit a high content of TILs. These cells are often linked to a good prognosis in terminally ill cancer patients (Curiel et al. 2004; Fridman et al. 2012).

9.2.3 *Inhibitory Cytokines: Another Major Hurdle in the TME*

T-cell function can be modulated by cytokines, which are proteins or peptides secreted by various cell types, including cancer cells and T-cells themselves. Cytokine secretion provides a way for the immune system cells to communicate with one another and to generate a response toward a target antigen. Many cytokines are pleiotropic, meaning they can have different, sometimes opposite effects over different cell types, under different secretion levels, and in different microenvironment compositions. Thus, they are of great interest regarding immunotherapy (Lee and Margolin 2011).

The tumor microenvironment is rich in inhibitory cytokines such as IL-4, IL-13, IL-10, and transforming growth factor (TGF) β. For example, TGF-β is a multifunctional cytokine that plays a major role in hematopoiesis, cell growth, differentiation, apoptosis, tumor development, and immune regulation (Hayashi et al. 2004; Larson et al. 2020). It has three isoforms—TGF-β1 (most common), TGF-β2, and TGF-β3. In the beginning, TGF-β inhibits tumorigenesis, but later in the presence of various oncogenic events, it can switch and act as a tumor promoter (Huang and Blobe 2016; Lebrun 2012). For example, among its immunosuppressive effects, it was shown to inhibit IL-1-dependent lymphocyte proliferation (Wahl et al. 1988) and to polarize macrophages to become immunosuppressive (Wu et al. 2010).

Another example is IL-10, which generally functions as an immunosuppressive cytokine, polarizing T-cell responses toward the T helper type 2 lymphocytes (Th2) phenotype (de Waal et al. 1993). IL-10 can enhance the growth of malignant clones of multiple myeloma and other B-cell lymphoproliferative diseases (Beatty et al. 1997). It is produced both by immune cells and tumor cells including cells from non–small cell lung cancers, melanomas, gliomas, leukemias, and lymphomas (Smith et al. 1994; Sato et al. 1996; Huettner et al. 1994; Mori and Prager 1998; Voorzanger et al. 1996).

9.2.4 *Cancer Metabolism: The Object of a Renewed Interest*

The metabolic composition of the tumor microenvironment could severely affect the metabolic immune cells' phenotype. Cancer cells induce mutations to help them survive and to gain control over tumor territory. There are many hallmarks of cancer cells, like angiogenesis, evading growth suppressors, resisting apoptosis, invasion, and overproliferation. The metabolic reprogramming of cancer is transcriptionally regulated by oncogenes and mutated tumor suppressors (Frezza 2020), leading to the development of mechanisms to cope with the lack of oxygen and nutrients in the tumor microenvironment. Healthy cells, under aerobic conditions, use the glycolysis pathway and exploit the mitochondria to exert oxidative phosphorylation (OXPHOS). Nevertheless, under conditions where oxygen is limited, glycolysis

and lactate production is favored (Tran et al. 2016a). Several cancer-associated mutations enable cancer cells to acquire and metabolize nutrients in a manner conducive to proliferation. In the presence of oxygen, cancer cells could complete the entire process of respiration though they often opt to convert glucose into lactate. This phenomenon is called "the Warburg effect" (Vaupel et al. 2019; Tran et al. 2016a).

Cancer cells grow more rapidly than the blood vessels to nourish them. Thus, as solid tumors grow, they are unable to obtain oxygen efficiently (Lu et al. 2002). In other words, they slowly end up experiencing hypoxia. Under these conditions, glycolysis leading to lactic acid fermentation becomes the primary source of ATP. Glycolysis is made more efficient in hypoxic tumors by the action of a transcription factor, hypoxia-inducible transcription factor (HIF-1α). Using only glycolysis may provide some advantages, such as helping cancer cells survive and grow by producing ATP more rapidly. Diverse metabolism changes, which can occur in cancer cells, may also activate oncogenes that allow them to avoid death (Kroemer and Pouyssegur 2008). In these competitive conditions, immune cells are at a disadvantage, which hampers their survival and persistence in the tumor vicinity, leading to a reduced antitumor response (Brand et al. 2016; Lim et al. 2020). In addition to inhibitory ligands such as programmed cell death protein 1 (PD-L1) or immunosuppressive cytokines, T-cells will generally encounter hypoxia, which, when sustained, often leads to tumor escape and progression; all mammalian cells that divide rapidly require high glucose uptake to sustain their proliferation. As a result, tumor cells, stromal cells, and immune cells must undergo fierce competition against the limited glucose in the natural environment (Shyer et al. 2020; Aksoylar et al. 2020). However, tumor cells may outcompete others as they can drive, for example, higher expression of the glucose transporters under situations of hypoxia, maintaining a high metabolic rate and proliferation, leading also to diminished T-cell antitumor activity (Gupta et al. 2020).

9.2.5 Inhibitory Receptors and Immune Checkpoints Inhibitors

Immune checkpoints are negative regulators of T-cell immune function and could help cancer cells to evade the immune system response. Immune checkpoint inhibitors (ICIs) are monoclonal antibodies (mAbs) that could prevent the attachment between an inhibitory receptor and its ligand, thus blocking inhibitory pathways in T-cells that can lead to tumor regression (Alsaiari et al. 2021; Meir et al. 2017; Ribas and Wolchok 2018). The emergence of immune checkpoint inhibitors, essentially including antiprogrammed cell death protein 1/programmed cell death ligand 1 (PD-1/PD-L1) and anticytotoxic T lymphocyte–associated antigen-4 (CTLA-4), and their use has heralded a new era in immuno-oncology. In T-cells, CTLA-4 is originally expressed as an intracellular protein, which after T-cell receptor (TCR)

activation and a costimulatory signal through CD28, can translocate to the cell surface. There, it outcompetes CD28 for binding to crucial costimulatory molecules (CD80, CD86) in T-cells. CTLA-4 could activate inhibitory signaling into the T-cell, ending T-cell proliferation and activation. The PD-1 receptor is a dominant-negative regulator of effector T-cells activated by its ligand PD-L1, expressed on the surface of tumor cells. Inflammation-induced PD-L1 expression in the tumor microenvironment mediates T-cell exhaustion or anergy (Pardoll 2012; Ribas 2015), leading to reduced responses against cancer cells. While immune checkpoint inhibitors have hastened a novel in the treatment of cancer patients, most of the latter will not respond to this kind of treatment (Haslam and Prasad 2019).

9.3 T-cell lymphocyte–Based Immunotherapy

9.3.1 Tumor-Infiltrating-Lymphocytes (TILs)

9.3.1.1 TIL Treatment

T-cell lymphocytes are one of the immune components with a high potential to eradicate cancer cells. Tumor-infiltrating lymphocytes are T-cells isolated from the tumor, which have the potential to recognize the expressed tumor antigen and specifically attack it. They also could be implicated in tumor type, stage, and prognosis (Dudley et al. 2003; Maibach et al. 2020; Zikich et al. 2016; Besser et al. 2020). Yet, their number can be insufficient in the TME, and expanding them ex vivo might be necessary to exploit their potential against tumors. For example, the adoptive cell transfer (ACT) of TILs (Rosenberg 2014), isolated from the tumor itself, can lead to the regression of regress solid tumors in advanced melanoma patients, with nearly a quarter of the treated individuals achieving durable complete responses (Rosenberg et al. 2011). Recent findings show also the successful application of TIL therapy to other types of cancer including cholangiocarcinoma (Tran et al. 2014), cervical cancer (Stevanovic et al. 2015), colorectal cancer (Tran et al. 2016b), and, lately, breast cancer (Zacharakis et al. 2018). TIL therapy exemplifies the strong curative potential of T-cells, but their correct isolation and expansion are crucial for the treatment success (Eisenberg et al. 2019).

Despite its aforementioned success (especially in melanoma), ACT therapy with autologous TILs bears some limitations that include, for example, the requirement to isolate and expand T-cells with antitumor 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. 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 antitumor activity. This might be due to the fact that melanoma tumors express a high number of mutated antigens (Cohen et al. 2015;

Prickett et al. 2016) that could help break self-tolerance and were also shown to harbor class II-MHC molecules (Walia et al. 2012; Restifo et al. 2012).

Other tumors, such as papillomavirus (HPV)-associated carcinomas and breast tumors, are infiltrated by T-cells, but the specificities and functions of the latter are unclear (Stanton and Disis 2016; Stevanovic et al. 2019; Zacharakis et al. 2018; Vihervuori et al. 2019). In most breast cancer subtypes, TILs are in low, intermediate, or high quantity and could be a marker for tumor prognosis. For example, each 10% decrease in stromal TILs resulted in a 20% increased risk of mortality in triple-negative breast cancer. An average of 13.2-year survival difference was observed between the majority ($> 75\%$) of patients with low ($< 14\%$ of TILs) or high ($\geq 14\%$ of TILs) frequency of CD8+ T-cells patients (Vihervuori et al. 2019).

Renal cell carcinoma (RCC) is also considered an immunogenic tumor that exhibits rich intratumoral lymphocytic infiltration. Still, it seems that in this case, T-cell activation is insufficient at the tumor site due to many immunosuppressive mechanisms induced in the microenvironment of RCC (Heidegger et al. 2019; Vuong et al. 2019; Mier 2019). 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 melanoma patients and their treatment could provide clues to elaborate an improved therapeutic regimen for ACT in RCC and other malignancies (Goedegebuure et al. 1995; Markel et al. 2009; Andersen et al. 2018).

9.3.1.2 TIL Therapy Implications and Future Promises

In most cancer patients, those naturally occurring TIL fail to destroy the tumor as they are outnumbered, subjected to constant immunosuppression, and due to other factors that are not fully understood (Zidlik et al. 2020; van den Berg et al. 2020). Additionally, the generation of a TIL culture(s) that proves reactive for each patient tumor is not always feasible and requires several weeks. During the last few years, the ACT of the activated TILs has demonstrated encouraging results in treating metastatic melanoma patients, a malignancy with a poor prognosis. Several studies performed by independent groups demonstrated that more than 50% of the melanoma patients treated with autologous TILs achieved an objective clinical response (Rosenberg et al. 2011; Nguyen et al. 2019; Besser et al. 2010) with more than 20% of the patients treated obtaining a complete remission (Rosenberg et al. 2011). These new clinical studies are designed to improve the TIL antitumor activity, growth, and expansion by generating "young TIL" cultures (Besser et al. 2010). 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 spends minimal time in culture by eliminating the individualized tumor reactivity screening step. As no further selection process is required, all established "young TIL" cultures are technically eligible for treatment (Tran et al. 2008). As immunomodulatory monoclonal antibodies show promise in the clinic/

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 (Tang et al. 2019). 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 (Gattinoni et al. 2009)) or by subjecting TIL cultures to different cytokines, such as IL-7, IL-15, or IL-21 alone or in addition to IL-2 (Refaeli et al. 1998; Zorro et al. 2020; Waldmann et al. 2020; Tian et al. 2016; Rosenberg 2014). Additionally, the rapid selection of tumor-specific T-cells either from TIL cultures or from the patient blood (based on antitumor reactivity) may contribute to generating a more personalized cellular therapeutic product (Cohen et al. 2015; Gros et al. 2014, 2016). The latter approach is based on the correct identification of T-cell reactivities often helped with the use of algorithms that can scan rapidly genomic data indicating potential neoepitopes (Besser et al. 2019). Recently, tumor-infiltrating lymphocyte therapies had positive results in solid tumors in clinical studies in phase I or phase II (Yu et al. 2020; Zhang and Wang 2019).

9.3.2 Genetic Engineering of T-Cells

TIL therapy, while promising, is not always feasible, due to the reduced presence of specific antitumor T-cells (if they even exist) and the relatively long time required to grow and expand the T-cells from patients (who often have limited life expectancy). Therefore, to generate a large number of functional tumor-specific T-cells, two main approaches have been developed to "de novo" genetically engineer the T-cells specificity against cancer, using either TCRs or CARs (Fig. 9.2).

9.3.2.1 TCR Structure: A Critical Determinant of T-Cell Function

T-cells can recognize specific epitopes that are presented by the MHC complex, via the interaction of their TCR. Indeed, following a process known as positive and negative selection in the thymus, T-cells clonally express a defined TCR demonstrating distinct specificity. The TCR is generally composed of α/β chains (only 5% approximately of the T-cells may display a TCR built by γ/δ chains). Both chains are composed of 2 Immunoglobulin-like domains that can be further divided into a variable and a constant region (VR and CR). The variable parts can interact with the MHC/antigen complex via the binding of 6 protruding loops (3 on α and 3 on β chains) termed CDRs (complementarity-determining regions). TCR constant regions are responsible for promoting the pairing between the α and β chains. Additionally, they can facilitate the interaction with the signaling complex composed of CD3 chains. In this regard, the α and β chains cannot signal by themselves when binding to their cognate antigen as they have a short cytoplasmic region. Thus, the TCR α/β

dimer is coupled to three CD3 dimers (a homodimer of CD3ζ, and 2 heterodimers of CD3γ and CD3ε, and of CD3δ and CD3ε). The CD3 complex can provide activation signals via phosphorylation of its ITAMs (immunoreceptor tyrosine–based activation motifs) contained in the cytoplasmic domains. In naïve T-cells (i.e., cells that yet have to encounter their cognate antigen), a supplementary (costimulatory) signal is typically required to properly activate them, leading to their survival and proliferation. Such signal can be mediated by the binding of the CD28 receptor to its ligand CD80/CD86 on the target cell. Cytokines may provide a third signal, which is destined to promote differentiation into different T-cell subsets (Rosenberg 2014).

9.3.2.2 TCR-Gene Transfer

While T-cell specificity is singularly 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 pioneer study by Steinmetz and colleagues back in 1986 demonstrated, for the first time, the feasibility of the TCR-gene transfer approach. In that work, T-cell specificity was redirected by genetic engineering, but it was intended primarily to study the receptor dynamics (Dembic et al. 1986). Later on, several studies with a more translational purpose demonstrated how (human) T-cells can be engineered to recognize specific antigens using TCR gene transfer using a melanoma-specific TCR in vitro (Clay et al. 1999a, b), followed by an in vivo study using a mouse model (Kessels et al. 2001). Morgan et al. reported in 2006 the results of the first clinical trial based on TCR gene therapy to treat metastatic melanoma patients. They made use of autologous peripheral blood lymphocytes (PBLs) that were retrovirally transduced with a MART-1 specific TCR (Morgan et al. 2006). MART1 is known as a TAA broadly expressed by melanomas (Riker et al. 2000). In this first pioneering TCR-gene transfer clinical trial, a MelanA/MART1-HLA-A*0201 restricted specific TCR termed DMF4 was isolated from a TIL clone and expressed in T-cells isolated from metastatic melanoma patients. HLA-A*0201 was chosen as the targeted MC allele, since it is expressed by 30–50% of the Caucasian population, which hypothetically made this approach clinically relevant to more than a third of melanoma patients (Weizman and Cohen 2016). Whereas only 2 patients out of 15 experienced an objective response, this clinical trial demonstrated the feasibility and potential usefulness of TCR-gene transfer. To further explore the potential of this strategy, a second clinical trial was conducted using this time a high-affinity TCR specific for MART1 termed DMF5 (Johnson et al. 2006). In this clinical trial, around 30% of the patients experienced an objective response. Interestingly, patients also developed oculo-vestibular side effects and a severe skin rash five days post infusion. MART1 is expressed in the eyes, ears, skin, and other pigmented tissues, and this expression pattern could be linked to off-target effects (Johnson et al. 2009). In the same work, another melanoma-specific high-affinity TCR specific for $gp100_{154–162}$ was used though it originated from HLA-A*0201 transgenic mice and the use of this TCR led to a response rate of 19% (Johnson et al. 2009). The use of TCR for nonmelanoma

tumors was also investigated in the past few years to extend this approach to other malignancies. Several studies were based on TCRs directed against germline or overexpressed antigens. For example, using a p53-specific TCR isolated also from HLA-A*0201 transgenic mice, 10 patients underwent TCR-gene transfer (Cohen et al. 2005). A single cholangiocarcinoma patient experienced a partial regression, and no major toxicity was noted though a nonmutated ubiquitous epitope was targeted in this trial (Davis et al. 2010). Interestingly, no noticeable relationship was found between p53-specific T-cell reactivity and p53 levels measured in these tumors (Theoret et al. 2008). However, additional studies revealed that T-cell reactivity could be correlated with p53 protein stability (Shamalov et al. 2017).

The choice of the targeted antigen is central to the success of such treatment and the reduced off-target effects by gene-engineered T-cell therapy (Tran et al. 2017); indeed, TCRs targeting tumor-associated antigens may engender serious and even fatal consequences. The use of a CEA-specific TCR led to severe colitis (Parkhurst et al. 2011) and patients treated with MAGE-A3 specific-TCR engineered T-cells were reported to suffer from leukoencephalopathy, coma, and lethal cardiac toxicity (Linette et al. 2013). Thus, it is therefore important to elaborate approaches to assist in determining potential cross-reactivities displayed by new TCRs (Stone et al. 2015; Hickman et al. 2016). Nevertheless, TCRs targeting the cancer-testis antigen NY-ESO1, such as derivatives of the 1G4 TCR, can lead to impressive clinical results with minimal toxicity. Objective responses ranging between 50% and 90% (the best to date to our knowledge for TCR-gene transfer treatments) were obtained using such an approach. Two studies reported the successful treatment of synovial cell sarcoma, multiple myeloma, melanoma patients when targeting NY-ESO-1 antigen with a specific TCR (Robbins et al. 2015; Rapoport et al. 2015). Additionally, viral antigens (that may be expressed by cancer cells) can be targeted: in a current phase I/II clinical trial (NCT02858310), metastatic HPV+ carcinoma patients are treated using an HPV-E7-specific TCR and this approach led to tumor regression (Nagarsheth et al. 2021).

Thus, TCR gene transfer has been proven to be an effective strategy to generate specific tumor-reactive T-cells, and this, without the requirement or limitations of isolating naturally occurring tumor-reactive T-cells. Additional factors that should be considered are the prolonged expression of TCR genes, the phenotype of T-cells to be engineered, the persistence of the TCR-modified T-cells after infusion, and the necessity to reach sufficient T-cell functional avidity (Manfredi et al. 2020).

Currently, many clinical and preclinical studies aim to evaluate the effects of modifying different steps of the ACT procedure. Some strategies have been used to modify T-cells' effector function. For example, changing/adding adhesion molecule expression on T-cells trafficking to tumor sites (Hinrichs et al. 2011). To increase T-cell proliferation, some researchers also transduced the IL-2 cytokine gene into lymphocytes (Bandyopadhyay et al. 2002). It is also possible to clone the patient's TCR after their remission with ACT therapy and then inject the tumor-specific TCR gene into autologous T-cells from other patients (Johnson et al. 2009; Parkhurst et al. 2017). However, this approach is limited by the TCR recognition to the tumor antigen exclusively in the context of the source patient's MHC molecule. Another

approach can use TCRs isolated from HLA-humanized mice that have been stimulated with a defined tumor antigen (Restifo et al. 2012; Klebanoff et al. 2016). Mice TCRs directed against tumor antigens type p53, CEA, and PMEL were used in clinical trials and demonstrated clinical benefit. Nevertheless, they noticed toxicity in the patients: the target tumor antigens were not specific enough to the tumor (Johnson et al. 2009; Parkhurst et al. 2011). Furthermore, we and others showed that TCR chains can be mutated to improve expression levels of the transduced TCR and diminish mispairing in TCR alpha and beta chains (Cohen et al. 2006, 2007; Audehm et al. 2019; Helmy et al. 2013; Kuball et al. 2007; Bethune et al. 2016; Haga-Friedman et al. 2012; Bialer et al. 2010).

9.3.3 Limits of TCR Gene Transfer and Ways to Address Them

The TCR can recognize antigens of several types: intracellular, cell surface, or neoantigen, all presented as short epitopes by tumor cells class I MHC molecules. However, cancer cells can evade the immune system by mutations or other judicious mechanisms. Challenges in the development of TCR technology include the selection of specific targets, the choice for appropriate TCRs, the screen to find the optimal binding TCR affinity, the safety evaluation and possible off-target effect, and also protein engineering to generate more stable TCRs with greater affinity (Spear et al. 2019; Merhavi-Shoham et al. 2012.

Indeed, the native TCR affinity to these cancer antigens may be low, especially when the targeted epitope is derived from a tumor-associated antigen (due to negative selection of highly reactive TCR against self-antigenic determinant). To improve the native TCR, a high-affinity TCR can be designed using protein engineering tools (Bialer et al. 2010; Zhao et al. 2007; Robbins et al. 2008) and encoded in T-cells by gene therapy engineering (Lo et al. 2020), which could enhance both specificity and affinity during the recognition of tumor cells by TCR. The construction of high-affinity TCR needs specific target identification. In parallel, one needs to screen specific polypeptides expressed or overexpressed by cancer cells and healthy cells to determine in vitro potential off-target reactivity. It is also critical to perform preclinical safety assays to avoid or minimize secondary effects like off-target effects and cross-reactivity. TCR modifications could be engineered in the constant regions to promote better pairing or in the CDR variable regions mutations to enhance affinity and specificity (Bialer et al. 2010). One example was the engineering of the 1G4 TCR specific for the New York esophageal squamous cell carcinoma antigen (NY-ESO-1) overexpressed in melanoma, multiple myeloma (MM), and sarcomas (Robbins et al. 2008; Cohen et al. 2007). Using such improved TCR in clinical trials has shown encouraging results in cancer patients, reaching up to 45% clinical response (Robbins et al. 2015).

It is important to mention that MHC restriction may limit TCR-T-cell therapy as, unlike CARs, TCR will recognize their cognate antigen if displayed by the correct MHC allele. Other hurdles may hamper TCR-T-cell therapy—there is a risk of hybridization (mismatch) between exogenous and endogenous TCR chains, which may induce some potentially rare recognition of autoantigens, leading to graft versus host disease (Audehm et al. 2019). 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 nonfunctioning TCR or a receptor with a new specificity that can prove self-reactive. Indeed, it was demonstrated in a mouse model how the formation of mixed TCRs can result in self-reactive T-cells that engendered autoimmune manifestations (Bendle et al. 2010; Bunse et al. 2014).

Several strategies have been devised to increase the expression of the introduced TCRs and are often based on molecular approaches aiming for better pairing/association of the α/β chains of the introduced-exogenous TCR (Aggen et al. 2012; Debets et al. 2016). For example, hybrid human TCRs that are composed of parts of/entire murine constant regions (Cohen et al. 2006; Bialer et al. 2010; Sommermeyer and Uckert 2010; Goff et al. 2010) mediated an improved expression of the transferred TCR. The inclusion of an additional disulfide bond within the constant region of the TCR (Cohen et al. 2007; Kuball et al. 2007; Sadio et al. 2020), molecular "knob into holes," inversions in the constant regions of the TCR chains (Voss et al. 2008), single-chain TCRs (Nakajima et al. 2019), and the use of TCR/CD3ζ fusion products (Sebestyen et al. 2008) were also demonstrated as potential pairing-optimization strategies (Howie et al. 2015; Spear et al. 2018; Carter et al. 2019). Since α/β and γ/δ TCR chains cannot mutually pair (Morath and Schamel 2020; Saito et al. 1988), the use of γδ T-cells that are transduced with an αβ TCR is also an alternative approach (Fichtner et al. 2020; van der Veken et al. 2009). To prevent TCR mispairing and generate self-reactive TCRs, swapping constant domains between the α and β chains of a therapeutic TCR has demonstrated a safer TCR gene therapy in mouse models (Bethune et al. 2016). Silencing the endogenous TCRs is another strategy that can be achieved by cotransferring siRNAs/shRNAs targeting the endogenous TCR (Ernst et al. 2020; Okamoto et al. 2009) or by making use of zinc-finger nucleases (ZFNs) that are specific for the endogenous TCR chains (Ernst et al. 2020; Provasi et al. 2012). Recently, several experiments used the CRISPR/Cas method as knockout of the endogenous TCR to increase the antigen sensitivity with an engineered TCR (Ernst et al. 2020; Singh et al. 2017), (also seen with CAR-CD19 T-cells (Stenger et al. 2020) in adoptive cell therapy (Legut et al. 2018)).

Costimulatory signals are also essential for T-cell activation and could be provided to engineered cells to enhance their function, cytokine secretion, and survival—this could be achieved by transducing TCR T-cells to also express 4-1BB (Daniel-Meshulam et al. 2013). Another approach that can exploit inhibitory receptors is to use intracellular domains of activating molecules with the extracellular domain of inhibitory one as we and others developed. For example, PD1/CD28 engineered T-cells combined with checkpoint blockade secreted significantly more

IFN-γ compared to control T-cells without PD-1/CD28 (Ankri et al. 2013; Andrews et al. 2019; Qin et al. 2019; Schlenker et al. 2017) and display improved antitumor activity *in vivo*. Similarly, studies on coinhibitory receptors like the TIGIT receptor used as a costimulatory switch receptor have shown encouraging results on T-cell antitumor function (Hoogi et al. 2019). Thus, TCR-T-cell therapy has shown some encouraging results and may fulfill in the next decade its full therapeutic potential.

9.4 CAR-T-Cells

9.4.1 CAR-T-Cell Therapy: Structure and Development

In parallel to the TCR-gene transfer approach, it is possible to redirect the specificity of T-cells using CARs. These are molecules that combine elements delivering the first and second signals. CAR-transduced T-cells recognize a specific antigen usually through an antibody-derived fusion protein such as a single-chain variable fragment (scFv) composed of the variable regions of the heavy (V_H) and light (V_L) chains of an antibody. Native tumor-specific receptors can be used as we and others demonstrated when using NK cells receptors such as Natural Cytotoxicity Receptors (NCRs) (Tal et al. 2014; Zhang et al. 2012; Eisenberg et al. 2017), NKG2D (Weiss et al. 2018) or SIGLEC receptors (Meril et al. 2020). In first-generation CARs, the scFv is directly fused to a hinge region (connecting the scFv to the transmembrane domain), a transmembrane domain that anchors the CAR on the cell surface, and a signaling intracellular domain to stimulate T-cell activation upon antigen binding (CD3ζ or FcRγ are commonly used). In second-generation CARs, there is an addition of a costimulatory signaling domain positioned before the CD3ζ or FcRγ domain, added to provide a second activating signal required to enhance the physiological T-cell response. Third-generation CARs include multiple costimulatory or signaling domains in tandem (Fig. 9.3).

9.4.2 Driving the CARs to the Clinic

CAR-T-cell therapy has become an important addition to the treatment portfolio of refractory or relapsed B-cell malignancies. In 2017, two CD19-targeted CAR-T-cell therapies, known by their brand names “Kymriah” and “Yescarta,” were the first CAR-T-cell therapies to be approved by the FDA after successful clinical trials in relapsed and/or refractory B-cell malignancies. The ZUMA-1 trial (NCT02348216) evaluated the anti-CD19 CAR-T-cell product “Yescarta” on patients with various types of B-cell lymphoma. In a relatively recent update, an objective response rate (ORR) of 83% and complete remission (CR) rate of 58% were reported (Locke et al. 2019; Maude et al. 2018; Roex et al. 2020). A second trial that led to FDA approval was the JULIET study (NCT02445248) in which the anti-CD19 CAR-T-cell product

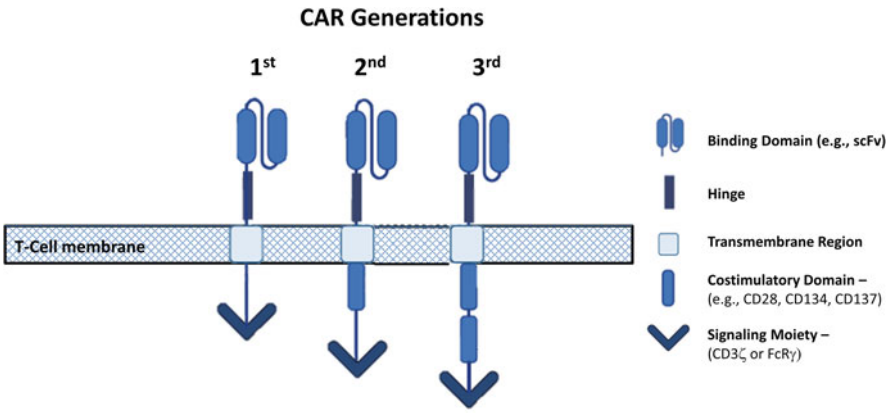

Fig. 9.3 Schematic representation of the different CAR generations

Kymriah was evaluated on adult patients with relapsed or refractory Diffuse Large B Cell Lymphoma (DLBCL). The reported overall response (OR(and CR rates were 52% and 40%, respectively (Schuster et al. 2019). Since their approval, data from patients who are not part of clinical trials has been gathered. In one postmarketing real-world study, regarding treatment with Yescarta, Nastoupil et al. reported considerable OR (81%) and CR (57%) rates after a median follow-up of 3.9 months (Nastoupil et al. 2020).

Building on the success of CAR-T-cell therapy in $CD19^{+}$ leukemia, it is being expanded to additional neoplastic diseases, both hematologic and nonhematologic. For example, extensive research is conducted to investigate CAR-T-cell therapies for multiple myelomas, one of which (idecabtagene vicleucel) was just approved by FDA as a new CAR T-cell therapy. The most widely used target antigen in CAR-T-cell studies for multiple myeloma is B-cell maturation antigen (BCMA) (D'Agostino and Raje 2020; Danhof et al. 2018; Cho et al. 2018a; Cohen 2018). In several reported clinical trials, (NCT02546167 (Cohen et al. 2019), NCT02215967 (Ali et al. 2016; Brudno et al. 2018) NCT02658929 (Raje et al. 2019), NCT03090659 (Xu et al. 2019), NCT03090659 (Zhao et al. 2018), ChiCTR-17,011,272 (Ma et al. 2019), the ORR range was 85–95% in most studies; only two of the mentioned studies, NCT02546167 and NCT02215967, reported lower ORRs of 48% and 58%, respectively. The median progression-free survival (PFS) observed with BCMA CAR-T-cell therapy was in the range of 1 year. Despite the relatively high ORR obtained with BCMA CAR-T-cell therapy, relapses were frequently observed. Dose escalation seems to be crucial to determine the optimal cell dose for the success of the therapy (Munshi et al. 2021). Nonetheless, a major problem is downregulation or loss of BCMA expression. Thus, one strategy to avoid BCMA-negative relapses combines administration of BCMA CAR-T-cells with inhibitors of the gamma-secretase enzyme, preventing it from cleavage BCMA from the MM cell surface (Pont et al. 2019).

Additional strategies are being developed to increase the potency of CAR-T therapy—for example, the dual CARs approach is being evaluated to improve response durability as seen by combining BCMA and CD19-specific CAR-T-cells. At a median follow-up of 179 days, 95% of the patients had an overall response, including 43% stringent complete responses and 14% complete responses (Yan et al. 2019). More studies are looking for other antigens to be targeted by CAR-T-cells such as CD38, SLAMF7, CD44v6, CD56, and GPRC5D, among others (Timmers et al. 2019).

Another B-cell malignancy and one of the first to be under investigation with CD19 CAR-T-cell therapy is Chronic Lymphocytic Leukemia (B-CLL). The total number of CLL patients reported being treated with CD19-targeted CAR-T-cell therapy is 134; most of them were in relapse after several lines of treatment. The different CAR-T-cell reports in CLL imply a lower efficacy for CLL than for DLBCL or Acute Lymphocytic Leukemia (B-ALL): CR, according to the IWCLL (International Workshop on Chronic Lymphocytic Leukemia) criteria, was obtained in only 20–30% of patients with an estimated 18-month PFS of 25%. This can be partially as a result of the exhausted immune system in CLL, which can decrease CAR-T-cell activation after transduction. Based on data that suggest improvement of CLL patients when receiving a combination of ibrutinib and CAR-T-cells, a prospective study is evaluating the efficacy of ibrutinib maintenance at the time of injection of the CAR-T-cells (NCT03331198) (Porter et al. 2011, 2015; Lemal and Tournilhac 2019; Turtle et al. 2017; Fraietta et al. 2018; Riches et al. 2013). As a direct result of its potential success, patenting activity for CAR-T-cell therapy has exponentially increased since 2013 (Jurgens and Clarke 2019), with the United States and China leading the way (together with more than two-thirds of the world share of CAR-T-cell applicants), followed by the UK, Germany, Japan, and France (Oluwole et al. 2020).

9.4.3 CAR Limits

To date, the success of CAR-T-cell therapy in hematological cancers has not been replicated in solid tumors, although a considerable effort is being invested. While some hurdles remain, extensive research has helped to identify several key factors contributing to the success of CAR-T-cell therapy:

Identifying the target antigen—ideally, the targeted antigen by the CAR-T-cell should be expressed exclusively on tumor cells to avoid an "on-target/off-tumor" effect. Second, it needs to present a sufficient expression level on target cells for CAR-T-cell detection and, third, to be distributed uniformly on tumor cells. Currently, CARs demonstrating the greatest success are directed against antigens expressed on B-cell lineage such as CD19, CD20, BCMA, etc.: those antigens are present also on normal B-cells. Nevertheless, this is an exceptional situation in which the outcomes of the "on-target/off-tumor" effect are manageable—B-cell aplasia followed by hypogammaglobulinemia can be managed by immunoglobulin

replacement therapy and after the CAR-T-cell population diminishes, the immune system can reconstruct the B-cell lineage (Jain et al. 2020). An occurrence such as that is yet to be found in solid tumors and cases of severe "on-target/off-tumor" toxicities have been reported. For example, a patient suffering from metastatic colon cancer was treated with CAR-T-cells directed against human epidermal growth factor receptor 2 (HER2) and died five days after the injection due to low expression of HER2 on epithelial cells of the lungs (Morgan et al. 2010).

Another caveat is the heterogeneity of antigen expression, as tumors are often composed of subsets of clones, presenting different antigens. Also, "antigen escape," which is the reduction of antigen expression on cancer cells, can affect treatment success. This potentially can be overpowered by using dual CAR logical gates, CARs, or Tandem CARs. Clinical studies of CAR-T-cell therapy for solid tumors can be directed against antigens as disialoganglioside GD2, HER2, epidermal growth factor receptor variant III (EGFRvIII), prostate-specific membrane antigen (PSMA), carcinoembryonic antigen (CEA), interleukin (IL)13R2, neural cell adhesion molecule L1 (NCAM-L1, CD171), receptor tyrosine kinase like orphan receptor 1 (ROR1), and B7H3. It can also be directed against glycolsylated antigens as we and other demonstrated (Tal et al. 2014; Eisenberg et al. 2017; Meril et al. 2020). So far, only minor success has been achieved when treating solid tumors with CAR, especially when compared to hematological malignancies. Besides common mechanisms engendering immunosuppression in T-cells, an important factor that needs to be considered is the binding affinity of the CAR, which can have an impact on both efficacy and safety. The strength of signaling was demonstrated to be a major determinant for CAR-T-cell therapy success (Feucht et al. 2019) as seen by an *in vivo* study regarding CAR-T-cells directed against ICAM-1, which is a TAA associated with many solid tumors, but also expressed on many normal tissues as an adhesion marker. The CAR was safer and more effective when treated with micromolar affinity CARs than with those with higher, nanomolar affinity, strengthening the need to evaluate the extent of a therapeutic window for each given CAR (Park et al. 2017; Min et al. 2017). Recently, the FDA granted fast-track status to an ICAM-1 specific CAR-T-cell product for the treatment of thyroid cancer.

Locating and reaching the tumor—To fight the cancerous cells, CAR-T-cells need to infiltrate the tumor site. When dealing with hematological cancers, CAR-T-cells interact with the malignant cells directly in the bloodstream. However, in solid tumors, several factors are making it hard for the CARs to reach their destination including chemokine–receptor mismatch that might hinder the ability of lymphocytes to follow a chemotactic gradient. For example, solid tumors can produce and secrete chemokines such as C-C chemokine ligand 2 (CCL2), while its compatible receptors, chemokine (C-C motif) receptor (CCR) 2b, and CCR4, are rarely expressed on the membrane of adoptive T-cells (Moon et al. 2011). Of note, showed it was possible to redirect T-cell migration by engineering those to express chemokine receptors (Sapoznik et al. 2012). Also, tumors can produce and secrete chemokines that attract immunosuppressive cells such as regulatory T-cells (T_{regs}). Other factors include physical barriers such as deformed and immature blood vessels called high endothelial venules, which are hypothesized to be necessary for T-cell

infiltration into the tumor (Ager 2017). One way to facilitate T-cell infiltration, for example, is to engineer them to secrete matrix-modifying enzymes such as heparinase (Caruana et al. 2015). Additionally, the access to certain tumor sites may be biologically restricted such as in the case of brain tumors though recent data demonstrated the practicability of natural killer T-cells engineered to coexpress GD2-CAR and interleukin-15 (IL-15) to expand *in vivo* and localized to metastatic sites in pediatric patients with stage IV relapsed/refractory neuroblastoma (Heczey et al. 2020).

Surviving in the tumor microenvironment—Even with suitable antigen and localization to the tumor site, the lymphocytes need to face obstacles in the tumor microenvironment such as the upregulation of checkpoint inhibitor proteins expression on tumor cells, presence of immune suppressor cells such as T_{regs}, MDSCs, and tumor-associated macrophages (Davoodzadeh et al. 2017; Son et al. 2017). As abovementioned, the immunosuppressive nature of the TME represents a major hurdle to CAR-T-cell success.

Toxicity—CAR-T-cell treatment has been associated with severe cytokine release syndrome (CRS or cytokine storm) and immune effector cell–associated neurotoxicity syndrome (ICANS; often referred to as neurotoxicity) in one-third of the treated patients (Morris et al. 2021). It has been linked to tumor burden and an uncontrolled immune response and release of inflammatory cytokines such as IL-1 and IL-6. As such, potential therapeutic interventions include the injection blocking antibodies for these cytokines, their receptors, or the use of small molecules limiting immune activation such as dasatinib.

9.4.4 CAR-T-Cells Variations

CAR and TCR engineered T-cells are considered a "living drug"—on the one hand, they have the potential for dynamic, rapid and extensive activation, and proliferation, which contributes to their therapeutic efficacy, and on the other hand, this may cause particular side effects and toxicities. Currently, clinically approved CAR designs do not enable control over CAR-T-cells following infusion, and the use of immunosuppressive drugs severely limits the time in which the CAR-T-cells are functional (Davila et al. 2014). Therefore, researchers are working to develop regulatory mechanisms that will enable the tracing (Meir et al. 2015) and control over CAR-T-cells *in vivo*. mRNA-electroporation to drive transient CAR expression (unlike genome integrating platforms) may be used to limit potentially harmful long-term effects linked to CAR expression (Barnard 1992). Still, to palliate the lack of sustained expression when using mRNA electroporation, repetitive infusions are needed to maintain the desired CAR-expressing T-cells numbers though a careful balance is needed as repeated infusions may bear a greater risk for anaphylactic reaction due to the CAR-T-cells (Maus et al. 2013).

Another way of regulation is tuning the affinity of the CAR to its antigen, aiming to preclude on-target/off-tumor toxicities from arising. Lowering the CAR affinity

may still provide the affinity-tuned CARs the ability to bind cancer cells that have a high antigen expression, while the healthy tissues with lower expression may be left unharmed (Caruso et al. 2015; Liu et al. 2015). A drawback for this method might be the escape of low-antigen-expressing cancerous cells as nonuniform antigen distribution on cancerous cells is common (Anurathapan et al. 2014). Also, transduction often leads to heterogeneous expression of the CAR protein, making it hard to ensure consistent behavior among individual CAR-T-cells as their avidity toward the antigen can vary. To overcome this problem, Cunanan *et al.* offered to integrate the CAR construct into the endogenous TCR alpha chain (TRAC) locus using CRISPR (Eyquem et al. 2017).

Active interference and inducible control approaches are also under investigation. For example, CAR-T-cells were designed to express a suicide gene when a specific molecule is administered. Antibody-dependent cytotoxicity can be achieved when administering Rituximab (antiCD20) aimed toward CAR-T-cells also expressing a codon-optimized CD20. The CARs were depleted from peripheral blood and secondary lymphoid organs of transplanted animals after Rituximab treatment. Their data suggests their optimized CD20 construct as a suicide gene for adoptive immunotherapy (Vogler et al. 2010). In another example, a safety switch approach for CD19 CAR-T-cells, based on human enzyme caspase 9 (Cas9) designed to be suited for conditional dimerization, was developed (Di et al. 2011). When exposed to a synthetic dimerizing drug, the inducible Cas9 becomes activated and leads to the death of the CARs designed to express this construct, leading to the rapid depletion of CAR-T-cells.

The process of CAR manufacturing can be costly and time consuming. Part of the reason is the need for individual cell manufacturing. Hence, researchers are trying to develop systems of "universal" CARs using models of adapter-mediated CARs—linking a molecule recognized by the CAR (an adapter) to a moiety that recognizes the tumor antigen. This suggests new possibilities such as titrated-administration of the adapter, thus ensuring better control of toxicities that may arise and changing the target antigen without reinfusion and manufacturing the T-cells (Urbanska et al. 2012; Rodgers et al. 2016; Cho et al. 2018b; Ma et al. 2016).

CARs can also be designed to be independently regulated by "Boolean logic gates." For example, a CAR-T-cell with an "AND gate" will be activated only when meeting concomitantly two antigens. This allows for better discrimination between tumor cells and healthy cells (Kloss et al. 2013; Srivastava et al. 2019). The "OR" logic gate enables CAR-T-cells to antitumor in the presence of either targeted antigen. It can be achieved by two independent CAR molecules or a pool mixed with different specific CAR-T-cells. This strategy can improve efficacy in case of an antigen loss from the cancer cell surface (Hegde et al. 2013; Ruella et al. 2016). As mentioned previously, when using CARs targeting TAA (especially in the case of solid tumors) off-target effect could be fatal. Thus, CARs designed with a "NOT gate" (also known as iCAR—inhibitory CAR) are receptors with an extracellular binding domain directed against antigen known to be expressed on healthy tissues, while the intracellular signaling moiety is derived from inhibiting molecules such as CTLA-4 or PD-1. By recognition of an antigen expressed on healthy tissue, the

inhibitory signal may countermand the signals from the CAR that recognize the target TAA expressed on both malignant and healthy cells (Fedorov et al. 2013), improving the safety of the approach.

9.5 Additional Strategies for T-Cell Engineering

9.5.1 Editing Platforms in Gene Engineering

As discussed above, viral vectors are expression platforms widely used for T-cell engineering to introduce and control the expression of the transgene. These are usually based on γ-retroviral vectors such as the MSCV (mouse stem cell virus) or MPSV (myeloproliferative sarcoma virus) (Uckert and Schumacher 2009; Nowicki et al. 2020) or lentiviral vectors (Gutierrez-Guerrero et al. 2020; Circosta et al. 2009; Moco et al. 2020; Jones et al. 2009; Yang et al. 2008). Due to their capacity to transduce nondividing cells, their resistance to gene silencing (Frecha et al. 2010), and their safer integration site profile (Levine et al. 2006; Bulcha et al. 2021), lentiviral vectors are generally considered more suitable than γ- retroviral vectors (Jones et al. 2009). Still, both viral vector types are efficient and currently used in the FDA-approved therapeutic products Axicabtagene ciloleucel (retrovirus) and Tisagenlecleucel (lentivirus). Nonviral methods for T-cell engineering such as the *Sleeping beauty* transposon system were developed to avoid the need for expensive large-scale production and safety testing (Clauss et al. 2018; Deniger et al. 2016; Peng et al. 2009). In this method, introducing both the transposase (originally identified in the genome of the extinct salmonid) and the transposon (DNA) in the target cell is necessary to be integrated into the host cell genome. While this approach originally achieved only low transgene expression levels (Peng et al. 2009), it has been constantly improved in the past years and this led to its evaluation in a CD19-CAR trial in which complete remissions were achieved (Kebriaei et al. 2016) without severe toxicities (Magnani et al. 2020).

As mentioned, manufacturing engineered T-cells *ex vivo* could be costly and time consuming. With the recent interest in nanoparticles carrying a genetic load (such as certain SARS-Cov2 vaccines), studies have shown encouraging results using nanoparticles to carry and deliver in vitro transcribed (IVT) CAR or TCR mRNA for transiently reprogramming of circulating T-cells, to recognize disease-relevant antigens in mouse models of human tumors (Parayath et al. 2020).

9.5.2 Beyond TCR and CAR Engineering

As antigen specificity is not the only factor determining the success of T-cell based therapy, T-cell engineering is studied beyond the scope of TCR and CAR modifications; a considerable effort is devoted to finding strategies to enhance the potency

of T-cells and to overcome the hurdles that T-cells face when challenged by cancer cells in the tumor microenvironment. Among those strategies are the use of dominant-negative receptors in which a mutated form of a receptor is abrogating the negative signaling cascade in cells that express this receptor (Foster et al. 2008; Bollard et al. 2018; Zhang et al. 2013; Bendle et al. 2013; Kloss et al. 2018; Fahlen et al. 2005; Qin et al. 2008; Quatromoni et al. 2012), the expression of chemokine receptors to facilitate the homing of engineered T-cells to the tumor sites based on the chemokines that are secreted by cancer cells (Moon et al. 2011; Sapoznik et al. 2012; Di et al. 2010; Kershaw et al. 2002; Craddock et al. 2010; Garetto et al. 2016; Siddiqui and Erreni 2016). T-cells can also be engineered to secrete constitutively stimulatory cytokines such as IL-2, IL-15, and IL-12 to help these cells survive *in vivo*, even in a hostile tumor environment (Hoyos et al. 2010; Treisman et al. 1995; Zhang et al. 2011; Hsu et al. 2005; Koneru et al. 2015a, b). As aforementioned, chimeric costimulatory or cytokine receptors (CSRs or CCRs) can be designed to reverse the immunosuppressive effects of inhibitory cytokines, since they possess an extracellular domain that binds to an immunosuppressive factor (e.g., ligand or cytokine), while the intracellular signaling domain is derived from an immune-activating molecule (CD28, 4-1BB, etc.) as we and others published for chimeric PD1/28 or TIGIT/28 (Ankri et al. 2013; Hoogi et al. 2019; Ankri and Cohen 2014; Leen et al. 2014; Wilkie et al. 2010; Mohammed et al. 2017; Lo et al. 2008; Vera et al. 2009; Markley and Sadelain 2010). Moreover, T-cells can be used to deliver specific cargo at the tumor sites such as checkpoint inhibitors (Li et al. 2017; Rafiq et al. 2018) that will increase *in situ* immune potency.

9.6 Conclusions

In the past 30 years, the adoptive transfer of T-cells has been establishing itself as a promising immunotherapeutic strategy for the treatment of advanced cancer. The basic idea that the patient immune system can be manipulated 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 must be defined/characterized to provide safer treatments targeting a broad spectrum of tumors. Improving the success rate of adoptive T-cell transfer will also require its combination with multimodal therapies targeting for instance the tumor microenvironment as well as immunosuppressive agents. Several studies also suggest that these concepts can be applied to treat other severe diseases than cancer. As a testimony of the promising potential of these cell-based therapeutic approaches, the number of clinical studies involving adoptive T-cell immunotherapy dramatically increased with, for example, close to seven hundred CAR-T-cells clinical trials (Picanco-Castro et al. 2020). Thus, adoptive T-cell immunotherapy is certainly consolidating its respected place in the "Hall of Fame" of personalized treatments.

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

Grazia Graziani, Lucia Lisi, Lucio Tentori, and Pierluigi Navarra

Contents

Abstract The immune checkpoint 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 antitumor 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 anti-CTLA-4 IgG1κ monoclonal antibody (mAb) that represents the first immune checkpoint inhibitor approved as monotherapy by FDA and EMA in 2011 for the treatment of unresectable/metastatic melanoma. In 2015, FDA also granted approval to ipilimumab monotherapy as adjuvant treatment of stage III melanoma to reduce the risk of tumour recurrence. The subsequent approved indications of ipilimumab for metastatic melanoma, regardless of BRAF mutational status, and

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other advanced/metastatic solid tumours always involve its use in association with the anti-programmed cell death protein 1 (PD-1) mAb nivolumab. Currently, ipilimumab is evaluated in ongoing clinical trials for refractory/advanced solid tumours mainly in combination with additional immunostimulating agents.

Keywords Immune checkpoint inhibitors · Ipilimumab · CTLA-4 · PD-1 · PD-L1 · Melanoma · Immunotherapy · NSCLC · BRAF · Monoclonal antibodies

Abbreviations

APC	antigen-presenting cells
CTLA-4	cytotoxic T-lymphocyte antigen-4
dMMR	mismatch repair deficiency
EMA	European Medicines Agency
FDA	Food and Drug Administration
FoxP3	transcription factor forkhead box protein P3
GM-CSF	granulocyte-macrophage colony-stimulating factor
HRQL	health-related quality of life
ICOS	inducible co-stimulator
IDO	indoleamine 2,3 deoxygenase
IL	interleukin
irAEs	immune-related adverse effects
irRC	immune-related criteria
LAT	linker for activation of T cells
mAb	monoclonal antibody
MHC	major histocompatibility complex
MSI-H	microsatellite instability high
MSI-L	microsatellite instability low
MSS	microsatellite stability
mWHO	modified World Health Organization
NIBIT	Italian Network of Tumour Biotherapy
NSCLC	non–small-cell lung cancer
OS	overall survival
PD-1	programmed cell death protein 1
PFS	progression-free survival
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PP2A	serine-threonine protein phosphatase 2A
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.

10.1 CTLA-4

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 or CD152) is an immune checkpoint that acts as negative regulator of T-cell-mediated immune responses, playing a critical role in autoimmunity suppression and immune homeostasis maintenance. It is induced after activation on $CD4^{+}Foxp3^{-}$ and $CD8^{+}Foxp3^{-}$ conventional (or effector) T cells but is constitutively expressed by $CD4^{+}Foxp3^{+}$ regulatory T cells (Tregs). However, CTLA-4 expression is not restricted to T cells, since it has been reported in B cells, dendritic cells, monocytes, granulocytes, $CD34^{+}$ stem cells, mouse embryonic cells, placental fibroblasts, and pituitary gland (Oyewole-Said et al. 2020). The CTLA-4 gene consists of four exons: exon 1 encodes the signal peptide sequence; exon 2, an IgV-like domain comprising the B7-binding domain; exon 3, the transmembrane region; and exon 4, the cytoplasmic tail. The CTLA-4 transcript can undergo alternative splicing, and four splice variants of CTLA-4 have been described that may account for the different CTLA-4 functions (Valk et al. 2008; Walker and Sansom 2015). Classically, 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 (i.e. mainly related to functions on Tregs).

CTLA-4 acts as a negative regulator of CD28-dependent T cell responses (Fig. 10.1). Differently from CD28, which is a surface receptor, CTLA-4 is mostly present in intracellular vesicles. It is constitutively present as a homodimer and does not appear to undergo conformational changes following ligand binding (Brown et al. 2019). 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 (APCs), 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 (Teft et al. 2006; Chattopadhyay et al. 2009). Like CD28 and the other co-stimulatory receptor inducible co-stimulator (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 (Fife and Bluestone 2008). Despite their structural and sequence similarities, CD28 and CTLA-4 differ in

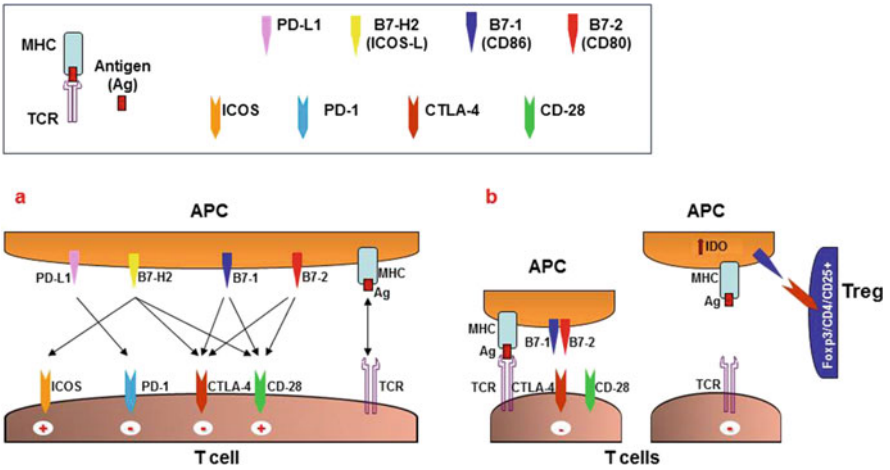

Fig. 10.1 CTLA-4 is a negative modulator of T cell activation. *a. Co-stimulatory and co-inhibitory molecules.* T cell activation is triggered when TCR binds to an antigen bound to the MHC on the surface of APC and it is completed by a second co-stimulatory signal, represented by the interaction between 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 co-stimulatory receptor and its ligand, B7-H2 (ICOS-L), has recently been proposed to bind also CD28 and CTLA-4. (The sign + represents a positive/activating signal; the sign - indicates a negative/inhibitory signal). *b. Inhibition of T cell activation*. Following T cell activation, CTLA-4 is upregulated 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 upregulation 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

their localization in T cells, being the former expressed at the cell surface both in resting and activated cells. CTLA-4 is, instead, upregulated on the surface of activated T cells in response to TCR/CD28 co-stimulation (Fife and Bluestone 2008). 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 (Qureshi et al. 2012). In particular, the YVKM motif mediates rapid endocytosis by interacting with the clathrin adaptor, activating protein 2 (AP-2). Endocytosis likely requires other motifs: the proline motif contributes to AP-2 binding and the C-terminal YFIPIN motif behaves as an alternative low endocytic adaptor (Teft et al. 2006). On the other hand, interaction with AP-1 has been associated with CTLA-4 degradation. The cytoplasmic domain of CTLA-4 controls its recruitment to lipid rafts and mediates interactions with the scaffold proteins T cell receptor–interacting molecule (TRIM) and linker for activation of X cells (LAX)

which affects CTLA-4 surface expression (Valk et al. 2008; Schneider and Rudd 2014). Moreover, the membrane proximal lysine motif seems to have a role in bringing CTLA-4 into complex with protein kinase C (PKC)-η. This association mediates recruitment of p21-activated kinase (PAK) in complex with GIT2 and PIX proteins that might promote cellular motility through focal adhesion disassembly, destabilizing the APC-T cell contacts (Kong et al. 2014). In addition, several signalling molecules, such as phospholipase D (PLD), ADP-ribosylation factor 1 (ARF-1), and T cell immune response cDNA 7 (TIRC7), have been described to be involved in the transport of CTLA-4 to the cell surface (Valk et al. 2008).

Once expressed on plasma membrane of activated T cells, CTLA-4 outcompetes with CD28 for the binding to B7 complex inhibiting T cell activation, because of decreased proliferation and impairment of CD28-mediated interleukin 2 (IL-2) secretion (Fife and Bluestone 2008). 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) (Rudd et al. 2009). 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 (Rudd et al. 2009). 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 by 14–3-3 proteins, preventing its interaction with the anti-apoptotic Bcl-xL and Bcl-2 proteins, and causes upregulation of Bcl-xL expression. In this way, Bcl-xL and Bcl-2 are free to mediate mitochondrial-dependent cell survival (Schneider et al. 2008b). In addition, CTLA-4 was reported to directly modify AKT activity (Schneider et al., 2008b). However, through the PI3K/AKT pathway, CTLA-4 favours T cell survival under conditions 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 (Schneider et al. 2006). 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. It has been suggested that the effects of CTLA-4 blockade by specific antibodies on T cell motility are not due to signalling but rather to the physical disruption of stable interactions between T cells and their targets (Brunner-Weinzierl and Rudd 2018).

Moreover, CTLA-4 inhibits the expression of lipid rafts, a clustering of glycosphingolipid enriched microdomain that is considered as an essential component of the immunologic synapse (Chikuma et al. 2003). Lipid rafts form on T cell surface a "platform" for signalling proteins crucial for proper TCR-mediated signalling. After TCR engagement, molecules, such as Lck, Fyn, PKCθ, 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 like Lck, Fyn, LAT, and TCR chain (Chikuma et al. 2003). Finally, CTLA-4 also blocks the formation of microclusters containing TCR and molecules needed for an effective transmission of signals from TCR (Schneider et al. 2008a).

A well-characterized extrinsic mechanism by which CTLA-4 may act as negative regulator of T cell responses is through the action of Tregs (Fig. 10.1), where CTLA-4 is constitutively expressed (Takahashi et al. 2000). 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 (Sakaguchi 2011). 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 upregulated 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 (Tai et al. 2012). Recently, it has been found that Treg suppression and anergy require the external domain of CTLA-4, which binds to co-stimulatory ligands on APCs, whereas TCR hyposignalling only requires CTLA-4 internal domain (Tai et al. 2012). Suppression of the activation of naïve T cells associated with Treg externalization of CTLA-4 can be mediated by its interaction with CD80/CD86 that triggers a reverse signalling in APC, causing upregulation of the indoleamine 2,3-dioxygenase (IDO), an enzyme involved in the catabolism of tryptophan and tumour immune evasion through kynurenine production. In fact, 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 (Grohmann et al. 2002; Mellor and Munn 2004; Fallarino et al. 2002; Grohmann et al. 2003). 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 (Fallarino et al. 2006).

CTLA-4 proteins have been shown to induce co-stimulatory blockade either by sequestering or removing co-stimulatory ligands from the APC surface. In fact, Tregs expressing CTLA-4 on the surface can induce the downregulation of CD80 and CD86 on APC, limiting the activation of naïve T cells via CD28 (Oderup et al. 2006). 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 co-stimulatory ligands are degraded inside CTLA-4-positive cells, depriving T cells of CD28-mediated co-stimulation (Qureshi et al. 2011).

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). In regard to immunosuppressive compounds that amplify 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 (Linsley and Nadler 2009; Su et al. 2012a). 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 initially approved in 2005 by Food and Drug Administration (FDA) and in 2007 by European Medicines Agency (EMA) for moderate-to-severe active rheumatoid arthritis in adults and, subsequently, for

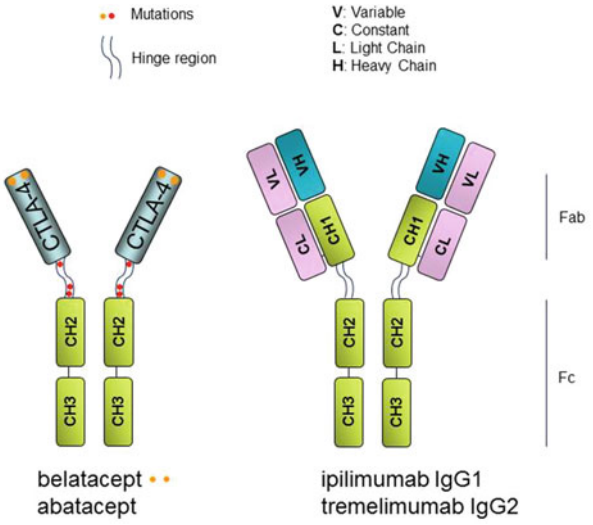

Fig. 10.2 CTLA-4 as a target for immunosuppressive or immunostimulating agents. Abatacept was generated by fusing the extracellular domain of human CTLA-4 to the Fc portion of human IgG1. The Fc portion is mutated at three sites (red dots), to eliminate effector functions of the Fc part. Belatacept was 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

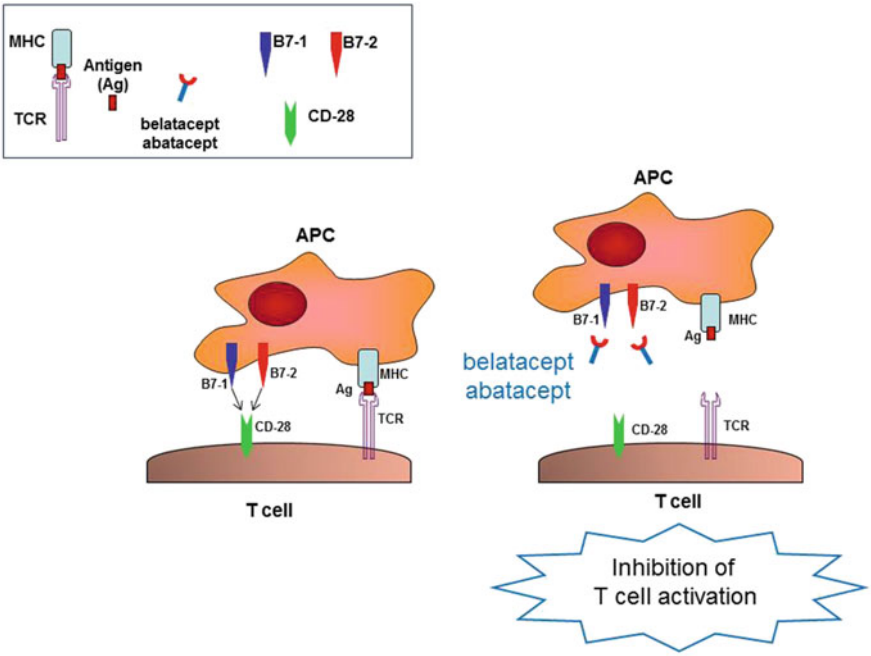

Fig. 10.3 Enhancement of CTLA-4 function. Belatacept or abatacept interferes with CD28/B7 pathway by binding to B7 molecules. Via their CTLA-4 portion, these recombinant proteins prevent the interaction of B7 with CD28 on T cells, thus inhibiting full T cell activation

moderate-to-severe active polyarticular juvenile idiopathic arthritis in paediatric patients and active psoriatic arthritis in adults (Linsley and Nadler 2009). According to the last updated paediatric investigation plan (PIP), subcutaneous abatacept is recommended for the treatment of chronic idiopathic arthritis (including rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, and juvenile idiopathic arthritis) (Brunner et al. 2020).

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 acute rejection of kidney transplantations (Su et al. 2012a) (Fig. 10.2).

On the contrary, 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 to 4 weeks of age (Waterhouse et al. 1995; Tivol et al. 1995). In vivo preclinical studies in the murine model indicated that

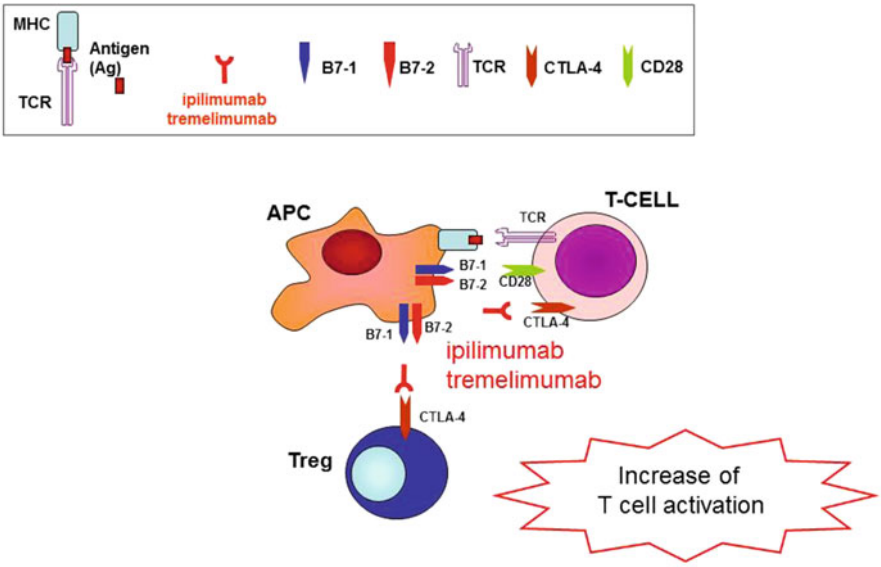

Fig. 10.4 Inhibition of CTLA-4 function. The mAbs ipilimumab and tremelimumab block CTLA-4 inhibitory signals, prolonging T cell activation and amplifying T cell–mediated immunity against tumours

administration of antibodies to CTLA-4 resulted in the rejection of tumours of different tissue origin, such as colon, prostatic and renal carcinomas, fibrosarcoma, lymphoma (Leach et al. 1996; Kwon et al. 1997; Yang et al. 1997; Korman et al. 2006; van Elsas et al. 2001). Moreover, recent in vivo preclinical studies have focused the attention on the role of CTLA-4 and programmed cell death protein 1 (PD-1) blockade. The dual blockade resulted in activation of both $CD8^+$ and $CD4^+$ T cells as well as of $CD103^+$ dendritic cells, supporting the induction of therapeutic synergistic effects (Beavis et al. 2018; Wei et al. 2019; Keilson et al. 2021).

Two monoclonal antibodies (mAbs) that block the inhibitory signal of CTLA-4 (tremelimumab and ipilimumab) have been developed for clinical use (Fig. 10.2). The antitumor 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 antitumor 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) (Peggs et al. 2009).

Tremelimumab (CP 675206; CP-675; CP-675,206; CP-675206; ticilimumab) is a fully human non-complement-fixing IgG2 mAb developed by Pfizer by using transgenic mice. Thereafter, AstraZeneca's MedImmune subsidiary has assumed the global development rights to this mAb. Tremelimumab is currently under clinical investigation for the treatment of a variety of tumours, both in the adult and paediatric populations, as monotherapy or in combination with other drugs among

which there is the anti-programmed cell death ligand 1 (PD-L1) mAb durvalumab (http://www.clinicaltrials.gov). In a previous phase 3 study, tremelimumab monotherapy, as first-line treatment in patients with advanced melanoma, failed to demonstrate an improvement in overall survival (OS) with respect to temozolomide or dacarbazine (Ribas et al. 2013). Among studies where tremelimumab is administered alone, some new indications are explored. For example a phase 2 trial has been designed to estimate tremelimumab activity in subjects with metastatic urothelial cancer with disease progression despite prior treatment with PD-1/PD-L1 blockade (NCT03557918). Regarding combination studies, a phase 2 study, in which 37 patients with metastatic melanoma received tremelimumab in combination with high doses of interferon α-2b, indicated that this treatment had an acceptable toxicity profile and promising antitumor efficacy that warranted further testing in randomized trials (Tarhini et al. 2012). Moreover, the combination of tremelimumab with durvalumab, evaluated in a phase 2 trial (NCT02870920) enrolling 180 patients with advanced/refractory colorectal cancer, was reported to prolong OS compared to the best supportive care (Chen et al. 2020a). However, in a phase 3 study recruiting 209 patients with extensive-stage small-cell lung cancer, the addition of tremelimumab to durvalumab plus platinum-etoposide in the frontline setting did not significantly improve OS versus platinum-etoposide, whereas durvalumab plus platinum-etoposide induced a significant survival benefit (Goldman et al. 2021). Negative results were also reported by a randomized, open-label phase 3 study (NCT02369874) in 280 patients with recurrent or metastatic head and neck squamous cell carcinoma treated with durvalumab plus tremelimumab or standard of care [the anti-epidermal growth factor receptor (EGFR) mAb cetuximab, a taxane, methotrexate, or a fluoropyrimidine]. In fact, neither durvalumab nor durvalumab plus tremelimumab significantly increased OS compared to the standard of care (Ferris et al. 2020). Recently, a phase 1 trial in a metastatic melanoma population was designed (http://www.clinicaltrials.gov) (NCT04223648) with the primary objective of assessing the ability of durvalumab plus tremelimumab to affect the number of PD-1$^+$ T cells in the tumour microenvironment. This mAb association has been also evaluated as neoadjuvant treatment for cisplatin-ineligible patients with high-risk localized urothelial carcinoma (NCT02812420). The results of this study indicated a 37.5% pathological complete response rate and down-staging to pT1 or less in 58% of patients who underwent complete tumour resection (Gao et al. 2020). A paediatric trial has also been designed to evaluate the safety and tolerability of durvalumab, as monotherapy or in combination with tremelimumab, at increasing doses in patients with advanced solid tumours and haematological malignancies (including lymphomas) and for whom no standard of care treatments are available (NCT03837899).

The following sections will focus on the pharmacological properties of ipilimumab and on the main results of clinical trials performed 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 IgG1κ mAb 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 (Keler et al. 2003). This product was used for phase 1 studies; for phase 2 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 initially approved as monotherapy 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. Subsequently, in 2015 and 2016, ipilimumab was approved by FDA and EMA, respectively, in combination with the anti-PD-1 mAb nivolumab for the treatment of advanced (unresectable or metastatic), *BRAF*-wild-type melanoma in adults. In 2016, the approval was expanded to include melanoma patients, regardless of *BRAF* mutational status. Approval of ipilimumab for melanoma treatment was then extended, in 2017 by FDA and in 2018 by EMA, to adolescents 12 years of age and older. For stage III melanoma, ipilimumab was FDA-approved (in 2015) also as adjuvant treatment after surgery of cutaneous melanoma with pathologic involvement of regional lymph nodes of more than 1 mm, to reduce the risk of tumour recurrence. The subsequent approved indications of ipilimumab for other tumour types always involved its use in association with nivolumab. In particular, the mAb combination was approved in 2018 by both FDA and EMA as first-line treatment of adult patients with intermediate- and poor-risk advanced renal cell carcinoma. In the same year, FDA granted accelerated approval to low-dose ipilimumab in combination with nivolumab for previously treated metastatic colorectal cancer characterized by microsatellite instability high (MSI-H) or mismatch repair deficiency (dMMR). In 2020, ipilimumab in combination with nivolumab received FDA approval for hepatocellular carcinoma in patients previously treated with the kinase inhibitor sorafenib. Always, in 2020, both FDA and EMA approved the mAb combination plus two cycles of platinum-based chemotherapy as first-line treatment of metastatic non-small-cell lung cancer (NSCLC) in adults whose tumours have no sensitizing EGFR mutations or ALK

translocation. For the same tumour type, FDA also approved the use of the mAb combination, without chemotherapy, in the frontline setting for PD-L1 positive (≥1%) NSCLC. Finally, ipilimumab plus nivolumab has been authorized by FDA for the first-line treatment of adult patients with unresectable malignant pleural mesothelioma (US Food and Drug Administration 2021; European Medicines Agency 2021).

When used in the treatment of unresectable/metastatic melanoma, both as monotherapy or in combination, the recommended dose of ipilimumab is 3 mg/kg administered as 90-minute intravenous infusion every 3 weeks for a total of four doses. Conversely, as adjuvant therapy, ipilimumab is given at 10 mg/kg, the first four doses administered every 3 weeks and the subsequent ones every 12 weeks for up to 3 years or until disease recurrence or unacceptable toxicity. In all the other indications, ipilimumab is administered as 30-minute intravenous infusion in combination with nivolumab. For renal cell carcinoma and MSI-H/dMMR colorectal cancer, the recommended dose is 1 mg/kg ipilimumab plus 3 mg/kg nivolumab administered on the same day every 3 weeks for the first four doses, followed by nivolumab 240 mg as single agent every 2 weeks or 480 mg every 4 weeks, until disease progression or unacceptable toxicity. In the case of hepatocellular carcinoma, the mAb schedule is the same with the exception that ipilimumab dose is higher than that of nivolumab (3 mg/kg and 1 mg/kg, respectively). For metastatic NSCLC, the recommended dose is 1 mg/kg ipilimumab every 6 weeks in combination with 360 mg nivolumab every 3 weeks, with 2 cycles of platinum-based chemotherapy administered every 3 weeks. The same mAb dosage (ipilimumab 1 mg/kg every 6 weeks with nivolumab 360 mg every 3 weeks) is used for unresectable malignant pleural mesothelioma. For PD-L1 positive NSCLC, ipilimumab is dosed at 1 mg/kg every 6 weeks and nivolumab at 3 mg/kg every 2 weeks. Treatment with both mAbs is recommended until disease progression, unacceptable toxicity, or up to 2 years in patients with no evidence of disease progression.

The pharmacokinetic profile of intravenous ipilimumab was studied in four monotherapy phase 2 trials on a total of 499 patients with advanced melanoma treated with up to four doses of 0.3, 3, or 10 mg/kg every 3 weeks (Feng et al. 2014). For the 0.3–10 mg/kg dose range, ipilimumab pharmacokinetics was linear and time-invariant. The values of peak concentration (Cmax), trough concentration (Cmin), 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 Cmax with the 3 mg/kg approved regimen ranges between 72 ± 33 μg/ml and 84.5 μg/ml, according to different studies (Wolchok et al. 2010a; Weber et al. 2008; Product information n.d.; Phan et al. 2003; Feng et al. 2014; Sanghavi et al. 2020). 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 Cmin concentration is 20 μg/ml. Prior to the second dose of 3 mg/kg the mean Cmin is 12 ± 7 μg/ml, and the concentration at steady-state is 21.8 ± 11.2 μg/ml (Product information n.d.; Phan et al. 2003). The terminal half-life of ipilimumab is 14.7 days (Weber et al. 2008; Product information n.d.). The

mean (percent 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%) (Product information n. d.). Ipilimumab clearance increases with increasing body weight, baseline serum lactate dehydrogenase (LDH), and albumin levels (Feng et al. 2014; Sanghavi et al. 2020); however, no dose adjustment is required for elevated lactate dehydrogenase or body weight. Moreover, the clearance decreases over time with a higher change when ipilimumab is administered in combination with nivolumab as compared to when it is used as monotherapy (Sanghavi et al. 2020).

10.3.1 Clinical Efficacy Studies with Ipilimumab

10.3.1.1 Malignant Melanoma

Melanoma is the most aggressive form of skin cancer. According to data of the American Cancer Society referring to people diagnosed with cutaneous melanoma between 2010 and 2016, the 5-year survival rates are 99%, 66%, and 27% for localized (stage I and II), regional (stage III) and distant metastatic melanoma, respectively. The median OS of patients with metastatic melanoma is low, and depends on metastasis localization. The worst prognosis is reserved to patients with brain or liver and gastrointestinal tract metastases. Before the approval of immune checkpoint and BRAF/MEK inhibitors, the median OS was ~5 months and the mean survival was ~9 months from diagnosis. The OS is strongly influenced by the number and location of metastases. The 9-month OS for patients with brain, digestive, lung, or extra-regional lymph nodes plus subcutaneous metastases was 10%, 17.5%, 65%, and 73%, with 2-year OS of 0%, 0%, 22%, and 27%, respectively (Sandru et al. 2014).

In the last decade, the therapeutic options for treating advanced melanoma are progressing rapidly. The first chemotherapeutic agent approved by FDA in 1975 for the treatment of metastatic melanoma was the DNA-methylating compound dacarbazine. The response rates with intravenous administration of dacarbazine were 15–25%, with median response durations of 5–6 months, but complete responses were 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 (Bafaloukos and Gogas 2004). The oral dacarbazine analogue temozolomide and the chloroethylating agent fotemustine have also been compared with dacarbazine, but none of these agents proved to be more efficacious (Middleton et al. 2000; Avril et al. 2004). Temozolomide has been approved by FDA and EMA only for the treatment of newly diagnosed glioblastoma and recurrent anaplastic astrocytoma. However, it was 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% (Tatar et al. 2013). Unfortunately, in a phase 3 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 (Chiarion-Sileni et al. 2011). 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 (http://www.clinicaltrials.gov; Tentori and Graziani 2009). In some European countries, fotemustine has been used for the treatment of brain metastases in melanoma patients; the reported overall response rate was 5.9% versus 0% with dacarbazine (Avril et al. 2004). However, the bone marrow toxicity induced by fotemustine is more severe than that caused by temozolomide.

In 1998, high doses of IL-2 were also approved by FDA in the United States, but not by EMA in Europe, for the treatment of the metastatic disease, based on the results of phase 2 studies showing its ability to induce durable responses in 5–7% of patients (Atkins et al. 1999). The IL-2 antitumor 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.

A meta-analysis of 42 phase 2 trials that completed accrual between 1975 and 2005 reported 1-year survival rates of about 25% for patients treated with a variety of chemotherapeutic protocols (Korn et al. 2008). Moreover, no accepted standard of care for second-line therapy was available and enrolment in a clinical trial was recommended. Before the approval of the first immune checkpoint inhibitor ipilimumab (in March 2011 by FDA and July 2011 by EMA) and the BRAF inhibitor vemurafenib (in August 2011 by FDA and February 2012 by EMA), no other agents had demonstrated better results than dacarbazine in phase 3 studies.

Vemurafenib (Zelboraf, Hoffman-La Roche) is a small-molecule kinase inhibitor that selectively targets mutated BRAF V600 and lacks activity against melanoma with wild-type BRAF (v-Raf murine sarcoma viral oncogene homolog B1), a threonine/serine protein kinase involved in the mitogen activation protein kinase (MAPK)-ERK pathway. Vemurafenib was approved for unresectable or metastatic melanoma with the *BRAF* V600E mutation as detected by an FDA-approved test. Mutations of BRAF [represented by valine substitution at amino acid 600 with glutamic acid (BRAF V600E, up to 90% of cases), lysine (V600K, 5–12%), and aspartic acid or arginine (V600D or V600R, $\leq$5%)] are present in about 50% of melanoma patients and cause an over-activation of the downstream MAPK/ERK pathway, involved in cell proliferation and survival (Davies et al. 2002; Bradish and Cheng 2014; Cheng et al. 2018). Differently from ipilimumab that is given intravenously for a total of four doses, treatment with vemurafenib requires continuous oral daily doses. In a phase 3 trial including untreated patients with metastatic melanoma carrying the *BRAF* V600E mutation, the OS 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 (Chapman et al. 2011). In previously treated

patients with *BRAF* V600E-mutant metastatic melanoma, vemurafenib induced clinical responses in more than half of patients with a median OS of 16 months (Sosman et al. 2012). The most commonly reported adverse effects of vemurafenib include arthralgia, rash, photosensitivity, fatigue, pruritus, alopecia, diarrhoea, nausea, and cutaneous squamous-cell carcinoma (Chapman et al. 2011; Sosman et al. 2012). Evidence on the clinical efficacy deriving from targeting BRAF V600E derives also from the results of a phase 3 trial with the other BRAF inhibitor dabrafenib (GSK-2118436, initially developed by GlaxoSmithKline) (Hauschild et al. 2012). Indeed in 2013, FDA and EMA approved dabrafenib (Tafinlar, Novartis) as monotherapy for the treatment of adult patients with unresectable or metastatic melanoma with a *BRAF* V600 mutation. Subsequent clinical studies have demonstrated that the antitumor efficacy of BRAF inhibitors could be enhanced by their use in combination with inhibitors of MEK, a downstream target of BRAF. Thus, in 2014, dabrafenib was approved for the same indication in combination with the MEK inhibitor trametinib (Mekinist, Novartis). The efficacy of this kinase inhibitor association was tested in a phase 3 trial where patients were randomly assigned to receive dabrafenib plus trametinib ($n = 211$) or dabrafenib monotherapy ($n = 212$). The 3-year progression-free survival (PFS) was 22% with the drug combination versus 12% with dabrafenib only, and the 3-year OS was 44% versus 32%, respectively. The 3-year OS with the drug combination reached 62% in the most favourable subgroup (normal lactate dehydrogenase and < 3 organ sites with metastases), whereas it was only 25% in the unfavourable subgroup (high lactate dehydrogenase levels) (Long et al. 2017). In addition, a pooled analysis of data from two clinical trials (COMBI-d NCT01584648 and COMBI-v NCT01597908) reported that first-line treatment with dabrafenib plus trametinib led to long-term benefit in approximately one-third of metastatic *BRAF*-mutated melanoma patients (4-year OS 37% and 5-year OS 34%) (Robert et al. 2019).

Similarly, in 2015, vemurafenib was approved in combination with the MEK inhibitor cobimetinib (Cotellic, Genentech) and an additional BRAF inhibitor, encorafenib (Braftovi, Array BioPharma), was approved in combination with the MEK inhibitor binimetinib (Mektovi, Array Biopharma) (Ascierto et al. 2016; Dummer et al. 2018). In parallel, a different profile of adverse events was reported for the kinase inhibitor combination therapy, with less skin-related events, but more gastrointestinal events (Yu et al. 2019).

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 and secondary activating NRAS mutations are frequently described (Fedorenko et al. 2011; Tentori et al. 2013). Interestingly, the cutaneous squamous-cell carcinomas and keratoacanthomas that develop in 15–30% of patients treated with vemurafenib or dabrafenib, frequently, show RAS mutations (Su et al. 2012b).

Clinical Studies with Ipilimumab as Monotherapy

The anti-CTLA-4 mAb, ipilimumab, represented the first treatment for metastatic melanoma that provided a long-term benefit, at least in a certain proportion of patients. The approval of ipilimumab by FDA was based on its ability to increase the OS with respect to vaccine with the gp100 peptide in a phase 3 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 (Hodi et al. 2010). This phase 3 study was the first randomized clinical trial showing increased OS in patients with metastatic melanoma (about 70% of patients had visceral metastases) and the first reporting efficacy as second-line treatment of melanoma. Patients were randomly assigned, 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 9 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. The median OS 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 OS of the combined treatment was identical to that of ipilimumab alone (Hodi et al. 2010). On the other hand, gp100 was afterwards found to increase the efficacy of IL-2 in patients with locally advanced stage III or IV melanoma (Schwartzentruber et al. 2011). 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 OS in the ipilimumab plus gp100, ipilimumab alone, and gp100 alone groups, respectively, were 43.6%, 45.6%, and 25.3% at 1 year, and 21.6%, 23.5%, and 13.7% at 2 years (Hodi et al. 2010). 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 (Wolchok et al. 2010b). Despite the NCT00094653/CA184–002 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 3 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 OS. The results, published three months later, indicated that four doses ipilimumab every 3 weeks in combination with dacarbazine (850 mg/m^2), significantly improved OS compared to dacarbazine plus placebo (11.2 months versus 9.1 months) in the front-line metastatic setting (Robert et al. 2011). 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 5 years (Maio et al. 2015).

Other studies with ipilimumab as monotherapy were the NCT01515189/CA184–169, CA184332, and CA184338 trials. The NCT01515189/CA184–169 was a phase 3, double-blind study enrolling patients with previously treated or untreated unresectable stage III or IV melanoma. A total of 727 patients were randomized to receive ipilimumab 3 mg/kg ($n = 362$) or ipilimumab 10 mg/kg ($n = 365$) every 3 weeks for four doses. In the 10 mg/kg group, the median OS was 16 months, whereas in the 3 mg/kg group, the median OS was 12 months. The median OS values in the subgroup with asymptomatic brain metastases at baseline were 7 months and 5.67 months at the doses of 10 mg/kg and 3 mg/kg, respectively (Ascierto et al. 2017). The CA184–332 ($n = 157$) and CA184–338 ($n = 273$) trials were instead two retrospective observational studies in chemotherapy-naive patients treated with ipilimumab 3 mg/kg that reported the following estimated survival rates: 1-year 44% and 59%, 2-year 26% and 39%, 3-year 22% and 31%, 4-year 22% and 26%, respectively (Schadendorf et al. 2015; https://ec.europa.eu/health/documents/community-register/2019/20190402144619/anx_144619_en.pdf).

Apart from the phase 3 registration trial used by FDA for ipilimumab approval (NCT00094653/CA184–002) where 10–15% of patients in each arm presented CNS involvement at baseline (Hodi et al. 2010), 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 OS is only 4 months (Davies et al. 2011). Previous case reports showed clinical benefits of ipilimumab for brain metastases from melanoma (Hodi et al. 2008; Schartz et al. 2010). Moreover, a phase 2 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 (Margolin et al. 2012). Moreover, the Italian Network of Tumour Biotherapy (NIBIT) 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 2 study (NIBIT-M1) for patients with metastatic melanoma, with or without brain metastases (Margolin et al. 2010; Di Giacomo et al. 2015a, b). 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 3 NIBIT-M2 trial (NCT02460068) (Di Giacomo et al. 2015a, b).

Conventional treatment options for melanoma brain metastases consist of surgical resection, whole brain radiation, and stereotactic radiotherapy. However, with major understanding of melanoma biology and development of more effective systemic treatments, with immune checkpoint inhibitors and BRAF inhibitors, as well as local therapies like stereotactic radiosurgery, the 1-year OS rate of patients with melanoma brain metastases has reached about 85% (Rishi and Yu 2020). When ipilimumab was

combined with radiotherapy, the abscopal effect was observed, a phenomenon related to activation of the immune system in which local radiotherapy is associated with the regression of metastatic lesions distant 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 (Postow et al. 2012; Stamell et al. 2013; Grimaldi et al. 2014; Chicas-Sett et al. 2017). Indeed, several phase 1/2 clinical trials are evaluating the combination of ipilimumab with radiation therapy and other drugs for the treatment of unresectable stage III or stage IV melanoma (http://www.clinicaltrials.gov). For further details, see the next section.

Ipilimumab monotherapy has also shown efficacy when administered as adjuvant treatment of high-risk stage III cutaneous melanoma after complete resection of regional lymph nodes (with metastasis >1 mm). The clinical trial [NCT00636168, European Organisation for Research and Treatment of Cancer (EORTC) 18,071] that led to ipilimumab FDA approval for this indication recruited 951 patients (treated with 10 mg/kg for every 3 weeks for four doses and then every 3 months for up to 3 years) and reported a significant increase of recurrence-free survival compared to placebo (26·1 months versus 17·1 months) (Eggermont et al. 2015). These patients also showed a significant long-term benefit, as indicated by the increase in the 5-year and 7-year OS (65.4% and 60% in the ipilimumab group versus 54.4% and 51.3% in the placebo group) and distant metastasis-free survival (48.3% and 44.5% versus 38.9% and 36.9%) (Eggermont et al. 2019, 2016). The immune-related adverse effects associated to this ipilimumab regimen were common, and some of them of particular concern leading to treatment discontinuation. Despite increased toxicity especially during the induction phase of ipilimumab administration, no clinically relevant deterioration in global Health Related Quality of Life (HRQOL) due to ipilimumab administration was observed. However, clinically relevant differences between ipilimumab and placebo arms were reported at week 10 since treatment start for specific symptoms (diarrhoea and insomnia) (Coens et al. 2017).

Clinical Studies with Ipilimumab in Combination with Nivolumab

Currently ipilimumab association with nivolumab is regarded as the most effective immunotherapy in patients with unresectable stage III and IV melanoma. The rationale for combining these two antibodies is based on their action at different phases of the T cell–mediated immune responses: CTLA-4 is operative during T cell priming, whereas PD-1/PD-L1 pathway acts during the effector phase mostly in the peripheral tissues (i.e. in the tumour environment) (Buchbinder and Desai 2016).

Approval of ipilimumab/nivolumab was based on the PFS results deriving from a phase 3 randomized, double-blind, double-dummy study (NCT01844505/CA209–067/CheckMate-067) assessing the safety and efficacy of the antibody combination for advanced (unresectable or metastatic) melanoma (Larkin et al. 2015). In particular, a total of 945 untreated patients were randomized to receive

ipilimumab 3 mg/kg plus nivolumab 1 mg/kg every 3 weeks for four doses ($n = 314$), nivolumab 3 mg/kg monotherapy every 2 weeks ($n = 316$), or ipilimumab 3 mg/kg monotherapy every 3 weeks for 4 doses ($n = 315$). Patients in the combination arm, after the first four doses of the two antibodies, received nivolumab 3 mg/kg as monotherapy every 2 weeks. Randomization was stratified by PD-L1 expression ($\geq 5\%$ versus $< 5\%$ tumour cell membrane expression), BRAF status, and M stage. The median PFS was 11.5 months in the ipilimumab plus nivolumab group, as compared with 2.9 and 6.9 months in the ipilimumab and nivolumab monotherapy groups, respectively. Significantly higher clinical benefit was observed in both nivolumab-containing groups compared to ipilimumab monotherapy, independently of PD-L1 expression, BRAF mutational status, or metastasis stage. In patients with PD-L1-positive tumours, no differences were observed between the median PFS of the nivolumab-plus-ipilimumab and nivolumab groups (14.0 months), whereas in patients with PD-L1-negative tumours, the PFS was longer in the combination therapy arm than in the nivolumab monotherapy arm (11.2 months versus 5.3 months). Subsequent analyses of this clinical trial have demonstrated a sustained long-term OS benefit deriving from the combination of ipilimumab with nivolumab (Wolchok et al. 2017; Hodi et al. 2018a; Larkin et al. 2019). In particular, both nivolumab-containing arms demonstrated a significantly improved PFS and OS benefit compared with ipilimumab alone. After 60 months of follow-up, the median OS was >60.0 months (median not reached) in the nivolumab-plus-ipilimumab group, 36.9 months in the nivolumab group, and 19.9 months in the ipilimumab group. Moreover, the 5-year OS was 52% in the nivolumab-plus-ipilimumab group, 44% in the nivolumab group, and 26% in the ipilimumab group (Larkin et al. 2019). The results of a multicentre randomized phase 2 study also indicated that the combination of nivolumab and ipilimumab was effective in untreated patients with asymptomatic brain metastases (Long et al. 2018). Improved long-term clinical benefit was also observed among patients with *BRAF*-mutated melanoma (Larkin et al. 2019). These results led to the expanded approval of ipilimumab plus nivolumab for unresectable/metastatic melanoma regardless of *BRAF* mutational status. Moreover, a matching-adjusted indirect comparison for analysing the efficacy of treatments from different trials demonstrated more durable clinical benefit, measured in terms of PFS and OS, among patients with *BRAF*-mutant melanoma treated with the antibody combination compared to those treated with BRAF/MEK inhibitors combination (Atkins et al. 2019; Tarhini et al. 2021). However, the optimal sequencing of kinase inhibitors and immunotherapy in the treatment of patients with *BRAF* V600-mutated metastatic melanoma has not been established, yet (Pavlick et al. 2019). Phase 2/3 clinical trials are ongoing to evaluate whether ipilimumab and nivolumab followed by BRAF/MEK inhibitors (dabrafenib plus trametinib or encorafenib plus binimetinib) are more effective than the reverse drug sequence (NCT02224781/DREAMseq, NCT02631447/SECOMBIT) or whether the targeted therapy with encorafenib plus binimetinib followed by or in combination with ipilimumab and nivolumab is more active than the sole immunotherapy or targeted therapy (NCT03235245/EBIN; NCT04655157/QUAD01). Moreover, the same drugs (encorafenib plus binimetinib plus nivolumab

versus ipilimumab plus nivolumab) are tested in another phase 2 study (NCT04511013) enrolling patients with melanoma brain metastases. In addition, a phase 2 study (NCT04562129) is investigating the effects of high-dose bolus IL-2 in combination with low-dose ipilimumab followed by nivolumab in patients with advanced inoperable stage III or stage IV melanoma who have failed prior anti-PD1 immunotherapy.

Different clinical trials are ongoing where ipilimumab/nivolumab combination therapy is added to different treatments. For instance phase 1 studies are evaluating the safety and tolerability of the CD40 agonistic mAb APX005M (sotigalimab) or of BMS-986205, an inhibitor of IDO, in combination with nivolumab and ipilimumab in patients with advanced melanoma (NCT04495257, NCT02658890). Moreover, a phase 1 clinical trial is studying a new type of personalized neoantigen vaccine (NeoVax) (Ott et al. 2017) plus a vaccine adjuvant (Montanide®) in combination with locally administered ipilimumab and systemic nivolumab as a possible treatment for advanced melanoma (NCT03929029).

As described above, ipilimumab has shown efficacy as adjuvant therapy after surgical removal of melanoma for patients with high-risk stage III or IV to reduce the risk of disease recurrence. Adjuvant treatment is recommended for patients with stage IIIB-C and may be considered for patients with IIIA melanoma. Anti-PD1 mAbs used as single agents (i.e. nivolumab or pembrolizumab) or BRAF/MEK inhibitors (dabrafenib/trametinib) for BRAF mutated tumours are currently regarded as the standard of care for resected stage III melanoma (Dimitriou et al. 2021). Conversely, for resected stage IV, nivolumab is the only approved agent (Weber et al. 2017; Ascierto et al. 2020). Interestingly, the results of a randomized, placebo-controlled, phase 2 clinical trial (NCT02523313/IMMUNED) in 167 patients with resected stage IV melanoma (with no evidence of disease after surgery or radiotherapy) treated with ipilimumab plus nivolumab ($n = 56$; 1 mg/kg nivolumab every 3 weeks plus 3 mg/kg of ipilimumab every 3 weeks for four doses, followed by 3 mg/kg of nivolumab every 2 weeks), nivolumab ($n = 59$; 3 mg/kg of inivolumab every 2 weeks plus ipilimumab-matching placebo during weeks 1–12), or double-matching placebo ($n = 52$) showed that in the combined antibody arm, the 2-year recurrence-free survival was higher than that reported in the nivolumab monotherapy or placebo arms (70% versus 42% and 14%) (Zimmer et al. 2020). Nevertheless, the reported rates of grade 3–4 adverse events were also higher in patients receiving ipilimumab plus nivolumab compared to those treated with nivolumab (71% versus 27%) (Zimmer et al. 2020). However, the announced results of a phase 3 trial (NCT03068455/CheckMate-915) aimed at evaluating adjuvant ipilimumab plus nivolumab versus nivolumab in stage IIIB/C/D or stage IV melanoma patients indicated that in the intention-to-treat population, the addition of ipilimumab to nivolumab did not improve the recurrence-free survival (Bristol Myers Squibb Announces 2020).

Despite adjuvant treatment (with immunotherapy or BRAF/MEK inhibitors), about 40% patients with macroscopic stage III melanoma relapse within 3 years (Ascierto et al. 2020; Hauschild et al. 2018; Eggermont et al. 2020). Thus, in order to improve the relapse-free survival, the ipilimumab/nivolumab combination was

evaluated as neoadjuvant therapy before tumour surgical resection. The pathological response obtained by this therapeutic approach may also help to guide the choice of the most appropriate adjuvant treatment. The phase 1 OpACIN (NCT02437279) and phase 2 NCT02519322 clinical studies showed that neoadjuvant ipilimumab plus nivolumab induced high pathologic response rates in patients with macroscopic stage III melanoma' (Blank et al. 2018; Amaria et al. 2018). The OpACIN study was performed in 20 melanoma patients with palpable stage III melanoma who were 1:1 randomized to receive ipilimumab 3 mg/kg and nivolumab 1 mg/kg, four courses after surgery ($n = 10$; adjuvant arm) or two courses before surgery and two courses after surgery ($n = 10$; neoadjuvant arm). Pathological responses were obtained in 7/9 (78%) patients receiving the neoadjuvant treatment (Blank et al. 2018). In addition, the NCT02519322 trial compared the activity of nivolumab plus ipilimumab ($n = 11$) versus nivolumab ($n = 12$) treatment and reported higher pathological complete response rates in the combined treatment arm (Amaria et al. 2018). More interestingly, no patients with a pathological response have relapsed after a median follow-up of 4 years (Rozeman et al. 2021). However, in both clinical studies, the combined treatment regimen was associated with high toxicity rates. The subsequent phase 2 OpACIN-neo study (NCT02977052) tested the efficacy and toxicity of three different dosing schedules of neoadjuvant ipilimumab plus nivolumab in 86 patients and identified two cycles of ipilimumab 1 mg/kg plus nivolumab 3 mg/kg as a tolerable and effective neoadjuvant dosing schedule (Rozeman et al. 2019). This neoadjuvant regimen without subsequent adjuvant therapy induced durable clinical benefit with 2-year relapse-free survival of more than 80%, encouraging its further evaluation in a phase 3 trial (Rozeman et al. 2021).

Finally, in addition to clinical studies on stage III and IV cutaneous melanoma, ipilimumab is also studied in combination with nivolumab for other melanoma types, such as uveal melanoma. Among these studies, the NCT04463368 NCT04283890 trials are recruiting patients with liver metastases from uveal melanoma, whereas the NCT03528408 trial aims at testing adjuvant ipilimumab and nivolumab in subjects with high-risk ocular melanoma.

10.3.1.2 Renal Cell Carcinoma

Renal cell carcinoma represents over 90% of kidney tumours. The 5-year survival rates reported by the American Cancer Society are 81%, 74%, 53%, and 8%, for stage I, II, III, and IV, respectively. Treatment of renal cell carcinoma depends on the stage and, in the last years, there have been substantial changes in the management of patients with advanced/metastatic disease, with upfront immunotherapy-based combinations plus anti-angiogenic kinase inhibitors (i.e. the anti-PD-1 pembrolizumab and anti-PD-L1 avelumab in combination with axitinib or the anti-PD-1 antibody nivolumab plus cabozantinib) showing superior activity over targeted agents as monotherapy (Rini et al. 2019; Motzer et al. 2019a; Choueiri et al. 2020, 2021). For intermediate- and poor-risk advanced renal cell carcinoma, ipilimumab in combination with nivolumab has been approved by both FDA and EMA and,

according to both ESMO and NCCN guidelines, is recommended as first-line therapy, based on the results of the CheckMate 214 (NCT02231749/CA209–214) trial. This phase 3, randomized, open-label study included patients with previously untreated, advanced/metastatic renal cell carcinoma with a clear-cell component. Of the 1096 patients enrolled in the trial, 847 had intermediate−/poor-risk disease. Patients were randomized to receive either ipilimumab 1 mg/kg in combination with nivolumab every 3 weeks for four doses, followed by nivolumab monotherapy 3 mg/kg every 2 weeks ($n = 550$), or sunitinib 50 mg daily ($n = 546$), administered orally for 4 weeks followed by 2 weeks off, every cycle. Treatment was continued as long as clinical benefit was observed or until unacceptable toxicity. In intermediate- and poor-risk patients, the clinical benefit was higher with ipilimumab plus nivolumab versus sunitinib, regardless of tumour PD-L1 expression levels (objective response rates: 42% versus 27%; at a median follow-up of 25.2 months, median OS: not reached versus 26.0 months). However, the magnitude of benefit was higher in the case of tumours with ≥1% PD-L1 expression (Motzer et al. 2018). This antibody combination was also found to be the most cost-effective treatment option compared with the other immunotherapy/kinase inhibitor combinations (Shay et al. 2021). Long-term follow-up analysis confirmed the durable efficacy of the nivolumab plus ipilimumab treatment with about 50% of patients being alive at 4 years in the intermediate- and poor-risk population (Motzer et al. 2019b, 2020; Albiges et al. 2020). Moreover, a meta-analysis aimed at indirectly comparing the efficacy and safety of currently available treatments for metastatic renal cell carcinoma using the data of six phase 3 randomized controlled trials indicated that pembrolizumab plus axitinib was the most efficacious first-line regimen, whereas nivolumab plus ipilimumab was the best tolerated treatment (Mori et al. 2021). An updated meta-analysis showed that nivolumab plus cabozantinib and lenvatinib plus pembrolizumab had the highest probability of providing better OS and PFS, and confirmed that nivolumab plus ipilimumab had the most favourable toxicity profile, being associated with the lowest rates of grade ≥ 3 treatment-related adverse effects. Moreover, in patients with high PD-L1 expression, this immune checkpoint inhibitor combination seemed to result in higher PFS and OS (Quhal et al. 2021).

Several phase 1–3 clinical trials are recruiting patients with advanced/metastatic renal cell carcinoma to evaluate ipilimumab plus nivolumab in combination with other drugs [e.g. sitravatinib, a kinase inhibitor targeting myeloid-derived suppressor cells and Tregs in the tumour microenvironment (NCT04518046), APX005M/sotigalimab (NCT04495257), cabozantinib (NCT04413123; NCT03937219), or the histone deacetylase (HDAC) inhibitor entinostat (NCT03552380)].

10.3.1.3 Colorectal Cancer

Colorectal cancer is the third most commonly diagnosed cancer and the second cause of cancer-related death. The 5-year survival rates are 91%, 72%, and 14% for localized, regional, and distant metastatic diseases, respectively. The first-line treatment of advanced/metastatic colorectal cancer is based on doublet chemotherapy

(5-flourouracil/folinic acid and oxaliplatin or irinotecan) alone or in combination with targeted agents [anti-EGFR or anti-vascular endothelial growth factor-A (VEGF-A) mAbs].

About 12% of sporadic colorectal cancer and 5% of the metastatic forms are characterized by MSI-H as a result of MMR dysfunction due to mutations or more frequently epigenetic methylation of MMR genes. MMR deficiency results in failure to repair DNA replication errors with consequent insertions or deletions in DNA repeat sequences (microsatellites) and acquisition of a "hypermutator" phenotype (Gupta et al. 2018). Tumours with MSI-H/dMMR show high mutational burden, expression of immunogenic neoantigens, and infiltration of cytotoxic T lymphocytes. Early-stage colorectal cancer with dMMR/MSI-H has better prognosis and longer survival compared to MMR-proficient and microsatellite stability (MSS) tumours, likely due to increased antitumor immune responses [reviewed in (Kloor and von Knebel 2016; Lizardo et al. 2020). However, adjuvant chemotherapy does not result in clinical benefit. In the case of metastatic colorectal cancer, MSI-H is associated with a worse disease-free survival and OS as compared to MSS (Venderbosch et al. 2014). MSI-H colorectal cancers also show a worse response to 5-fluorouracil, compared to tumours with low-frequency MSI (MSI-L) or with MSS, due to reduced recognition and processing by the MMR of 5-fluorouracil-induced DNA damage (Kim et al. 2016; Wensink et al. 2021). Moreover, upregulation of immune checkpoints in tumour cells with MSI-H/dMMR may help them to evade the control by the immune system. In this context, immunotherapy with immune checkpoint inhibitors has demonstrated high clinical activity with durable responses in patients with metastatic colorectal cancer including heavily pre-treated patients. Thus, the anti-PD-1 pembrolizumab and nivolumab have been granted approval for MSI-H/dMMR unresectable or metastatic colorectal cancer, the former for the first-line treatment, whereas the latter for previously treated patients in combination with low-dose ipilimumab (Cohen et al. 2021).

The approval of the nivolumab/ipilimumab combination followed the results of the NCT02060188/CKECKMATE-142 trial, a multicentre, non-randomized, multicohort, open-label study conducted in patients with metastatic colorectal cancer who had disease progression during or after treatment with chemotherapy, to investigate the activity and safety of nivolumab monotherapy or its combination with ipilimumab in patients with MSI-H and non-MSI-H tumours. Patients enrolled in the ipilimumab and nivolumab MSI-H cohort received ipilimumab 1 mg/kg and nivolumab 3 mg/kg every 3 weeks for four doses, followed by nivolumab 3 mg/kg as a single agent every 2 weeks (Overman et al. 2017, 2018; Morse et al. 2019). A total of 119 patients were enrolled in the ipilimumab plus nivolumab cohort and at a median follow-up of 13.4 months, the 1-year PFS and OS rates were 71% and 85%, respectively (Overman et al. 2018).

Recent data suggest that nivolumab plus ipilimumab may be effective also in the first-line setting and the results reported for 45 patients on the same NCT02060188/CKECKMATE-142 trial, after 29 months of follow-up, showed a durable disease control rate with 69% overall response rate; median PFS and OS were not reached (Lenz et al. 2020). A phase 3 study (NCT04008030/CheckMate-8HW) is ongoing in

order to compare the efficacy of nivolumab plus ipilimumab with nivolumab monotherapy and with conventional chemotherapy. Currently, the NCCN guidelines recommend nivolumab in combination with ipilimumab, as first-line treatment options for patients with MSI-H/dMMR metastatic colorectal cancer if they are candidate for intensive therapy (Benson et al. 2021).

10.3.1.4 NSCLC

About 85–90% of all lung cancers are NSCLC; at an advanced stage, standard chemotherapy only marginally improves OS. Platinum-based combination therapy was the standard of first-line care for patients with advanced NSCLC, with a median OS of 8–12 months. The advent of immunotherapy and kinase inhibitors for tumour-specific mutations has changed the therapeutic approaches of NSCLC, and several immune checkpoint inhibitors have been evaluated and approved for this indication.

One of the first studies where ipilimumab was tested in NSCLC is NCT00527735/CA184–041 trial. 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. The results of this phase 2 trial indicated that phased ipilimumab plus paclitaxel and carboplatin improved PFS (phased ipilimumab 5.1 months versus concurrent ipilimumab 4.1 months or chemotherapy alone 4.2 months). Median OS were 12.2, 9.7, and 8.3 months, respectively (Lynch et al. 2012). A phase 3 trial has also tested the impact of the phased regimen on OS in NCSLC with squamous histology when used as first-line treatment (NCT01285609/CA184–104). However, the addition of ipilimumab to chemotherapy did not result in prolonged OS compared with chemotherapy alone (Govindan et al. 2017). 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 2 study NCT00527735/CA184–041 (Lynch et al. 2012). However, a systematic review showed that the association of etoposide/platinum (gold standard therapy for ED-SCLC) plus ipilimumab did not have any positive impact on OS or PFS (Chen et al. 2020b).

Conversely, ipilimumab plus nivolumab was approved as first-line treatment of patients with metastatic NSCLC whose tumours express PD-L1 $\geq$ 1% based on the results of part 1a of the NCT02477826/CheckMate 227 study. This randomized, open-label, phase 3 trial comprised two parts: part 1 and 2. In turn, part 1 was divided into part 1a and 1b, enrolling patients with PD-L1 expression in $\geq$1% ($n = 1189$) and in <1% of tumour cells ($n = 550$), respectively. After 1:1:1 randomization, patients were treated with nivolumab (3 mg/kg every 2 weeks) plus ipilimumab (1 mg/kg every 6 weeks), or nivolumab monotherapy (240 mg every 2 weeks in part 1a and 360 mg every 3 weeks in part 1b), or platinum doublet chemotherapy every 3 weeks for up to four cycles. The results of the part 1 study indicated that in patients with tumours expressing PD-L1 $\geq$ 1% treatment with ipilimumab plus nivolumab induced longer OS (median OS: 17.1 versus 14.9 months; 2-year OS rates: 40.0%

versus 32.8%) and PFS (10.5% versus 4.6% at 2 years) compared to chemotherapy. The combined antibody treatment was also more effective than nivolumab monotherapy, although the trial was not powered to compare the two regimens (Hellmann et al. 2019). The combination of ipilimumab plus nivolumab resulted also in more durable and clinically significant improvements of patient-reported outcomes that provide information on symptoms and health status by collecting the data directly from patients (Reck et al. 2019). In part 2 of the NCT02477826/CheckMate 227 study, previously untreated patients with advanced NSCLC, regardless of PD-L1 expression level, were randomized 1:1 to receive platinum doublet chemotherapy alone ($n = 378$) or in combination with nivolumab ($n = 377$). However, the primary endpoint of OS in patients with non-squamous NSCLC was not met (Bristol-Myers Squibb Provides 2019).

On the other hand, ipilimumab in combination with nivolumab and 2 cycles of platinum-based chemotherapy was approved for the first-line treatment of metastatic NSCLC in adults whose tumours have no sensitizing EGFR mutations or ALK translocation, regardless of PD-L1 expression. Approval for this indication was based on the results of pre-specified interim analysis of the NCT03215706/CA209-9LA/CheckMate 9LA study. This randomized phase 3 trial compared the safety and efficacy of ipilimumab 1 mg/kg every 6 weeks in combination with nivolumab 360 mg every 3 weeks and 2 cycles of platinum-based chemotherapy ($n = 361$) versus 4 cycles of platinum doublet chemotherapy administered every 3 weeks (carboplatin or cisplatin plus pemetrexed for non-squamous and carboplatin/cisplatin plus paclitaxel for squamous) ($n = 358$). Randomization was stratified by histology (squamous versus non-squamous), tumour PD-L1 expression level ($\geq 1\%$ versus $< 1\%$), and gender. The results demonstrated a statistically significant clinical benefit in terms of OS for patients treated with the immunotherapy-containing regimen irrespective of tumour histology or PD-L1 expression. At the time of interim analysis, the median OS was 14.1 in the experimental groups versus 10.7 months in the control group (Paz-Ares et al. 2021). Finally, a recent systematic review that analysed 16 studies involving 8278 advanced NSCLC patients, including 10 immunotherapy combinations, indicated nivolumab plus ipilimumab combined with chemotherapy as the immunotherapy associated with the best OS in patients with PD-L1 $< 1\%$. Conversely, pembrolizumab plus chemotherapy was associated with the best OS in patients with PD-L1 $\geq 1\%$ (Liu et al. 2021). In this context, a real-life study is recruiting NSCLC patients to describe the outcomes, safety, and quality of life of the first-line treatment with nivolumab plus ipilimumab in combination with two cycles of chemotherapy (NCT04794010).

Phase 1/2 trials are investigating ipilimumab plus nivolumab compared to chemotherapy for earlier NSCLC stages (I-IIIA or II-III) (NCT03158129; NCT04013542) or ipilimumab in combination with other agents [e.g. with the EGFR inhibitor osimertinib for locally advanced or metastatic EGFR mutated NSCLC (NCT04141644)].

10.3.1.5 Hepatocellular Carcinoma

Hepatocellular carcinoma is the most common form of liver cancer accounting for 90% of tumours. In the majority of cases, it develops in patients with cirrhosis and is frequently associated with hepatitis B and C viral chronic infection, alcohol, diabetes, and other metabolic diseases. The overall 5-year survival is 10–12%, improving up to 70–80% in patients undergoing surgical treatment including hepatic resection and liver transplantation. Unfortunately, most patients are diagnosed with unresectable, advanced stage disease and have a poor prognosis. Early-stage hepatocellular carcinoma unsuitable for surgery can be cured by image-guided radiofrequency ablation, whereas intermediate-stage can be treated with palliative locoregional treatment with or without transarterial chemoembolization. Systemic therapy is administered to patients ineligible for or progressing on local regional treatment.

The management of hepatocellular carcinoma has markedly improved in the last few years. Presently, six systemic therapies (kinase inhibitors and mAbs, including an anti-PD-L1) have been approved by FDA and EMA (i.e. sorafenib, lenvatinib, regorafenib, cabozantinib, ramucirumab, and atezolizumab plus the anti-VEGF-A bevacizumab) and three additional immunotherapies have received accelerated FDA approval (nivolumab, pembrolizumab, and nivolumab plus ipilimumab). Until 2018, the multitargeted, anti-angiogenic kinase inhibitor sorafenib was the only agent able to significantly increase OS in the front-line setting (Cabibbo et al. 2019). Afterwards, the kinase inhibitor lenvatinib and the anti-PD-L1 antibody atezolizumab plus bevacizumab were also approved as first-line agents, showing the former agent non-inferiority and the latter combination superiority compared to sorafenib (Kudo et al. 2018; Finn et al. 2020). Conversely, regorafenib, cabozantinib, and the anti-VEGF-A receptor 2 ramucirumab were approved as second-line therapy after progression on sorafenib (Llovet et al. 2021).

The approval of ipilimumab in association with nivolumab derived from the results of the NCT01658878/CHECKMATE-040 trial (arm A), the same phase 1/2 clinical study that led to the previous approval of nivolumab as monotherapy for patients with hepatocellular carcinoma who progressed on or were intolerant to sorafenib (El-Khoueiry et al. 2017). This study evaluated the activity of three dosing regimens of the antibody combination in 148 patients randomized 1:1:1 to receive the following treatments: ipilimumab 3 mg/kg in combination with nivolumab 1 mg/kg (every 3 weeks for four doses), followed by nivolumab 240 mg every 2 weeks (arm A); nivolumab 3 mg/kg plus ipilimumab 1 mg/kg (every 3 weeks for 4 doses), followed by nivolumab 240 mg every 2 weeks (arm B); or nivolumab 3 mg/kg every 2 weeks plus ipilimumab 1 mg/kg every 6 weeks (arm C). The dosing regimen tested in arm A induced the highest complete response rate, median OS (22.8 months), and 2-year survival rates (48%), likely due to the higher starting ipilimumab dose compared to the other arms. However, nivolumab plus ipilimumab regimens (in particular, the one tested in arm A) were associated with higher rates of adverse

events than those previously reported with nivolumab monotherapy (Yau et al. 2020).

Presently, a phase 3 trial (NCT04039607/CheckMate 9DW) is recruiting patients with advanced hepatocellular carcinoma to compare the OS of nivolumab plus ipilimumab as first-line therapy in untreated patients versus standard of care (i.e. sorafenib or lenvatinib). In parallel, another phase 3 trial (NCT04340193/CheckMate 74 W) is enrolling patients with intermediate-stage liver cancer to compare the effectiveness and safety of nivolumab with and without ipilimumab in combination with trans-arterial chemoembolization. In the same clinical setting, a phase 2 single-arm, open-label clinical trial is recruiting patients, not eligible for surgical resection or transplantation, to determine the efficacy of the two antibodies combined with cabozantinib (NCT04472767). Nivolumab/ipilimumab combination therapy is also evaluated in phase 1 or 2 trials as neoadjuvant treatment in hepatocellular carcinoma patients prior to liver resection (NCT03682276/PRIME-HCC; NCT03510871) (Pinato et al. 2021).

10.3.1.6 Malignant Pleural Mesothelioma

Malignant pleural mesothelioma is a highly aggressive cancer, often unresectable at diagnosis. In the majority of cases, it is associated with asbestos exposure several decades before symptoms arise. Although regarded as a rare cancer, its incidence is increasing worldwide. The average age at diagnosis is approximately 72 years and the 5-year survival rate is about 10%, reflecting the dismal prognosis of mesothelioma. Among the different histological subtypes of mesothelioma, the epithelioid subtype has the worst prognosis. The standard first-line treatment is chemotherapy with cisplatin or carboplatin plus pemetrexed that however does not markedly improve long-term survival. For patients with early-stage disease, the role of radical surgery is still a matter of debate (i.e. surgery combined with adjuvant chemotherapy, radiotherapy, or both) due to high perioperative mortality (Kim et al. 2019). Median OS with standard first-line options is about 13 months, with the best outcome for the epithelioid subtype (Vogelzang et al. 2003).

In 2020, FDA has approved as first-line treatment of unresectable malignant pleural mesothelioma the combination therapy of ipilimumab plus nivolumab, regardless of histological subtypes, based on the results of an open-label phase 3 trial (NCT02899299/CHECKMATE-743) (Baas et al. 2021). A total of 605 patients were randomized 1:1 to receive either ipilimumab 1 mg/kg every 6 weeks and nivolumab 3 mg/kg every 2 weeks for up to 2 years ($n = 303$), or cisplatin 75 mg/m^2 and pemetrexed 500 mg/m^2, or carboplatin 5 AUC and pemetrexed 500 mg/m^2 administered every 3 weeks for 6 cycles ($n = 302$). Stratification factors for randomization included tumour histology (epithelioid versus sarcomatoid or mixed histology subtypes). With a median follow-up of 29.7 months, a statistically significant improvement in OS was observed in patients treated with immunotherapy compared to those treated with chemotherapy (median OS: 18.1 versus 14.1 months; 2-year OS rates: 41% versus 27%). Moreover, survival

advantage was similar in patients with both non-epithelioid and epithelioid histologies. Grade 3 or 4 serious treatment-related adverse events were higher with nivolumab plus ipilimumab than with chemotherapy (Baas et al. 2021).

Ipilimumab plus nivolumab is also evaluated as second-line treatment after disease progression on first-line standard platinum doublet chemotherapy (NCT04300244) or as neoadjuvant therapy for resectable tumours (NCT03918252).

10.3.1.7 Phase III Clinical Trials in Prostate Cancer and Other Solid Tumours

The standard of care for hormone-sensitive (or hormone-naïve) metastatic prostate cancer is androgen deprivation therapy via medical castration [i.e. with the gonadotropin-releasing hormone (GnRH) agonist/analogues leuprolide or goserelin or with the GnRH antagonist degarelix] in combination with docetaxel or abiraterone, a pregnenolone derivative that irreversibly inhibits CYP17A (a key enzyme in androgen synthesis) or androgen receptor antagonists, such as enzalutamide and apalutamide. For metastatic castration-resistant prostate cancer, besides docetaxel, abiraterone, or enzalutamide, a number of additional agents are available, especially for the second-line treatment. Among these, cabazitaxel (a semisynthetic taxane derivative), sipuleucel-T [an autologous antigen-presenting cell vaccine loaded with prostate acid phosphatase conjugated with granulocyte-macrophage colony-stimulating factor (GM-CSF)], poly(ADP-ribose) polymerase (PARP) inhibitors (olaparib, rucaparib) for tumours with germline or somatic BRCA mutations, the anti-PD-1 antibody pembrolizuamb for MSI-H/dMMR tumours, have all been approved for pretreated patients. The treatment choice depends on whether patients were or were not previously exposed to docetaxel and/or novel hormone therapy (i.e. abiraterone or enzalutamide). In this clinical setting, ipilimumab has shown some activity in several phase 1/2 clinical trials, as single agent (Small et al. 2007) and in combination with GM-CSF (Fong et al. 2009) or radiotherapy (Slovin et al. 2009).

Two multicentre randomized phase 3 studies, both with OS as primary endpoint, have been conducted in chemotherapy-naïve or post-docetaxel patients with metastatic castration-resistant prostate cancer. Based on previous data supporting a role for irradiation to enhance immune responses, one of these studies compared radiotherapy followed by ipilimumab (10 mg/kg) versus radiotherapy plus placebo in patients who had received prior treatment with docetaxel, but no significant differences were found between the two groups (NCT00861614/CA184–043) (Kwon et al. 2014). The other phase 3 trial has tested the same ipilimumab dose versus placebo in asymptomatic or minimally symptomatic chemotherapy-naïve patients; also in this case, ipilimumab did not improve OS (NCT01057810/CA184–095) (Beer et al. 2017).

Based on the previously reported synergy between the anti-CTLA-4 antibody in combination with GM-CSF-secreting tumour-cell vaccines, a phase 1 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) (van den Eertwegh et al. 2012). Moreover, another phase I study (NCT00113984/NCT00124670) with escalating doses of ipilimumab plus PSA-Tricom vaccine, a poxviral-based vaccine targeting the prostate-specific antigen and containing three T cell co-stimulatory molecules (CD58, CD80, and ICAM1), has shown that this treatment is tolerable and safe (Madan et al. 2012).

Failure of ipilimumab to improve OS in metastatic castration-resistant prostate cancer has been attributed to the fact that this tumour is generally regarded as "immunological cold," being characterized by low somatic mutation frequency and few tumour-infiltrating T cells. However, in prostate cancer patients, ipilimumab was found to increase tumour infiltration by T cells and to induce a compensatory upregulation of the PD-L1 and VISTA inhibitory molecules (Gao et al. 2017). Thus, it has been hypothesized that in prostate cancer, the combination of the anti-CTLA4 antibody with an anti-PD-1 might be more effective and ipilimumab 3 mg/kg plus nivolumab 1 mg/kg has been evaluated in a phase 2 trial (NCT02985957/CheckMate 650). Analysis of the results obtained after enrolling the first 90 patients (out of the 270 planned) showed antitumor activity of the combined immunotherapy both in chemotherapy-naive and chemotherapy-experienced patients (25% and 10% overall response rates; 19.0 and 15.2 months median OS, in the pre-chemotherapy and post-chemotherapy cohorts) (Sharma et al. 2020).

The combination of ipilimumab with nivolumab is also tested in phase 3 clinical trials for other solid tumours, such as advanced sarcoma, glioblastoma, resectable or advanced/metastatic gastric and gastroesophageal junction cancer, advanced head and neck cancer, unresectable or metastatic urothelial cancer.

For metastatic or unresectable advanced sarcoma of rare subtype, previously treated with an anthracycline-based regimen (except in those cases for which standard therapy is not available), the efficacy (PFS) of ipilimumab plus nivolumab will be compared with that of pazopanib alone (both treatment administered for up to 12 months) (NCT04741438). For head and neck cancers, the antibody combination or nivolumab alone will be tested as adjuvant maintenance therapy, after radiotherapy, in patients with surgically treated, locally advanced squamous cell carcinoma and compared to standard adjuvant radiotherapy and cisplatin-based chemotherapy (NCT03700905).

The NCT04396860 trial is a phase 2/3 study testing immunotherapy with ipilimumab plus nivolumab versus standard chemotherapy (temozolomide) in patients with newly diagnosed glioblastoma with O^6-methylguanine DNA-methyltransferase (MGMT) promoter methylation (known predictive marker of response to temozolomide), after surgery and radiotherapy. The trial will recruit 485 patients and primary outcomes are PFS for the phase 2 and OS for the phase 3.

In patients with oesophageal and gastroesophageal junction adenocarcinoma who are undergoing surgery, the usefulness of nivolumab and ipilimumab in addition to standard of care chemotherapy and radiation therapy is tested in a phase 2/3 trial, having as primary outcomes the pathologic complete response and the disease-free survival (NCT03604991). Moreover, the NCT02872116/CheckMate649 study is

comparing the combination of the two antibodies followed by nivolumab, with nivolumab plus chemotherapy (oxaliplatin/5-fluorouracil/leucovorin or oxaliplatin/capecitane) or chemotherapy alone (NCT02872116).

In the case of urothelial cancer, ipilimumab plus nivolumab is compared to nivolumab plus chemotherapy (gemcitabine and cisplatin/carboplatin) (NCT03036098).

Finally, several phase 2 studies are also testing the association of ipilimumab plus pembrolizumab in different solid tumours.

10.4 Criteria to Evaluate the Efficacy of Immune-Checkpoint Inhibitors

The clinical experience with ipilimumab has indicated that the Response Evaluation Criteria in Solid Tumours (RECIST, version 1 and 1.1), typically used by oncologists to define tumour response and disease progression, are not suitable for assessing the clinical responses to immunotherapy. 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 and the subsequently approved immune checkpoint inhibitors, due to their 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; d) a response in the presence of new lesions (Wolchok et al. 2009). 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 (Ribas et al. 2009). Therefore, RECIST 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 explains why OS benefit with immunotherapy is often not fully reflected in RECIST-based PFS or overall response rate.

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 (Wolchok et al. 2009). These criteria have been evaluated

in large studies involving 487 patients with advanced melanoma treated with ipilimumab (Wolchok et al. 2009). Patients with new lesions, but with a decrease in size of baseline lesions, will not necessarily be considered to have progressive disease. They will be, instead, considered responders and continue to receive immune checkpoint inhibitors, with possible long-term benefits. The irRC allowed identifying additional patients with favourable survival who were, instead, considered having progressive disease according to the RECIST criteria. Moreover, irRC helped to understand why low response rates (~10%) obtained with ipilimumab monotherapy in metastatic melanoma translated into long-term survival in about 20% of patients (Hodi et al. 2010). The irRC are based on bidimensional measurements of target lesions, whereas RECIST criteria, largely used for solid tumours, utilize unidimensional measurements. Thus, in order to more easily compare the efficacy and effectiveness of anti-cancer agents, in 2013, a unified method for assessing tumour response was developed (irRECIST) that incorporated irRC but used a monodimensional approach to evaluate the efficacy of immunotherapies (Nishino et al. 2013). However, the irRECIST criteria showed some limitations; moreover, they were not consistently applied or were further modified, depending on the clinical protocol, leading to inconsistencies across studies. Thus, other RECIST 1.1 for immune-based therapeutics (iRECIST) and immune-modified RECIST (imRECIST) criteria have been designed for better capturing tumour response to immunotherapies evaluated in clinical trials (Seymour et al. 2017; Hodi et al. 2018b). However, further clinical studies are needed to validate these criteria in comparison with the standard RECIST 1.1 criteria and to establish whether they might have a role in regulatory approval of new immunotherapeutic agents.

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 warnings about the risk of severe and fatal irAEs due to T cell activation and proliferation (Product information n.d.). 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 programme consists of 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 1–3 trials of ipilimumab in 1498 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 (Ibrahim et al. 2011). In the registration

phase 3 trial (NCT00094653/CA184–002), the most common irAE was diarrhoea at any grade in 27–31% of the patients receiving ipilimumab (Hodi et al. 2010). 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 (Revicki et al. 2012). Analysis of the safety profile of patients alive after 2 years of the phase 3 trial NCT00324155/CA184–024, in which ipilimumab plus dacarbazine was compared to dacarbazine plus placebo, indicated a low rate of irAEs in the ipilimumab containing arm and that irAEs were medically manageable according to established guidelines (Thomas et al. 2012). Indeed, algorithms are available for the correct management of irAEs which depends on the severity of adverse effects (Kähler and Hauschild 2011).

A meta-analysis (including 11 clinical trials with 7088 patients) compared irAEs induced with anti-CTLA-4 antibodies (ipilimumab and tremelimumab) with those reported with control therapies (placebo, chemotherapy, radiation therapy, or vaccine). This study indicated an increased risk of severe irAEs with immune checkpoint inhibitors, predominantly at the dermatological, gastrointestinal (diarrhoea and colitis), endocrine (hypophysitis, hypothyroidism, adrenal insufficiency, and hypopituitarism), and hepatic (hepatitis, elevated alanine aminotransferase, and elevated aspartate aminotransferase) levels. The most common severe organ-specific irAEs were gastrointestinal (diarrhoea 9.8% and colitis 5.3%). However, no increased risk of haematological abnormalities or severe musculoskeletal disorders was observed compared with control therapies (Xu et al. 2019).

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 (Wolchok et al. 2010a). The mainstay of irAE treatment consists of immunosuppression with glucocorticoids or, in case of long-lasting and/or refractory toxicities, other immunosuppressant agents (e.g. mycophenolate mofetil, cyclophosphamide, and the anti-tumour necrosis factor mAb infliximab). The majority of irAEs resolve with systemic administration of glucocorticoids; 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 glucocorticoids on ipilimumab antitumor efficacy has not been established in large-scale trials. Some retrospective studies or case reports did not show unfavourable effects of steroid treatments on the antitumor efficacy of ipilimumab (Thumar and Kluger 2010; Harmankaya et al. 2011; Graziani et al. 2012). However, a retrospective analysis in 98 melanoma patients who had ipilimumab-induced hypophysitis indicated that treatment with high-dose glucocorticoids reduced survival compared to low doses (Faje et al. 2018).

Several trials have reported a possible correlation between grade 3 and 4 irAEs with the clinical efficacy of ipilimumab (Attia et al. 2005; Lutzky et al. 2009), suggesting that tumour regression is associated with the development of autoimmunity. Interestingly, a recent meta-analysis indicated a positive association between the development of irAEs and overall response rate, PFS, and OS in patients treated

with immune checkpoint inhibitors (including ipilimumab and nivolumab or pembrolizumab), regardless of disease site, type of antibody, and irAE. Grade 3 or higher toxicities resulted in better overall response rate, but worse OS (Hussaini et al. 2021). Nevertheless, based on a real-world clinical experience with ipilimumab (3 mg/kg) outside of a clinical trial, efficacy did not appear related to irAE occurrence (Ascierto et al. 2014). Moreover, clinical benefit has been observed also in patients who did not develop irAEs (Lutzky et al. 2009).

Finally, in terms of irAEs, more attention should be paid to the combination of ipilimumab and nivolumab. In fact, although the combination showed improved antitumor activity compared to monotherapy, it carried a higher risk of all grade irAEs. In fact, in a phase 1 study, treatment-related grade 3 or 4 toxicities were seen in 53% of patients receiving the concurrent regimen (Wolchok et al. 2013). In the Checkmate-067 trial that led to the approval of the ipilimumab/nivolumab combination for metastatic melanoma, the rate of grade 3–4 toxicities was 55% with the combination compared with 16% and 27% with nivolumab and ipilimumab monotherapy, respectively (Larkin et al. 2015). Pooled safety data from 1551 melanoma patients indicated that irAEs occurred more frequently with ipilimumab 3 mg/kg plus nivolumab 1 mg/Kg ($N = 407$) than with ipilimumab ($N = 357$) or nivolumab ($N = 787$) used as monotherapy. Moreover, irAEs have a shorter time-to-onset and are generally more severe (Hassel et al. 2017). A recent meta-analysis recruiting 2544 patients confirmed this trend, being nivolumab plus ipilimumab associated with statistically significant higher risk of all-grade adverse events and of drug discontinuation due to all grade adverse events, as compared to either ipilimumab or nivolumab used as single agents (Abdelhafeez et al. 2020). Although more frequent and of higher grade, the toxicities reported with the antibody combination were similar to those observed with either single agent. Moreover, these irAEs, when early recognized and timely managed before severe or life-threatening situations occur, are frequently reversible (Linardou and Gogas 2016). Management of irAEs associated with immune checkpoint inhibitor combination requires following the same established algorithms described for the monotherapy regimens [reviewed in (Friedman et al. 2016).

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 (Hodi et al. 2003). All grade rash and pruritus were more common with the combined treatment than with ipilimumab (Almutairi et al. 2020). Skin eruptions and pruritus usually do not require skipping a dose or discontinuation and resolve with topical glucocorticoids or urea-containing creams and antipruritic

agents. Severe rash (grade $\geq$ 3) should be treated with oral glucocorticoids (Rovers and Bovenschen 2020).

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% (Beck et al. 2006). Treatment of mild diarrhoea is symptomatic, with loperamide, oral hydration, and electrolyte substitution. For persistent or grade $\geq$ 2 diarrhoea, infection must be excluded by stool cultures and sigmoidoscopy or colonoscopy is indicated to confirm or rule out colitis (Weber et al. 2012). When ipilimumab was combined with nivolumab, the rate of grade 3/4 diarrhoea was not higher than that detected with ipilimumab alone (Larkin et al. 2019).

In the presence of grade 2 diarrhoea or colitis, treatment with immune checkpoint inhibitors should be withheld and budesonide, a locally acting glucocorticoid 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 $\geq$ 2 gastro-intestinal irAEs (Weber et al. 2009). In patients with severe diarrhoea or colitis (grade $\geq$ 3), ipilimumab should be permanently discontinued. These patients require high-dose intravenous steroids (e.g. methylprednisolone or dexamethasone) or, in case of steroid-refractory colitis, infliximab or, in alternative, vedolizumab, an integrin antagonist that is able to reduce gastrointestinal inflammation by blocking lymphocyte interaction with endothelial cells (Dougan et al. 2021).

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 histological changes observed with ipilimumab-related hepatitis are similar to those detected with acute viral and autoimmune hepatitis (Kleiner and Berman 2012). Combined immunotherapy is associated with statistically significant higher risk of hepatitis compared to patients treated with each single antibody, with about one quarter of patients developing this irAE (Reynolds et al. 2018).

Prompt treatment with glucocorticoids is required with prednisone or methyl-prednisolone. If serum transaminase levels do not decrease within 48 hours after the beginning of systemic steroids, other immunosuppressive agents such as

mycophenolate, azathioprine, or tacrolimus may be required (Weber et al. 2012; Dougan et al. 2021). Unlike colitis, infliximab is contraindicated for the management of hepatitis, since this antibody can cause hepatotoxic effects.

10.5.4 Endocrinopathies

The endocrinopathies provoked by ipilimumab may affect the pituitary gland (panhypopituitarism or hypophysitis), thyroid (thyroid dysfunctions resulting in hypothyroidism that can be preceded by thyrotoxicosis), adrenal glands (primary adrenal insufficiency), and pancreas (type 1 diabetes mellitus) [reviewed in (Wright et al. 2021)]. Among the endocrine dysfunctions induced 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 that need to be excluded. Most patients present with headache, fatigue, asthenia, lethargy, nausea, vertigo, behaviour changes, 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 (Juszczak et al. 2012). The posterior pituitary lobe is rarely affected, resulting in diabetes insipidus. Treatment of endocrine irAEs includes high-dose steroid therapy and appropriate hormone replacement that should be undertaken in consultation with an endocrinologist (Kähler and Hauschild 2011; Weber et al. 2012). Unlike most of the other irAEs, hypophysitis takes a long time to resolve and in many cases persists, requiring lifelong therapy.

Based on pooled data of the CheckMate-067 and CheckMate-069 trials for advanced melanoma, immune-related endocrinopathies were observed more frequently (~30% of patients) when ipilimumab was used in association with nivolumab compared to antibody monotherapies (ipilimumab 12% and nivolumab 11%). Moreover, in about 20% of cases, the thyroid gland was affected (as compared to ~5% with ipilimumab and ~ 10% with nivolumab). Pituitary dysfunction was also reported more frequently with the antibody combination (9% versus 4.2% and 0.4 with ipilimumab or nivolumab monotherapy, respectively). Adrenal insufficiency was observed in 4% of cases, and half of these cases were of grade 3–4 (versus 2% with nivolumab and not reported with ipilimumab in these studies). Endocrine disorders in the combination group generally appeared after 1–4 months from treatment start (Hassel et al. 2017). Type 1 diabetes was also reported in melanoma patients treated with ipilimumab after nivolumab although with a low frequency (Omodaka et al. 2018; Zezza et al. 2019). The higher incidence of endocrinopathies (especially thyroid dysfunction, hypophysitis, and primary adrenal insufficiency) with ipilimumab plus nivolumab as compared to antibody monotherapy was also

confirmed by a meta-analysis of 101 studies involving 19,922 patients (de Filette et al. 2019).

10.5.5 Other irAEs

Immune-related pancreatitis has been reported in less than 1.5% of patients treated with ipilimumab and generally manifested as an asymptomatic increase of amylase and lipase (Attia et al. 2005). Elevated lipase and amylase were also described in about 1% of patients treated with ipilimumab plus nivolumab, occurring after about 7 weeks of treatment (Hassel et al. 2017). Diffuse lymphadenopathy and a sarcoid-like syndrome have been reported anecdotally (Berthod et al. 2012; Vogel et al. 2012; Eckert et al. 2009). Transient peripheral neuropathies, both sensory and motor, associated with ipilimumab, have been noted in less than 1% patients (Weber et al. 2012). A case of acquired haemophilia A was diagnosed in a patient with metastatic melanoma 2 months after the introduction of ipilimumab and was related to ipilimumab therapy (Delyon et al. 2011). In addition, ipilimumab plus nivolumab is associated with an increased risk of all-grade pneumonitis compared to monotherapy (Huang et al. 2019).

10.6 Conclusions

Ipilimumab is the first immune checkpoint inhibitor that has established the clinically relevant role of immunotherapy for cancer treatment. Indeed, this antibody was the first agent to improve the survival of patients with unresectable/metastatic melanoma significantly increasing OS. Before ipilimumab approval, no other agents had demonstrated better results than dacarbazine-based chemotherapy in phase 3 clinical trials. About one-third of melanoma patients achieved clinical benefit from ipilimumab treatment, and some of the responses were long-lasting (~20% 5-year survival). The most impressive property of ipilimumab was represented by the ability of a short-course treatment (4 doses) to increase OS in a subset of heavily pre-treated patients with metastatic melanoma (Hodi et al. 2010).

In the last decade, the therapeutic indications of ipilimumab have been progressed rapidly (Fig. 10.5). After the initial approval as monotherapy for *BRAF*-wild type advanced/metastatic melanoma or after tumour surgical resection (adjuvant), all the other approved indications of ipilimumab require its association with the anti-PD-1 antibody nivolumab. In fact, significantly higher response rate and longer progression-free survival and OS were obtained with nivolumab plus ipilimumab or nivolumab alone compared to ipilimumab, regardless of *BRAF* mutational status. Moreover, this antibody association has shown activity in patients with melanoma brain metastases. The other approved indications of ipilimumab plus nivolumab include, in chronological order, the following: first-line treatment of

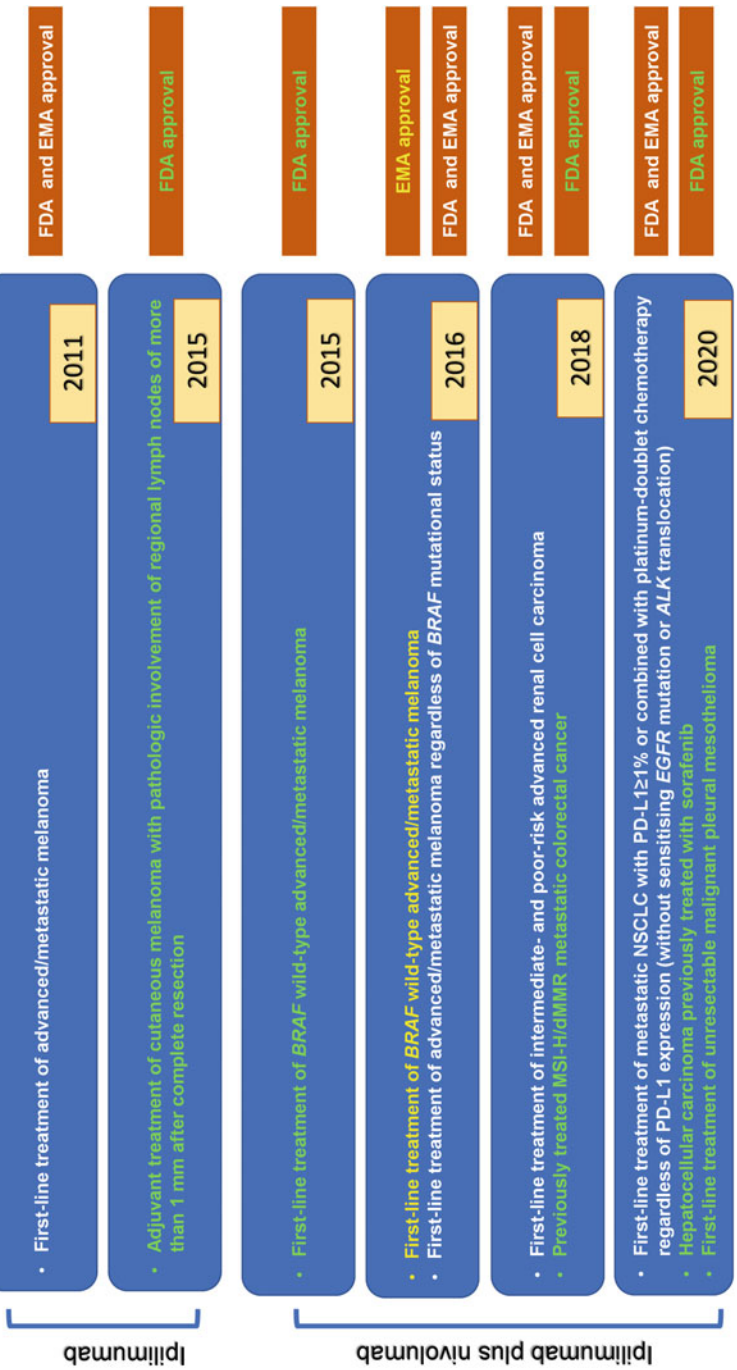

Fig. 10.5 Approved indications of ipilimumab as monotherapy or in combination with nivolumab. See the text for further details

intermediate- and poor-risk advanced renal cell carcinoma, previously treated MSI-H/dMMR metastatic colorectal cancer, hepatocellular carcinoma previously treated with sorafenib, first-line treatment of metastatic NSCLC with PD-L1 $\geq$ 1% or combined with platinum-doublet chemotherapy regardless PD-L1 expression, and first-line treatment of unresectable malignant pleural mesothelioma. In some of these clinical settings, the combination of ipilimumab plus nivolumab has shown clinically meaningful benefit compared to immune checkpoint monotherapy or to chemotherapy, in terms of progression-free survival and/or OS. For instance, besides metastatic melanoma, durable efficacy of the nivolumab plus ipilimumab treatment was reported in patients with renal cell carcinoma, with about 50% of patients being alive at 4 years in the intermediate- and poor-risk population. In NSCLC patients with tumours expressing PD-L1 $\geq$ 1%, ipilimumab plus nivolumab induced longer progression-free survival rates (10.5% versus 4.6% at 2 years) compared to chemotherapy.

However, the improved clinical efficacy of the ipilimumab-nivolumab combination comes to the cost of increased incidence of systemic toxicity, carrying a higher risk of all-grade irAEs compared to single-agent treatment. The immune-related toxicity needs a prompt diagnosis and management according to product-specific guidelines to adequately treat irAE which sometimes can be also life-threatening. The use of a specified treatment algorithm has substantially reduced drug-related deaths and has required an accurate training of physicians who will use this agent and other immune checkpoint inhibitors.

The clinical experience with ipilimumab indicates that patients receiving an immune checkpoint inhibitor 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 anti-CTLA-4 or anti-PD-1/PD-L1 antibodies. 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.

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