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Alexander Birbrair *Editor*

Tumor Microenvironment

Hematopoietic Cells – Part B

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Hematopoietic Cells – Part B

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This book is dedicated to my mother, Marina Sobolevsky, of blessed memory, who passed away during the creation of this volume. Professor of Mathematics at the State University of Ceará (UECE), she was loved by her colleagues and students, whom she inspired by her unique manner of teaching. All success in my career and personal life I owe to her.

My beloved mom Marina Sobolevsky of blessed memory (July 28, 1959–June 3, 2020) and my father Lev Birbrair.

Preface

This book's initial title was "Tumor Microenvironment." However, due to the current great interest in this topic, we were able to assemble more chapters than would fit in one book, covering tumor microenvironment biology from different perspectives. Therefore, the book was subdivided into several volumes.

This book "Tumor Microenvironment: Hematopoietic Cells – Part B" presents contributions by expert researchers and clinicians in the multidisciplinary areas of medical and biological research. The chapters provide timely detailed overviews of recent advances in the field. This book describes the major contributions of different hematopoietic components in the tumor microenvironment during cancer development. Further insights into these mechanisms will have important implications for our understanding of cancer initiation, development, and progression. The authors focus on the modern methodologies and the leading-edge concepts in the field of cancer biology. In recent years, remarkable progress has been made in the identification and characterization of different components of the tumor microenvironment in several tissues using state-of-art techniques. These advantages facilitated identification of key targets and definition of the molecular basis of cancer progression within different organs. Thus, the present book is an attempt to describe the most recent developments in the area of tumor biology which is one of the emergent hot topics in the field of molecular and cellular biology today. Here, we present a selected collection of detailed chapters on what we know so far about the hematopoietic components in the tumor microenvironment in various tissues. Eleven chapters written by experts in the field summarize the present knowledge about distinct hematopoietic components during tumor development.

Fabrizio Mattei and colleagues from Instituto Superiore di Sanità discuss the role of eosinophils in the tumor microenvironment. Karan Kohli and Venu G. Pillarisetty from the University of Washington describe dendritic cells in the tumor microenvironment. Jason B. Williams and Thomas S. Kupper from Harvard Medical School compile our understanding of resident memory T cells in the tumor microenvironment. Camille Guillerey from The University of Queensland updates us with what we know about tumoral NK cells. Caroline Imbert and Daniel Olive from Inserm, France, summarize current knowledge on γδ T cells in tumor microenvironment. Dario A.A. Vignali and colleagues from the University of Pittsburgh School of Medicine address the importance of regulatory T cells in the tumor microenvironment. Huichun

Zhan and Kenneth Kaushansky from Stony Brook School of Medicine talk about the hematopoietic stem cells microenvironment in myeloproliferative neoplasms. Aarthi Rajesh and Merilyn Hibma from the University of Otago focus on Langerhans cells in the tumor microenvironment. Angélica Aponte-López and Samira Muñoz-Cruz from Universidad Nacional Autónoma de México give an overview of the mast cells in the tumor microenvironment. Markus Maeurer and colleagues from Champalimaud Center for the Unknown present the role of B cells in the gastrointestinal tumor microenvironment. Finally, Samuel Cheshier and colleagues from the University of Utah School of Medicine introduce what we know so far about the role of microglia within brain tumors.

It is hoped that the articles published in this book will become a source of reference and inspiration for future research ideas. I would like to express my deep gratitude to my wife Veranika Ushakova and Mr. Murugesan Tamilselvan from Springer, who helped at every step of the execution of this project.

Belo Horizonte, Minas Gerais, Brazil Alexander Birbrair

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Eosinophils in the Tumor Microenvironment

1

Fabrizio Mattei, Sara Andreone, Giancarlo Marone, Adriana Rosa Gambardella, Stefania Loffredo, Gilda Varricchi, and Giovanna Schiavoni

Abstract

Eosinophils are rare blood-circulating and tissue-infiltrating immune cells studied for decades in the context of allergic diseases and parasitic infections. Eosinophils can secrete a wide array of soluble mediators and effector molecules, with potential immunoregulatory activities in the tumor microenvironment (TME). These findings imply that these cells may play a role in cancer immunity. Despite these cells were known to infiltrate tumors since many years ago, their role in TME is gaining attention only recently. In this chapter, we will review the main biological functions of eosinophils that can be relevant within the TME. We will discuss how these cells may undergo phenotypic changes acquiring pro- or antitumoricidal properties according to the surrounding stimuli. Moreover, we will analyze canonical (i.e., degranulation) and unconventional mechanisms (i.e., DNA traps, exosome secretion) employed by eosinophils in inflammatory contexts, which can be relevant for tumor immune responses. Finally, we will review the available preclinical models that could be employed for the study of the role in vivo of eosinophils in cancer.

Keywords

Angiogenesis · Cancer · Cationic proteins · $CD8^+$ T cells · Cytotoxicity · Eosinophil · Exosomes · Extracellular Traps · Immune regulation · Lymphangiogenesis · Mast cell · Mouse models · Tumor Immunity · Tumor Microenvironment · Tumor prognostic value

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List of Abbreviations

AEC	Absolute eosinophils count
ANGPTs	Angiopoietins
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
APCs	Antigen-presenting cells
A. fumigatus	*Aspergillus fumigatus*
BECs	Blood endothelial cells
BAL	Bronchoalveolar lavage
CAFs	Cancer-associated fibroblasts
CEL	Chronic eosinophilic leukemia
DCs	Dendritic cells
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
EPX	Eosinophil peroxidase
E. coli	*Escherichia coli*
EXO	Exosomes
EV	Extracellular vesicles
FGF	Fibroblast growth factor
FPR-1	Formyl peptide receptor-1
HSC	Hematopoietic stem cell
HMGB1	High Mobility Group Box 1
HD	Hodgkin's disease
HES	Hypereosinophilic syndrome
ICIs	Immune checkpoint inhibitors
ILC2	Innate lymphoid cells
IFN	Interferon
IL	Interleukin
iNKT	Invariant natural killer T
LIAR	Local immunity and/or remod eling/repair
LECs	Lymphatic endothelial cells
MBP	Major basic protein
MCA	Methylcholanthrene
mAb	Monoclonal antibody
M-MDSC	Monocytic myeloid-derived suppressor cells
MVB	Multivesicular bodies
MDSC	Myeloid-derived suppressor cells
NK	Natural killer () cells
NO	Nitric oxide
NOG	NOD/Shi-*scid*/IL-2Rγ^{null}
NSCLC	Non-small cell lung cancer
OSCC	Oral squamous cell carcinoma
OS	Overall survival
PRR	Pattern recognition receptor
PlGF	Placenta growth factor
PDGF	Platelet-derived growth factor
PMN-MDSC	Polymorphonuclear myeloid-derived suppressor cells
PD-1	Programmed cell death-1
PSF	Progression-free survival
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
Siglec-8	Sialic-binding immunoglobulin like lectin 8
S. aureus	*Staphylococcus aureus*
Th2	T helper 2
TSLP	Thymic stromal lymphopoietin
T_{REG}	T regulatory cell
TAM	Tumor-associated macrophages
TATE	Tumor-associated tissue eosinophilia
TILs	Tumor-infiltrating lymphocytes
TME	Tumor microenvironment
TK	Tyrosine kinase
VEGF	Vascular endothelial growth factor

1.1 Introduction

Eosinophils are rare blood circulating granulocytic cells representing 1–3% of total leukocyte population under physiological condition. Paul Ehrlich in 1879 first described blood eosinophils by their unique staining properties with acidic dyes, such as eosin and Luxol fast blue [1]. These cells originate and differentiate in the bone marrow in response to IL-5, together with IL-3 and GM-CSF, which support both maturation and survival of eosinophils [2]. In addition, IL-33 sustains eosinophilopoiesis at various levels, promoting survival, maturation, and functional activation [3]. During bone marrow development, IL-33 both expands eosinophil precursors expressing the IL-5Rα and induces systemic IL-5 production, thus fueling the eosinophil maturation [4].

Upon response to certain inflammatory conditions (i.e., allergies, parasitic infections, and autoimmune diseases), eosinophils can rapidly

expand and can infiltrate inflamed tissues, where they play diverse roles in inflammatory responses. Eosinophils are well known to infiltrate the tumor microenvironment (TME), and this condition is referred to as tumor-associated tissue eosinophilia (TATE). The role of TATE in human cancers is still controversial [5, 6]. However, recent clinical observations in melanoma patients undergoing immunotherapy targeting the immune checkpoints CTLA-4 and PD-1 have unraveled a predictive role of eosinophil counts for therapeutic response [7]. These findings suggested that eosinophils might be regarded as possible prognostic/predictive biomarkers in cancer immunotherapy, thus repositioning this immune cell population at the forefront of cancer immunology research.

1.2 The Tumor Microenvironment: A Dynamic System with Multiple Interacting Players

The definition of tumor microenvironment (TME) originates from the dynamic interaction of the host immune system with the forming and growing tumor. This continuously evolving milieu is the result of the constant cross-talk between cancer cells and immune cells through the release of soluble factors that shape the phenotype of both cell types [8]. The TME is composed of a number of resident and nonresident cell types, as well as extracellular factors, and each cell component has a distinct role in this complex scenario [9]. When the TME is in its initial stage, resident tumor cells instruct the TME for the formation of blood vessels that allow the access of nutritive factors, cell-derived vesicles, and immune cells. Pericytes and endocytes, key cellular components of the blood vessel architecture, are considered resident cells in the TME [9] and play a relevant role in angiogenesis. In particular, a type-2 (Nestin$^+$) subset of pericytes has been identified that promotes normal and tumoral angiogenesis [10]. Cancer-associated fibroblasts (CAFs), a heterogeneous subset of several cell types, are resident cells that play an important role in tumorigenesis. These cells produce and release several mediators, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and cytokines, important to generate the 3D stromal architecture of blood vessels and of the TME itself [11].

The immune cells infiltrating the TME in solid cancers are heterogeneous, and their roles depend on the site, grade, and stage of malignancy. This is in part due to the fact that within the TME the patterns of soluble mediators (cytokines, chemokines, angiogenic, lymphangiogenic, and growth factors) and cellular receptors dynamically change and thus influence the homing and phenotype of immune cells [9].

Tumor infiltrating lymphocytes (TILs) are associated with antitumor activity, whose frequency often correlates with a favorable prognosis in cancer patients. In particular, CD8$^+$ T cells are often present as infiltrating cells in solid cancers, where they can exert potent and selective cytotoxic action on tumor cells [12]. However, an important fraction of TILs is represented by regulatory CD4$^+$ T (T_{REG}) lymphocytes with opposite effects on cancer progression. Indeed T_{REG} are endowed with potent pro-tumoral effects when infiltrating the TME and are considered a target for immunotherapeutic strategies [13]. Recently, a novel subset of tissue-resident memory CD69$^+$CD103$^+$ T cells (T_{RM}), either CD4$^+$ or CD8$^+$, has been reported to play a crucial role in preventing the development and spread of solid tumors and has been associated with favorable outcomes in cancer patients. T_{RM} cells may mediate tumor protection by promoting tumor-immune equilibrium through the secretion of cytokines and/or via CD103-enhanced tumor cell killing [14]. Natural killer (NK) cells, an innate immune subset with potent cytotoxic function, also contribute to tumor rejection [15].

Several dendritic cell (DC) subsets may be found in variable frequencies in the TME of various solid cancers, where they are deputed to tumor antigen (Ag) presentation and cross-presentation in lymphoid organs and in the TME itself [16]. Certain chemotherapeutic drugs pro-

mote the release of immunogenic signals from dying tumor cells, which are perceived by DC and promote a cascade of events that stimulate an anticancer immune response [17]. Among these signals, the ligand Annexin-a1 released by dying tumor cells was shown to bind formyl peptide receptor-1 (FPR-1), expressed by DC, acting as signal for the correct positioning of DC in proximity of dying cancer cells within TME. This Annexin-a1/FPR-1 axis enabled stable DC-corpse interactions, and subsequent engulfment and Ag cross-presentation by DC [18]. Ag cross-presentation for $CD8^+$ T-cell cross-priming is mainly carried out by Batf3- and Irf8-dependent type 1 conventional DCs, a subset of DC expressing the markers CD103 and CD8α [19]. $CD8^+$ T-cell cross-priming is promoted by type I IFNs signaling on CD8α DC and is required for antitumor immunity in vivo [20]. Type I IFNs act on CD8α DC prolonging Ag retention after engulfment of tumor apoptotic cells leading to efficient $CD8^+$ T-cell cross-priming [21]. In addition, DC can interact with innate and innate-like immune cells, including NK, invariant natural killer T (iNKT), and γδ T cells, amplifying direct and indirect antitumoral responses through a mutual cross-talk [22]. On the other hand, some tolerogenic DC contribute to the generation of T_{REG} and engage in a cross-talk, thus favoring the establishment and maintenance of an immunosuppressive TME that inhibits antitumor immunity [23].

Myeloid cells represent a major fraction of infiltrating immune cells. Tumor-associated macrophages (TAM) play a major role in tumor progression. TAM are distinguished into two major subsets: classically activated M1 with antitumor functions and pro-inflammatory M2 that supports tumor progression [24]. The balance of frequencies in infiltrating M1 and M2 TAMs often dictates the tumor fate and is a prognostic factor for patients [25]. Myeloid-derived suppressor cells (MDSC) are immature-like myeloid cells capable of strong immunosuppressive activity. Based on their phenotype marker expression and morphology, these cells can be subdivided into two subgroups, monocytic MDSC (M-MDSC) and granulocytic or polymorphonuclear MDSC (PMN-MDSC) due to their morphological (but not functional) resemblance with monocytes and granulocytes, respectively [25]. Both types of MDSC infiltrate the TME, where they act as potent suppressors of $CD4^+$ and $CD8^+$ T lymphocytes while favoring recruitment of T_{REG} cells. In addition, MDSC promote tumor cell stemness, angiogenesis, and metastasis [25]. Mast cells, [26], neutrophils [27], eosinophils [6], and basophils [28], historically recognized for their involvement in allergy and inflammation, are now being repositioned for the recently discovered role in cancer. The function and role of eosinophils within the TME will be covered in detail below.

1.3 General Properties of Eosinophils

For many years, eosinophils have been mostly appreciated for two aspects of immune response: the ability to fight parasites and their contribution to allergic inflammation [29, 30]. This is because eosinophils produce a wide array of toxic granule proteins and pro-inflammatory mediators that lead to tissue damage [31]. Indeed, eosinophils exert potent cytotoxic functions through the production and release of cationic proteins, such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxide (EPX), and eosinophil-derived neurotoxin (EDN). Furthermore, eosinophils secrete a wide array of soluble mediators, including cytokines, chemokines, and angiogenic and lipid mediators, contributing to immune regulation, tissue remodeling, and many other processes [30].

Eosinophil degranulation can occur via different cellular mechanisms [32]. Eosinophils adherent to parasites have been shown to degranulate through classical exocytosis, a process involving granule fusion with the plasma membrane that creates a pore through which the total granule content is secreted into the target cell. In contrast, piecemeal degranulation enables the release "piece-by-piece" of specific granule-stored proteins, such as cytokines and chemokines, and is thought to be the main secretion mode during chronic inflammatory responses. As a third mode

of secretion, eosinophils may undergo cytolysis, a process involving extracellular release of intact granules with rupture of plasma membrane. Eosinophils may also undergo cytolytic cell death with extrusion of nuclear materials, such as histones and DNA, and extracellular expulsion of intact granules entrapped in DNA nets, named DNA traps [33, 34].

According to the *LIAR* hypothesis formulated by James Lee, eosinophils are homeostatic cells that regulate Local Immunity And/or Remodeling/Repair during both steady state conditions and disease, especially associated with tissue injury [35]. Hence, besides the destructive effects, eosinophils also participate in resolution of inflammation, tissue repair, remodeling, and homeostasis, through the release of a variety of pro-fibrotic (i.e., TGF-β), growth factors (i.e., FGF-2, NGF, and VEGF), and matrix metalloproteinases. In addition, eosinophils participate in the modulation of adaptive immune responses [36]. Eosinophils can induce the recruitment of Th2 cells [37] and T_{REG} cells [38] through the production of the chemokines CCL17 and CCL22. Moreover, eosinophil-derived CXCL9 and CXCL10 recruit Th1 cells [37, 39] and $CD8^+$ T cells [40–42]. Eosinophil cationic proteins can have immunostimulatory activities; for example, EDN can both attract and induce the maturation of DC into a Th2-promoting phenotype [43]. Stimulation of eosinophils with CpG-ODN results in degranulation and induction of DC maturation in a cell contact independent manner via MBP [44]. Furthermore, EPX activates DC in vitro and in vivo, inducing mobilization to lymph nodes and Th2 priming [45]. Eosinophils play an active role in the induction and expansion of Th2 type of immune response, through the production of IL-4, IL-5, IL-13, and IL-25 participating in allergic reactions, parasitic infections, and autoimmune disorders [46]. Eosinophils can also produce, store, and secrete Th1-associated pro-inflammatory cytokines (i.e., IFN-γ, TNF-α and IL-12) and T_{REG}-associated mediators (i.e., IDO, IL-10, and TGF-β), thus demonstrating their versatile immunoregulatory role [46]. Following activation with cytokines, such as GM-CSF, IL-4, IL-5, or IFN-γ, eosinophils upregulate MHC class II and co-stimulatory molecules (CD80, CD86, and CD40) and can act as non-professional Ag presenting cells (APC) stimulating Ag-specific $CD4^+$ T-cell proliferation and Th2 cytokine production in vitro and in vivo [47].

Eosinophils are equipped with a variety of surface receptors fundamental for their function and localization within inflamed tissues [30]. These include pattern recognition receptors (PRRs), such as TLR1–5, TLR7, TLR9, NOD1, NOD2, Dectin-1, and RAGE, that recognize specific molecular components associated with pathogens or danger signals and allow a rapid pro-inflammatory response to insults through production of cytokines, chemokines, and granule cationic proteins [48]. Eosinophils also express receptors for many cytokines (IL-2R, IL-3R, IL-4R, IL-5Rα, IL-9R, IL-10R, IL-13R, IL-17R, IL-23R, IL-27R, IL-31R, IL-33/ST2, TSLPR, GM-CSFR, IFNγR, TGF-βR), chemokines (CCR1, CCR3, CCR4, CCR5, CCR6, CCR8, CCR9, CXCR2, CXCR3, CXCR4), formyl peptide receptors-1, −2, −3, and a variety of integrins and adhesion molecules (CD11a/CD18, CD11b/CD18, CD11c/CD18, CD49d/CD29, CD49f/CD29, ICAM-1), which drive eosinophil transmigration from the bloodstream to inflamed tissues [49, 50].

Eosinophils express various receptors for immunoglobulins, complements, proteases, and lipid mediators, such as leukotrienes and prostaglandins. Sialic-binding immunoglobulin-like lectin 8 (Siglec-8) and its ortholog murine Siglec-F are hallmark receptors for eosinophils that function as inducers of apoptosis associated with reactive oxygen species (ROS) production following antibody cross-linking [51, 52], especially when eosinophils are pre-activated with cytokines [53, 54]. Administration of Siglec-F mAb in vivo results in selective ablation of blood and tissue eosinophils in mice through induction of apoptosis [55]. EGF-like module containing mucin-like hormone receptor-1 (EMR1), the human ortholog of mouse F4/80, is a receptor highly specific to mature human eosinophils [56]. Targeting EMR1 with a specific mAb enhanced NK-mediated killing of human eosinophils

in vitro and induced eosinophil depletion in monkeys [57]. In addition, eosinophils express various adhesion molecules and integrins (i.e., CD49d/29, CD49f/29, CD11b/18, CD11a/18, CD11c/18, CD11d/18, and CD49d/β7) that are upregulated upon activation and mediate eosinophil migration and effector functions [58]. Overall, these features endow eosinophils with multiple roles as effectors and regulators of different immune responses.

1.4 Eosinophils in Allergic Diseases and Infections

Eosinophils play a prominent role in Th2-related pathologies, and tissue eosinophilia is associated with inflammation in respiratory allergies, atopic dermatitis, eosinophilic esophagitis, and gastroenteritis [30]. Allergic asthma is often associated with skewing of naïve Th cells toward Th2 phenotype and activation of eosinophils. In the latter condition, referred to as eosinophilic asthma [59], eosinophils are recruited in the airways by Th2 cytokines (i.e., IL-5) and chemokines (i.e., eotaxin-1/CCL11). Airway epithelial cells activated by different stimuli (e.g., allergens, superallergens, viral and bacterial proteins, tobacco smoke, and so on) release the alarmins IL-25, IL-33, and TSLP that promote Th2 polarization with massive production of IL-4, IL-5, and IL-13 [60]. Recent evidence suggests that these alarmins stimulate type 2 innate lymphoid cells (ILC2), which also secrete IL-4, IL-5, and IL-13 and subsequently recruit eosinophils to the inflamed tissue [61].

Eosinophils are key players in airway inflammation contributing to the so-called T2 asthma pathogenesis by damaging the epithelium and orchestrating the immune response [62]. It is believed that the pathophysiologic effects of eosinophils in allergic inflammation are caused by the release of cationic proteins, ROS, lipid mediators, proteases, and pro-inflammatory cytokines. The mechanisms triggering eosinophil degranulation in inflamed tissues are not fully understood. However, recent evidences have shown that epithelial cell-derived alarmins may play a role. In mouse models of allergic asthma, accumulation of eosinophils in the lung and ensuing allergic inflammation are strongly inhibited by blockade of IL-33/ST2 signaling pathway [63–66], while they are exacerbated by administration of recombinant IL-33 [67]. In patients, a rare IL-33 loss-of-function causes reduced number of eosinophils in blood and protects against asthma [68]. Furthermore, IL-33 is a potent activator of eosinophils in vitro, enhancing adhesion and promoting degranulation [69–71]. These data strongly suggest that IL-33 not only drives eosinophilia but also stimulates eosinophil effector functions in allergy. TSLP, another epithelial-derived alarmin involved in allergic inflammatory response [60], can promote degranulation and survival of eosinophils through STAT5 phosphorylation [72]. In mice, intradermal administrations of TSLP resulted in the induction of a systemic Th2-skewed inflammatory response, which was dependent on the presence of eosinophils [73]. A monoclonal antibody (mAb) targeting TSLP (tezepelumab) is currently under development for the treatment of different forms of severe type 2 asthma (i.e., eosinophilic and non-eosinophilic) [74, 75].

A canonical function of eosinophils is to provide protection against parasitic helminths. Eosinophils also participate in the host defense against other pathogens, such as bacteria, viruses, and fungi [49, 76]. The mechanisms accounting for the antiparasitic role of eosinophils in vitro include direct killing through the release of cytotoxic proteins (MBP, EPX, ECP, EDN) [77, 78]. Furthermore, eosinophils can present parasite-specific Ags to T cells in vivo, leading to the polarization of Th2 response and increase of Ag-specific IgM concentration [79]. However, studies in mouse models of helminth infection have yielded contrasting results, and the role of eosinophils in host response to parasites in vivo remains controversial [49]. In vitro, eosinophils adhere to the fungus *Alternaria alternata* by binding of the integrin CD11b to β-glucan, a component of the fungal cell wall, resulting in degranulation and release of the cationic proteins MBP and EDN [80]. In 1978, DeChatelet and coworkers first reported a bactericidal activity of

eosinophils, showing that these cells can phagocytize *Escherichia coli* or *Staphylococcus aureus* as efficiently as neutrophils via hydrogen peroxide production [81]. Furthermore, human eosinophils can release extracellular DNA traps in response to *Aspergillus fumigatus* in vitro, via a cytolytic mechanism that depends on the Syk tyrosine kinase pathway and CD11b [82]. Eosinophils exploit extracellular traps of mitochondrial DNA and granule proteins also to kill bacteria both in vitro and in vivo [34]. These catapult-like released traps protected from microbial sepsis in a model of intestinal inflammation, unravelling the importance of eosinophils for maintaining the intestinal barrier function after inflammation-associated epithelial cell damage.

Eosinophils play a role in host defense against single-stranded RNA viruses, such as respiratory syncytial virus (RSV), by exploiting the ribonuclease activity of the granule proteins EDN and ECP [83]. In vitro, degranulation of eosinophils following RSV-infected pulmonary epithelial cells is dependent on CD18-mediated cell contact [84]. In addition, binding of rhinovirus to eosinophils via ICAM-1 causes phenotypic activation of eosinophils promoting their ability to present viral Ags to Ag-specific T cells, causing T-cell proliferation and secretion of IFN-γ [85]. In vivo, eosinophils promote the clearance of RSV by stimulation of the TLR-7-MyD88 pathway, which triggers degranulation and expression of IRF-7, IFN-β, and iNOS [86]. Furthermore, infection of eosinophils with pulmonary viruses can result in the release of proinflammatory mediators, such as IL-6, IP-10, CCL2, and CCL3 [87]. The precise mechanisms by which eosinophils interact with viruses and contribute to host antiviral immunity remain to be clarified.

1.5 Repositioning Eosinophils in Cancer

The increase of eosinophils in cancer patients has been known for over a century. Pioneering studies in the 1980s described tumor-infiltrating eosinophils in human gastric cancers and suggested their good prognostic value for prolonged survival [88, 89]. In addition, eosinophils were reported to exert cytotoxic function against breast cancer cells in vitro [89], and blood eosinophil counts inversely correlated with risk of recurrent disease in breast cancer patients [90]. In cancer patients undergoing immunotherapy with IL-2, eosinophils were expanded and acquired an activated phenotype, with increased degranulation, survival, and antitumor cytotoxicity [91–93]. Despite these early evidences supporting the presence of eosinophils in TME of many human cancers, the role of eosinophils in cancer has been largely overlooked for long time. Recent data indicate that these cells are potent immune effectors and regulators within the TME with potential prognostic/predictive role in human cancers.

1.5.1 Role of Eosinophils in Hematologic Tumors

The role of eosinophils in hematologic tumors is still unclear. Andersen and coworkers showed that the eosinophil counts below 0.16×10^9 /L can be associated with acute myeloid leukemia and myelodysplastic syndrome. By contrast, eosinophil counts above 0.16×10^9 /L were associated with myeloproliferative neoplasms [94]. Several studies have reported the association of peripheral blood and tissue eosinophilia with Hodgkin's disease (HD) [95]. Eosinophilia in peripheral blood is associated with a positive prognostic factor [96]. Eosinophils can be found in lymph nodes of HD patients, but their prognostic relevance remains controversial [95]. It is unclear why tissue eosinophilia can be found only in some subsets of HD patients. Tumor cells can produce different eosinophil-attracting molecules such as IL-5 and eotaxins [97, 98]. In nodular sclerosing Hodgkin's disease, tissue eosinophilia was shown to represent a poor prognostic indicator. In fact, binding of eosinophil-secreted CD30 ligand to CD30 on tumor cells was shown to trigger NF-kB activation and consequent proliferation of tumor cells [99].

Verstovsek and coworkers highlighted the efficacy of alemtuzumab in the treatment of

patients affected by hypereosinophilic syndrome (HES) or chronic eosinophilic leukemia (CEL) refractory to standard therapies [100]. Alemtuzumab induced a relevant decline of the absolute eosinophilic count and the percentage of eosinophils in the peripheral blood in all 11 patients examined. This study demonstrated that alemtuzumab decreased blood eosinophils and improved patients with HES and CEL refractory to standard therapy. These case studies suggest, on one hand, that resistance to standard therapy may be in part due to the hypereosinophilia and, on the other hand, that treatment of these refractory patients with alternative therapies may be successful and is partly associated with a decrease of blood eosinophilia [100]. These data have been confirmed by Strati and coworkers, who reported that alemtuzumab is a useful treatment for CEL patients with hypereosinophilia [101].

1.5.2 Role of the Eosinophils in Solid Tumors

Several studies have shown an improved prognosis of patients with TATE or evidence of eosinophil degranulation in various types of solid tumors. Caruso and coworkers described TATE in human gastric adenocarcinoma, with tumor cells in close proximity to eosinophils displaying signs of autophagic cell death [102]. The same group described the presence of degranulating eosinophils in human advanced gastric carcinoma. In these ultrastructural studies, deposition of extracellular granules from apoptotic eosinophils either free in the tumor stroma [103] or within the cytoplasm of gastric carcinoma cells was observed [104].

An antitumoral role of eosinophils has been described in colon cancer, melanoma, lung cancer, and oral squamous cell carcinoma [6]. In melanoma mouse models, eosinophils play a clear antitumoral role both in restraining tumor growth and in preventing lung metastasis onset [40, 41, 105]. Lucarini et al. demonstrated the positive influence of eosinophils on CD8+ T cells for the local tumor, and their tumor cytotoxicity activity on lung metastasis [41]. Importantly, eosinophil count in peripheral blood is correlated with a good prognosis in patients with metastatic melanoma undergoing immunotherapy with immune checkpoint inhibitors targeting CTLA-4 [106, 107] or PD-1 [108]. Furthermore, in a study involving 173 patients, it was shown that peripheral blood eosinophilia is a good prognostic marker correlating with prolonged survival in patients with metastatic melanoma independently from any treatment [109].

In mouse and human colorectal cancer, Munitz and colleagues elegantly demonstrated an antitumorigenic role of eosinophils during tumor development [110]. By analyzing human biopsies, they found an inverse correlation between tumor stage and intratumoral eosinophil counts. In $Apc^{min/+}$ mice, which develop spontaneous intestinal adenomas, eosinophils were recruited into tumors during induction of inflammation-induced colorectal cancer and played an essential role in tumor rejection, independently of $CD8^+$ T cells [110].

In non-small-cell lung cancer (NSCLC), a recent report by Tanizaki and colleagues showed an important correlation between peripheral blood eosinophil counts and patients' survival. They showed that an increase in absolute eosinophil count (AEC) ($\geq$ 150 /μl) is linked to a better progression-free survival (PSF) and overall survival (OS) in patients treated with nivolumab, an anti-PD-1 mAb. For this reason, they suggested that absolute eosinophil count can be a biomarker for this kind of treatment [111]. Dorta and colleagues reported an antitumor role of eosinophils in oral squamous cell carcinoma (OSCC). Indeed, they demonstrated that patients with a lower number of TATE have lower probability to survive [112].

By contrast, elevated eosinophils in the TME seem to be correlated with poor survival in cervical carcinoma patients [113]. This pro-tumoral activity of eosinophils may be related to the microenvironment of this type of cancer. In this regard, eosinophils may be polarized to a phenotype that promotes tumor growth and reduces tumor cell death [114]. The ensemble of these evidences suggests that the role of eosinophils in tumorigenesis is cancer dependent. Alternatively,

as discussed below, different subsets of eosinophils may exist that play divergent roles in tumorigenesis, depending on the tumor histotype.

1.6 Antitumoral Mechanisms of Eosinophils

Tumor-infiltrating eosinophils have the potential to control tumor progression, exerting direct and indirect antitumoral activities, through secretion of a variety of soluble mediators. Recruitment of eosinophils to the TME can be driven by several chemokines (eotaxin-1/CCL11, eotaxin-2/CCL24, eotaxin-3/CCL26, and RANTES) that activate the CCR3 receptor highly expressed on eosinophils [6]. The alarmin IL-33, locally expressed by epithelial and tumor cells, can also promote eosinophil recruitment through stimulation of tumor-derived eosinophil-attracting chemokines [71]. IL-33, together with IL-5, may also prolong the life span of eosinophils at site of tumor growth [6].

Eosinophils accumulate early within tumor necrotic areas of experimental tumors, and this event is accompanied with degranulation and release of MBP and EPX [115, 116]. In fact, danger signals released by necrotic cells, particularly High Mobility Group Box 1 (HMGB1), can induce eosinophil migration, adhesion, survival, and degranulation with release of granule cationic proteins and ROS that promote oxidation and thus inactivation of necrotic material and tumor-promoting inflammation [116]. Moreover, eosinophil-derived MBP can inhibit the activity of heparanase, an endoglycosidase involved in remodeling the extracellular matrix that enhances tumor growth, angiogenesis, and the formation of metastasis [117].

1.6.1 Direct Antitumor Activity of Eosinophils

Eosinophils are equipped with granule proteins endowed with potent cytotoxic activity [118]. MBP can mediate tumor toxicity through disruption of membrane lipid bilayers [119]. ECP is cytotoxic for Hodgkin lymphoma tumor cells [120], inhibits the proliferation of oral squamous cell carcinoma [121], and induces apoptosis of bronchial epithelial cells by caspase-8 activation [122]. Furthermore, EPX and EDN exhibit cytolytic activity against human colorectal carcinoma cell lines [123]. These findings support the hypothesis that eosinophil cationic proteins can exert direct antitumor activities. Vadas and coworkers reported that exposure of eosinophils to CSF or GM-CSF activates antibody-dependent cell-mediated cytotoxicity (ADCC) against EL-4 and BW thymoma cells, as well as P815 mastocytoma cells. Importantly, eosinophils needed direct contact with P815 cells to induce killing, suggesting a contact-dependent cytotoxicity [124]. Murine eosinophils also could induce apoptosis of A20 murine lymphoma cells through the release of granzyme B [125]. Furthermore, eosinophils activated by cross-linking of the 2B4/CD244 receptor exhibited tumoricidal activities against human B lymphoma cells [126]. In addition, human eosinophils can directly kill human colon carcinoma cells via release of granzyme A in a mechanism dependent on the integrin CD11a/CD18 and on IL-18 [123, 127]. These in vitro results suggest that tumor cytotoxic function of eosinophils require cell–cell contact through various adhesion molecules and integrins.

Whether eosinophils mediate tumor cytotoxicity in vivo remains to be demonstrated. Presence of degranulating eosinophils and of eosinophil-specific granules within tumor cytoplasm has been reported in human gastric cancer biopsies [103, 104]. In mouse melanoma models, immunotherapy with the “alarmin” IL-33 promotes the expansion and tumor infiltration of eosinophils, which play an essential role in antitumor responses mediated by IL-33 and prevents the onset of pulmonary metastasis. Terminal differentiation of bone marrow-derived eosinophils with IL-33 results in the generation of highly activated cells, compared to classical eosinophils differentiated with IL-5, revealed by increased aggregation in clusters (Fig. 1.1). IL-33-activated eosinophils highly resembled pulmonary eosinophils recruited by IL-33 in vivo and exhibited upregulation of granzyme B and potent tumor

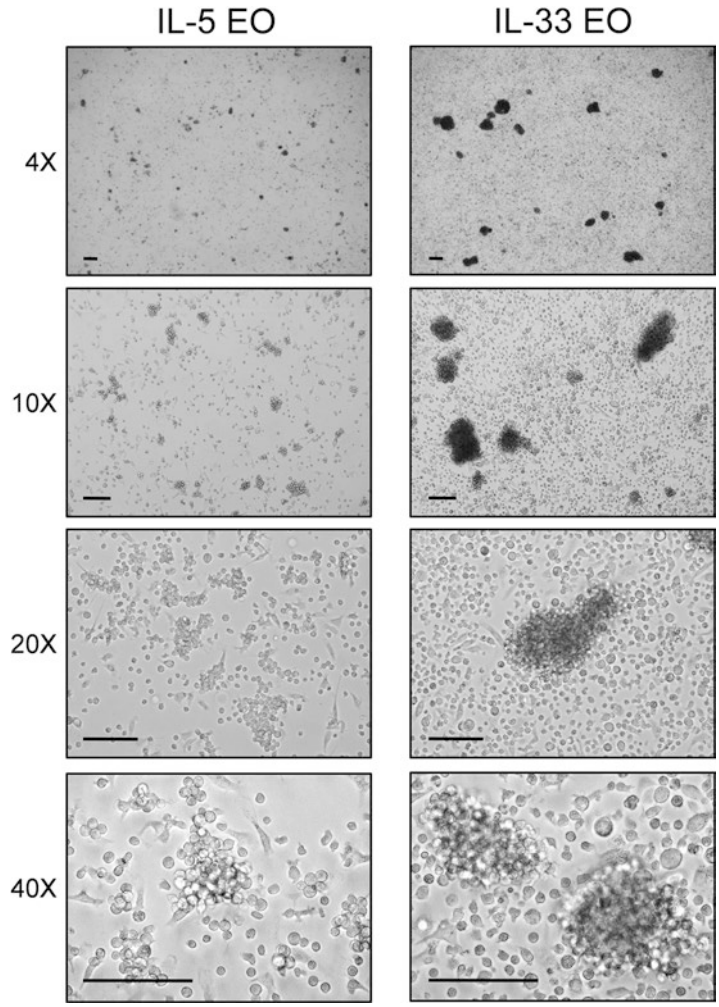

Fig. 1.1 Terminal differentiation of bone marrow–derived eosinophils with IL-33 yields highly activated cells. Bone marrow cells from tibiae and femurs of C57Bl/6 mice were cultured for 4 days in a medium containing 100 ng/ml SCF and 100 ng/ml FLT-3 L, followed by 10 ng/ml IL-5. From day 10, cells were supplemented every other day with either IL-5 or IL-33 (100 ng/ml) in order to generate IL-5 eosinophils (IL-5 EO) or IL-33 eosinophils (IL-33 EO), as described previously [36]. On day 16, when fully differentiated eosinophils were generated, microphotographs were obtained by EVOS-FL microscope. Images at the indicated magnifications show IL-33 EO aggregating in clusters, as opposed to IL-5 EO, indicative of highly activated phenotype. Scale bars, 100 μm

cytotoxicity in vitro [6, 41]. Furthermore, IL-33-activated eosinophils are able to establish a large number of cell conjugates with different tumor cell lines (B16.F10 melanoma, MC38 colon carcinoma, TC-1 lung adenocarcinoma and MCA205 fibrosarcoma) leading to efficient tumor cell killing in vitro and in vivo [71]. IL-33 promoted the tumoricidal functions of eosinophils in a cell adhesion-dependent manner through the integrin CD11b/CD18 and by inducing lytic granule convergence, with polarization of eosinophil effector proteins (ECP, EPX, and granzyme B) to the tumor-eosinophil immune synapse [71], in a similar mechanism operated by NK cells [128]. These observations demonstrate that IL-33 can potently stimulate eosinophil-dependent direct tumor cell killing by targeted degranulation, as schematized in Fig. 1.2.

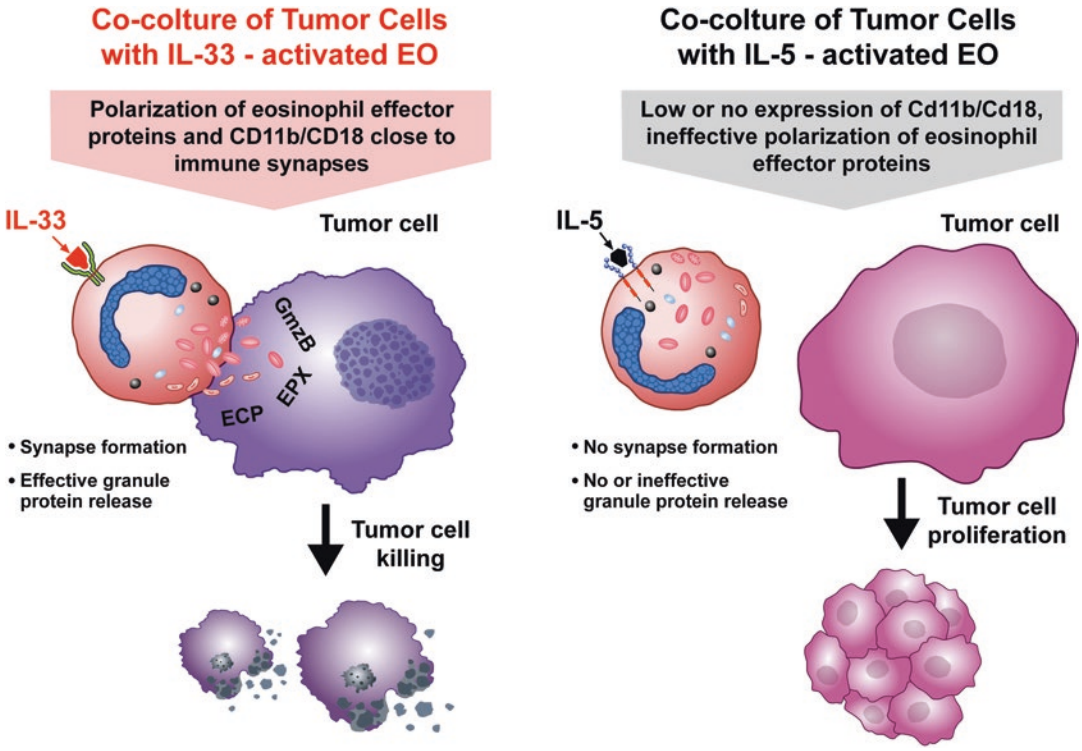

Fig. 1.2 IL-33 promotes the activation of CD11b/CD18 adhesion-dependent granule polarization in eosinophils within the immune synapse. Upon coculture with tumor cells, eosinophils (EO) activated with IL-33 through its specific receptor complex ST2/IL1RAP form stable EO-tumor cell conjugates. This event is mediated by CD11b/CD18-dependent adhesion and synapse-polarized degranulation of eosinophil toxic proteins (EPX, ECP, granzyme B), resulting in efficient tumor cell killing. By contrast, activation of eosinophils with IL-5, through its receptor complex IL5RA/IL3RB subunit, fails to induce tumor cell adhesion and subsequent degranulation, thus sustaining tumor cell proliferation

1.6.2 Indirect Antitumoral Function of Eosinophils: Interaction with Other Immune Cells within the TME

Studies in preclinical models indicate that tumor-infiltrating eosinophils may affect indirectly tumor growth. In mouse models of melanoma, it has been shown that infiltrating eosinophils promote the recruitment of tumor-reactive $CD8^+$ T cells through expression of the T-cell-attracting chemokines CCL5, CXCL9, and CXCL10 [40, 41]. Another indirect antitumor mechanism operated by eosinophils is their ability of influencing the tumor angiogenesis in TME. Human eosinophils can produce in vitro several proangiogenic molecules [129–132]. In vivo eosinophils induce vessel normalization by increasing the expression of adhesion molecules, such as VCAM-1, and by polarizing TAM toward M1-like macrophages, which produce smaller amounts of pro-angiogenic factors, compared to M2 macrophages [40]. Finally, as discussed above, eosinophils may function as nonprofessional APC, although whether they do so within the TME remains to be demonstrated.

Both solid and hematologic tumors are associated with the accumulation of peritumoral and/or intratumoral mast cells, suggesting that these cells can help to promote and/or limit tumorigenesis [133]. Interestingly, human mast cells and eosinophils were both identified and named by Paul Ehrlich [134, 135]. These cells have distinct progenitors and differ morphologically, ultra-

structurally, immunologically, and biochemically. However, mast cells and eosinophils can form the "allergic effector unit" and can be found in proximity in TME of several tumors [135]. Therefore, it is likely that eosinophils have the capacity to modulate mast cell functions and vice versa. For example, ECP and MBP [136] and VEGFs released by activated eosinophils [137] can modulate mast cell functions. These bidirectional interactions between eosinophils and mast cells and vice versa might be relevant in TME.

1.7 Functional Plasticity of Eosinophils in Cancer

Eosinophils display the potential to interact with the tumor moiety. This feature stems from the ability of eosinophils to change their phenotype in response to stimuli present in the TME, such as cytokines, inducing variable responses. In addition, eosinophils have the capacity to release extracellular vesicles, which may shape the TME. For these reasons, eosinophils can be considered as cells endowed with a certain functional plasticity constantly remodeling the TME.

1.7.1 Role of Cytokines in Shaping Eosinophil Phenotype within the TME

It has been suggested that at least four subsets of murine eosinophils exist depending on their tissue localization, maturation, and type of immune response triggered [138]. The first subset is represented by eosinophil progenitors, which are immature eosinophils undergoing hematopoiesis in situ. They express the receptors for IL-5, IL-33, and TSLP, the latter two regulating eosinophil homing to inflamed tissues and activation. Steady-state or tissue-resident eosinophils, which were only characterized in the lung parenchyma of mice, are resting cells expressing intermediate levels of Siglec-F and with donut-shaped nucleus. A third subset (i.e., type 1 eosinophils) was described as interstitial/stromal cells in morphogenetic and type 1 immunity contexts. These eosinophils display similar surface markers as steady-state eosinophils, such as Siglec-F^{int}, but have pluri-lobated nucleus without vacuolization. The fourth subset (i.e., type 2 eosinophils) is characterized in the epithelium in type 2 immunity contexts, such as allergic asthma and chronic colitis. These eosinophils exhibit pluri-lobated nucleus, vacuolized cytoplasm and high expression of Siglec-F. The relative roles of these subsets of eosinophils in cancer immunity are unknown. This is because these cell types have been characterized mainly morphologically and by the expression of some surface markers. Thus, gene expression profiles and single-cell RNA sequencing of tumor-infiltrating eosinophils in various settings may help to define these cells in relation to their functional (pro- or antitumorigenic) role.

Some studies have reported that cytokines may shape the phenotype of eosinophils, determining their polarization and tumor immune responses induced. Reichman and colleagues reported that in experimental colorectal cancer, intratumoral eosinophils exhibit an IFN-γ-related signature, which prevented the development of colorectal cancer in mice. Furthermore, activation of resting peritoneal eosinophils with IFN-γ potentiated their ability to kill colorectal cancer cells in vitro [110]. Similarly, activation of eosinophils with IFN-γ plus TNF-α induced upregulation of T-cell-attracting chemokines (CCL5, CXCL9, and CXCL10), IFN-γ, TNF-α, and NOS2. These activated eosinophils reduced tumor growth, through recruitment of tumor-reactive CD8 T cells, when adoptively transferred in melanoma-bearing mice [40]. These data suggest that IFN-γ may skew eosinophils toward a type 1 immunity-promoting phenotype.

IL-33 is another important cytokine that may affect eosinophil phenotype within the TME. In experimental tumors, IL-33 promotes antitumor immunity through expansion and activation of eosinophils in vivo [41, 105]. In melanoma-bearing mice, depletion of eosinophils by an anti-Siglec-F antibody injections abrogated the therapeutic efficacy of IL-33, indicating that eosinophils were indispensable for IL-33 antitumoral function [41]. Exposure to IL-33 in vivo

induced in tumor-infiltrating eosinophil gene transcripts, which differ in the primary tumor site and pulmonary metastasis. At the primary tumor site, IL-33-recruited eosinophils expressed T-cell-attracting chemokines (CCL5, CXCL9, and CXCL10), but not effector molecules. Conversely, at the pulmonary site, eosinophils expressed high levels of granzyme B and IFN-γ, but not T-cell-attracting chemokines. These findings suggested indirect or direct antitumor functions of eosinophils at the primary or pulmonary site, respectively. Furthermore, in vitro activation of eosinophils with IL-33 resulted in upregulation of effector molecules (i.e., granzyme B), Th-1 (i.e., IL-12, TNF-a), and Th-2 cytokines (i.e., IL-10, IL-13) [41, 71]. Thus, IL-33 may polarize eosinophils to a mixed type 1 and 2 immunity phenotype that promotes antitumoral function.

Several lines of evidence indicate that IL-5 may not support antitumor reactions in eosinophils. An early study showed that IL-5 gene-transfected tumors promote eosinophil recruitment but not antitumor immunity [139]. In a Meth-A fibrosarcoma model, intratumoral injection of OK-432 (a derivative of penicillin) and fibrinogen induces local production of IL-5 that recruits in the tumor tissue eosinophils which, however, did not play a relevant role in tumor regression [140]. In a different model, IL-5 could facilitate experimental lung metastasis of various cancer cells by creating an allergic inflammatory environment with CCL22-producing eosinophils that recruited T_{REG} cells [38]. By contrast, IL-5 plays a major role in driving the recruitment of eosinophils at primary and metastatic sites, promoting antitumor responses in models of hepatocellular carcinoma [141], methylcholanthrene-induced fibrosarcoma [98], and melanoma [142]. These apparently contrasting findings are compatible with the hypothesis that IL-5 can play distinct or even opposite roles in modulation of tumorigenesis. The role of IL-5 in shaping the phenotype of eosinophils in TME from different cancers needs to be addressed more extensively. Fig. 1.3 summarizes the possible role of subsets of eosinophils in the TME and their modulation by cytokines (i.e., IFN-γ, IL-5, IL-33).

1.7.2 Role of Extracellular Vesicles in the Biological Activity of Eosinophils within the TME

There is compelling evidence that eosinophils can release exosomes (EXO) and other extracellular vesicles (EV). EXO represent a small population of vesicles produced by any kind of cell and reflect the molecular signature (made up of lipids, nucleic acids, and proteins) of their producing cells. Through transfer of their bioactive molecules from the cell of origin to the target cell or tissue, EXO contribute to intercellular communication and represent an important diagnostic biomarker in pathological conditions [143].

Although intercellular communication appears to be the one of the most important functions of EV, they also have specific molecules related to their biogenesis. In fact, EXO have been defined by their size, density, and expression of specific biomarkers such as proteins involved in targeting and adhesion (tetraspanins, integrins, adhesion molecules), multivesicular bodies (MVB) biogenesis and secretion-associated proteins (ALIX, Rab GTPase), chaperone proteins, and others. The distinction between eosinophilic granules and EV becomes increasingly difficult, due to many shared molecules expressed, such as CD63 [144], and to the fact that granules can also be found intact extracellularly as membrane-bound, ligand-responsive structures [145]. Eosinophil EXO include/contain a series of cationic proteins (MBP, EPX, EDN, ECP), miRNA, mRNA, cytokines, chemokines, enzymes, and lipid mediators whose activities can mediate and autoregulate eosinophil biological functions. EXO-stored products are released from eosinophils through different mechanisms: classical exocytosis providing for exosome fusion with the plasma membrane and exosome embedding within target cell [146]. The release of eosinophil-derived EXO content in the receiving cell can condition the most important biological cell activities (transcription, translation, regulation by posttranscriptional or translational modifications), leading to drastic phenotypic variations in the receiving cell.

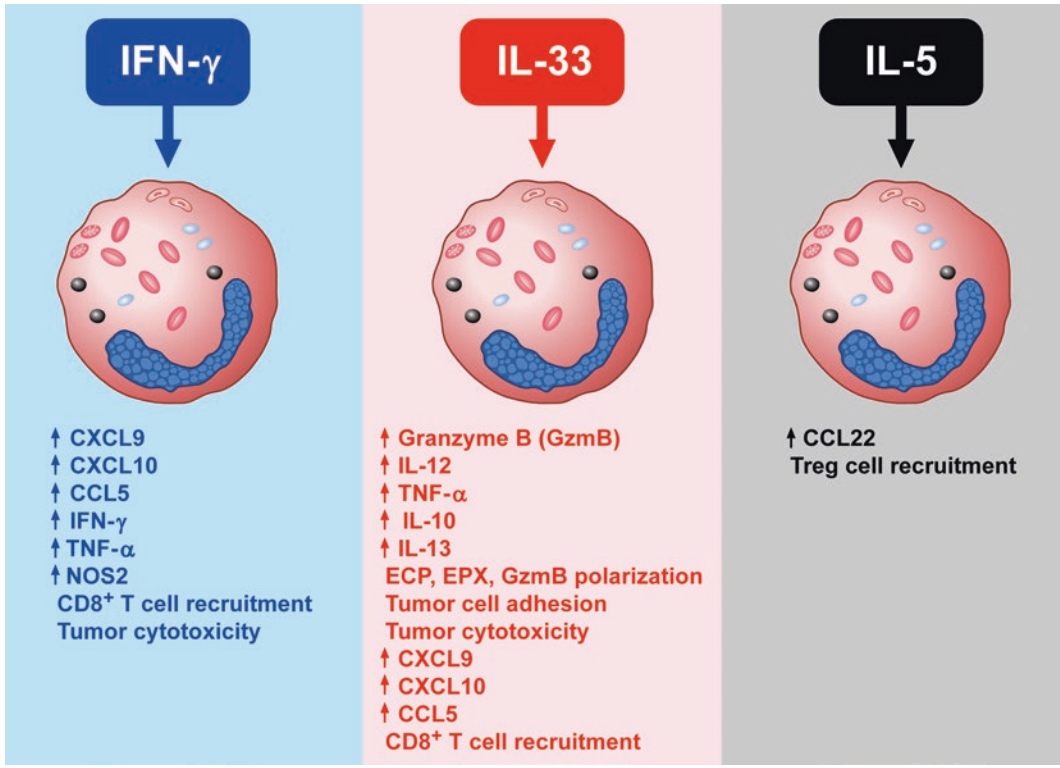

Fig. 1.3 Modulation of eosinophil phenotype by cytokines. Activation of eosinophils with IFN-γ results in the expression of T-cell-attracting chemokines (CXCL9, CXCL10, and CCL5), effector molecules (TNF-α, IFN-γ, and NOS2), promoting both CD8 T-cell recruitment and tumor cytotoxicity. Similarly, IL-33 triggering leads to upregulation of T-cell-attracting chemokines (CXCL9, CXCL10, and CCL5), effector molecules (granzyme B and TNF-α), Th-1 cytokines (IL-12), and Th-2 cytokines (IL-10 and IL-13), as well as to granule protein polarization toward immune synapses. These traits favor eosinophil-mediated direct and indirect (CD8 T-cell-mediated) antitumor activities. In contrast, activation of eosinophils with IL-5 induce the expression of the chemokine CCL22 that recruits T_{REG} cells, which may promote tumor progression

Evidences suggest that eosinophil EXO, secreted into the extracellular microenvironment and delivered to different locations within the body, participate in multiple processes and pathologies, including asthma. Eosinophil EXO from asthmatic patients can influence the functions of structural lung cells, modifying several processes and changing the expression profile of various pro-inflammatory molecules [147]. Furthermore, eosinophil-derived EXO can increase nitric oxide (NO) and ROS production in eosinophils themselves, thus autoregulating eosinophil functions [148]. The role of eosinophil-derived EXO in cancer progression is unknown and deserves investigation. By using electron microscopy, Feng and coworkers demonstrated that pericytes, like eosinophils, are equipped with internal vesicles that can be released outside of the cell [149]. Since pericytes represent an important component of vessels involved in the modulation of angiogenesis, it is conceivable that eosinophils and pericytes interact within the TME through the release of EV.

1.8 Regulatory Functions of Eosinophils: The Complex Role of the Angiogenesis

Angiogenesis and lymphangiogenesis are complex processes requiring a finely tuned balance between stimulatory and inhibitory signals [150–152]. The

formation of new blood and lymphatic vessels occurs vigorously during embryogenesis but is restricted in adults [150]. In adults, angiogenesis and lymphangiogenesis are limited to sites of chronic inflammation [64], tissue injury or remodeling [153], and cancer [154]. The association between angiogenesis/lymphangiogenesis and tumor growth was of great interest during the last decades for the implications of the nature of tumors and the possibility to inhibit cancer growth and the formation of metastasis by blocking angiogenesis/lymphangiogenesis [155]. The interest in angiogenesis/lymphangiogenesis increased during last years for several reasons. Chronic low-grade inflammation is an essential hallmark of cancer [8] and several immune cells can be involved, directly and indirectly, in the modulation of angiogenesis and lymphangiogenesis [6, 156–163]. The latter observation led to the recognition that the interactions between immune cells and the vascular system are involved in a multitude of cancers [164].

Angiogenesis is initiated by activation of vascular endothelial growth factor receptor 2 (VEGFR2), expressed on blood endothelial cells (BECs) by vascular endothelial growth factor-A (VEGF-A). Cancer cells are an important source of VEGF-A and other pro-angiogenic mediators [6, 165, 166]. Immune cells in TME increase VEGF-A availability during the angiogenic switch [159]. Angiogenesis and lymphangiogenesis require the participation of additional molecules, such as angiopoietins (ANGPTs) [167]. VEGF-A signaling through VEGFR2 is the major angiogenic pathway. VEGF-C and VEGF-D, mainly through the engagement of VEGFR3 on lymphatic endothelial cells (LECs), induce lymphangiogenesis in tumors and stimulate the formation of metastasis [168,169]. The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF) [159]. VEGF-A signaling through VEGFR2 activates angiogenesis by inducing the survival, proliferation, sprouting, and migration of BECs. VEGF-A also increases endothelial permeability [160, 170] and induces inflammation [157, 163, 171]. There are several splicing isoforms of VEGF-A (121, 165, 189, and 206), which differ in their binding to matrix and to co-receptor. $VEGF\text{-}A_{121}$ is freely diffusible, whereas $VEGF\text{-}A_{165}$, $VEGF\text{-}A_{189}$, and $VEGF\text{-}A_{206}$ bind to heparin and heparin proteoglycans on cellular surfaces and extracellular matrices [172].

VEGF-A is primarily known for its essential role in physiologic and pathologic angiogenesis [173] and also retains lymphangiogenic properties [174] by binding to VEGFR2/VEGFR3 heterodimer receptor [175]. VEGF-A modulates lymphangiogenesis also indirectly by recruiting immune cells (e.g., macrophages, mast cells) that produce VEGF-C and VEGF-D [161, 163]. PlGF, expressed in the placenta, heart, and lungs, has four isoforms (PlGF1–4) [176, 177]. VEGF-B is highly expressed in heart, skeletal muscles, and brown fat in adults and has two major isoforms in humans: $VEGF\text{-}B_{167}$ binds to heparin proteoglycans, whereas $VEGF\text{-}B_{186}$ does not bind heparin and is more soluble [178]. PlGF and VEGF-B bind with high affinity to VEGFR1 whose tyrosine kinase (TK) activity is weak and downstream signaling poorly understood [179]. VEGFR1 is expressed on BECs, some immune cells, and pericytes, and its TK activity is required for cell migration toward VEGFs or PlGF [157, 171, 180]. VEGF-B and PlGF are angiogenic in certain pathophysiological settings [181]. VEGF-B can modulate coronary vessel growth and cardiac hypertrophy and lipid metabolism [177, 182].

The VEGF-C/VEGFR3 signaling pathway is the main pathway implicated in lymphangiogenesis [183]. VEGF-C is crucial for the survival, proliferation, and migration of LECs [184]. VEGF-D also binds VEGFR3 to promote lymphangiogenesis [168].

Angiopoietins (ANGPTs) also play an important role in modulating angiogenesis and lymphangiogenesis. In humans, the ANGPT/Tie system consists of two cell-surface TK receptors (TIE1 and TIE2) and two ligands ANGPT1 and ANGPT2. TIE2, primarily expressed on BECs, binds both ANGPTs, whereas TIE1 is an orphan receptor that can modulate ANGPT1, expressed by perivascular cells (i.e., pericytes), and sustains BEC survival. By contrast, ANGPT2, secreted by BECs, acts autocrinally and paracrinally as TIE2 ligand to promote angiogenesis and lymphangiogenesis [185]. Several chemokines, produced by

immune and nonimmune cells, also play a role in the modulation of angiogenesis and antiangiogenesis [158].

Human eosinophils produce several angiogenic factors such as VEGF-A [129, 137], fibroblast growth factors (FGF-2) [40, 130], CXCL8/IL-8 [131], and osteopontin [132]. Human eosinophils also produce MMP9 [186–188]. Eosinophils have been detected in metastatic lymph nodes of cancer patients, but the production of lymphangiogenic factors by these cells should be further addressed.

1.9 Mouse Models to Investigate the Role of Eosinophils in Cancer

Several mouse models have been developed for the study of the functional role of eosinophils. Most of these experimental models have been employed mainly in the study of respiratory diseases, such as asthma and eosinophilic esophagitis. These models can be transgenic, genetically engineered, target-specific, and humanized.

1.9.1 Transgenic Mouse Models

A relevant transgenic mouse model used to assess phenotypic features of eosinophils in the host is represented by PHIL transgenic mouse model of study. PHIL mice were first described by Lee and colleagues as a transgenic line of mice with a complete ablation of eosinophils and the contemporary presence of a fully functional hematopoietic compartment [189]. These mice have been generated by replacing the eosinophil peroxidase (EPX) with the Diphtheria toxin A chain (DT) and by exploiting the cytocidal property of DT. When host eosinophils undergo maturation or activation of the transcription factors devoted to the expression initiation of EPX in PHIL transgenic mice, the promoter transcribes the replaced DT sequence, thus selectively ablating eosinophils [189]. These mice have allowed to establish the contribution of eosinophils to the resolution of inflammatory responses in experimental pulmonary allergies [190], experimental colitis [191], and to pathology and protection against parasites [78].

An alternative mouse model for studying eosinophil functions in vivo is the ΔdblGATA1 transgenic mice. Deletion of a high-affinity GATA site in the GATA-1 promoter results in a complete ablation of the eosinophil lineage without affecting the development of the other GATA-1-dependent lineages, such as erythroid, megakaryocytic, and mast cell [192]. These mice have been used to demonstrate a protective role for eosinophils in a methylcholanthrene (MCA)-induced fibrosarcoma tumor mouse model [98]. However, besides eosinophil deficiency, ΔdblGATA1 mice were subsequently reported to display numerical and functional aberrancy in basophils [193], thus raising concerns on their specificity for eosinophil-specific studies.

IL-5 transgenic mice display an overrepresented eosinophil compartment due to the insurgence of eosinophilia in the host. These hypereosinophilic mice display abnormally high presence of eosinophils in bone marrow, spleen, and peritoneal exudate, compared to controls. Simson and coworkers proposed a role for eosinophils in tumor immunosurveillance by using the IL-5 transgenic mice with elevated levels of circulating eosinophils [98]. Similarly, Kataoka and coworkers exploited these IL-5 transgenic mice to demonstrate an antitumor activity of eosinophils in hepatocellular carcinoma [141].

1.9.2 Target-Specific in Vivo Models

Depletion mechanisms to generate selected target-specific mouse models have been developed to produce in vivo eosinophil-targeted models of study. Repeated systemic injections of an anti-Siglec-F polyclonal antibody that functionally inhibits the activity of Siglec-F protein and selectively induces apoptosis in eosinophils result in eosinophil ablation in mice [55]. Recently, this Siglec-F-based functional depletion of eosinophils in mice has been employed to study the role of eosinophils in cancer. By using this approach, three independent groups demon-

strated the essential role of eosinophils in antitumor response in mouse models of melanoma and other cancers [40, 41, 105].

1.9.3 Genetically Engineered in Vivo Models

Initially, genetically modified mouse models with impaired eosinophil development and/or function, although not eosinophil-specific, were described. These include mice deficient for the eosinophilic cytokine IL-5 [194] or its receptor [195], which are characterized by the absence of eosinophilia upon Th2 cell-inducing stimuli. Furthermore, mice with a double deletion of CCL11 and CCL24 genes are characterized by a severe diminished eosinophil recruitment in response to allergic stimuli [196]. These models were largely employed in allergy and respiratory disease research.

Subsequently, mice deficient for eosinophil-specific granule proteins MBP and EPX were described. MBP-1$^{-/-}$ mice were generated by truncating the MBP-1 gene, thus producing a dysfunctional protein containing the exons 1 and 2 but lacking the exons 2, 3, and 4. This model is characterized by a reorganization of eosinophil secondary granule structures and by a marked reduction of the eosinophil numbers in lung parenchyma and bronchoalveolar lavage [197]. Similarly, EPX$^{-/-}$ mice were generated by a targeted disruption of the EPX gene, in which the normal EPX gene was replaced with a dysfunctional EPX sequence lacking the exons 7, 8, and 9. EPX$^{-/-}$ mice displayed an altered structure of eosinophil secondary granules and a remarkable reduction of eosinophils in lung BAL [198]. Interestingly, a recent report described the generation of a double knockout mouse model for both MBP and EPX (MBP$^{-/-}$ EPX$^{-/-}$ mice). These mice are featured with eosinophil deficiencies similar to those observed in animals deficient of EPX or MBP only, but represent an advance in the implementation of an in vivo model to investigate eosinophil pathophysiology [199]. These models are largely utilized for research in allergy, inflammation, and respiratory diseases and could be successfully employed in anticancer research [200].

1.9.4 Humanized Mouse Models

The development of humanized mouse models best recapitulates the pathology of human diseases and thus represents a major goal to understand the role of eosinophils in human cancer. Recently, a novel IL-3/GM-CSF/IL-5 Tg NOD/Shi-scid-IL2rγnull (NOG) model, a mouse strain in which human eosinophil differentiation is induced from HSC, was reported [201]. In this mouse strain, the authors established a human asthmatic inflammation model by intratracheal administration of human IL-33. This enabled to study the Th2 responses specifically in a human context, including the eosinophil-dependent responses in asthma. By using humanized NSG mice adoptively transferred with human CD34^{+} hematopoietic stem cells, Arnold and collaborators showed that following infection with gastrointestinal bacteria, eosinophils are recruited to the tissue where they reduce inflammation by suppressing Th1 immune responses [202]. Thus, humanized mouse models represent a valid opportunity to investigate specifically the roles of eosinophils in different human cancers.

1.10 Concluding Remarks and Outstanding Questions

There is compelling evidence that eosinophils are potent effector and immunoregulatory cells in TME of experimental and human cancer [49, 203]. Several studies have reported that eosinophilia can be associated with a favorable prognosis in a variety of solid and hematologic tumors [6]. By contrast, a limited number of studies indicate a protumorigenic role of eosinophils [6, 114]. The potentially dual role (i.e., protumorigenic and antitumorigenic) of eosinophils raises several fundamental questions. First, there is the possibility that the role of eosinophils and their mediator is cancer-specific (e.g., influenced by different TMEs). Alternatively, different sub-

sets of eosinophils and/or different eosinophil-derived mediators can play distinct or even opposite roles in tumorigenesis. There is already evidence, at least in mice, of the existence of different subsets representing different stages of maturation of eosinophils [202, 204, 205]. Recent fate mapping experiments demonstrate that macrophages [206] and mast cells [207, 208] form a highly heterogeneous population of immune cells, similar to T cells [209]. Future studies should address the possible roles of plasticity/hypothetical subtypes of eosinophils by single-cell RNA-seq, together with analyses of encoded proteins.

Studies of eosinophils are usually performed on cells isolated from peripheral blood where O_2 and nutrients are abundant and pH neutral. By contrast, eosinophils in TME are embedded in a hostile metabolic setting characterized by hypoxia, accumulation of lactate, potassium and adenosine, and low pH [210–214]. Thus, the biochemical and functional characteristics of peritumoral and intratumoral eosinophils likely differ from those of peripheral blood eosinophils.

Experimental models have started to provide evidence that eosinophils and their mediators can play a protective role by inhibiting tumor growth and the formation of metastasis in different cancers [40, 41]. Several mouse models have been characterized for the evaluation of the pathophysiological roles of eosinophils. Different groups have used target-specific mouse models to demonstrate the antitumorigenic role of eosinophils [40, 41, 105]. Humanized mouse models best recapitulate human disease and will represent a useful tool to evaluate the role of eosinophils and their mediators in different human cancers.

Immunotherapy with mAbs targeting immune checkpoint inhibitors (ICIs) (e.g., CTLA-4, PD-1/PD-L1 network) has revolutionized the therapies of an increasing number of solid and hematologic tumors [215, 216]. Unfortunately, these therapies are effective only in a percentage of patients, and there is urgent need of biomarkers predictive for ICI-based immunotherapy [217]. There is some evidence that baseline peripheral blood eosinophils represent a useful biomarker for prognosis of melanoma [109] and NSCLC [111]. Future studies should evaluate the predictive value of different subsets of peripheral blood eosinophils (e.g., low and high density) in response to ICIs in different cancers.

A deeper insight into the immunological and molecular mechanisms regulating the link between tumor-infiltrating eosinophils and tumor cells could lead to the identification of new prognostic/predictive biomarkers, as well as a wider view of cancer immunotherapy, in an even more personalized therapeutic approach.

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Dendritic Cells in the Tumor Microenvironment

2

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Abstract

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) of the immune system. They capture foreign antigens and can present them to lymphocytes, that is, T cells and B cells, to activate them. DCs are the most potent of all immune cells at inducing the adaptive immune system. Thus, the presence of DCs at the anatomical site of the immune challenge is imperative for the immune system to mount an effective immune response. From the anatomical site of the immune challenge, DCs cargo antigens to the draining lymph nodes, specialized immune organs where adaptive immunity is generated. DCs are heterogeneous as a type of immune cell, and various subsets of DCs have been reported and their functions described. In this chapter, we discuss various aspects of DC development and function. We further discuss how various tumor microenvironments can affect DC development, function, and migration, thus evading a strong adaptive immune response.

Keywords

Dendritic cells · Antigen-presenting cells · Lymph nodes · Tumor microenvironment · Anti-tumor immunity · Danger signal · Toll-like receptors · Cytokines · Migration · Chemotaxis · Development · Longevity · Subsets · Immunosuppression · Tertiary lymphoid organs

2.1 Introduction

Dendritic cells (DCs) play a crucial role in initiating and modulating adaptive immune responses during infections, allergies, autoimmune disorders as well as maintain T-cell homeostasis in steady-state conditions. Depending on the immune challenge, they can initiate or enhance an immune response. Moreover, they can tolerize or suppress the immune system toward innocuous antigens. DCs can infiltrate solid tumors, capture, and process tumor antigens and transport them to the draining lymph node (LN) to initiate an effective adaptive immune response against the tumor, by priming and expanding naïve T cells to become anti-tumor effector T cells.

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Although many other antigen-presenting cells (APCs) such as macrophages also contribute to this process, DCs are considered the most proficient of all APC types. Additionally, DCs can modulate the tumor microenvironment to influence the recruitment of other immune cell populations into the tumor. DCs are very heterogeneous and can be classified into many subsets depending on factors including their phenotype, specialized function, localization in peripheral tissue, lineage, etc. Even though the exact role of every DC subset in generating anti-tumor immunity has not yet been fully deciphered, DCs, in general, are an important arm in adaptive immune responses against tumors. However, a tumor environment can present many hurdles in the scheme of DC-mediated anti-tumor immunity. Here, we review different aspects of DC biology and how DC function can be influenced by tumors.

2.2 Tumor-Infiltrating Dendritic Cells Mediate Anti-tumor Immunity

For the generation of efficient anti-tumor immunity professional APCs need to capture tumor-derived antigens, process them to form a complex with major histocompatibility complex (MHC) molecules, migrate to the draining LN, and finally present them to cognate CD4 or CD8 T cells [1]. Presentation of antigen to T cells can lead to two possible outcomes. Either the T cells can be tolerized, that is, they become quiescent and/or get converted into an immune regulatory T cell, or they can be activated to mount a response against the immune threat [2]. The latter outcome is desired for an effective anti-tumor immune response and for that APCs, either alone or in co-operation with other APC types, need to provide three signals to the cognate T cells [3].

- Signal one is the peptide:MHC complex. APCs capture exogenous antigens such as tumor-derived antigens and process them efficiently to eventually present them as a complex with MHCI or MHCII molecules to present them to CD8 T cells or CD4 T cells, respectively. The process of internalization of exogenous antigens to be processed and presented as a complex with MHCI complex to CD8 T cells is called "cross-presentation" and this process is important to generate effective anti-tumor immunity. Among all APC types, DCs are considered most proficient at antigen presentation, especially cross-presentation.
- Signal two are co-stimulatory signals. APCs can present p:MHC complexes to T cells, but, in the absence of any inflammatory signal, it might not necessarily lead to T-cell activation. However, during an infection or other inflammatory conditions, APCs can sense the danger, as they express specific receptors which can bind to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) and up-regulate the expression of co-stimulatory molecules such as CD40 and CD86. The ligands for these receptors are expressed on T cells eternally. An APC when presents p:MHC complexes in conjunction with co-stimulatory molecules leads to efficient activation of T cells. DCs express an array of receptors to detect PAMPs and DAMPs including toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectins, etc., which enable them to detect danger signals and over-express co-stimulatory molecules, making them capable of effectively activating T cells toward anti-tumor response [4].
- Signal three is activating cytokines. Along with receiving signal one and two concurrently from a single APC type, T cells need inflammatory cytokines such as IL-12 and IL-15 which boost and sustain their effector status and enable them to keep expanding [5]. Certain subsets of APCs, depending on the environment, can provide these signals along with signal 1 and signal 2. However, a new concept of APC co-operation is emerging which suggests that, while a single APC can provide signals 1 and 2 to the cognate T cells, another myeloid cell can serve as a source of signal 3 during an inflammatory

challenge [6, 7]. Whether this co-operation between myeloid cells exists in the tumor microenvironment is a challenging research question.

DCs are considered as the most proficient APCs that provide signals 1, 2, and 3 to T cells and thus initiate adaptive immune responses against tumor antigens. However, to do so, differentiated DCs must be able to infiltrate the tumor microenvironment from neighboring tissues. Precursors of DCs which originate in the bone marrow (BM) can also enter the tumor parenchyma and differentiate in situ. While it is still unclear which path DCs take to infiltrate tumors, their presence in the tumor is beneficial toward anti-tumor immunity. In clinical samples, the presence of DCs in the primary tumor site has been correlated with increased survival in many tumor typesincluding ovarian carcinoma, head and neck tumors, pancreatic adenocarcinoma, lung, and breast cancer [8–11].

2.3 Migration of DCs to and from Tumor Parenchyma

Chemotaxis is a major mechanism utilized by immune cells for directional migration. DCs exhibit classic directional migration engaging their chemokine receptors to move toward gradients of the corresponding chemokine ligands. DCs are pliable in their expression of chemokine receptors and, depending on the subset, anatomical location and pathophysiological condition, they can express varying levels of multiple chemokine receptors [12]. DCs do not develop fully at their site of origin, that is, BM, and complete their differentiation program in peripheral tissues [13]. For instance, skin DCs develop from DC precursors traveling from BM into the skin via blood. In the tissue DCs capture antigens, follow the chemokine gradients to reach terminal lymphatics, and through the lymphatic vessels enter the nearest LN. Unlike other immune cell populations, DCs, except pDCs, migrate dominantly through the lymphatic system [12]. This scheme of DC development and migration raises an important question regarding the migration of DCs into solid tumors, that is, do terminally differentiated DCs migrate from surrounding tissue into the tumor? or if DC precursors migrate into the tumors and differentiate in situ. Assuming either possibility, another key aspect that needs investigation is what chemokine gradients do DC or DC precursors follow to reach into the tumor parenchyma. DC migration toward the LN has been well characterized. C–C Motif Chemokine Receptor (CCR)7 on DCs is the dominant chemokine receptor which guides DCs toward gradients of C–C Motif Chemokine Ligand (CCL)19/21 on the way to LN and also from the boundaries of the LN into the deep T-cell zone of the LN. However, steps involved in the migration of DCs/DC precursors toward non-lymphoid tissues have only been partly characterized [14]. Adoptive transfer experiments revealed that DCs can adhere to skin venules by attaching to certain selectins expressed on endothelial cells; however, attachment to selectins is only the start of a multistep adhesion cascade and subsequent intravasation [15]. All the steps entailed in DC migration from blood to tissue are not fully characterized for solid tumors. This is a challenge, as tumors are diverse and there might not be a common adhesion and chemotactic axes that guide DCs into solid tumors. Nonetheless, certain chemokine ligands that have been seen in certain tumors can attract DCs. These chemokines can be secreted either by the tumor themselves such as CCL4 or immune cells within the tumor [16]. For example, CD8 T cells or NK cells can secrete CCL5 and CXCL1 which can attract DCs expressing the corresponding chemokine receptor [17]. In various settings, it has been demonstrated that immune cells reaching a site can secrete chemokines to attract other types of immune cells as a part of the ongoing immune response. Thus, it seems likely that certain immune cells in tumors have the potential to attract other immune cells, and thus the role of other immune cells in attracting DCs into the tumor environment must be further investigated.

2.4 Longevity of DCs in the Tumor Microenvironment

A study, investigating the life of major immune cell populations revealed that while B cells and T cells can live up to 20 days after they leave their site of origin, DCs have a relatively short life of around 3 days in lymphoid and non-lymphoid tissues at steady state [18]. However, the ratio of DCs to other immune cells remains constant, which suggests that DCs have a high turnover. However, in many tumors, the decreased presence of DCs can be attributed to factors released in the tumor microenvironment, which can affect the lifespan of DCs in the tumor. These factors include mucins, gangliosides neuropeptides, and nitric oxide. A study showed that gangliosides including GM3 and GD3 induce apoptosis of DCs in vitro [19]. These gangliosides are found at high concentrations in melanoma patients. The presence of these gangliosides in melanoma explains the lower number of DCs in malignant melanoma as compared to that in benign skin lesions. MUC2 mucins derived from conditioned medium of LS180 cells, a colorectal cancer cell line, can cause apoptosis of mature DCs [20]. Overexpression of mucins, large extracellular proteins that are heavily glycosylated with complex oligosaccharides, is associated with many epithelial cancers. Thus, considering these unfavorable conditions, the life of DCs could be much different compared to healthy tissue.

2.5 Immunosuppression of DCs in Solid Tumors

Some tumors, such as Merkel Cell Carcinoma (MCC), are initiated by oncoviruses, and principally these viruses can provide danger signals to DCs which could trigger them to their path toward initiating anti-tumor immunity. However, most solid tumors do not provide any inflammatory or danger signals to infiltrating DCs, unless somehow the tumor microenvironment can derive inflammatory signals. Besides a lack of adequate inflammatory signals, a solid tumor environment can actively suppress DC-mediated anti-tumor immunity. Across many studies, various mechanisms have been postulated by which tumor or tumor components can suppress DC activity. For instance, in a mouse model of ovarian tumor, tumor cells induced the activation of transcription factor Xbox binding protein (XBP) which caused endoplasmic reticulum stress in DCs which impeded their ability to prime T cells [21]. Cancer cells by initiating the B-catenin signaling pathway can also limit DC recruitment into tumors [22]. Interleukin (IL)-10 is a widely known immunosuppressive cytokine, and tumor cells, as well as other components of the tumor environment such as tumor-associated macrophages (TAMs), can secrete IL-10 [23]. $CD103^+$ tumor-infiltrating DCs have a high expression of IL-10 receptor and, in response to sensing IL-10, they can downregulate the production of IL-12, a cytokine known to enhance $CD8^+$ T-cell proliferation and effector function [24]. IL-10 can also skew the differentiation of monocytes toward immunosuppressive macrophages instead of monocyte-derived DCs [25]. The role of IL-10 in the suppression of anti-tumor immunity is a broad area of investigation and the studies describing its mechanisms have been covered in this review [26].

Tumor cells can also secrete thymic stromal lymphoprotein (TSLP) which induces OX40 OX40L expression in DCs [27]. OX40L-expressing DCs induce a type 2 immune response which is not as potent an anti-tumor response as a type 1 response. Plasmacytoid dendritic cells (pDCs), which are known to secrete high amounts of type 1 interferon during viral infections, can also limit their production of type 1 interferon when immunoglobulin-like transcript 7 (ILT7) on pDCs engages with bone marrow stromal antigen 2 (BST2) on tumor cells [28]. Tumors secrete growth factors and produce chemokines that help sustain the tumor and even metastasize. A prominent growth factor that is over-produced in many tumors is Vascular Endothelial Growth Factor (VEGFA). Tumors expressing VEGFA can cause "angiogenic switch" which means that new vasculature can develop which will support tumor growth and metastasis [29]. Besides supporting

tumor growth, VEGF is an immunosuppressant for DCs [30]. Treatment of DCs with VEGF results in inhibition of their maturation [31, 32]. Some tumor-associated chemokines that can also cause similar inhibition of DC maturation include CCL2, C-X-C Motif Chemokine Ligand (CXCL)1, and CXCL5 [33]. In a mouse model of Ovarian cancer, Programmed cell death-1 (PD-1) was expressed on tumor-infiltrating DCs [34]. PD-1 is generally expressed on T cells which are exhausted in solid tumors or during chronic viral infections [35]. The same study further showed that these PD-1$^+$ DCs could block T-cell proliferation. Factors that induce PD-1 expression on DCs are largely unknown. It is tempting to assume that the same factors that induce PD-1 expression on T cells would do the same for DCs. Nonetheless, anti-PD-1 therapy which is the most commonly applied immunotherapy for solid tumors could potentially work by blocking the immunosuppressive activity of PD-1-expressing DCs. Another marker of exhaustion expressed generally on T cells, that is, T-cell Ig and mucin domain 3 (Tim-3), was also expressed on tumor-infiltrating DCs in mouse models for Lewis lung cancer tumors and colorectal adenocarcinoma. Nucleic acids (NA) from dying tumor cells can lead to NA-mediated anti-tumor immunity. Tim-3-expressing DCs were shown to suppress NA-mediated anti-tumor immunity [36]. Thus, much like T cells, DCs can also become dormant or immunosuppressive in solid tumor microenvironments and this can be reflected by the expression of certain markers. Besides inducing immunity, DCs also play a pivotal role in maintaining immune tolerance to innocuous antigens. In the steady state, some subsets of DCs such as migratory DCs and pDCs have been shown to specialize in inducing immune tolerance, by transporting peripheral harmless antigens to secondary lymphoid organs such as lymph nodes and priming T cells to an anergic, that is, non-responsive state, or even convert naïve CD4$^+$ T cells into regulatory T cells [36]. Many solid tumors have a relatively high frequency of Tregs, which adds to the immunosuppressive environment. In mice and rats bearing melanoma, it was shown that a fraction of DCs accumulated in the draining LNs and these DCs were proficient in inducing expansion of Treg cells.

2.6 DC Subsets in Tumors

DCs are a heterogeneous group of cells and depending on the anatomical location and physiological condition many DC subsets can be identified [37]. However, recently a study that attempted to relate DCs between species and anatomical locations broadly grouped all DC subsets into five subsets, that is, cDC1s and cDC2s for conventional DCs, pDCs, Langerhans cells, and MoDCs [38]. These subsets express exclusive markers that can be used to identify them. For instance, in both mice and humans, cDC1s express C-type lectin endocytic receptor CLEC9A and chemokine receptor XCR1, whereas cDC2s express CD1c, a transmembrane glycoprotein [39]. Many studies have investigated the above subsets for specialized roles. While this remains a topic of intense investigation, some specialized roles have been specifically ascribed to individual subsets. For instance, in various settings including infection and tumor models, it has been shown that cDC1s are specialized at cross-presentation of exogenous antigens and crucial for inducing an effective CD8 T-cell response [40–42]. Since the cross-presentation of exogenous antigens in the context of MHC-I molecules to CD8 T cells is essential to generate effective anti-tumor immunity, the presence of cDC1s in tumors is believed to be beneficial to anti-tumor immunity [43]. Indeed, defect in cDC1s in the tumor microenvironment has been shown to negatively impact tumor immunosurveillance in many tumor models [44–46]. However, cDC1s are the minority DC type in tumors, as well as lymphoid and non-lymphoid tissues, making them a precious immune population. However, more studies across different cancer types looking at relative cDC1 frequencies in solid tumors and their impact on patient survival and tumor progression are warranted.

In mouse tumor models, specific depletion of cDC1s impairs the CD8 T cell-mediated anti-tumor response and the ability to reject trans-

planted tumors [47]. Across many studies, much evidence suggests that cDC1s in tumors are the most proficient of all DC subsets at cross-presentation and some other key unique features of cDC1s make them the most prominent DC type for generating anti-tumor immunity. For instance, cDC1s produce CXCL9/10, chemokine ligands that can recruit effector and memory T cells expressing the corresponding chemokine receptor CXCR3 [48]. cDC1s have been shown to efficiently capture, process, and transport tumor-derived antigens to the draining LN, and ex vivo experiments showed that they were most proficient at stimulating and activating T cells [49]. cDC1s also produce high amounts of IL-12, an inflammatory cytokine that has been shown to enhance the CD8 T cell- and NK cell-mediated cytotoxicity [50]. cDC1s are also most sensitive and responsive to type I interferons, as they enhance their cross-presentation capacity in response to stimulation by type I interferons [51]. Due to the above features, cDC1s are regarded as the DC type essential for mounting effective anti-tumor immunity. However, it is important to realize that the anti-tumor functions of cDC1s have been ascribed to them based on mouse tumor models. Besides the obvious caveat of species-specific discrepancies, there are other issues with mouse models that could complicate the interpretation of results. For instance, none of the models could exclusively deplete cDC1s. The most common model uses knocking out of Basic Leucine Zipper ATF-Like Transcription Factor 3 (BATF3), which is crucial for cDC1 development. However, the same transcription factor is also crucial in the development of other DC types. Similarly, some of the genetic ablation models have not been able to exclusively deplete cDC1s and thus better models are warranted to support the above findings [52]. In addition to caveats of the mice models, discrepancies between the profile and function of cDC1s in mice and humans still leaves some doubt about the exclusive specialty of cDC1s in generating anti-tumor immunity. For instance, a few studies showed that other DC subsets, for example, the cDC2 subset, produced more IL-12 than cDC1s in response to certain adjuvants [53]. Also, while in mouse models, cDC1s were shown to efficiently capture and transport tumor-derived antigens, cDC1s in humans were shown not to efficiently capture antigens from the parenchyma of non-lymphoid tissues to the draining LNs [54]. The study also shows that cDC1s express a lower level of CCR7, a receptor pivotal for the migration of DC from peripheral tissues to LNs. Thus, in summary, while experimental models have highlighted the importance of cDC1s in anti-tumor immunity, more data regarding the alignment of the phenotype and function of DC subsets across species, especially in different cancer types, are warranted to endorse the notion that cDC1s are the specialized DC subset in cross-presentation and generating anti-tumor immunity.

cDC2s are the more abundant population in lymphoid and non-lymphoid tissues [54]. Unlike cDC1s, their role in cancer is much less established, perhaps because they are considered less efficient at cross-presentation than cDC1s. However, studies have shown that cDC2s are present in solid tumors and can migrate to draining LNs [55]. The current belief is that while cDC1s are more potent at cross-presentation and priming CD8 T cells, cDC2s are better activators of CD4 T cells [54]. Effective priming of CD4 T cells for an anti-tumor response is crucial as a large body of literature emphasizes the role of CD4 T cells in helping CD8 T cells to infiltrate and kill tumor cells [56]. Thus, cDC2s, although not considered specialized at cross-presentation, serves as a crucial arm in anti-tumor immune responses by effectively priming CD4 T cells. This idea is corroborated by studies that show that the presence of gene signature of cDC2s directly correlates with better survival for cancer patients [56, 57].

pDCs were initially discovered as cells specialized to produce type-1 interferon in response to viral ligands [58]. However, afterward, many reports postulated a role of pDCs in driving both central and peripheral tolerance [59–63]. Currently, the consensus is that pDCs can be tolerogenic or immunogenic depending on their environmental stimuli. While type 1 interferons have a clear role in anti-viral immunity, they can be both anti-tumor and pro-tumor [64]. pDCs can

contribute to anti-tumor immunity by serving as APCs and via type 1 interferon production, but there have been many mechanisms described through which various solid tumors can induce the tolerogenic or regulatory function of pDCs to promote tumor growth. Demoulin, S et al. in their review describe mechanisms by which various tumors can induce tolerogenic function and inhibit immunogenic functions of pDCs [65].

2.7 DCs Help in Anti-tumor Immunity: Beyond T-Cell Priming

Besides their classic function of antigen presentation, DCs also play a key role in the recruitment of other immune cells. In a landmark study, it was shown that LNs of mice lacking DCs are much smaller than those of wild-type (WT) mice [66]. Mechanistic experiments in the same study further showed that DCs modulate the phenotype of specialized endothelial venules of the LN, called high endothelial venules (HEVs) which are portals through which other immune cells including T cells and B cells can enter into the LN from the bloodstream. DCs induced the expression of multiple adhesion molecules on endothelial cells to which immune cells can adhere as the first step in their intravasation into the LNs. A similar phenomenon is observed in ectopic lymphoid structures that form in certain pathophysiological conditions, such as persistent pathogenic infections, or autoimmune diseases, such as rheumatoid arthritis, to generate local immunity [67]. The neogenesis of tertiary lymphoid structures (TLS) has been described in many models and across many of these studies; DCs have been shown to play a pivotal role in aiding genesis of TLS [68]. Even in cancer patients, a positive correlation between the presence of DCs and the presence of TLSs has been demonstrated [8, 10, 61–63]. While the role of TLS in cancer immunity is still an area of investigation, the majority of the correlation studies show that the presence of TLS in the tumor is positively correlated with patient survival for several different cancer types. After their genesis, TLS can play several roles including serving as a site for DC-T cell priming, and somatic hypermutation. Additionally, TLS provide the necessary adhesion molecules and chemokines to serve as a portal for the recruitment of immune cells into the tumor [72–74]. Thus, by aiding the formation of TLS in cancer, intra-tumoral DCs play a pivotal role in the recruitment of immune cells into the tumor microenvironment, akin to their role in LN.

Besides inducing the maturation of endothelial cells of TLS, DCs themselves can modulate the chemotactic environment of tumors to further assist in lymphocyte recruitment. For instance, in a mouse model of melanoma, it was shown that DCs are the chief source of the chemokine CXCL10 which is a ligand for CXCR3. CXCR3–CXCL10 chemokine axis plays a pivotal role in the migration of effector T cells into the tumor [75].

2.8 Summary

Dendritic cells are a heterogeneous population of cells and research into the specialized function of different DC subsets continues. Ultimately, as the development and function of each subset becomes more clear, specific DC subsets can be targeted to either enhance or suppress immunity. Additionally, a clear understanding of the development requirements of DC subsets will also enable us to skew in vitro generated DCs toward one type or other, which then can be used as therapeutics. Migration of DCs and DC precursors into tumors is not well studied. Unlike lymphoid organs, solid tumors might not have the necessary lymphatic structure and chemokine gradients to allow for the migration of DCs from the adjacent healthy tissue. Thus, even if appropriate DCs can be generated in vitro or identified in vivo, one has to think about their route to the tumor parenchyma. It is also crucial to understand better tumor-derived factors that can lead to suppression of DC activity or their death or cause aberrant development of DC precursors. Strategies to make DCs immunogenic and refractory to tumor-derived apoptotic factors can help in the effort to use DC-based therapeutics to treat cancer (Fig. 2.1).

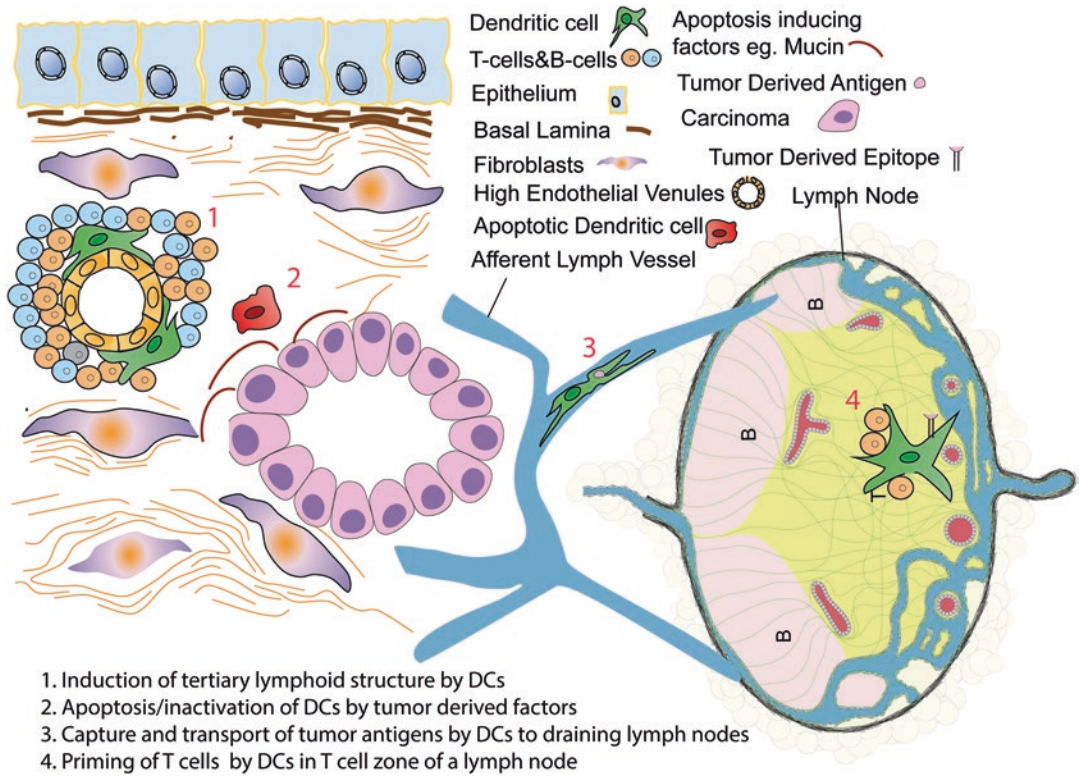

Fig. 2.1 Scheme of DC-mediated anti-tumor immunity and tumor-derived factors which can impede this process

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3 Resident Memory T Cells in the Tumor Microenvironment

Jason B. Williams and Thomas S. Kupper

Abstract

Tissue-resident memory T (T_{RM}) cells are strategically positioned within the epithelial layers of many tissues to provide enduring site-specific immunological memory. This unique T-cell lineage is endowed with the capacity to rapidly respond to tissue perturbations and has a well-documented role in eradicating pathogens upon reexposure. Emerging evidence has highlighted a key role for T_{RM} cells in cancer immunity. Single-cell approaches have identified T_{RM} cells among other $CD8^+$ tumor-infiltrating lymphocyte (TIL) subsets, and their presence is a positive indicator of clinical outcome in cancer patients. Furthermore, recent preclinical studies have elegantly demonstrated that T_{RM} cells are a critical component of the antitumor immune response. Given their unique functional abilities, T_{RM} cells have emerged as a potential immunotherapeutic target. Here, we discuss T_{RM} cells in the framework of the cancer-immunity cycle and in the context of the T cell- and non-T cell-inflamed tumor microenvironments (TME). We highlight how their core features make T_{RM} cells uniquely suited to function within the metabolically demanding TME. Finally, we consider potential therapeutic avenues that target T_{RM} cells to augment the antitumor immune response.

Keywords

Tissue-resident memory T cells · Cancer · Immunity · Microenvironment · Immune exclusion · T cell dysfunction · Antigen-presenting cells · Immunotherapy

3.1 Introduction

While the immune response to cancer is incompletely understood, cytotoxic $CD8^+$ T lymphocytes are thought to be the fundamental antitumor effector cells. The presence of a $CD8^+$T-cell infiltrate is a positive prognostic marker in most types of solid cancer [1–3] and can predict clinical response to immune checkpoint blockade therapy [4, 5]. Most of these therapies aim to increase the frequency and function of tumor-infiltrating T lymphocytes (TILs) by inhibiting the negative regulatory pathways present in the tumor microenvironment (TME). Despite recent advances in immunotherapy to harness the power of these

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cells, cures remain rare, and only a subset of patients exhibit durable responses. At present, a fundamental objective in the field of immunotherapy is to understand the biological mechanisms behind the lack of clinical response and to develop new therapeutic approaches to overcome these obstacles.

Research over the past 20 years has revealed a network of checkpoints impeding effective T cell-mediated tumor destruction [6]. This checkpoint network can be framed into two generalized phases: T cell homing to and entry into the tumor site and overcoming the immunosuppressive TME. Once within a tumor tissue, a T cells' ability to function requires adaptation to a challenging environment. Furthermore, different cancer tissues exhibit distinct local microenvironments. Even different metastases within the same patient may create distinct milieus capable of influencing T-cell function [7]. As such, T cells must be adaptable to survive and operate in diverse and difficult environments. The availability of nutrients and oxygen, local cytokine and chemokine concentrations, extracellular matrix components, adhesion molecules, and commensal microbials are among many other physiological variables. Within this framework, a recently discovered T-cell subset, called tissue-resident memory T (T_{RM}) cells, has emerged as a critical T-cell lineage in the fight against cancer, in part because its most distinctive feature is its ability to persist and function within demanding tissue microenvironments.

Memory T cells enter tissues in response to external stimuli (for example, infection or inflammation) and a subset remain as long-lived permanent residents in that tissue. In this process, after pathogen encounter, naïve T cells are primed in the draining lymph node by dendritic cells carrying antigen from the site of infection. Naïve T cells differentiate into effector T cells, which then migrate to the infected tissues to clear the pathogen. After clearance of the infection, the majority of effector T cells die or leave the tissue, but some differentiate into T_{RM} cells that impart long-term localized T-cell immunity with the capacity to rapidly respond to subsequent infection. T_{RM} cells reside in most major organs and are abundant in epithelial barrier tissues such as the skin, lung, kidneys, and gastrointestinal and reproductive tracts [8–10]. Commitment to tissue of residence distinguishes T_{RM} from effector T (T_{EFF}) cells, effector memory T (T_{EM}) cells, and central memory T (T_{CM}) cell subsets. It also differentiates T_{RM} cells functionally; they can persist in tissues for years [11, 12], display heightened response kinetics [13, 14], exhibit a unique metabolism [15], and are transcriptionally distinct compared to other T-cell memory subsets [10, 16]. These characteristics are a reflection of an adaptation to survive and function in the local environment and highlight T_{RM} cells as a potentially targetable population for the development of novel immunotherapies in the fight against cancer. In this chapter, we will discuss the fundamental properties of T_{RM} cells in the context of cancer and approaches to target T_{RM} cells as a cancer therapy.

3.2 Core Features of T_{RM} Cells

Bunkered in barrier tissue at the interface between the host and environment, T_{RM} cells are strategically positioned to react quickly to tissue perturbations, such as infection, injury, or cancer. T_{RM} cells are not immobile within tissues: instead, they migrate locally along components of the extracellular matrix and blood capillary to patrol resident tissue for signs of barrier disruption, such as cellular distress signals produced in response to pathogen insult or from cellular death or injury. T_{RM} cells display a wide range of migratory behavior, which is likely dictated by the local anatomical properties. For instance, in the dense regions of the epidermis, T_{RM} cells take on a dendritic morphology and patrol at a slower rate (0.5–1.5 μm min^{-1}) [17–19] compared to T_{RM} cells in the myometrium of the female reproductive tract (10 μm min^{-1}) [20] or liver sinusoids [21]. This localized surveillance by T_{RM} cells in tissues after pathogen encounter is a critical feature of immune memory and anamnestic protection against repeat pathogen exposure.

The commitment to remain in tissue after pathogen clearance is a defining feature of T_{RM}

cells and has been elegantly demonstrated in transplantation and parabiosis experiments. In one set of foundational studies, tissues from virally infected mice containing T_{RM} cells were transplanted into naïve congenic recipients, where they were restrained within the graft [13, 22, 23] and exhibited protective functions upon reinfection [13, 23]. In another set of experiments, by fusing the circulatory system of a virus-immune mouse with a virus-naïve parabiont, T_{RM} cells were found in the tissues of the immune parabiont but not the naïve parabiont, whereas T memory cells equilibrated between parabionts in secondary lymphoid organs (SLO, e.g., spleen and lymph nodes) [13, 22, 24, 25].

Tissue retention is maintained by two main mechanisms: expression of adhesion molecules, and a lack of responsiveness to cytokines and chemokines that direct cells back into circulation. Important for the capacity to remain in tissues are cell surface receptors that also function as useful markers to define and identify T_{RM} cells. In this role are an array of adhesion molecules, some upregulated rapidly after T-cell activation (e.g., CD44) that are important for tissue entry, while others are tissue-specific and are only expressed once T cells gain access to the tissue. While the exact mechanistic roles adhesion molecules play are still being investigated, especially in the case of cancer, evidence indicates that they are critical for T_{RM} formation but are not always required for maintenance [26]. Two well-studied adhesion molecules, the α_E integrin CD103, which pairs with β7, and the α1 integrin CD49a, which pairs with β1 and together are called VLA-1, are found at variable frequencies on epithelium-localized T_{RM} cells across many tissues [19, 26–30]. $CD8^+$ and $CD4^+$ T_{RM} cells can be found in the dermal and basement membrane regions but are more likely to be CD103 negative [19, 30]. While CD103 is critical for T_{RM} cell formation in epithelial tissues, it was found to be dispensable for T_{RM} maintenance in models of intestinal infection [31, 32]. Intriguingly, CD103 expression was found to be enriched on $CD8^+$ TILs in epithelial-derived tumors [33–35] and is important for anti-tumor activities of $CD8^+$ TILs [33, 36, 37]. Studies inhibiting VLA-1 or CD103 by using blocking antibodies or gene-deficient T cells highlighted the importance of these integrins for generating T_{RM} cells against tumors [38] and led to a loss of tumor control [39].

Aiding in tissue retention is the refractory nature of T_{RM} cells to mediators that signal T cell egress from tissues into the afferent lymphatics. Egress is mainly dictated by sphingosine-1-phosphate (S1P) gradients, which are established by vascular endothelial cells. Accordingly, T_{RM} cells display minimal levels of the S1P receptor ($S1P_1$, *SIPR1*) [40]. Continued unresponsiveness to S1P is promoted by CD69, a C-type lectin that interferes with the function of $S1P_1$, and is expressed on many T_{RM} cells, especially those in the skin [28]. Moreover, T_{RM} cells lack surface expression CCR7, a lymphoid tissue homing chemokine receptor, and CD62L (L-selectin), responsible for tethering to high endothelial venules for entry into lymph nodes (LN). Caution should be exercised for solely using cell surface markers, the most common being CD103 and CD69, for the identification of T_{RM} cells. CD103 is expressed on only a subset of T_{RM} cells and is more enriched in epithelial sites compared to other organs in the body [28]. While CD69 seems to be expressed on the majority of epidermal-localized T_{RM} cells, this does not hold true for other sites such as the kidney and liver [28]. Furthermore, $CD69^+$ T cells were found to recirculate in the LNs of mice [41]. Therefore, in addition to phenotypic characterization, residency should be tested functionally by complementary methods including parabiosis, transplantation, in vivo intravascular antibody staining, and in situ labeling of cells [42].

An important feature of T_{RM} cells is their ability to provide accelerated protection against repeatedly encountered pathogens. This function relies on their strategic location in barrier tissues, where pathogens are most likely to be encountered, and where epithelial cancers originate. When a disruption in homeostasis is recognized by T_{RM} cells, they rapidly produce effector molecules that not only impede the spread of the pathogen but also alarm and mobilize the surrounding tissue into an anti-pathogen state. In response to local secretion of alarmins such as

ATP and IL-33, T_{RM} cells are reactivated and induce a strong effector cytokine program [40, 43]. Reactivated T_{RM} cells can upregulate perforin and granzyme B and efficiently kill target cells [8, 30], secrete IFN-γ, TNF-α, and IL-2, and activate dendritic cells (DCs), NK cells, and other resident $CD4^+$ and $CD8^+$ T cells [44]. The role of IFN-γ is particularly important for protection. In response to IFN-γ, nearby cells upregulate interferon-stimulated genes, which are critical for resisting pathogen spread and also activate the endothelium to express adhesion molecules that support recruitment of immune cells. Furthermore, it was found that TNF-α plus IFN-γ do the latter synergistically, which induced the recruitment of T_{CM} and T_{EM} cells from peripheral blood [45]. Reactivated T_{RM} cells also contribute directly to the recruitment of other immune cells by producing chemokines [14, 30]. These mechanisms by which T_{RM} perform their protective functions against pathogens naturally engender the question of whether these same functions are also used against neoplasms. This question remains largely uninvestigated, although recent reports in addition to circumstantial evidence point to an important role for T_{RM} cells in antitumor immunity. In the following sections, we will first focus on the similarities and differences between the T_{RM} lineage and $CD8^+$ TILs. In particular, we review how T_{RM} cells may be generated against tumor antigens, the phenotypic features of the T_{RM} cells compared to TILs, and the transcriptional and metabolic profiles of T cells within tumors. We explore how T_{RM} cells fit within the framework of the T cell- and non-T cell-inflamed TME and finally, we end with current immunotherapeutic strategies under investigation to target or augment the antitumor T_{RM} cell response.

3.3 Immunologic Memory to Tumors

The immune system plays a critical role in protecting the host from cancer [46]. The innate sensing of tumor cell-derived factors can lead to an adaptive T-cell response through the presentation of tumor-associated antigens generated from genetic mutations and epigenetic changes that occur during carcinogenesis [47]. Spontaneously primed $CD8^+$ T cells can home to tumor sites and accumulate there, even if tumors are not completely eliminated [48, 49]. In fact, an extensive body of work over the past 20 years suggests that T cells frequently prune neoplastic cells from healthy tissue throughout life [46, 50, 51]. Under this premise, it follows that tumor antigen-specific T_{RM} cells likely form in response to tumors.

Early evidence of immunologic memory against cancer was uncovered in mice that received autologous tumor transplantation. After resection of 3-methylcholanthrene (MCA)-induced tumors, mice were protected against a subsequent challenge with the same tumor cells but not those derived from a different MCA-induced tumor [52, 53]. These studies were followed by a conceptual proposal by Burnet and Thomas, who independently posited that an evolutionary-driven feature of the immune system is to detect and eliminate incipient tumors that arise in tissues susceptible to genetic mutations, such as the highly proliferative epithelium [54, 55]. This theory evolved into what is known today as "cancer immunoediting" [56]. However, it is still unclear as to the relative involvement of different effector and memory T-cell subsets in the antitumor immune response.

Circumstantial evidence from human tumor biopsies pointed toward T_{RM} cells as a major T-cell population found within tumors. $CD8^+$ TILs isolated from biopsies of human solid tumors often express markers characteristic of residency, including CD103 [33, 34, 57], VLA-1 [39], and CD69 [33, 57]. Although phenotypic similarity alone is not adequate to identify T_{RM} cells, transcriptional profiling of $CD8^+$ TILs from human tumors has revealed a T_{RM} cell gene signature, in at least a subpopulation of $CD8^+$ TILs [33, 34, 58]. The detailed study of the T_{RM} cell response to cancer has been hampered by a lack of robust mouse models. Mice are kept in abnormally hygienic Specific Pathogen Free (SPF) barrier facilities, the most common mouse husbandry practice, and hence lack key characteris-

tics of the human immune systems due to a lack of exposure to common pathogens. Perhaps most importantly, SPF laboratory mice contain fewer T_{RM} cells and have a naïve immune phenotype more similar to the immune profile of human neonates rather than adults [59, 60]. SPF facilities also prevent exposure to environmental carcinogens, which may be important for the natural pruning of neoplastic cells and the formation of T_{RM} cells against future cancers. Several studies have circumvented this limitation by populating the skin with T_{RM} cells before tumor challenge by vaccination [61, 62], resection of a growing tumor [38], or epicutaneous engraftment of tumor cells [63]. These studies revealed that T_{RM} cells can indeed form against tumors and if the tumor is successfully eradicated, the resulting T_{RM} cells display antitumor properties that are distinct from those of circulating memory T cells.

It is important to keep in mind that the successful initiation of an immune response against an established tumor will inevitably lead to a heterogenous $CD8^+$ TIL population, which includes T_{RM} and circulating effector and memory cells. This heterogeneity makes understanding the biology of a given $CD8^+$ TIL subset very difficult, as there is no current way to identify T_{RM} cells versus newly recruited T cells expressing T_{RM}-like phenotypic markers. In addition, it remains unknown whether bona-fide T_{RM} cells can form within an established tumor, as structural and molecular features within tumors differ from normal tissues. Nevertheless, it is clear that many tumors are populated by $CD8^+$ TILs that display a tissue-resident phenotype, and recent studies in mice clearly demonstrate a role for T_{RM} cells in antitumor immunity.

3.4 Tumor-Antigen Specificity

The three tenets of cancer immunoediting describe different phases of the antitumor immune response: elimination, equilibrium, and escape [64]. During the elimination phase, nascent transformed cells are effectively pruned by the immune system from otherwise healthy tissue. This presents a dichotomy regarding the formation of T_{RM} cells against neoplastic tissue: those that successfully eliminate nascent transformed cells thus preventing tumor development, and those that form after a tumor is established, during the equilibrium or escape phases. In the latter situation, the cues within the TME influencing T_{RM} cell formation are likely to be different from those experienced in normal tissue. The immunological mechanisms pertinent to T_{RM} cell formation in these two scenarios remain to be elucidated.

Certain steps and features in the cancer-immune cycle are required regardless of the environmental cues influencing T_{RM} differentiation, and most important of these is tumor antigen reactivity.

At the core of the endogenous antitumor immune response are T cells that have the ability to recognize tumor-specific antigens. Tumor antigens can fall under three main classifications: tumor-specific, tumor-associated, and cancer-testis antigens [65]. Tumor-specific antigens, also referred to as neoantigens, are absent from normal cells and recognized as foreign by the host immune system. They are derived from non-synonymous driver or passenger mutations and viral genes [66]. Since tumors are derived from normal self-tissues, how neoantigens arise and how the innate immune system initiates an effector rather than a tolerogenic adaptive immune response had been unclear. Recent data have indicated that many tumor-specific antigens are derived from mutational processes that also drive oncogenesis. Defects in DNA repair machinery, exposure to mutagens (e.g., UV light and tobacco smoking), and abnormalities in enzymes that modify DNA can lead to somatic mutations, genome translocations, and alterations in gene expression as part of the process of carcinogenesis [47]. These processes lead to diverse mutational landscapes, with some commonly mutated oncogenes or tumor-suppressor genes that are characteristics of certain cancer types but also defined by a spectrum of unique mutations specific to an individual tumor [67].

It is clear that multiple cancer types can be infiltrated by $CD8^+$ T cells, and in many cases, a proportion of these T cells are tumor-antigen

specific. However, recent evidence questions this central dogma by suggesting that tumor residency does not always translate to tumor-specificity. Profiling T-cell reactivity among $CD8^+$ TILs in human tumors with MHCI tetramers or cloning TCRα/β pairs from intratumoral T cells revealed that nearly all tumors analyzed were infiltrated by both virus-specific and tumor-specific $CD8^+$ T cells [68–71]. To distinguish bystander from tumor-reactive T cells, expression of the ATP catabolizing ectonucleoside, CD39, was found to be a promising marker for tumor reactivity. Expression of CD39 correlated with T_{RM} genetic signatures and with co-expression of other resident markers, namely CD69 and CD103 [34, 69, 72]. Deciphering whether a TIL expressing T_{RM} phenotypic markers is a recent immigrant or resident cell remains a challenging problem in the field, especially with human tumor samples. Nevertheless, it is clear that many tumors are populated with tumor-reactive $CD8^+$ TILs that display a tissue-resident phenotype.

3.5 Innate Immune Factors Regulating Antitumor T-Cell Responses

Local activation of APCs is a required initiating step for a productive adaptive T-cell response against tumor antigens. The innate signaling pathways involved in this activation step were first hinted in transcriptome profiling of human tumors, where a type I IFN gene signature was found to correlate with a T-cell infiltrate [48, 73]. Mice deficient in genes involved in IFN signaling, IFNAR, and STAT1, could not control immunogenic tumors [49, 74]. Ultimately, the required APC cell population receiving the type I IFN signals were mapped to a rare population of CD8α positive classical dendritic cell (cDC1). cDC1s are known for their ability to cross-present antigens and are developmentally dependent on the transcription factors Batf3 and IRF8 [75, 76]. cDC1s are also important for the generation of T_{RM} cells in the skin and lung. *Batf3*-deficient mice exhibit blunted T_{RM} development in the skin after intradermal vaccinia virus (VACV) immunization [77]. Given the essential role cDC1s play in priming $CD8^+$ T cells against tumor antigens, it is likely that they are also critical for T_{RM} formation against tumors; however, detailed studies addressing this question remain to be performed.

The functional role for type I IFNs prompted the next level question regarding the nature of the damage-associated molecular pattern (DAMP) that could induce type I IFN production in a sterile tumor without pathogen exposure. Early studies identified several DAMPs that could be released by stressed or dying tumor cells that subsequently could lead to productive T-cell priming. For example, high-mobility group protein B1 (HMGB1) binding to TLR4 and extracellular ATP binding to the P2X7 purinergic receptor triggering activation of the NLRP3 inflammasome were both reported to induce DC maturation and subsequent activation of anti-tumor T cells [78, 79]. In apparent contrast to inducing DC maturation, extracellular ATP can also impact T_{RM} cell maintenance. T_{RM} cells can express P2RX7, which induces cell death when triggered [80]. However, attrition of T_{RM} cells can be fine-tuned by their ability to regulate local ATP concentrations through the action of the ectoenzyme CD39. Beyond HMGB1 and ATP, tumor-derived DNA was found to be a potent initiator of the endogenous antitumor immune response [81, 82]. DCs recruited to the TME were found to take up tumor-derived DNA leading to stimulator of interferon genes (STING)-dependent production of type I IFNs [83]. Besides DCs, endothelial cells of the tumor vasculature were also reported to produce type I IFNs in response to STING activation [84]. STING signaling not only activates DCs but also induces the upregulation of adhesion molecules on endothelial cells of the tumor-associated vasculature, a critical step for T-cell extravasation into the tumor [85]. STING is an endoplasmic reticulum adaptor that is activated by cyclic dinucleotides generated by cGAMP synthase [86]. The mechanism by which tumor-derived DNA can gain access to the cytosol to activate the STING pathway has yet to be elucidated. But consistent with this mechanism, immunogenic tumors fail to be rejected and grow

progressively in mice lacking STING and spontaneous priming of CD8+ T cells against tumor antigens is nearly ablated [81, 82, 87]. The cellular cues alerting the immune system of a nascent tumor are just starting to be uncovered. Mouse models highlight an important role for sensing tumor-derived DNA. Whether this occurs in the absence of spontaneous tumor cell death remains to be determined.

3.6 Antigen-Presenting Cells: The Gatekeepers of the Antitumor T-Cell Response

The process by which DCs are initially recruited to the tumor site is not fully understood and likely depends on the chemokine repertoire produced by tumor cells or the surrounding tissue. Alternatively, a subset of DCs exists at steady-state in barrier tissues, such as the CD103+ variety found in the skin [88], and therefore local activation may not require DC recruitment from the periphery. In support of this notion, it was found that CD103+ DCs were uniquely capable in the uptake of tumor antigens and trafficking to the lymph node to prime CD8+ T cells (Fig. 3.1) [89]. Regardless of the mode of DC recruitment, tumor cells can acquire the ability to produce chemokines that can contribute to their own growth, survival, and metastasis [90]. Genomic aberrations such as oncogenic pathways can impact the array of chemokines expressed. In a melanoma model, B-Raf pathway activation led to the production of the chemokine CCL4, which contributed to the recruitment of cDC1s in a CCR5-dependent manner [87]. Taken together, it is likely that both tissue-localized and circulatory DCs can effectively prime CD8+ T cells, indicating another layer of control for effective T-cell responses. Nevertheless, delineating the anatomical logistics of DC activation will be important for determining which DC populations can be effectively targeted as a cancer therapy.

After DC activation by type I IFN at the site of tumor formation, DCs traffic to the draining lymph node to prime tumor-antigen-specific naïve T cells. T-cell priming encompasses a complex series of spatial, biochemical, transcriptional, proliferative, and differentiation events that engender clonal populations of activated T cells with effector and tissue-homing programs [91]. The tissue-homing program is characterized by expression of specific chemokine receptors responsible for correctly trafficking T cells to the site of an infection or tumor and aid in entry into the tissue. The CXC-chemokine receptor 3 (CXCR3) has an intriguing role, as it was shown to be important for T-cell entry into many inflamed peripheral sites [92]. Adoptively transferred antigen-specific CXCR3-deficient CD8+ T cells failed to traffic to infected skin [26], vaginal epithelium [93, 94], and melanoma [95]. Antibody-mediated blockade of CXCR3 prevented T-cell infiltration in a model of pancreatic ductal carcinoma [96]. It must be noted that CXCR3 was not required for tissue infiltration in all experimental systems. For instance, CXCR3-deficient mice displayed similar numbers of skin-infiltrating T cells after cutaneous VACV infection and B16F10 [97] tumor engraftment [98], suggesting the existence of compensatory mechanisms. Beyond its role in aiding T-cell entry into tissues and tumors, CXCR3 was also shown to play an active role in T_{RM} generation within tissues [26], possibly through influencing intra-tissue migration [99] and cell–cell interactions [100, 101]. Consistent with this latter role, CD8+ TIL interactions with cDC1s were mediated by CXCR3, which was critical for the effectiveness of anti-PD-1 blockade in mice [98].

A strong correlation between the presence of CD8+ T cells and expression of the CXCR3 ligands, CXCL9 and CXCL10, has been observed across a range of tumor types [48, 102, 103]. The source of these chemokines in early recruitment of CD8+ T cells was initially attributed to tumor cells [48, 104] or activated keratinocytes [26]; however, in a murine model of oncogene-induced melanoma, cDC1s were identified to be the major source of CXCL9 and CXCL10 within the TME and those DCs were required to recruit activated CD8+ T cells to the tumor site in a CXCR3-dependent manner [87]. Similar results were found in an engraftable tumor model using a dual

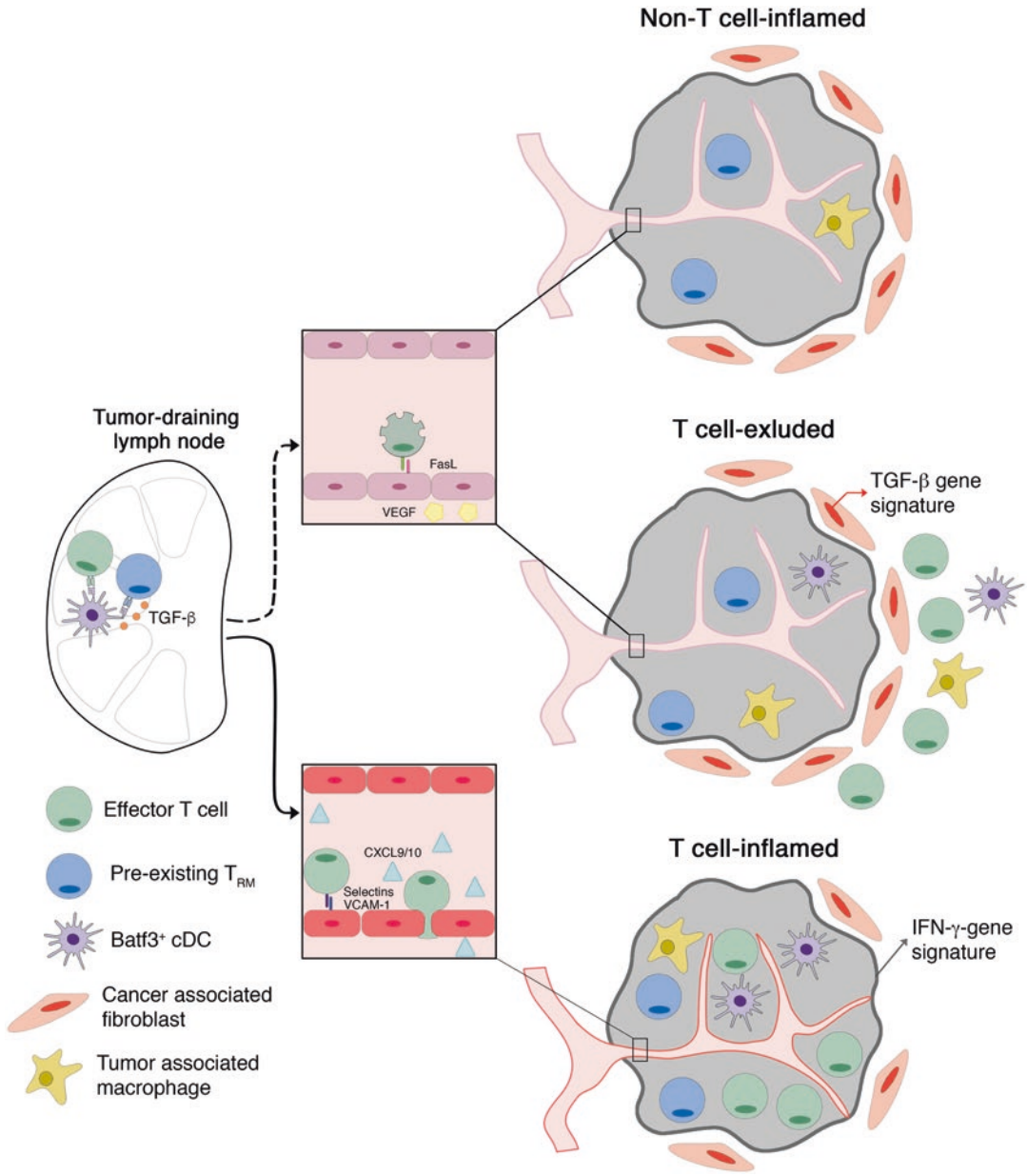

Fig. 3.1 T_{RM} cells in the context of T cell-inflamed, T cell-excluded, and non-T cell-inflamed tumors. DCs acquire tumor antigens and traffic to the LN where they prime T cells. Studies in mice indicate that the Batf3-lineage DC is particularly critical in this process. TGF-β signaling in the LN can precondition T cells to become T_{RM} cells upon entering their target tissue prior to tumor development. This suggests the possibility that preexisting T_{RM} cells are present in many tumors, despite the failure at a later time to recruit new T cells. T_{RM} cells may be an actionable target to disrupt the non-T cell-inflamed phenotype. T cell infiltration into tumors is regulated by many factors. A lack of T cell priming can occur when DCs are not recruited to the TME, for instance in the case of tumor-intrinsic β-catenin activation (non-T cell-inflamed). The tumor vasculature can also diminish T cell influx when adhesion molecules are not upregulated or induce T cell apoptosis via Fas-FasL interaction (both T cell-excluded and non-T cell-inflamed). TGF-β in the TME can act on cancer-associated fibroblasts to exclude T cells to the marginal area (T cell-excluded). In response to proper homing signals, such as CXCL9 and CXCL10 and adhesion molecule expression on vascular endothelium, T cells migrate into the tumor leading to the T cell-inflamed phenotype. This phenotype exhibits an IFN-γ-gene signature, which correlates with responsiveness to checkpoint blockade therapy

reporter for CXCL9 and CXCL10, where $CD11b^+$ DCs were also producing these chemokines [98]. In summary, DCs play a critical role in regulating the antitumor immune response, acting as sentries for detecting the initial cellular cues provided by nascent tumors. The cDC1 subset is critical for priming and recruiting $CD8^+$ T cells to the tumor site, and for providing a stimulus to T cells within the tumor.

3.7 T_{RM} Cell Commitment, Maintenance, and Function

T-cell memory encompasses not just the antigen for which a TCR is specific, but also the anatomic site of T-cell activation. The initial site of T-cell priming imprints chemokine receptors and adhesion molecules that biases migration to a specific tissue or organ where the pathogen is first encountered [105]. Upregulation of tissue-specific chemokine and adhesion molecules occurs after T-cell stimulation in coordination with molecular cues encountered in secondary lymphoid organs (SLOs) [106]. For example, in conjunction with TGF-β and retinoic acid, DCs that emigrate from intestinal tissue induce expression of $\alpha_4\beta_7$ and CCR9 on T cells within the mesenteric lymph nodes, guiding T cells to inflamed sites within the intestine [107].

Migratory potential seems to be lost over time as T_{EFF} cells isolated from the spleen 7 days after LCMV infection failed to generate T_{RM} cells [22]. This raises the question of whether T_{RM} cells are restricted to only populate sites of infection. In this scenario, the T_{RM}-arm of immunological memory would be compromised if the pathogen is re-encountered elsewhere in the body. Addressing this question, it was found that T_{RM} cells populated distant non-infected skin sites in response to skin-localized vaccinia virus infection [24]. In addition, overlapping TCR repertoires were found between T_{CM} and T_{RM} cell populations after skin immunization, which points to a common naive T-cell precursor and suggests that T_{CM} cells can serve as a reservoir for the formation of T_{RM} cells upon re-challenge [108]. In support of this notion, T_{CM} cells possess stem-like properties [109], a feature shared with T_{RM} cells, and after transfer into a naïve host, T_{CM} cells can differentiate into T_{RM} cells upon re-challenge [61]. These findings revealed additional pathways for the formation of T_{RM} cells, providing protection at sites secondary to the initial pathogen encounter. It also suggested that at least part of the T_{RM} genetic program is initiated in SLOs, while the final commitment steps occur in tissue. This is supported by a recent study proposing a mechanism where naïve T cells are preconditions to become T_{RM} cells after interacting with cognate antigen, cDCs, and TGF-β in the LN [110]. The full extent of the genetic determinants permissive for T_{RM} development initiated in the LN remains to be elucidated.

In the early phases after T-cell priming and entry into target tissue, T cells are endowed with the capacity to patrol and eliminate infected or cancerous cells. Signals within the peripheral tissue environment aid in this transformation by heightening cytotoxic capacity and cellular motility. One example is TGF-β, which has a well-documented role in the induction of the T_{RM} phenotype. In response to TGF-β, T cells entering epithelial tissue upregulate CD103 and downregulate the transcription factor KLF2, a promoter of S1PR1, which together enforce tissue residence [40]. CD103 is not only important for retaining T cells within tissues, it contributes to T-cell movement toward tumor regions [36], enhances cytotoxic functions against tumor cells [111], and can convey survival signals [26, 29]. These functional roles may help explain why the intratumoral expression of CD8 and CD103 is a more robust prognostic indicator of overall survival and predictor of response to anti-PD-1 therapy than CD8 alone [33, 34, 112]. However, an alternative possibility for this observation is that CD103 may identify the critical cDC1 population, which has been linked to the efficacy of anti-PD-1 therapy [98, 113].

After successful elimination of infected cells, T cells undergo a contraction phase where they can die by apoptosis, enter the circulation to become T_{CM} or T_{EM} cells, or remain in the tissue

to become T_{RM} cells. The selection of cells entering the T_{RM}-lineage may not entirely be stochastic, but likely depends on the differentiation state, expression of pro-survival cytokine receptors and adhesion molecules, and transcriptional regulation. For example, homeostatic cytokines, namely IL-7 and IL-15, are important for T_{RM} formation. However, their requirements are heterogenous among different tissues. IL-15 and IL-7 signaling is critical for T_{RM} formation in the skin, kidney, and liver, but not for the female reproductive tract, pancreas, or small intestine [114–117].

During the T-cell response to a pathogen, it remains unclear when commitment to the T_{RM} lineage occurs. Highly differentiated cells that are marked by high KLRG-1 and low IL-7R expression fail to differentiate into T_{RM} cells [26, 32, 117]. However, using a KLRG-1 lineage tracing mouse model, it was found that T cells which downregulated KLRG-1 but retained IL-7R expression during the contraction phase were able to differentiate into T_{RM} cells [117]. Thus, expression of KLRG-1 does not exclude cells from entering the T_{RM} cell fate and its downregulation before T_{RM}-formation may indicate escape from a terminally differentiated state. Consistent with this notion, T_{RM} cells do not express KLRG-1 at steady state and local antigen persistence is not required for T_{RM} maintenance in some tissues [29, 118]. On the other hand, KLRG-1 is upregulated after antigen stimulation [119]; therefore, it is intriguing that $CD8^+$ TILs generally do not express KLRG-1 despite the presence of local cognate antigens [120, 121]. However, in response to checkpoint blockade therapy or agonistic antibodies against co-stimulatory receptors, KLRG-1 is upregulated, which correlates with greater antitumor activity and indicates a transition into a more effector-like state [122, 123]. In summary, T_{RM} differentiation may be initiated in the LN and finalized in the tissue. The steps toward T_{RM} commitment involve local cytokine signals, which can vary among different tissues. The exact mechanism driving differentiation and commitment, as well as markers identifying T_{RM}-precursors, remains to be elucidated.

3.8 T_{RM} Cells and Tumor Immune Exclusion

Despite the ability of the immune system to recognize cancer cells, not all patients respond to checkpoint blockade therapy. Anti-PD-1/PD-L1 therapy exhibits an almost bimodal response. Some patients experience complete eradication of tumors, while a majority derive little or no clinical benefit. To explain this dichotomy, gene expression profiling across all cancer types has revealed that tumors can be classified into three major subsets, the T cell-inflamed, T cell-excluded, and non-T cell-inflamed based on the relative abundance of T cell-related transcripts (e.g., CD8A, GZMA, PRF1, and IFNG) and location of T cells relative to the tumor core (Fig. 3.1) [4, 124–127]. By segregating tumors this way, it was found that the majority of patients responding to checkpoint blockade therapy contained a T cell-inflamed tumor phenotype [4], suggesting that in these patients, the immune system has been restrained while remaining primed for reinvigoration. However, some patients within this subset fail to respond to checkpoint blockade therapy, indicating that additional resistance mechanisms must be overcome to achieve effective clinical responses [128, 129].

The non-T cell-inflamed subtype of tumor is remarkably devoid of immune cell signatures, including the negative regulatory pathways normally seen in the T cell-inflamed tumor. Exclusion of T cells from the tumor may result from the breakdown of key events required for successful T-cell recruitment. These include innate immune activation, chemotaxis, and extravasation into the tumor or surrounding tissue, and penetration from peritumoral space into the tumor bed. The mechanisms behind the evolution of non-T cell-inflamed tumors are currently under intense investigation and recent observations have helped to understand this phenotype. Genetic events with oncogenic potential include those that increase immune evasion. For example, activation of the Wnt/β-catenin pathway led to a loss of chemokines critical for the recruitment of the

Batf3-DC lineage to the tumor site, and thus a failure to activate the innate immune system [127, 130]. Inactivating mutations or deletions of PTEN led to activation of the PI3K-AKT pathway and subsequent decrease in tumor cell autophagy, thereby diminishing innate immune activation and T-cell priming [131]. The non-T cell-inflamed TME appears to be independent of nonsynonymous mutation load, indicating that a lack of immunogenic T-cell antigens does not drive exclusion [132]. However, tumor evolution in response to immune pressure can lead to tumor cells with defects in antigen presentation machinery. Many of these cases were documented from patients that developed acquired resistance to immunotherapies through loss of function mutations in B2M and HLA genes [133–137]. Neoantigen loss also contributes to immune resistance through selective killing of tumor subclones or gene silencing via epigenetic processes and chromosomal deletions can lead to an overall decrease in tumor immunogenicity [138, 139].

The tumor-associated vasculature is also a critical barrier regulating T-cell infiltration [140]. A network of arterioles, capillaries, and postcapillary venules provide avenues for T cells to enter the tumor peritumorally though the tumor stroma or intratumorally through the tumor parenchyma. Unlike peritumoral blood vessels, which can be derived from exiting normal endothelium, intratumoral vessels are often found to be immature [141]. This immaturity is driven by rapid angiogenesis when the metabolic demands of the tumor surpass the supply of the local vasculature. In response, tumors produce angiogenic factors, including vascular endothelial growth factor (VEGF), angiopoietins, and thrombospondins to induce the formation of new blood vessels. These angiogenic factors contribute to leaky and chaotically organized vessels, which often express low levels of adhesion molecules (e.g., E/P-selectin, ICAM-1/2, VCAM-1, and VAP-1) [141–146], fail to respond to inflammatory stimuli [147, 148], and can express FasL to directly kill antitumor T cells [149]. Additional players in tumor angiogenesis are pericytes, which surround blood vessels and contribute to new blood vessel formation and immune cell trafficking. Pericyte phenotype and coverage along the tumor vasculature are often found to be abnormal when compared to normal adjacent vasculature [150]. Interestingly, immune cells can interact with pericytes in a positive feedback loop resulting in normalization of blood vessels and immune-favorable changes in the TME such as hypoxia mitigation [151]. Similarly, genetic deletion of the G-protein signaling component Rsg5 induced pericyte-mediated vasculature normalization and increased T-cell recruitment [152]. These studies indicated that pericytes associated with the tumor vasculature have abnormal activity and distribution. Normalization of pericyte function to promote T-cell infiltration into the TME may be a potential therapeutic approach. Entry into the TME via the peritumoral route also contains hurdles for T cells. Stroma surrounding the tumor often contains immune-suppressive cell populations such as cancer-associated fibroblasts (CAFs), myeloid-derived suppressor cells, and tumor-associated macrophages. CAFs sterically inhibit T-cell ingress into the tumor through the synthesis of a dense extracellular matrix [153, 154]. Overall, endothelial cell immaturity, anergy, and pericyte abnormality diminish T-cell infiltration directly into the tumor parenchyma, diverting T cells to enter via perivascular routes, where the dense ECM of the tumor stroma can border T cells.

When considering immune exclusion in the context of T_{RM} cells, there is the possibility that T_{RM} cell localization at tumor sites precedes tumor formation. Given the patrolling nature of T_{RM} cells and their relative abundance within tissues (a recent study found around 500 T_{RM} cells per mm^3 in healthy human skin [19]), the question arises as to how some tumors can apparently develop without T cells present. One possibility lies in how T-cell infiltration is calculated. While methods may differ, quantification of T-cell infiltrations from RNASeq data is generally scored based on relative expression of a T cell-related gene signature. Therefore, non-T cell-inflamed samples may contain RNA transcripts below a defined threshold, but this does not translate to a complete lack of T cell-related transcripts. Thus, T cells may be present at low frequency in non-T

cell-inflamed tumors, which may represent a pre-existing T_{RM} cell population. This is consistent with recent findings that in some T cell-containing tumors the majority of T cells are specific for commonly encountered viruses (e.g., EBV, CMV) and not tumor antigens [69, 70]. However, studies quantifying T-cell infiltration by histology clearly identify tumors that are devoid of T cells, termed "immune-desert", or T cells that are retained to the peritumoral area, termed "immune-excluded" [6]. These phenotypes suggest that tumors may actively exclude T cells.

Active exclusion of T cells may involve sequestering T cells to peritumoral regions via coinhibitory receptor interactions such as PD-1:PD-L1. Biopsies taken before and after anti-PD-1/PD-L1 therapy show an increase in tumor penetration after treatment [155]. Another possibility is adhesion molecule-mediated retention of T cells in the stroma. Recent studies provide evidence for a role of TGF-β in driving peritumoral T-cell retention. While TGF-β exerts positive immune effects on T_{RM} cell differentiation and function, the abundance of *TGFB* transcripts in the tumor also correlates with poor prognosis in multiple cancer types [156–158]. Indeed, the role of TGF-β in cancer immunity is complex and contextual, exhibiting pleotropic effects on cancer, stromal, and immune cells within the tumor [159]. TGF-β can be co-opted by cancers to promote their progression by evading the growth-inhibitory effects through inactivating mutations in the TGF-β signaling pathway and maintaining the immune suppressive effects on surrounding stroma and immune cells. In particular, TGF-β signaling in CAFs was associated with poor prognosis in colorectal cancer [160, 161]. Furthermore, transcriptome analysis of tumors from patients with metastatic urothelial cancer refractory to the PD-L1 antagonist atezolizumab had an enrichment for genes involved in the TGF-β signaling pathway. This enrichment correlated an immune-excluded phenotype [162]. Similar evidence was found in a genetically engineered mouse model of colorectal cancer. In this model, combinatorial oncogenic mutations led to metastatic tumors with an immune excluded phenotype and TGF-β transcriptional signature. Interfering with TGF-β via blocking antibodies or a small molecule inhibitor for TGFBR1 redistributed T cells into the intratumoral zone and sensitized mice to PD-L1 blockade therapy [163]. Taken together, an interesting relationship emerges between the T_{RM}-promoting and the immune-excluding effects of TGF-β, where the sum of the effects results in retainment of immune cells to peritumoral regions.

3.9 T-Cell Dysfunction

The defining features of T_{RM} cells: tissue residency, tissue patrol, and rapid response to stimulus, have mainly been described under conditions of tissue homeostasis after pathogen clearance. In the context of chronic infection or persistent antigen exposure, less is known about T_{RM} differentiation or how the T_{RM} genetic program is influenced. However, it is well known that T cells isolated from tumors or from secondary lymphoid organs during chronic viral infections are dysfunctional or exhausted. Much of the knowledge surrounding T-cell dysfunction is derived from in vivo models of chronic viral infections, in particular clone 13 LCMV. In this model, antigen is continuously present, which drives the breakdown of immunological memory formation and pushes responding T cells into a state termed exhaustion, which is characterized by a gradual and sequential loss of effector functions [164–166]. Furthermore, continuous TCR signaling induces an NFAT-driven transcriptional program, promoting the expression of inhibitory receptors, including PD-1 [167, 168], which in turn blunts CD28 co-stimulatory receptor signaling in a SHP2-dependent manner [169]. Other in vivo studies found that SHP2 was dispensable for promoting exhaustion; thus, similar phosphatase-recruiting inhibitory receptors may compensate [170]. In fact, both tumor-antigen specific TILs and exhausted virus-specific $CD8^+$ T cells upregulate and maintain expression of an array of co-inhibitory receptors, including CTLA-4, TIM-3, TIGIT, and LAG-3, in addition to PD-1 [120, 121, 171–173]. Engagement of these receptors has been shown to blunt proliferation and

cytokine production by T cells, and blocking interactions between these receptors and their corresponding ligands can restore T-cell function [120, 174, 175]. Due to these key features that parallel chronic infection and cancer, persistence of antigen and expression of inhibitory receptors on T cells, it has long been proposed that dysfunctional CD8+ TILs resemble virally exhausted CD8+ T cells. Some studies have found similarities between these two cellular states [121, 176]. Other studies have found significant differences. For example, despite expression of inhibitory receptors, CD8+ TILs were found to not be functionally inert and retained the capacity to proliferate, produce cytokines, and lyse target cells [120, 174, 177]. Under the latter premise, it is unclear why a tumor is not controlled by the immune system despite a tumor-reactive T-cell infiltrate with tumoricidal properties. This question and the discrepancy surrounding T-cell functionality between chronic viruses and cancer have been looming in the background for many years [178] and not until recently, with the technological advances of single-cell genomic analyses, has a more encompassing picture emerged.

Investigations into CD8+ TIL biology using single-cell RNA sequencing (scRNA Seq) technology have revealed previously unappreciated transcriptional heterogeneity. Clustering of single cells based on the expression of core genetic signatures suggests a developmental continuum, at least for some T-cell subsets. This type of analysis has revealed similar CD8+ TIL subsets across many human cancers including human lung cancer [179–181], breast cancer [58, 182], liver cancer [183], colorectal cancer [184], and human and mouse melanoma [176, 185, 186]. In addition, the immune cell infiltrate appears to differ significantly depending on the tumor tissue type, the individual patient, and even among different metastasis sites within the same host [7]. Host genetics and environmental influences such as the composition of microbiota can have a profound impact on the transcriptional landscape of tumor-infiltrating immune cell populations [187, 188]. Despite much observed heterogeneity, these studies revealed several key CD8+ TIL populations that are commonly found across many tumor types. One of the most abundant T-cell types are dysfunctional CD8+ TILs, characterized by expression of inhibitory (e.g., Pdcd1, Havrc2), co-stimulatory (e.g., Tnfrsf9), effector cytokines (e.g., Ifng, Gzmb), and cell cycle genes. This population usually contains many expanded clones, suggesting tumor specificity, and is often actively proliferating. Generally, within this population is a subset with a transcriptional signature similar to T_{RM} cells that has been described in mice and humans [58, 179, 180, 186]. However, the precise relationship between T_{RM} cells and infiltrating T cells is not well characterized. Memory T-cell populations, including T_{CM} and T_{EM} were identified as having lower expression of inhibitory receptors while retaining expression of effector molecules (e.g., Gzmk and Prf1) and in the case of T_{CM}, expressing genes important for circulating among secondary lymphoid organs (e.g., Sell and Ccr7). Finally, a new stem-like T-cell population was found, which was characterized by the expression of genes that promote self-renewal properties (e.g., Tcf1) [189].

A primary goal of checkpoint blockade is to reinvigorate T cells into a state of potent effector function. It follows that determining the T-cell populations responding to checkpoint blockade can have important clinical implications for selecting patients who are more likely to respond. An analysis of immune cell infiltrates responding to anti-PD-1 therapy revealed that the presence of the CD8+ TCF7+ stem-like TIL population indicated a greater probability of a clinical response [185]. Furthermore, studies have suggested an interplay between the stem-like and dysfunctional CD8+ TIL populations, where the stem-like cells act as long-lived progenitors for the dysfunctional population [176, 190]. Since the stem-like population expresses intermediate levels of PD-1, it is thought that they are antigen experienced. Furthermore, PD-1 was found to promote survival of this population by preventing overstimulation [191]. This population expresses SLAM6 and CXCR5 and has antigen-independent self-renewal properties [176, 192]. In response to anti-PD-1 therapy, T cells from the stem-like population differentiate and expand into effector

cells with a dysfunctional phenotype [176, 185, 192]. Integration of these datasets indicates that transitional response to anti-PD-1 therapy first gives rise to potent cytotoxic T cells, which overtime enter into a dysfunctional state [192–194]. In line with this evidence, TCR sequence analysis revealed an overlap between the dysfunctional and stem-like T-cell populations, suggesting a clonal relationship. In addition, adoptive transfer of the stem-like TIL population into tumor-bearing mice further demonstrated their transition into the dysfunctional phenotype [176, 185]. Importantly, dysfunctional $CD8^+$ TILs can also respond to checkpoint blockade. In one proposed mechanism, it was found that $CD8^+$ TILs undergo a futile cycle of proliferation and apoptosis at steady state, which was reversed by agonistic anti-4-1BB plus anti-PD-L1 antibody treatment or by inhibiting Fas–FasL interactions [174, 175]. Similarly, targeting co-stimulatory receptors such as 4-1BB, GITR, and OX40, or other co-inhibitory receptors like LAG-3, both of which are absent or lowly expressed on stem-like TILs, can restore $CD8^+$ TIL function and induce tumor control [195]. Taken together, blocking PD-1 may stimulate $PD\text{-}1^+$ $TCF7^+$ stem-like $CD8^+$ TILs, giving rise to potent effector cells. Although these cells may eventually enter into a dysfunctional state, dysfunctional cells can be reinvigorated in response to the same anti-PD-1/PD-L1 therapy. Developing a more comprehensive understanding of the clonal and functional relationships between the stem-like population, T_{RM} cells, and dysfunctional $CD8^+$ TILs is an investigational priority with considerable therapeutic implications.

In response to cognate antigen recognition, T cells undergo a metabolic switch from oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) to aerobic glycolysis and glutaminolysis. While energetically less efficient, this allows for the rapid production of biosynthetic molecules such as nucleotides, amino acids, and lipids that are required for clonal expansion and the acquisition of effector functions [196]. Similarly, cancer cells use more aerobic glycolysis and glutaminolysis compared to normal cells to support their rapid growth. This, in combination with poor angiogenesis, nearly depletes exogenous glucose and fills the tumor with hypoxic regions. In addition, uncontrolled cell growth and necrosis can lead to a buildup of byproducts such as lactate and extracellular potassium that can interfere with T-cell function [197, 198]. Accordingly, after entering the TME, T cells undergo profound metabolic changes in response to competition with tumor cells for nutrients. For instance, in melanoma and renal cell carcinoma, $CD8^+$ TILs exhibited severely diminished glycolysis and mitochondrial function [199, 200]. In a preclinical model, $CD8^+$ TILs in tumors exhibiting low glycolytic activity maintained tumoricidal activity, while those in high glycolytic tumors did not. These data highlight a local competition for glucose that can impede the antitumor functions of T cells and impair immunotherapy [201, 202]. Due to these metabolic constraints, $CD8^+$ TILs developed the altered metabolism necessary to support survival and function. For example, in response to hypoxia and hypoglycemia, $CD8^+$ TILs upregulate PPAR-α signaling and increase FAO of exogenous lipids and decrease glycolysis. When treated with a PPAR-α agonist, $CD8^+$ T cells displayed enhanced cytolytic function after adoptive transfer into a tumor-bearing host [203]. A shift to FAO after entering the TME is also partially due to PD-1 signaling, which can inhibit glycolysis and promote FAO [204]. In the face of a metabolically hostile TME, T_{RM} cells may therefore be ideally suited to function within a tumor. As T_{RM} cells differentiate, they adapt to the metabolic constraints and available energy resources in their residing tissue. For example, T_{RM} cells that reside in the skin epidermis, which is avascular and relies on diffusion for nutrients [205, 206], have altered their metabolism to function with less oxygen and glucose. This is accomplished through mitochondrial beta oxidation using exogenous FAs scavenged from the surrounding environment [15, 207]. Reliance on FAs could benefit T_{RM} cells inside solid tumors as the lipid content is generally higher compared to normal tissue [208]. In summary, TIL function is impeded by the immunosuppressive and metabolically challenging TME, which can push TILs into a

state of dysfunction. Checkpoint blockade therapy can reinvigorate TILs and understanding which TIL populations respond is critical for designing new therapies. Evidence suggests that both the stem-like and dysfunctional TIL populations contain the capacity to respond. T_{RM} cells may be a component of the responding populations; however, further investigation is needed to characterize the nature of T_{RM} cell responses. Nonetheless, T_{RM} cells are prime targets for checkpoint blockade therapy due to their ability to function under the metabolic constraints within their tissue of residence.

3.10 Targeting T_{RM} Cells in Cancer Immunotherapy

The magnitude of the T-cell infiltrate in tumors is a major determinant of effective immunotherapy, including checkpoint blockade. Patients with low or no T-cell infiltrate are generally less likely to respond. Designing new therapeutic interventions to augment the chances of a response is a principal goal for researchers and clinicians. Targeting T_{RM} cells may provide new therapeutic avenues by either directly augmenting T_{RM} cell function or inducing the recruitment of peripheral T cells (Fig. 3.2). In fact, T_{RM} cells may be a component of the T-cell pool that is reinvigorated in response to checkpoint blockade. In their core genetic signature, T_{RM} cells can express a range of co-inhibitory receptors, such as PD-1, TIM-3, and LAG-3, as well as costimulatory receptors, such as 4-1BB and ICOS [10, 15, 26, 209]. Since T_{RM} cells are poised to rapidly respond, expression of these co-inhibitory receptors on T_{RM} cells is thought to limit unwarranted activation. In a mouse model of contact hypersensitivity, antibody blockade of PD-1 and TIM-3 exacerbated T_{RM}-driven skin inflammation in response to allergen rechallenge. Further, in response to viral challenge, T_{RM} cells were found to proliferate in situ generating a secondary pool of T_{RM} cells [17, 210]. These observations suggest that T_{RM} functions can be augmented in response to checkpoint blockade.

T_{RM} cells can also be targeted by vaccination. Cancer vaccine therapies can provoke two different T_{RM} responses by (i) priming new T-cell infiltration into the tumor and (ii) activating T_{RM} cells already present in the tumor at the time of vaccination. The recruitment of new effector T cells was the goal of many cancer vaccine trials, which have only shown limited efficacy [211]. One possible explanation may involve the route of administration. It is well documented that intramuscular vaccination induces the formation of circulating memory T cells, but only weakly induces T_{RM} cells in tissues [212, 213]. It is now evident that manipulation of the target tissue is needed to induce the proper homing and inflammatory signaling required for T_{RM} formation. Vaccine administration to mucosal sites, for instance, through intranasal, cervicovaginal, or skin scarification routes, more robustly generates T_{RM} cells at the site of vaccination [214–216]. In a preclinical model of orthotopic head and neck or lung cancer, intranasal, but not intramuscular, vaccination protected nearly all mice from tumor growth when given prophylactically, and inhibited tumor growth in the therapeutic setting [216, 217]. Similarly, vaccination by skin scarification was sufficient to slow tumor growth and synergized with circulating memory T cells [61]. Site-specific vaccination may also provide a means to disperse T cells to other unmanipulated sites, such as in the case of skin scarification [24]. This phenomenon may provide a means to promote T-cell infiltration when in situ vaccination is not possible and may help explain cases of abscopal tumor regression [218].

Other approaches combine systemic immunization with tissue-specific stimulation. In these "prime and pull" strategies, the T_{RM} precursor frequency is increased either by adoptive transfer of activated antigen-specific T cells or by subcutaneous vaccination, which is followed by antigen-independent stimulation of the target tissue to recruit and promote T_{RM} cell formation. For example, after subcutaneous vaccination to induce a circulating memory T-cell response, intravaginal injection of CXCL9 and CXCL10 resulted in the recruitment and formation of T_{RM}

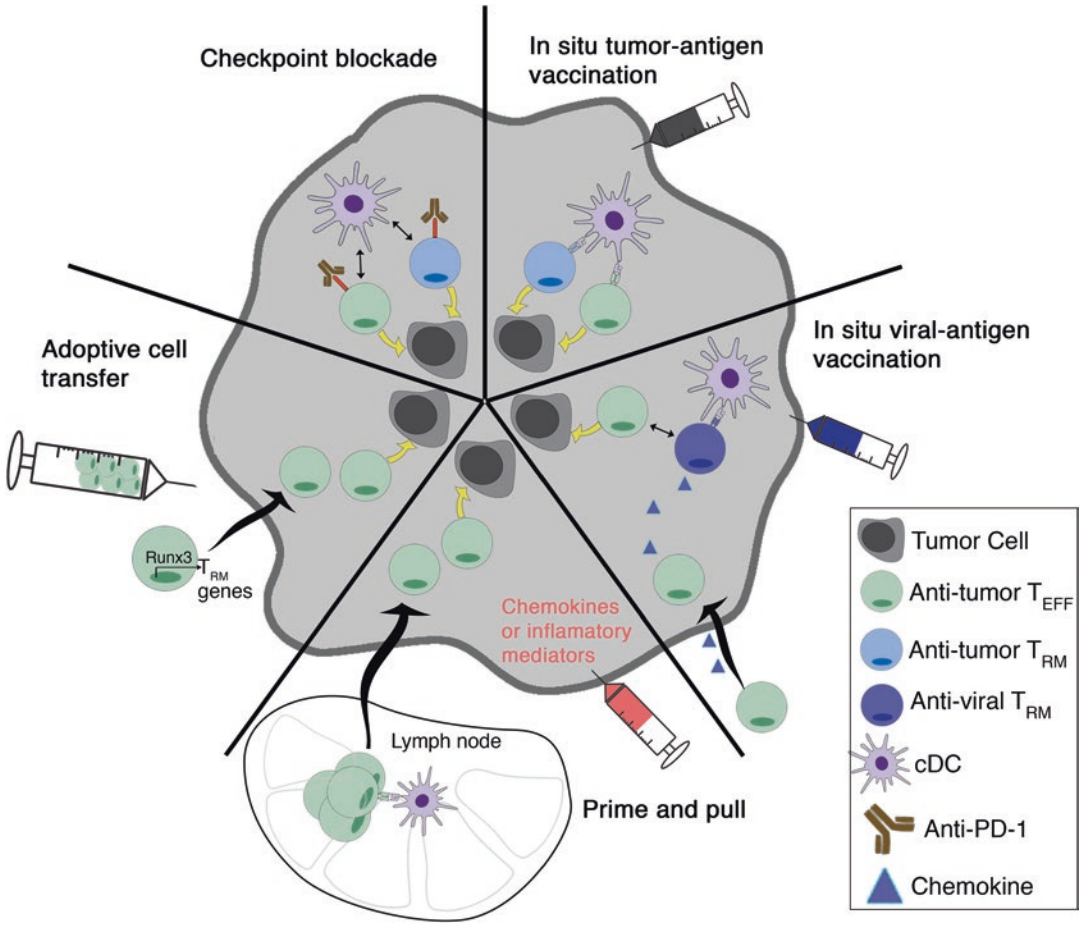

Fig. 3.2 Immunotherapeutic strategies that target T_{RM} cells to induce tumor control. T_{RM} cells may be one of the responding intratumoral T cell populations to checkpoint blockade, such as anti-PD-1, which augments their tumoricidal functions. Interactions between T cells and cDCs via CXCR3 are important for anti-PD-1 efficacy. In situ tumor vaccination with tumor antigens may activate preexisting T_{RM} cells and infiltrating T_{EFF} cells. In situ vaccination could also be used to activate anti-viral T_{RM} cells to induce positive immune changes within the TME. Such changes may include production of chemokines to recruit T_{EFF} cells or cytokines that support T_{EFF} cell functions. The "prime and pull" strategy, which has been successfully used to recruit T cells into tissues, could be adapted to recruit T cells to the tumor. In this strategy, the frequency of circulating tumor antigen-specific T_{EFF} cells is increased by immunization. Chemokines or inflammatory mediators are then injected into the tumor tissue to recruit these T_{EFF} cells. Finally, T cells extracted from the patient could be modified to express T_{RM} genes, for example, by promoting Runx3 activity, which may improve T cell infiltration and function after adoptive transfer back into the patient

cells in the vaginal tissue [94]. Likewise, adoptively transferred activated T cells were effectively recruited to the skin by topical application of the contact sensitizer dinitrofluorobenzene (DNFB) [17].

While cancer vaccination strategies show great promise, they are only possible when tumor antigens are known. Identifying neo-antigens and formulating personalized cancer vaccines remain logistically challenging and expensive. An alternative approach is to target virus-specific T cells within the tumor to incite favorable changes in other host cells found within the TME. This approach may be more feasible because for many common pathogens, immunogenic peptides are known and tumors often contain virus-specific T cells. Indeed, a recent study found that T-cell immunity against commensal papillomavirus

was critical for controlling development of skin tumors in response to chemically- or UV-induced carcinogenesis [219]. Further, activating pre-existing antiviral immunity can enhance antitumor immunity. Rosato et al. [71] showed that reactivation of VSV-specific $CD8^+$ T cells by intratumoral peptide injections could delay tumor growth and synergized with anti-PD-L1 antibody therapy.

One key to therapeutic strategies designed to enhance T-cell infiltration into tumors may lie in understanding and promoting the specific DC populations and attendant T-cell transcriptional programs that imprint T_{RM} precursor behavior. Recent data suggest that T cells in secondary lymphoid organs can be conditioned by migratory DCs to become T_{RM} cells in a TGF-β-dependent manner [110]. Promoting T_{RM} precursor characteristics can also be accomplished by manipulating transcriptional activity directly in T cells. Runx3 was found to program $CD8^+$ T cells for tissue residency and adoptive transfer of T cells overexpressing Runx3 augmented T-cell accumulation in the tumor while enhancing their anti-tumor activity [220]. These studies suggest that peripheral T cells can be programed to become T_{RM} cells by promoting genes and cellular pathways that regulate T_{RM} development. This may be an attractive approach to potentiate adoptive cell transfer therapies.

3.11 Concluding Remarks

T_{RM} cells are a unique lineage of T cells with specialized functions endowing them with the capacity to adapt and survive in their tissue of residence. Their high abundance in most peripheral tissues and ability to rapidly respond to stimuli make them prime targets for cancer immunotherapies. Studies in mice have clearly demonstrated a role for both peripheral T cells and T_{RM} cells in antitumor immunity. However, a lack of cellular markers defining T_{RM} cells from other infiltrating effector T-cell subsets has hindered determining their composition within human tumors. Recent single-cell transcriptome analyses have revealed that most immune-infiltrated tumors contain T cells with a T_{RM}-like genetic profile. Further, the abundance of T cells with T_{RM} cell characteristics often correlates with a favorable outcome and several lines of evidence suggest that T_{RM} cells may be an important population activated by anti-PD-1 therapy.

Cancer vaccination is one type of therapy that can activate intratumoral T_{RM} cells. However, the route of administration is a critical component influencing the effectiveness of this approach. While in situ vaccination has shown great potential, the immune-suppressive TME can diminish its effect. Furthermore, it remains to be determined how the TME affects T_{RM} differentiation and whether newly infiltrated T cells can become bona fide T_{RM} cells with their specific functional qualities. Understanding these influences, including which immune-inhibitory pathways are active in the TME, will be important for deciding which therapy will best synergize with in situ vaccination. Vaccinating against common viral antigens can activate preexisting T_{RM} cells to induce positive immune changes in the TME and sensitize the tumor to checkpoint blockade therapy. On balance, T_{RM} cells possess the desired functional characteristics that can be harnessed to eliminate tumors, and the study of T_{RM} cell biology in the context of cancer is nascent and a worthy endeavor.

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NK Cells in the Tumor Microenvironment

4

Camille Guillerey

Abstract

Natural killer cells are powerful effectors of innate immunity that constitute a first line of defense against cancer. NK cells express an array of germline-encoded receptors which allow them to eliminate transformed cells and spare normal, healthy cells. Owing to their ability to kill circulating tumor cells, NK cells play a major role in the protection against cancer metastases. There is also convincing evidence that NK cells protect against some hematological cancers such as acute myeloid leukemia. However, the importance of NK cells for the control of established solid tumors is rather uncertain. Several mechanisms impede NK cell-mediated elimination of solid tumors, starting with the incapacity of NK cells to infiltrate the core of the tumor. In addition, immune escape mechanisms are at play in both solid and hematological cancers. These include the immunoediting of tumor cells and aberrant chronic inflammation that renders NK cells ineffective. In this chapter, I review the phenotypic characteristics of NK cells within the tumor microenvironment. Furthermore, I describe the mechanisms by which NK cells contribute to antitumor immunity. Finally, I review the different immune-evasion factors that impair NK cell activity against cancer.

Keywords

Natural killer cells · Cancer immunology · Innate immunity · Immune escape · Immunoediting · Immunosurveillance · Immunotherapy · NK cell receptors · Cytokines · Metastases · Leukemia · T cells · Dendritic cells · ILCs · Immune checkpoints

4.1 Introduction

Natural killer (NK) cells are the cytotoxic members of the innate lymphoid cell (ILC) family [49]. They were discovered in the mid-1970s for their ability to rapidly kill tumor cells without pre-activation [69, 70]. In opposition to other ILCs which are tissue resident, NK cells are blood-circulating cells that screen our body for damaged or stressed cells. Because they can detect and respond immediately to the very early signs of tumor transformation or infection, NK cells constitute a first line of defense against viruses and cancer [136].

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Belonging to the innate immune system, NK cells are devoid of recombination activating gene (RAG)-rearranged receptors and do not mediate antigen-specific responses. Instead, the molecular basis for NK cell-mediated recognition of tumor cells is ensured by a panel of germline-encoded surface receptors [88]. Some of these receptors bind to stress-induced molecules and transmit activation signals, while other deliver inhibitory signals upon binding to "normal self" molecules (e.g., class I molecules of the major histocompatibility complex, MHC-I). Therefore, NK cell activation and the outcome of NK cell interaction with a potential target cell is tightly regulated by the fine balance of positive and negative signals NK cells receive from their receptors.

In the last few years, major progress has been made in understanding NK cell heterogeneity. Human NK cells are commonly divided into $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ subsets [26]. While $CD56^{bright}CD16^{-}$ NK cells are usually referred to as the immunoregulatory, cytokine-responsive subset, $CD56^{dim}CD16^{+}$ NK cells show potent killing activity and secretion of IFN-γ when stimulated with target cells [79]. In healthy donors, $CD56^{bright}$ NK cells represent 5–10% of peripheral blood NK cells, but these cells are more abundant in tissues. According to the linear model of NK cell differentiation, $CD56^{bright}$ NK cells constitute the precursors of $CD56^{dim}$ NK cells [106]. However, other models have been proposed, whereby different NK cell subsets arise from distinct lineages [24]. The question of equivalence between mouse and human NK cell subsets has occupied scientists for more than a decade [57]. It is only recently that high throughput single-cell RNA sequencing allowed the establishment of a correspondence across the two species [29]. NK1 cells, defined as human $CD56^{dim}$ NK cells or mouse $CD27^{-}CD11b^{+}$ NK cells, constitute a cytolytic subset and express a 5-gene signature (GZMB, PRF, EMP3, ITGB2, and EB2). The NK2 subset, defined as human $CD56^{bright}$ NK cells or mouse $CD27^{+}CD11b^{-}$ NK cells, is characterized by the expression of a single gene (XCL1, encoding a chemoattractant). It is important to keep in mind that NK cell diversity expands far beyond these two main subsets. Indeed, mass cytometry analysis of 28 NK cell receptors revealed a remarkable degree of heterogeneity with an estimation of 6000–30,000 phenotypic NK cell subsets in the peripheral blood of a given individual [60].

Owing to their ability to identify and rapidly kill genetically stressed or transformed cells, NK cells are crucial protagonists of anticancer responses. However, according to the dogma, NK cells eliminate newly arising tumors or metastases but are rather ineffective against established large solid tumors. Developing tumors employ a myriad of mechanisms to escape from NK cell-mediated immunosurveillance leading to limited access of NK cells to the tumor bed, alterations of NK cell phenotypes and functions and loss of immunogenicity impeding tumor cell recognition by NK cell receptors. In this chapter, I review the phenotypic characteristics of NK cells within the tumor microenvironment. Furthermore, I describe the mechanisms by which NK cells contribute to antitumor immunity. Finally, I review the different immune-evasion factors that impair NK cell activity against cancer.

4.2 Tumor Infiltration by NK Cells

NK cells have been shown to infiltrate primary tumors of solid cancers [16], as well as metastases [55, 105] and tumor-infiltrated lymph nodes [2]. However, NK cell infiltration of most solid tumors is rather scarce [116], and the majority of studies reported reduced NK cell infiltrate in malignant tissues when compared with corresponding nonmalignant tissues (Table 4.1). Several factors are likely to influence the degree of NK cell tumor invasion. An important factor is the tumor localization since in healthy individuals, organs are differentially populated by NK cells [16]. However, comparison of metastases from different cancer types revealed that the nature of the cancer cells greatly influences the degree of NK cell infiltration. Indeed, higher NK cell infiltrates have been reported in lung metastases of renal cell carcinomas when compared

Table 4.1 Comparison of NK cell infiltration levels between malignant and corresponding nonmalignant tissue

Cancer	Method	Phenotype	Tumor tissue	Normal tissue	Infiltration	Reference
Breast cancer	FC	$CD3^-$ $CD56^+$	Primary tumor	Healthy mammary tissue	↘ =[a]	Mamessier et al. [80]
Colorectal carcinoma	IH	$NKp46^+$	Primary tumor	Adjacent normal mucosa	↘	Halama et al. [56]
Colorectal carcinoma	FC	$NKp46^+$	Liver metastasis	Normal liver tissue	↘	Halama et al. [56]
Gastric and colorectal cancers	FC	$CD3^-$ $CD56^+$	Liver metastasis	Normal liver tissue	↘	Gulubova et al. [55]
Endometrial cancer	FC	$CD3^-$ $CD56^+$	Primary tumor	Adjacent normal tissue	↘	Degos et al. [32]
Esophageal cancer	FC	$CD3^-$ $CD56^{dim}$	Primary tumor	Normal mucosa	↘	Izawa et al. [66]
Gastric cancer	FC	$CD3^-$ $CD56^{dim}$	Primary tumor	Normal mucosa	↘	Izawa et al. [66]
Melanoma	FC	$CD3^-$ $CD56^+$	Tumor infiltrated LN	Ipsilateral tumor-free LN	↗	Ali et al. [2]
Non-small-cell lung carcinoma	FC	$CD3^-$ $CD56^+$	Primary tumor	Nonmalignant lung tissue	↘	Esendagli et al. [40]
Several[b]	FC	$CD3^-$ $CD56^+$	Primary tumor	Corresponding normal tissue	=	Carrega et al. [16]

FC flow cytometry, *IH* Immunohistochemistry, *LN* lymph node
↘ NK cell infiltration is decreased in tumor compared with normal tissue, = NK cell infiltration is similar in tumor and normal tissue, ↗ NK cell infiltration is increased in tumor compared with normal tissue
[a]Similar proportions but decreased numbers
[b]Colorectal, lung, stomach, breast, adrenal gland, and kidney cancers

with those of colorectal carcinomas [105]. A high variability between patients has also been observed. For instance, in endometrial cancer, intra-tumoral NK cells have been detected in 60% of patients and are absent in the remaining 40% [132]. The localization of NK cells within the tumor is likely to impact on NK cells' ability to control cancer progression. In this regard, immunohistochemistry analyses of primary tumors from non-small-cell lung cancer patients revealed that NK cells mostly localized at the invasive margin of the tumor and did not appear to be in direct contact with the tumor cells [17, 98]. These observations suggest that NK cell-mediated protection against established solid tumors is limited by NK cells' inability to reach their targets.

Although historically high levels of NK cell infiltration have been associated with favorable prognostic factors and better survival, many reports used CD57 as an NK cell-identifying marker (Table 4.2). Since CD57 only stains a subset of NK cells, and this marker is also expressed by T cells, additional studies should be performed to confirm the prognostic value of NK cell infiltrate. NKp46 is currently considered the most reliable marker to identify NK cells [87]. In colorectal carcinoma, high $CD57^+$ infiltrate is associated with good prognosis while $NKp46^+$ infiltrate has no prognostic value [105]. Data to date point to a correlation between high NK cell infiltration and better prognosis in renal cell carcinoma [105, 114], but NK cell infiltrate does not seem to influence the outcome of non-small-cell lung cancer patients [98]. Interestingly, a recent report suggested that the prognostic benefit of tumor-infiltrating NK cells may depend on the concurrent expression of ligands for NK cell receptors in the tumor microenvironment [132]. This study showed that, in endometrial cancer, NK cell presence in the tumor was associated with enhanced disease-free survival only when human leukocyte antigen (HLA)-E (a ligand for the inhibitory NK cell receptor NKG2A and the activating receptor NKG2C) was expressed at high levels. However, if HLA-E expression was

Table 4.2 Association between NK cell infiltration and cancer prognosis

Tumor	Method	NK cell identification	Prognostic factor	Prognostic of high NK cell infiltrate	Cohort size (patient number)	Reference
Non-small-cell lung cancer	FC	$CD3^-CD56^+$	Tumor size	–[a]	28	Carrega et al. [17]
Renal cell carcinoma	FC	$CD3^-CD56^+$	Distant metastasis	+	34	Schleypen et al. [114]
Non-small-cell lung cancer	FC	$CD3^-CD56^+$	Overall survival	None	30	Platonova et al. [98]
Endometrial cancer	IC	$NKp46^+$	Disease-free survival	+/– [b]	303	Versluis et al. [132]
Colorectal cancer (lung metastases)	IC	$NKp46^+$	Overall survival	None	140	Remark et al. [105]
Renal cell carcinoma (lung metastases)	IC	$NKp46^+$	Overall survival	+	52	Remark et al. [105]
Squamous cell lung carcinoma	IC	$CD57^+$	Overall survival	+	50	Villegas et al. [133]
Gastric carcinoma	IC	$CD57^+$	Overall survival	+	146	Ishigami et al. [65]
Colorectal carcinoma	IC	$CD57^+$	Overall and disease-free survival	+	157	Coca et al. [25]
Pulmonary adenocarcinoma	IC	$CD57^+$	Overall survival	+	150	Takanami et al. [126]
Lung adenocarcinoma (brain metastasis)	IC	$CD57^+$	Time free of intracranial disease	None	20	Vaquero et al. [130]

+ High NK cell infiltration is associated with good prognostic factors or better survival, – High NK cell infiltration is associated with poor prognostic factors or lower survival

[a]Larger tumors have higher percentages of NK cell infiltration

[b]In cases of normal HLA-E expression, disease free survival is reduced when NK cells are present, whereas in cases of upregulated HLA-E expression, disease free survival is increased when NK cells are present

normal, the presence of NK cells was associated with worse prognosis.

The mechanisms driving NK cell infiltration of tumors are not fully understood and are likely to vary depending on the cancer type and the tumor localization. An accumulation of $NKp46^+$ or $CD3^-CD56^+$cells (encompassing NK cells and subsets of helper ILCs) within a malignant tissue might result from a combination of cell recruitment from the blood stream and expansion of tissue-resident populations. The primary chemokine receptors involved in NK cell migration to inflammatory stimuli are CCR2, CCR5, CXCR3, and CX3CR1 [48]. Those allow NK cells to respond to the pro-inflammatory chemokines CCL2, CCL3, CCL5, CCL8, CCL9, CCL11, CCL13, CXCL9, CXCL10, CXCL11, and CX3CL1. The CXCR3/CXCL10 axis has been identified as a major mechanism driving NK cell accumulation in subcutaneous mouse tumors, as well as in xenograft models of melanoma [140, 141]. CXCR3, together with CXCR4, might also regulate NK cell localization to the bone marrow in the context of multiple myeloma [100]. Since $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets differ in their expression of chemokine receptors [48], specific chemokine microenvironments may lead to the preferential accumulation of one subset. In melanoma patients, the CXCR2/CXCL8 axis was suggested to mediate the recruitment of $CD56^{dim}$ NK cells to the metastatic lymph node while the CCR2/CCL2 axis would recruit

CD56^{bright} NK cells and CCL19/CCL21 would attract both subsets through CCR7 [2]. Moreover, chemerin, a chemoattractant protein that acts through the receptor CMKLR1 expressed by CD56^{dim} NK cells [48], has been identified as an NK cell-attracting factor in mouse cancer models [92, 93]. However, the detection of NK cell-homing factors within the tumor microenvironment is not always associated with intra-tumor NK cell infiltrate. For instance, despite elevated levels of NK cell-attracting chemokines, colorectal carcinomas are poorly infiltrated by NK cells [56]. This suggests that additional signals might be required to allow NK cell accumulation within the tumor bed and/or that inhibitory mechanisms might prevent NK cells from entering the tumor or from surviving within the tumor microenvironment. In this regard, an interesting study established that NK cell intrinsic expression of heparanase, an enzyme known to degrade heparan sulfate proteoglycans of the extracellular matrix, was absolutely necessary for tumor invasion and immunosurveillance in mice [102].

4.3 Phenotype of Intra-Tumoral NK Cells

A vast number of studies reported dysregulated proportions of CD56^{dim}CD16^{+} and CD56^{bright}CD16^{-} NK cells infiltrating solid tumors (Table 4.3). However, some studies used peripheral blood as a comparison. In this context, the higher percentages of CD56^{bright} or CD16^{-} NK cells observed in esophageal cancer and renal cell carcinoma might only reflect differences between tissue and blood-circulating NK cells [114, 142]. Direct comparison between malignant and corresponding nonmalignant tissue revealed increased CD56^{bright} NK cell infiltrate in lung and breast cancers [16, 17, 80]. By contrast, higher percentages of CD56^{dim} NK cells were found in tumor-infiltrated lymph nodes of melanoma patients, when compared with control non-infiltrated lymph nodes [2]. This last observation was associated with decreased CD56^{dim} NK cells in the peripheral blood of melanoma patients, suggesting that, in these patients, CD56^{dim} NK cells are recruited from the blood circulation to the tumor-colonized lymph node. Distortion in the proportions of CD56^{bright} and CD56^{dim} NK cell subsets has also been observed in the blood of hematological cancer patients (Table 4.4). The proportion of CD56^{bright} cells is increased in B cell lymphomas [131] while it is decreased in acute myeloid leukemia [23].

Distortion in NK cell subset representation is accompanied by changes in the expression of NK cell receptors (Table 4.5). The severity of these alterations varies depending on the type of malignancy. Significant differences in NK cell receptor expression have been observed in non-small-cell

Table 4.3 Proportions of CD56^{bright} NK cells infiltrating solid tumors

Cancer type	CD56^{bright} in tumor	Control tissue	Reference
Esophageal cancer	↗ (30%)	Peripheral blood (10%)	Zheng et al. [142]
Non-small-cell lung cancer	↗ (40%)	Peritumoral lung tissue (20%)	Carrega et al. [17]
Breast cancer	↗ (34%)	Healthy mammary tissue (15%)	Mamessier et al. [80]
Endometrial cancer	= (10%)	Adjacent tissue (15%)	Degos et al. [32]
Colorectal cancer	= (60%)	Normal tissue (60%)	Carrega et al. [16]
Lung cancer	↗ (50%)	Normal tissue (10%)	Carrega et al. [16]
Stomach cancer	= (50%)	Normal tissue (70%)	Carrega et al. [16]
Breast cancer	↗ (30%)	Normal tissue (5%)	Carrega et al. [16]
Kidney cancer	= (40%)	Normal tissue (40%)	Carrega et al. [16]
Metastatic lymph node of melanoma	↘ (50%)	Control lymph node (70%)	Ali et al. [2]

The percentages of CD56^{bright} NK cells among total NK cells in tumors and control tissues are indicated
↗ percentages of CD56^{bright} NK cells are increased compared with control, = no significant difference observed between tumor and control, ↘ percentages CD56^{bright} NK cells are decreased compared with control

Table 4.4 Percentages of total NK cells and proportions of CD56^{bright} NK cells in the blood of hematological cancer patients

Cancer type	Total NK cells	CD56^{bright} NK cells	Reference
Healthy	10%	10%	Cooper et al. [26]
Hodgkin lymphoma	↘ (6.2%)	↗ (38%)	Vari et al. [131]
Diffuse large B-cell lymphoma	↘ (5%)	↗ (28%)	Vari et al. [131]
Childhood B-cell acute lymphoblastic leukemia	↘ (3.3%)	=	Rouce et al. [108]
Acute myeloid leukemia	↘ (<1%)	↘(1.3%)	Costello et al. [28], Chretien et al. [23]
Chronic myeloid leukemia	↘ (5.10%)	=	Chen et al. [20]
Multiple myeloma	=	=	Konjevic et al. [72]

The first line indicates the proportions in the blood of healthy volunteers for comparison
↘ decreased compared with healthy controls, = no significant difference observed when compared with healthy controls, ↗ increased compared with healthy controls

Table 4.5 Alterations of NK cell receptor expression in cancer patients

	Melanoma metastatic LN	NSCLC	Breast cancer	HL and DLBCL	Childhood B-ALL	MM	AML
NKG2D		=	↘		=	=	=
DNAM		↘	↘			↘	↘
NKp46		=	=	=	↘	=	↘
NKp30		↘	↘	=	=	=	↘
NKp44		↗			=		↘
NKp80		↘					
CD16	↗	↘	↘	↘		↘	↘
2B4		↗	↘			↘	
KIR	↗	↘			=		
NKG2A		↗	↗		↗		↘
CD69	↗	↗		=			
HLA-DR		↗		=			
Reference	Ali et al. [2]	Carrega et al. [17], Platonova et al. [98]	Mamessier et al. [80]	Vari et al. [131]	Rouce et al. [108]	Fauriat et al. [41], El-Sherbiny et al. [38]	Costello et al. [28], Sanchez-Correa et al. [111]

For solid tumors (i.e., melanoma, NSCLC, breast cancer), the phenotypes of tumor-infiltrating NK cells were compared to those of NK cells from corresponding normal tissue. For hematological cancers (i.e., HL and DLBCL, B-ALL, MM, and AML), the phenotypes of patient peripheral blood NK cells were compared to those of healthy donors
LN lymph node, *NSCLC* non-small cell lung cancer, *HL* Hodgkin lymphoma, *DLBCL* diffuse large B cell lymphoma, *B-ALL* B-cell acute lymphoblastic leukemia, *MM* multiple myeloma, *AML* acute myeloid leukemia
↘ decreased expression, = no significant difference, ↗ increased expression

lung carcinomas [17, 98] and in breast cancers [80]. An interesting study comparing NK cells at different stages of breast cancer showed that NK-cell phenotypic alterations accompanied tumor progression and were much more pronounced at metastatic stages than in benign mammary tumors [80]. Among hematological cancers, the most drastic alterations have been observed in acute myeloid leukemia [28, 111], while only minor modifications were reported in B-cell lymphomas [131]. The most common alterations are the downregulation of activating receptors such as DNAM-1, NKG2D, and CD16 [38, 80, 98]. These alterations may hamper the immunosurveillance role of NK cells. Moreover, the decreased expression of CD16 observed in most solid and hematological cancers (that is likely to partially result from reduced proportions of CD56^{dim}CD16^{+} NK cell subset) has important therapeutic implications since CD16 is the

receptor mediating antibody-dependent cellular cytotoxicity (ADCC), a process involved in the mechanism of action of some monoclonal antibody (mAb) therapies [71].

Alterations of NK cell receptor expression and subset representation might be the result of defective maturation since $CD56^{bright}$ NK cells are known to differentiate into $CD56^{dim}$ NK cells [61, 106]. The $CD56^{bright}KIR^{-}CD16^{-}$ NK cell phenotype observed in gastrointestinal sarcomas is consistent with a blocked maturation process [33]. By contrast, increased percentages of mature NK cells characterized by a $CD56^{dim}KIR^{+}CD57^{+}$ phenotype have been detected in the blood of patients with acute myeloid leukemia [23]. The terminal differentiation marker CD57 was also found to be upregulated on NK cells in metastatic lymph nodes of melanoma patients [2]. While CD57 identifies mature NK cells with high cytotoxic potential and responsiveness to CD16 stimulation [77, 90], an alternative maturation pathway characterized by the loss of DNAM-1 expression on $CD56^{dim}$ NK cells has recently been reported [125]. Peripheral blood DNAM-1^{-} NK cells, which were found to arise from DNAM-1^{+} NK cells, are poorly functional in terms of cytokine secretion and killing capacity. Interestingly, increased proportions of the dysfunctional DNAM-1^{-} NK cells were observed in the blood of B-cell lymphomas patients, and overrepresentation of this terminally differentiated subset might contribute to cancer escape from immunosurveillance.

Furthermore, tumor-infiltrating NK cells often exhibit an activated/exhausted phenotype. Upregulation of the activation markers CD69, NKp44, and HLA-DR has been observed on NK cells infiltrating non-small-cell lung carcinoma or melanoma metastatic lymph nodes [2, 17, 98]. The upregulation of checkpoint molecules such as PD-1, Tim-3, TIGIT, and LAG-3, often regarded as a sign of exhaustion, has been detected in esophageal and endometrial cancers, as well as in B-cell lymphoma patients [32, 131, 142].

4.4 Regulation of NK Cell Responses Within the Tumor Microenvironment

NK cell-mediated killing is a tightly regulated process involving an array of germline-encoded surface receptors that screen the spectrum of ligands expressed on the surface of the target cell [136]. Moreover, cytokines and pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) modulate NK cell activity (Fig. 4.1).

4.4.1 Tumor Cell Recognition Through NK Cell Receptors

The "missing-self" recognition, which refers to the detection of cells lacking self MHC-I molecules, is a major functional feature of NK cells. This phenomenon was proposed almost 30 years ago [76] to explain NK cells' ability to spare healthy cells (expressing normal levels of MHC-I molecules) and to react against stressed or transformed cells that have downregulated MHC-I molecules as an immune-escape mechanism [44]. The "missing-self" recognition is mediated by killer cell immunoglobulin-like receptors (KIRs) in humans and the Ly49 receptor family in mice [81]. The KIR and Ly49 families include various members with distinct binding affinity for different allelic variants of MHC-I molecules. In addition to KIRs/Ly49 receptors, the CD94/NKG2A receptor complex (expressed by both human and mouse NK cells) delivers negative signals upon binding to nonclassical MHC-I molecules (HLA-E in human and Qa-1 in mice). NK cell autoreactivity is further controlled through a process called NK cell education, which ensures that fully responsive NK cells express at least one inhibitory receptor that is specific for a self-MHC-I ligand [118].

The lack of inhibitory signals is usually not sufficient to trigger NK cell cytotoxicity or cytokine production. Indeed, NK cell activation

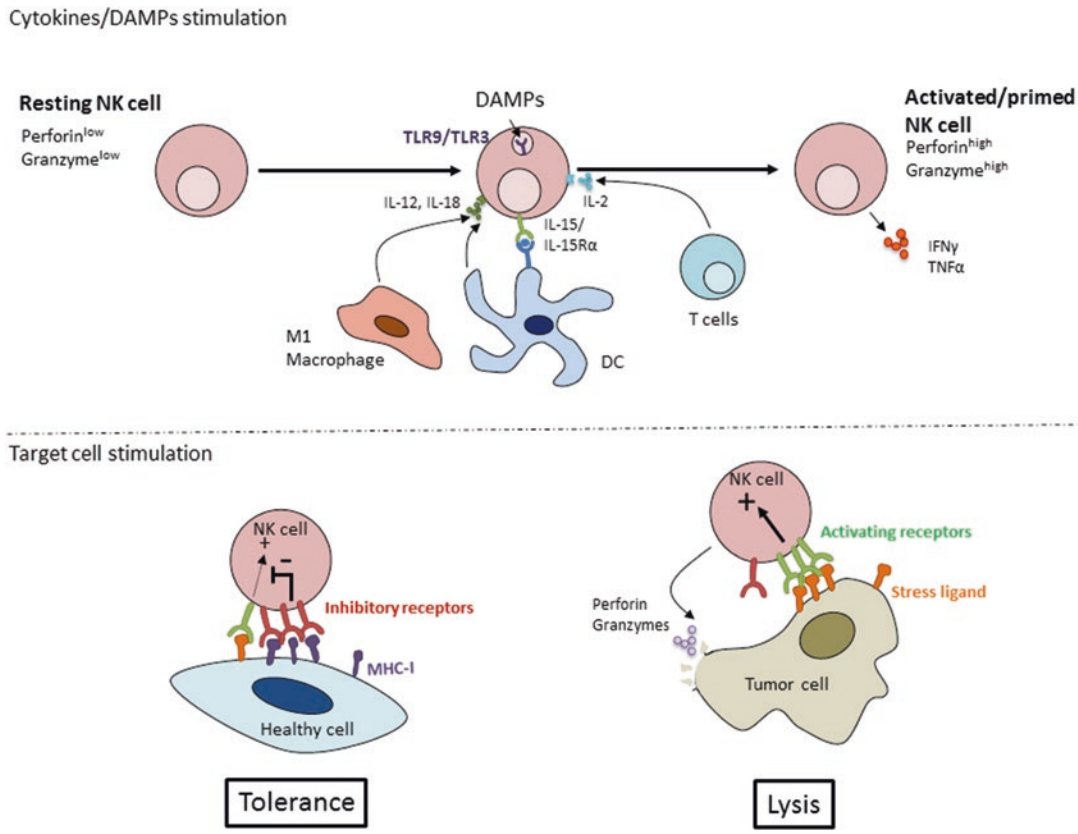

Fig. 4.1 NK cell activation. NK cells receive activation signals from soluble factors present in the tumor microenvironment (upper panel) or from their receptors upon interaction with their potential target cells (lower panel). Several cell types (including M1-polarized macrophages, DCs, and T cells) contribute to NK cell activation via the production of cytokines such as IL-2, IL-12, IL-15, and IL-18. These cytokines can induce NK cells to secrete IFN-γ or TNF-α. They also increase the killing potential of NK cells by augmenting their expression of effector molecules (perforin and granzyme). The outcome of NK cell interaction with a potential target cell is determined by the dynamic balance between the positive and negative signals transmitted by NK cell receptors. Inhibitory receptors recognize self-MHC-I molecules while activating receptors bind to stress-induced ligands upregulated on the surface of malignant cells. DAMP damage-associated molecular pattern, TLR Toll-like receptor, DC dendritic cell, MHC-I class I major histocompatibility complex

requires activating signals delivered by cell-surface receptors recognizing ligands expressed on the surface of stressed, damaged, or transformed cells [134]. NKG2D and natural cytotoxicity receptors (NCRs) are activating receptors playing an important role in cancer immunosurveillance. NKG2D recognizes MHC-I homologous molecules (e.g., MICA, MICB, and ULBPs in humans; Rae-1, Mult-1, and H60 in mice) and signals through the adapter DAP10 to induce NK cell degranulation and cytokine production [103]. The cellular expression of NKG2D ligands is regulated by DNA-damage pathways [45] and experiments in mice have revealed that NK cells play a crucial role in the rejection of tumor cells overexpressing NKG2D ligands [35]. The NCR family includes NKp30, NKp44, and NKp46 [88]. All NCRs are expressed by human NK cells while only NKp46 is expressed in mice. Moreover, NKp44 is not expressed on resting NK cells but is upregulated after activation. NCRs contain a transmembrane domain that interacts with signaling adaptor proteins containing immunoreceptor tyrosine-based activation motifs (ITAMs) and thereby mediate NK cell activation [73]. While the first NCR ligands identified were

of viral origin, several NCR ligands contributing to NK cell lysis of tumor cells have now been identified. All three NCRs can recognize tumor membrane-associated heparan sulfates [58]. Other NCR ligands expressed by tumor cells include B7-H6 [12] and HLA-B-associated transcript B (BAT3) [99]; both of them are recognized by NKp30. NKp44L, a shorter isoform of the mixed-lineage leukemia 5 protein, has been identified as a cancer cell-expressed ligand for NKp44 [9]. Recently, platelet-derived growth factor (PDGF)-DD, a soluble factor secreted by many tumors, has also been shown to stimulate NK cell functions through NKp44 [7]. In vivo experiments using mice modified to express NKp44 (NCR2-Tg mice) demonstrated that NKp44-PDGF-DD interactions induced IFN-γ and TNF-α secretion by NK cells, resulting in tumor cell growth arrest [7]. It is noteworthy that although NCRs are commonly seen as activating receptors, they can sometime deliver negative signals. For instance, proliferating cell nuclear antigen (PCNA), an NKp44 ligand often overexpressed by cancer cells, has been shown to inhibit NK cell functions [107]. While activating signals through NKp44 are mediated via the adapter DAP12, PCNA initiates a signaling cascade through the immunoreceptor tyrosine-based inhibitory motifs (ITIM) domain located in the NKp44 cytoplasmic tail. Alternative splicing of the NKp44 mRNA results in three isoforms; the NKp44-1 isoform but not NKp44-2 nor NKp44-3 harbors ITIM in its cytoplasmic portion. Consequently, in acute myeloid leukemia, poor survival has been associated with an NKp44-1 splice variant profile [117]. Similarly, alternative splicing of the NCR3 gene gives rise to three isoforms of NKp30 [33]. While NKp30a was shown to stimulate NK cell cytotoxicity and both NKp30a and NKp30b could stimulate the production of IFN-γ, NKp30c induced the secretion of the immunosuppressive cytokine IL-10 and has been associated with poor outcome in gastrointestinal cancers.

Receptors that bind to nectin and nectin-like family proteins have emerged as critical regulators of NK cell functions [83]. Those receptors include CD226 (DNAM-1), T-cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) and CD96 (TACTILE) and are expressed by both T cells and NK cells. CD226, TIGIT, and CD96 all bind to CD155 (also called PVR), a ligand expressed on antigen-presenting cells and activated lymphocytes, as well as non-hematopoietic cells and tumor cells from variable origin. In addition, TIGIT and CD226 bind to CD112 (nectin-2), and TIGIT also interacts with CD113. CD226 is an adhesion molecule that delivers co-stimulatory signals to NK cells [96]. Experiments in mouse tumor models indicated an important role for DNAM-1 in cancer immunosurveillance [51, 62]. By contrast, TIGIT and CD96 are inhibitory receptors that have emerged as new targets for immunotherapy [19, 36, 124].

4.4.2 Soluble Factors Regulating NK Cell Activity

4.4.2.1 Cytokines

Cytokines such as IL-2, IL-12, IL-15, IL-18, and type I IFNs are critical regulators of NK cell functions [143]. Within the tumor microenvironment, these cytokines are most likely to be secreted by the immune infiltrate, and particularly myeloid cells and T cells [31, 119]. IL-15 is essential for NK cell ontogeny, while both IL-2 and IL-15 promote NK cell survival, proliferation, and cytotoxic activity. For instance, IL-2-activated human NK cells are more sensitive to receptor stimulation [13]. In mice, the trans-presentation of IL-15 on IL-15Rα by dendritic cells (DCs) was found critical for the acquisition of IFN-γ-producing and killing capacities by NK cells, a process termed "NK priming" [78]. CIS (encoded by the gene Cish) is a negative regulator of IL-15 signaling; and Cish$^{-/-}$ mice were reported to be resistant to experimental metastasis, a phenotype that was associated with enhanced NK cell activity [34]. The importance of IL-15 for NK cell priming was also demonstrated in humans as brief exposure to IL-15 was found to improve the antitumor function of CD56^{bright} NK cells [138]. Cytokines such as IL-12 or IL-18 have little activity on NK cells when used on their own. However, IL-12 and

IL-18 synergize with other cytokines to potentiate NK cell functions. For instance, the combination of IL-12, IL-15, and IL-18 induces "memory-like" NK cells with increased responsiveness to IL-2 [74].

4.4.2.2 DAMPs

Some chemotherapy or radiotherapy treatments may stimulate the release of DAMPs that are recognized by Toll-like receptors (TLRs) and induce innate immune responses [4]. Human NK cells express the endosomal receptors TLR3 and TLR9, which allow responses to double-stranded RNA and CpG oligonucleotides, respectively [121]. Interestingly, the KIR receptor KIR3DL2 was found to interact with CpG oligonucleotides [120]. Data suggested that KIR3DL2 may act as a transporter that shuttles CpG oligonucleotides from the surface to the endosomes where TLR9 is localized. Moreover, NK cells isolated from $Tlr3^{-/-}$mice are hyporesponsive to in vitro cytokine stimulation [50]. Various studies performed in mice established the potential of TLR-ligand administration to enhance antitumor NK cell responses [1, 7, 21]. However, the importance of direct sensing of DAMPs by NK cells remains unclear. By contrast, there is evidence that TLR ligands stimulate NK cell functions in an indirect manner via DCs [75].

4.5 Anticancer Roles of NK Cells

Evidence for anticancer functions of NK cells has been mostly provided by mouse cancer models using NK cell-depleting antibodies or NK cell-deficient mouse strains (reviewed in [53, 54]). The most convincing data supporting a role of NK cell in the immunosurveillance of human cancers come from an epidemiology study of the Japanese population [64]. This study revealed that individuals with low cytotoxic activity of peripheral blood lymphocytes against K562 target cells have an enhanced risk of cancer development. Additional evidence for a protective role of NK cells against human cancers comes from clinical studies involving acute leukemia patients for whom the transfer of allogeneic KIR-mismatched NK cells appeared to be beneficial [30, 109, 110]. NK cells protect against tumor progression through various mechanisms (Fig. 4.2). Those include cytotoxic activity and the secretion of cytokines such as IFN-γ or TNF-α that may directly act on the tumor cells to decrease their survival or proliferation. Moreover, NK cells play an important role in shaping the immune response by interacting with cells from either the innate or the adaptive immune system.

4.5.1 NK Cells Provide Direct Protection Against Cancer Cells

NK cells directly eliminate cancer cells through the release of cytotoxic granules or by engaging death receptors. Both modes of killing require the formation of an immune synapse between the NK cell and the target cell. The initiation, maintenance, and termination of the immune synapse is a highly regulated process that determines the ability of NK cells to consecutively kill multiple targets [89]. Granule-mediated cellular toxicity appears as a major mechanism of cancer immunosurveillance [68, 129]. This pathway is entirely dependent on the pore-forming protein perforin, which allows serine proteases called granzymes and other granule-contained lytic molecules to reach the cytosol of the target cell, leading to the cleavage of several substrates and the consequent initiation of pro-apoptotic cascades [137]. In addition, NK cells can express the death receptors FasL and TNF-related apoptosis-inducing ligand (TRAIL). FasL and TRAIL bind to their receptors – Fas (CD95) and DR-4/DR-5, respectively – on the surface of the target cell and thereby initiate the formation of the death-signaling inducing complex, ultimately leading to the apoptosis of the target cell [97]. Although death-receptors do not appear as crucial as the granule-exocytosis pathway [122], experiments in mice suggested that FasL and TRAIL could contribute to NK cell-mediated control of liver metastasis [37, 127].

As different modes of cancer cell death lead to the release of different activating factors [43], the

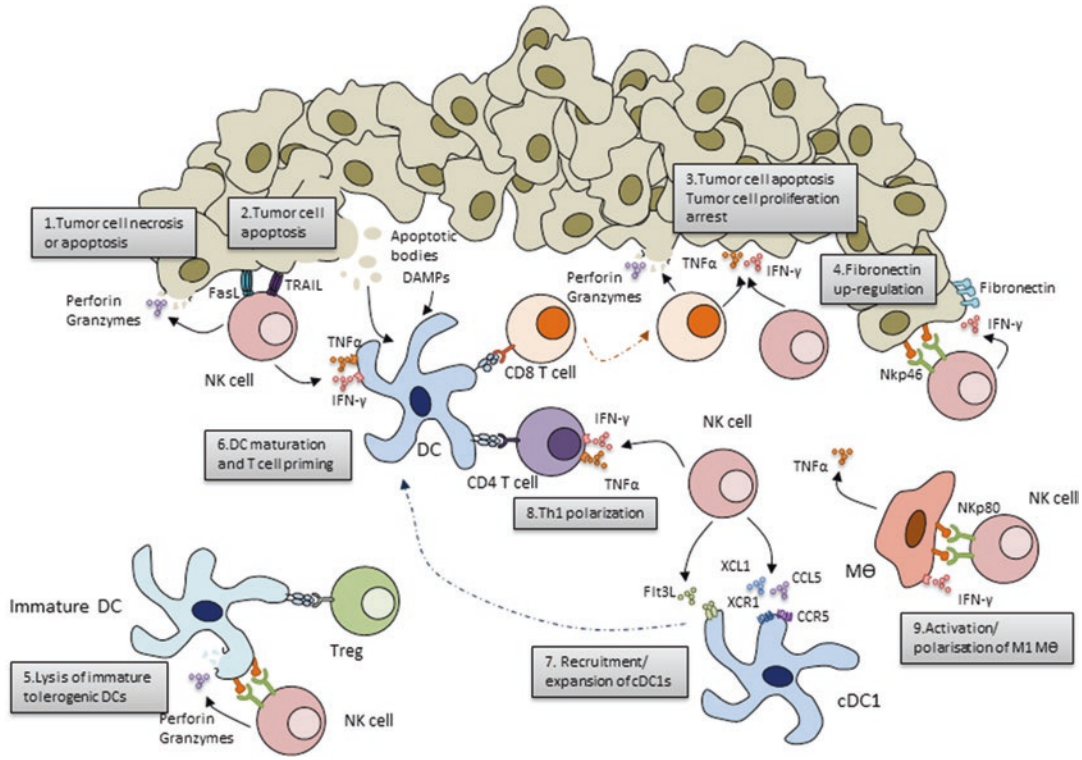

Fig. 4.2 Antitumor functions of NK cells. NK cells directly eliminate cancer cells through the release of perforin/granzyme-containing granules (1) or through TRAIL/FasL that interact with death receptors on target cells (2). In addition, NK cells secrete IFN-γ and TNF-α that may exert direct antiproliferative or pro-apoptotic effect on tumor cells (3). IFN-γ secreted by NK cells also induce the upregulation of fibronectin-1 on tumor cells and thereby decrease their metastatic potential (4). Tumor cell killing by NK cells leads to the release of apoptotic bodies and DAMPs that facilitate antigen uptake and T cell priming by DCs. Furthermore, NK cells directly interact with DCs to promote protective T cell responses. NK cells eliminate immature tolerogenic DCs (5) or induce their maturation through the release of IFN-γ and TNF-α (6), thereby ensuring that T cells receive adequate co-stimulatory signals. NK cells also release XCL1, CCL5, and Flt3L that promote cDC1 accumulation within the tumor (7). Finally, the secretion of IFN-γ and TNF-α promotes the development of protective type 1 responses, including Th1 polarization of CD4 T cells (8) and M1 polarization of macrophages (9). DC dendritic cell, MΘ monocyte/macrophage, DAMPs damage-associated molecular patterns

way NK cells kill their targets may greatly influence the immune response. While the granule exocytosis pathway can induce the target cell to die by necrosis or by apoptosis, the Fas-FasL pathways only kill via apoptosis [5]. Moreover, a recent in vitro study demonstrated that human NK cells use the granule exocytosis pathway during the first killing events and the death receptor pathway for their last killing event [101]. In this last study, granzyme B-mediated death was faster and mostly non-apoptotic, whereas death-receptor-mediated killing resulted in the slow apoptosis of the target cell.

IFN-γ production by NK cells has broad immune and non-immune-mediated antitumor effects. Many tumors are IFN-γ-sensitive, and disruption of IFN-γ signaling in these tumors increases their tumorigenicity [63]. IFN-γ can exert direct antiproliferative, anti-metabolic, and proapoptotic effects on IFN-γ-sensitive tumors. Moreover, a recent study using the mouse B16 melanoma model identified a novel mechanism by which NK cell-mediated IFN-γ production prevents metastasis [47]. In this study, IFN-γ production by NK cells triggered through NKp46 induced the upregulation of fibronectin-1 on

tumor cells; this led to architectural remodeling of the primary tumor and decreased metastasis.

4.5.2 NK Cells Shape the Ongoing Immune Response

NK cells secrete many soluble factors including IFN-γ, TNF, IL-6, GM-CSF, and CCL5. NK cell-derived cytokines and chemokines can influence both the innate and adaptive arms of the immune system. In addition, NK cells can establish cellular contacts with other immune subsets and thereby orient the immune response. For instance, the NK cell-activating receptor NKp80 binds to AICL to induce TNF production by monocytes [139]. NK cells have the ability to stimulate, shape, and terminate adaptive immune responses [3]. In mice, blood NK cells recruited to inflamed lymph nodes through CXCR3 constitute a source of IFN-γ that is critical for the Th1 polarization of $CD4^+$ T cells [82]. DCs are professional antigen-presenting cells responsible for the priming of T-cell responses [85]. NK cells are known to control T-cell responses through their interaction with DCs [31]. NK cells can enhance T-cell priming by promoting DC maturation and by eliminating immature and potentially tolerogenic DCs. Furthermore, the direct killing of tumor cells by NK may facilitate the uptake of antigenic material by DCs. In mice, NK cells activated by $MHC\text{-}I^{low}$ tumors were found to stimulate IL-12 production by DCs, which ultimately resulted in protective $CD8^+$ T-cell responses [86]. However, not all DC subsets are equivalent. Conventional (c)DC1s (characterized by the marker CD141 in humans and CD103 or CD8α in mice) constitute a small subset of DCs that are essential for the initiation of cytotoxic T cell responses [59, 67]. Recent studies performed in mouse tumor models indicated that NK cells play an important role in promoting the intra-tumoral accumulation cDC1s, a process that relies on the production of CCL5, XCL1, and/or Fms-related tyrosine kinase 3 ligand (Flt3L) by NK cells [8, 11]. Although correlative data suggest that similar mechanisms may be at play in humans, additional research is required formally to establish the importance of the NK cell-cDC1 axis in cancer patients.

4.6 Escape from NK Cell-Mediated Immunosurveillance

Although there is convincing evidence that NK cells kill newly transformed cells and protect against formation of metastases, they seem to have little effect against established cancers. This paradox can be explained by the multiple mechanisms that tumors have supplanted to evade NK cell surveillance. Among the various cellular and molecular pathways contributing to cancer evasion from NK cell-mediated immunosurveillance, some modifications reduce the immunogenicity of the tumor cells while others alter NK cell functions (Fig. 4.3).

4.6.1 Cancer Immunoediting by NK Cells

As they eradicate the most immunogenic malignant cells, immune cells sculpt the tumor and select the most aggressive variants. This process is known as "cancer immunoediting" [115]. Within a tumor population, clones that develop mutations rendering them more resistant to immune attacks will be preferentially selected. Because of their high mutational rate, tumor cells can rapidly adapt to their environment and accumulate changes that allow them to escape from the immune system. Such changes include the modulation of surface receptors to go unnoticed, the intrinsic resistance to immune killing, and the acquisition of highly immune-suppressive properties. All these modifications hinder NK cell-mediated eradication of tumors.

The formal evidence of NK cell-mediated immunoediting was provided by the comparison of the immune properties of carcinogen-induced tumors arising in $RAG2^{-/-}$ and $RAG2^{-/-}$ x $\gamma_c^{-/-}$ mice [91]. This study showed that the vast majority of the cancer cell lines developing in NK cell-deficient $RAG2^{-/-}$ x $\gamma_c^{-/-}$ mice were rejected when transplanted into wild-type (WT) mice. Similarly, WT mice rejected cell lines derived from $RAG2^{-/-}$ mice treated with NK cell-depleting Abs (anti-NK1.1) but not those derived from control Ab-treated $RAG2^{-/-}$ mice. These

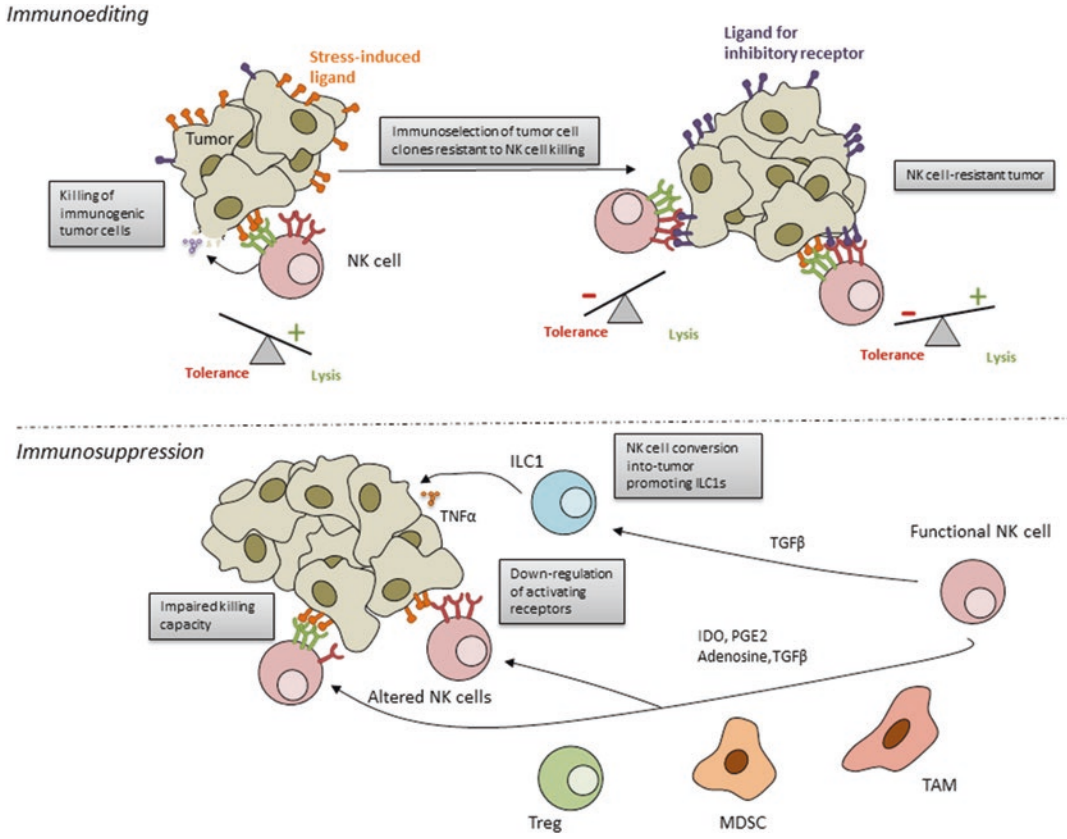

Fig. 4.3 Mechanisms leading to tumor escape from NK cell-mediated immunosurveillance. Immunoediting: NK cell-mediated killing of highly immunogenic tumor clones leads to the outgrowth of tumor variants that are poorly recognized by NK cells, either because they have downregulated ligands for activating NK cell receptors or because they have upregulated ligands for inhibitory NK cell receptors. Immunosuppression: the tumor microenvironment is highly immunosuppressive. Tregs tolerogenic immune cells comprise regulatory T cells, MDSCs myeloid-derived suppressor cells, TAM tumor-associated macrophages. Soluble factors such as prostaglandin E2 (PGE2), indoleamine 2,3 dioxygenase (IDO), adenosine, and TGFβ contribute to hamper NK cell functions

data demonstrate that tumors developing in the absence of NK cells are more immunogenic. In addition, another group observed that carcinogen-induced tumors arising in mice lacking the NK cell receptor NKp46 expressed NKp46 ligands, while those ligands were undetected on tumors originating in WT mice [39]. Indications that NK cells can sculpt the phenotype of human tumors come from the comparison of the expression of ligands for NK cell receptors at different disease stages. In colorectal cancer patients, NKG2D ligands are frequently expressed on tumors from tumor-node-metastasis stage I patients, but their expression is progressively reduced on stage II, III, and IV tumors [84].

The phenomenon of NK cell-mediated immunoediting seems to be particularly prominent in hematological cancers. Bone marrow tumors from early stages myeloma patients, which express NKG2D ligands ULBPs and/or MICA and low levels of MHC-I molecules, are readily killed by autologous NK cells [14]. By contrast, cell lines from pleural effusions of late myeloma patients are NK cell-resistant, a phenotype that is associated with increased MHC-I and decreased NKG2D ligand expression. Finally, recent work established that AML leukemic stem cells (that are chemo-resistant and responsible for disease relapse) can be distinguished from the bulk AML cells by their absence of NKG2D ligand

expression [94]. It was shown that leukemic stem cells also displayed reduced expression of the DNAM ligands CD112 and CD155 and were resistant to NK cell-mediated killing. These data highlight how a specific subpopulation of tumor cells may prevent cancer eradication by NK cells.

4.6.2 Suppression of NK Cell Responses Within the Tumor Microenvironment

Aberrant inflammation within the tumor microenvironment may favor cancer evasion from NK cell-mediated control. Several cell types contribute to the establishment of an immunosuppressive microenvironment poorly favorable to NK cell functions. Tumor-associated stromal cells, tumor-infiltrating immunosuppressive cells, or the tumor cells themselves may alter NK cell activity, either through cell-to-cell contacts or through the release of soluble factors [6, 135]. Immunosuppressive cells known to infiltrate tumors comprise regulatory T cells (Tregs), tumor-associated macrophages (TAM), and myeloid-derived suppressor cells (MDSCs). All these cell types have been shown to decrease NK cell functions through a wide range of mechanisms [135].

NK cells from cancer patients often display aberrant expression of NK cell receptors (Table 4.5). The mechanisms leading to the downregulation of major activating NK cell receptors (NKp30, NKp44, NKG2D, and DNAM-1) have been reviewed elsewhere [135]. In ovarian cancer patients, decreased expression of activating NK cell receptors has been associated with impaired killing activity [15]. Moreover, reduced expression of effector molecules such as perforin, granzymes, or TRAIL is a common feature of tumor-infiltrating NK cells that has been associated with decreased cytokine production and cytotoxicity (Table 4.6).

Soluble factors known to decrease NK cell antitumor activity include shed ligands for activating NK cell receptors (e.g., soluble MICA), prostaglandin E2 (PGE2), indoleamine 2,3 dioxygenase (IDO), extracellular adenosine, and TGFβ [6, 135]. In breast cancer patients, TGFβ1 secreted by tumor stromal cells was identified as a main factor driving NK cell dysfunction [80]. TGFβ can also be expressed on the surface of Tregs, and data obtained from gastrointestinal and metastatic cancer patients together with mouse models indicated that membrane-bound TGFβ inhibits NK cell functions [46]. Moreover, TGFβ may alter NK cell activity indirectly, for instance by promoting DCs with tolerogenic properties [128]. Finally, TGFβ was shown to promote the conversion of mouse NK cells into cells with a phenotype resembling type 1 helper ILC (ILC1s) [27, 42]. In opposition to mouse NK cells which are $CD49a^{-}CD49b^{+}$ and express the transcription factor Eomes, ILC1s are $CD49a^{+}CD49b^{-}Eomes^{-}$. The signal-transducer SMAD4 was found essential for the maintenance of NK cell identity through its role in restricting noncanonical TGFβ signaling [27]. SMAD4 deficiency largely impaired NK cell lytic activity and their secretion of IFN-γ in response to cytokines or target cells. Moreover, it was suggested that ILC1-derived TNF facilitates tumor escape from the innate immune system [42]. Another member of the TGFb family, activin-A, was found to signal through SMAD2/3 in NK cells, leading to suppressed cellular metabolism, impaired proliferation, and increased expression of ILC1-related markers [104]. Adenosine, an immunosuppressive purine metabolite that accumulates in hypoxic conditions, is another important factor that limits NK cell functions in the tumor microenvironment. Blockade of the adenosine receptor A_{2A} has been shown to enhance mouse NK cell cytotoxicity and control of experimental lung metastasis [10]. However, a recent study on human NK cells showed that adenosine targets specific cellular pathways and induces reprogramming of NK cell functions rather than a broad inhibition of NK cell activity [18]. Surprisingly, in this study, adenosine induced a profound inhibition in glycolysis and glycolytic capacity of IL-12/IL-15 stimulated NK cells but increased their production of IFN-γ. Additional work is necessary to better understand the effects of adenosinergic signaling on NK cells in the tumor microenvironment.

Table 4.6 Impaired functions of tumor infiltrating NK cells

Tumor	Expression of effector molecules	Cytokine production	Killing activity	Comparison	Reference
NSCLC	↘ Perforin	= PMA-ionomycin	↘ K562 cells	Peritumoral tissue and peripheral blood	Carrega et al. [17]
Renal cell carcinoma	↘, =[a] Granzymes A, B and perforin		=[b] K562 cells	Peripheral blood	Schleypen et al. [114]
NSCLC		↘ Autologous tumor cells	↘[c] Autologous tumor cells	Peripheral blood	Platonova et al. [98]
Endometrial cancer	↘ Granzyme B	↘ PMA-ionomycin	↘[c] PMA-ionomycin	Tumor-adjacent tissue	Degos et al. [32]
Breast cancer	↘ Perforin, granzymes and TRAIL	↘ K562 cells mAb-coated cells	↘[c] K562 cells mAb-coated cells	Healthy mammary tissue	Mamessier et al. [80]

Comparison of tumor-infiltrating NK cell functions with those of peripheral blood or corresponding healthy tissue
↘ decreased expression/function, = no significant difference. For the cytokine production and killing activity, the type of stimulation is specified
[a]Only NK cells from poorly infiltrated tumors, but not those from highly infiltrated tumors, had decreased expression of effector molecules
[b]Only NK from highly infiltrating tumors
[c]Only degranulation capacity was assessed

In the last decade, immune checkpoints have emerged as crucial immune regulators that inhibit the functions of cancer infiltrating T cells [95]. There is newly accumulated evidence that immune checkpoints might also impair NK cell responses [22, 112]. For instance, peripheral blood NK cells from Hodgkin lymphoma patients were found to express elevated levels of the PD-1 immune checkpoint; and it was suggested that, in these patients, tumor-associated monocytes and macrophages may inhibit NK cell activity through the PD-1/PD-L1 pathway [131]. The expression of two other immune checkpoints, TIGIT and Tim3, was elevated on the surface of tumor-resident CD103$^+$ NK cells in women with endometrial cancer [32]. Importantly, patients with lymph node invasions exhibited higher percentages of TIGIT- or Tim3-expressing NK cells, suggesting that checkpoint expression on NK cells may correlate with the severity of the disease. Similarly, in esophageal cancer patients, higher percentages of Tim3$^+$ NK cells were observed in the peripheral blood and tumor of patients with pathological parameters for poor prognosis [142]. Tim3$^+$ NK cells expressed lower levels of mRNAs encoding for the effector molecules perforin, granzyme, and IFN-γ. Moreover, in patients with myelodysplastic syndrome, TIGIT was found to hinder NK cell cytolytic ability by interacting with its ligand, CD155, expressed on MDSCs [113].

4.7 Future Directions

NK cells are powerful anticancer agents and constitute promising therapeutic targets. Unlike T cells, they are not MHC-restricted, they do not require clonal selection, and they are safe in allogeneic settings. Several approaches have been proposed to exploit NK cells in cancer patients [52, 123]. Patients can receive autologous or allogeneic NK cells, and genetic engineering of adoptively transferred NK cells may further improve cancer control. Moreover, several drugs have been developed to redirect NK cell killing such as bispecific or trispecific killer engagers (Bikes or Trikes). Finally, "NK cell-specific"

immune checkpoint inhibitors such as lirulumab (that targets KIRs) or monalizumab (that targets NKG2A) may improve anticancer responses by releasing signals suppressing NK cell activity. However, NK cell infiltration of the tumor bed and the preservation of NK cell functions within the local tumor microenvironment are prerequisites for the success of the aforementioned therapies. Successful therapies may include combinations of agents to ensure that fully active NK cells unrestricted access to tumor cells. In line with NK cell role in early defenses, efforts should also be focused on enhancing NK cell cross-talk with other immune cells for the initiation of adaptive immune responses and the establishment of long-lasting memory T-cell responses.

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γδ T Cells in Tumor Microenvironment

5

Caroline Imbert and Daniel Olive

Abstract

Gamma delta (γδ) T cells which combine both innate and adaptive potential have extraordinary properties. Indeed, their strong cytotoxic and pro-inflammatory activity allows them to kill a broad range of tumor cells. Several studies have demonstrated that γδ T cells are an important component of tumor-infiltrated lymphocytes in patients affected by different types of cancer. Tumor-infiltrating γδ T cells are also considered as a good prognostic marker in many studies, though the presence of these cells is associated with poor prognosis in breast and colon cancers. The tumor microenvironment seems to drive γδ T-cell differentiation toward a tumor-promoting or a tumor-controlling phenotype, which suggests that some tumor microenvironments can limit the effectiveness of γδ T cells.

The major γδ T-cell subsets in human are the Vγ9Vδ2 T cells that are specifically activated by phosphoantigens. This unique antigenic activation process operates in a framework that requires the expression of butyrophilin 3A (BTN3A) molecules. Interestingly, there is some evidence that BTN3A expression may be regulated by the tumor microenvironment. Given their strong antitumoral potential, Vγ9Vδ2 T cells are used in therapeutic approaches either by ex vivo culture and amplification, and then adoptive transfer to patients or by direct stimulation to propagate in vivo. These strategies have demonstrated promising initial results, but greater potency is needed. Combining Vγ9Vδ2 T-cell immunotherapy with systemic approaches to restore antitumor immune response in tumor microenvironment may improve efficacy.

In this chapter, we first review the basic features of γδ T cells and their roles in the tumor microenvironment and then analyze the advances about the understanding of these cells' activation in tumors and why this represent unique challenges for therapeutics, and finally we discuss γδ T-cell-based therapeutic strategies and future perspectives of their development.

Keywords

γδ T cells · Lymphocytes · Immunity · Microenvironment · Tumor · Recruitment ·

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5.1 Classification of γδ T Cells

The γδ T cells are a subgroup of T cells that possess a TCR composed of the γ and δ chains, and they are, therefore, distinguished from their αβ T-cell counterparts by utilizing a distinct set of somatically rearranged variable (V), diversity (D), joining (J), and constant (C) genes.

During thymic ontogeny, γδ T-cell subsets originating from a common lymphoid precursor cell emerge before αβ T cells to represent the predominant CD3+ population during fetal development. In humans, their frequency then decreases after birth, while αβ T cells progressively predominate. Interestingly, γδ T cells comprise up to 60% of circulating T lymphocytes in some nonprimate species (i.e., sheep, cattle, rabbits, and chicken), while in human peripheral blood (PB), they account for 1–5% of CD3+ T cells, which raises questions about the evolutionary processes and the biology of this subset [1]. However, unlike αβ T cells, our knowledge of γδ T-cell development is relatively limited and controversial [2].

Although important information has been obtained by studies in mice, this chapter focuses on human γδ T cells. Human γδ T cells can be divided into two main subsets based on their TCR δ chain expression: γδ T cells expressing the Vδ1 chain, which are most often present in tissues, and γδ T cells expressing the Vδ2 chain, which predominate in peripheral blood and secondary lymphoid organs [3]. The Vδ1 chains are predominantly associated with the VγI gene family (Vγ2/3/4/5/8) chains, whereas a majority of Vδ2 T cells coexpress the VγI (Vγ9) chain. Human Vδ3 and Vδ5 γδ T cells comprise only minor subsets of T lymphocytes, which are, respectively, present in tissues and peripheral blood (Table 5.1). Again, in contrast with αβ T cells and despite three decades of research since their discovery, the nature of γδ TCR-mediated ligand recognition remains poorly defined.

5.1.1 Vδ1 T Cells

Vδ1 T cells are the predominant tissue-associated γδ T-cell subset in humans. They are mainly found in the skin, gut, spleen, and liver and are involved in maintaining the integrity of epithelial tissue. These cells can recognize signs of cellular dysregulation, including viral infection and transformation. Indeed, during HIV infection, Vδ1 T-cell numbers are increased in PB and the normal Vδ2/ Vδ1 ratio is inverted, suggesting a potential involvement of Vδ1 T cells in antiviral immunity [13, 14]. Moreover, Ravens et al. showed that acute cytomegalovirus (CMV) infection following stem cell transplantation (SCT) can drive expansion of Vδ1 T cells [15].

The Vδ1 T-cell ligands discovered until now are CD1c, CD1d, MICA/B, ULBPs, "Staphylococcal enterotoxin B" (SEB), and B7-H6. In particular, the CD1-family proteins present in endogenous lipids act as markers of malignant transformation (CD1c) [16] or viral infection (CD1d) [17]. It has also been demonstrated that human intestinal epithelial Vδ1 T cells respond to tumor cells overexpressing MICA/B and ULBPs through the synergistic actions of TCR and NKG2D [18]. In addition, these cells respond to the SEB superantigen but not to the "Staphylococcal enterotoxin A" (SEA) thanks to their non-αβ TCR [19]. Finally, B7-H6, a member of the B7 family that is expressed in tumors, but not healthy tissues, is also recognized by Vδ1 T cells through the expression of "natural killer cell p30-related protein" (NKp30) [20, 21].

5.1.2 Vδ2 T Cells

In the blood of most healthy individuals, T cells expressing the Vδ2Vγ9 T cells account for 50–90% of the γδ T-cell population. Vδ2Vγ9 T-cell activation by phosphoantigens will be extensively explained in Sect. 5.3.

Table 5.1 Structural subsets of human γδ T cell

Structural subset	Paired Vγ gene	Distribution	Activation stimulus and/or γδ TCR ligands	References
Vδ1	Vγ2, Vγ3, Vγ4, Vγ5, Vγ8, Vγ9	Skin, gut, spleen, liver, and PB	MICA/B, ULBPs, CD1c, CD1d, SEB, and B7-H6	[4–6]
Vδ2	Vγ9	PB	Phosphoantigens, BTN3A1, F1-ATPase, hMSH2, MICA/B, ULBPs, TSST-1, Nectin-like-5	[7–10]
Vδ3	Vγ2, Vγ3	Liver, gut, chronic infections, and leukemia	CD1d	[11]
Vδ5	Vγ4	PB	EPCR	[12]

5.1.3 Non-Vδ2 and Non-Vδ1 T Cells

Human γδ T cells expressing the Vδ3 TCR comprise a minor lymphocyte subset in the blood but are enriched in the liver and in patients with some chronic viral infections and leukemias. In the same way as Vδ1 T cells, Vδ3 T cells recognize CD1d. Upon activation, they kill CD1d + target cells; release Th1, Th2, and Th17 cytokines; and induce maturation of dendritic cells into antigen-presenting cells (APCs). Thus, Vδ3 T cells are glycolipid-reactive T cells with distinct antigen specificities but functional similarities to natural killer T cells [11]. Furthermore, Vγ4Vδ5 T cells recognize stressed cells through TCR binding to the endothelial protein C receptor [12].

5.2 γδ T Cells in Tumor Microenvironment

Recently, the role of γδ T cells in tumor immunity has received considerable attention and research. Studies highlight that these cells are an important component of the immune effector cells that contribute to the tumor immunosurveillance against many types of tumors, especially in hematologic malignancies [22] in addition to solid cancers like melanoma [23], breast [24, 25], prostate [26, 27], and pancreas in an inflammatory context [28] (Table 5.2). Although their activation mechanisms differ, both Vδ1 and Vδ2 T-cell subsets can exert potent antitumor effects.

5.2.1 γδ T Recruitment into the Tumor Microenvironment

Human γδ T cells have been shown to migrate in vitro toward several chemokines, such as CCL2, CCL3, CCL4, CCL5, CXL10, CXCL11, and CXCL12 [29, 30]. In another study, which compared the chemotactic response of human αβ and γδ T cells, γδ T cells underwent transendothelial chemotaxis in response to the chemokines MCP-1, RANTES, MIP-1α, and MIP-1β, but not to interleukin (IL)-8 and IP-10 [31]. So far, little is known about chemokines that mediate γδ T-cell recruitment to tumor beds. In this context, chemokines can be expressed by tumor cells and other cells, including immune cells and stromal cells. Contrasting with the previous result, IL-10 secreted by breast cancer cells has been shown to attract regulatory γδ T cells [32]. Moreover, two studies have also highlighted a critical role for CCR2/CCL2 in γδ T-cell infiltration in B16 melanoma tumors [33, 34]. However, a detailed analysis on the expression of chemokine receptors on γδ T cells and secreted chemokines in tumor microenvironments is lacking and needs investigation.

5.2.2 γδ T-Cell Subsets in Tumor Immunity: The Good Guys and the Bad Guys

In melanoma patients, an elevated frequency of circulating as well as tumor infiltrating Vδ2Vγ9 T cells in early-stage tumors was correlated with

Table 5.2 γδ T-cell function in tumors

γδ T-cell subset	Cancer type	Results	Reference
Antitumor functions			
All γδ T cells	Myeloma	γδ T cells express the natural cytotoxicity receptor natural killer p 44 and show cytotoxic activity against myeloma cells	[22]
Vδ1 and Vδ2 T cells	Melanoma	γδ T cells are capable of killing melanoma cell lines in vitro and percentages of Vδ2 cells correlate with early stage of development of melanoma and absence of metastasis	[23]
Vγ2Vδ2 T cells	Breast	γδ T cells limit in vitro growth of most breast tumor cells by inhibiting their survival and inducing apoptosis	[25]
Vγ2Vδ2 T cells	Prostate	γδ T cells mediate innate antitumor activity against human prostate cancer cells in vitro	[26]
Pro-tumor functions			
All γδ T cells	Breast	Intratumoral γδ T-cell numbers were inversely correlated with breast cancer prognosis	[37]
γ2Vδ2 T cells	Colorectal	γδ T cell promotes the accumulation and expansion of myeloid-derived suppressor cells which lead to tumor progression	[38]
γδ T17 cells	Gall bladder	γδ T17 cell infiltration induces angiogenesis and is associated with poor survival	[39]

decreased mortality and disease relapse. Additionally, Meraviglia et al. showed with transcriptomic analysis that patients with colorectal tumor containing abundant γδ T cells had significantly longer 5-year disease-free survival rate, suggesting an efficacy of these cells in controlling tumors at a very early stage [35]. Of particular interest, a recent correlation between the molecular profile of the tumor immune microenvironment and prognosis in a large number of human tumors indicated that the presence of infiltrating γδ T cells was the strongest predictor of positive outcome [36].

Although the antitumor function of γδ T cells is well established, they can also promote tumor growth under certain circumstances (Table 5.2). For example, intratumoral γδ T-cell numbers are positively associated with advanced tumor stages and are inversely correlated with both relapse-free survival and overall survival of breast cancer patients [37]. However, these latter publications were recently challenged (A. Hayday, AACR 2019, unpublished).

Based on their function, γδ T cells can be divided into two subsets: effector γδ T cells with an antitumor role and regulatory γδ T cells which promote tumor progression (Fig. 5.1).

5.2.3 γδ T Cells as Foes in Tumor Development

γδ T cells have a broad array of effector functions that reflect their major involvement in the antitumor response. They can kill transformed cells, through four pathways that involve cytokine production, the release of cytotoxic effector molecules, such as perforin and granzymes, the engagement of death-inducing receptors, and antibody-dependent cell-mediated cytotoxicity (ADCC).

First, γδ T cells are an important early sources of interferon (IFN)-γ and tumor necrosis factor (TNF)-α, two cytokines known to inhibit cancer growth through special enhancement of antitumor immunity and the inhibition of angiogenesis [40]. Moreover, it has been demonstrated that γδ T cells can induce dendritic cell (DC) maturation through TNF-α production [41]. The secretion of these cytokines is promoted by several stimuli, including phosphoantigen stimulation, TCR agonist, ligands of NKG2D, and certain cytokines.

Second, after migrating to the local tumor environment, γδ T cells can lyse cancer cells through the perforin–granzyme pathway. Indeed, inhibiting the perforin–granzyme secretion capacity of Vγ9Vδ2 T cells reduces the lysis of

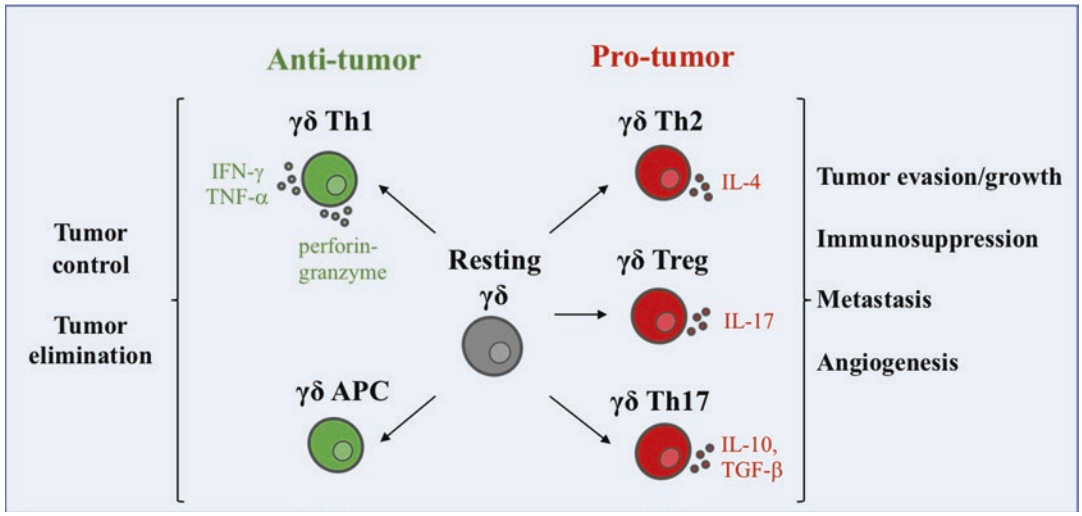

Fig. 5.1 Antitumor and pro-tumor functions of γδ T cell

aminobisphosphonate-sensitized MCF-7 breast tumor cells [42].

Third, γδ T cells upregulate the expression of Fas ligand (Fas-L) and TNF-related apoptosis-inducing ligand (TRAIL) and, therefore, enhance the tumor-killing activity in the Fas- or TRAIL-receptor-sensitive tumors.

Finally, human γδ T cells also express CD16, which can bind to the Fc region of immunoglobulin G deposited on tumor cells, leading to their lysis by ADCC [43]. In this context, and knowing that PD-1 is expressed by most follicular lymphoma-infiltrating γδ T lymphocytes, recently, Rossi et al. highlighted that boosting of γδ T-cell-mediated ADCC was due to PD-1 blockade in FL [44].

Intriguingly, γδ T cells also exhibit an antigen-presenting capacity. Similar to dendritic cells (DCs), Vγ9Vδ2 T cells are able to respond to signals from microbes and tumors and prime CD4+ and CD8+ T cells [45]. Moreover, not only can γδ T-APCs cross-present antigens to CD8+ T cells [46], but also activated γδ T cells are able to phagocytose tumor antigens and apoptotic or live cancer cells, possibly through the scavenger receptor CD36, to mount a tumor antigen-specific CD8+ T-cell response [47].

5.2.4 γδ T Cells as Friends in Tumor Development

Even though γδ T cells demonstrate potent antitumor capacity, they can paradoxically also exert protumor effects, through direct or indirect strategies, that subvert cytotoxic antitumor immunity. The differentiation of unique subpopulations of Vγ2Vδ2 T cells with immunosuppressive features can be induced in the presence of specific stimuli, such as in the tumor-established microenvironment. Vγ2Vδ2 T cells may display Th2, Th17, or Treg-like profile and produce IL-4, IL-17, or IL-10 and transforming growth factor (TGF)-β [32, 39].

γδ T17 cells are the major source of IL-17, which plays an immunosuppressive role in cancer. Indeed, in human colorectal cancer, γδ T17 cells promote the accumulation and expansion of myeloid-derived suppressor cells [38]. In addition, IL-17 producing γδ T cells induce angiogenesis and are associated with poor survival in gall bladder cancer patients [39].

Furthermore, it has been demonstrated that Vγ2Vδ2 T cells can polarize toward FOXP3+ γδ Treg cells following stimulation with TGF-β and IL-15 in vitro. In this context, a specific recruitment of γδ regulatory T cells into tumors is

induced by IL-10 secreted by breast cancer cells, thereby suppressing T-cell responses and DC maturation [32]. In addition, Kühl et al. showed that in vitro peripheral γδ T cells have a more potent regulatory potential than αβ Treg cells regarding T helper cell suppression [48]. In another study, Vδ1 T cells have been reported to be strongly secreted TGF-β, which can induce the epithelial to mesenchymal transition resulting in an increase in cancer invasiveness [48].

Unexpectedly, a recent report has shown that TGF-β augments the cytotoxic effector activity of short-term expanded Vδ2 T cells when purified γδ T cells are activated with specific pyrophosphate antigens and IL-2 or IL-15 in the presence of TGF-β [49].

5.2.5 γδ T-Cell Plasticity

In response to cytokine stimulation, like conventional T cells, γδ T cells are capable of modifying their functions. They can convert their functions into an antitumor or protumor phenotype depending on the cytokines present in the tumor microenvironment (Fig. 5.2). Consequently, an artificial switch toward an antitumoral tumor microenvironment could improve the efficacy of γδ T cells. In this way, IL-15 could be a good candidate to favor antitumor microenvironment. Indeed, this cytokine can promote both innate and adaptive immune reactions by stimulating CD8+/CD4+ T cells and natural killer cells (NK) while showing no effect in inducing activation-associated death among effector T cells and NK cells [50]. Furthermore, the addition of IL-15 to γδ T-cell cultures resulted in a more activated phenotype, higher proliferative capacity, and an increased cytotoxic capacity [51]. Finally, combining IL-15 with γδ T immunotherapy could be a promising strategy to enhance antitumor immune therapy.

5.3 Vδ2Vγ9 T-Cell Activation by Tumor Cells

Researchers first thought that γδ T cells, like αβ T cells, recognized peptides bound to major histocompatibility complex (MHC) molecules; however, there was poor evidence that antigens were physically presented to γδ T cells in classical way [52]. Indeed, it was later demonstrated that Vδ2Vγ9 T cells are specifically activated by phosphoantigens (pAgs) without the requirement for antigen processing, presentation, and MHC restriction, which strengthens their therapeutic interest by the absence of alloreactivity. Despite the major advance represented by this discovery, how such antigens are presented by tumors cells to Vδ2Vγ9 T cells remains a mystery to be elucidated. Discoveries in the αβ T-cell activation mechanism are now successfully exploited in the clinic, especially for antigen-specific vaccination against infectious diseases and cancer. In the same way, elucidating the molecular mechanisms of the fascinating γδ T-cell activation mechanism could open the way to an increase in clinical applications.

5.3.1 Sensing of Cellular Stress by Vδ2Vγ9 T Cells

Numerous studies have highlighted that Vδ2Vγ9 T-cell numbers in peripheral blood strongly increase in response to a variety of infectious diseases including not only bacterial infections, such as tuberculosis, sarcoidosis [53], salmonellosis [54], brucellosis [55], infections caused by protozoal parasites such as leishmaniasis [56], malaria [57], and toxoplasmosis [58], but also viral infections like HIV (early stages) [59] and Epstein-Barr virus (EBV) [60]. Expansion of Vγ9Vδ2 T cells has also been observed in patients with lymphoid malignancies [61]. These expansions can be reproduced in vitro by stimulating Vγ9Vδ2 T cells with certain cancer cell lines or cells treated with microbial extracts [62]. Interestingly, activation of Vδ2Vγ9 T cells is dependent on the expression of the Vδ2Vγ9 TCR, and this reactivity is transferable with the TCR [63, 64]. Whereas αβ T-cell activation is dependent on protein components, in vitro assays showed that Vγ9Vδ2 T expansion is mediated by protease-resistant and phosphatase-sensitive components hereafter called phosphoantigens (pAgs) [65].

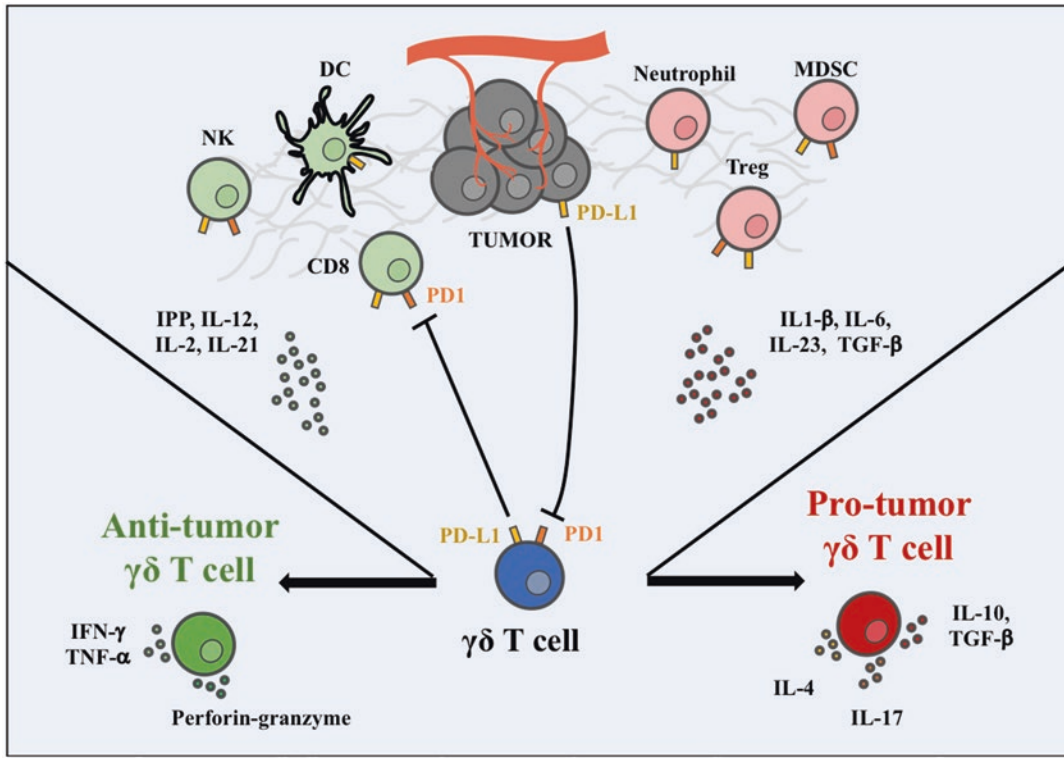

Fig. 5.2 γδ T plasticity in tumor microenvironment

5.3.2 The Mysterious Phosphoantigens

pAgs are characterized as small molecules containing a phosphate moiety with a variable organic group. pAgs can be classified as "exogenous" when they are from a microbial origin or "endogenous" when they are from a mammalian origin.

Exogenous pAgs were first identified in extracts from *Mycobacterium tuberculosis* [66]. Most common among them is the 4-hydroxy-3methyl-but-2-enyl pyrophosphate (HMBPP) produced through the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway found in most eubacteria and apicomplexan protozoa. Exogenous pAgs stimulate with 1000-fold more efficiency than their endogenous counterparts, therefore preventing autoreactivity in normal cells where the concentration should be below threshold for stimulation [67, 68].

Endogenous pAgs are products of isoprenoid synthesis, the building blocks of which are the isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate. In cancer cells, such as in the Hodgkin B-cell lymphoma cell line Daudi and breast adenocarcinoma cells, it has been demonstrated that IPP is strongly accumulated due to hyperactivity of HMG-CoA reductase, the rate-limiting enzyme in the mevalonate (MVA) pathway (Fig. 5.1) [69]. This accumulation compensates for low potency of endogenous pAgs and reaches the threshold that leads to the Vδ2Vγ9 T-cell activation.

In mammals and most animals, IPP is synthesized via the mevalonate pathway whose manipulation can turn human cells toward Vδ2Vγ9 T-cell activators. Indeed, cells pulsed with aminobisphosphonates (e.g., zoledronate or pamidronate) become potent activators of primary Vδ2Vγ9 T cells and Vδ2Vγ9 T TCR transduced cells most likely as a consequence of IPP accumulation after inhibition of the IPP metabolizing farnesyl pyrophosphate synthase (FPP synthase) (Fig. 5.3).

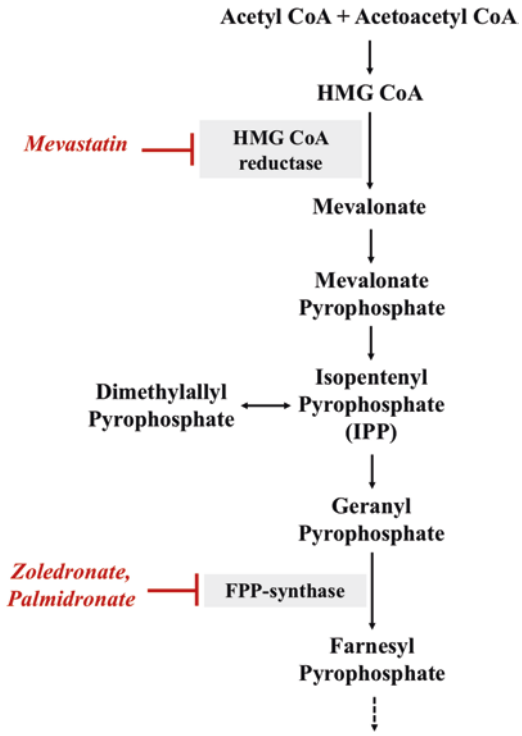

Fig. 5.3 Mevalonate metabolic pathway. (Adapted from [70])

Exogenous pAgs such as HMBPP could prove useful in the composition of vaccines involving γδ T-cell-mediated immunity, although their very low abundance in natural sources limits such applications. To overcome this, a phosphorylated bromohydrin (BrHPP) analog that mimics the biological properties of natural pAgs has been synthetized [71].

The discovery of pAgs role was a groundbreaking step in our understanding of Vγ9Vδ2 T cells; however, the molecular link that connects accumulation of endogenous (tumor-derived) or exogenous pAgs to activation was still missing. First, it was thought that pAgs could bind the γδ TCR directly, but the recent discovery of the crucial role of butyrophilin-3 (BTN3A) rather points toward an indirect recognition of pAgs.

5.3.3 BTN3A

Butyrophilins (BTNs) contain one or two extracellular Ig domains that exhibit some structural features of the B7 family of co-receptors and are considered to be B7-related proteins, which thus suggests that they possess immunological functions. The BTN3A subfamily, which includes three members in humans: BTN3A1, BTN3A2, and BTN3A3, shares a high structural homology for the extracellular domain composed of two immunoglobulin extracellular domains (IgV and IgC). However, the three isoforms differ substantially in their intracellular domains; whereas BTN3A1 and BTN3A3 contain a B30.2 domain, BTN3A2 lacks this domain. Moreover, the intracellular region of A3 has a unique extension C-terminal to its B30.2 domain and, thus, differs to the one of A1 [72].

5.3.3.1 The Crucial Role of BTN3A

When treated with the 20.1, a BTN3A agonist antibody, the three isoforms confer an activating signal to Vγ9Vδ2 T cells, suggesting the involvement of their extracellular domains in the activation process. This phenomenon was restricted to the Vγ9Vδ2 population in peripheral blood mononuclear cells (PBMCs), with no effect on αβ T cells or Vδ1 T cells. Moreover, addition of the 20.1 antibody to a panel of human tumor/transformed cell lines induced potent activation of Vγ9Vδ2 T cells [73]. However, only the BTN3A1 isoform mediates pAg-induced activation, a feature which requires the presence of its intracellular component containing a B30.2 domain. Surprisingly, the BTN3A3 isoform, which also contains a B30.2 domain, cannot stimulate in a pAg-dependent manner [73, 74]. Importantly, in 2014, Sandstrom et al. demonstrated that the B30.2 intracellular domain of BTN3A1 is a sensor for detecting changes in pAg metabolite concentrations and is associated with immobilization of the BTN3A extracellular domains. Interestingly, they identified a single amino acid difference in the B30.2 domain of the nonstimulatory BTN3A3 that, when mutated to the corresponding residue in BTN3A1, conferred the ability to bind pAg and to activate Vγ9Vδ2 T cells. These results suggest a mechanism through which intracellular recognition, and not extracellular presentation, of pAgs is essential to mediating Vγ9Vδ2 T-cell stimulation [75]. Despite these discoveries, BTN3A1 alone fails to activate

Vγ9Vδ2 T cells. Indeed, Vantourout et al. demonstrated recently that BTN3A2 regulates the subcellular localization of BTN3A1 and is, thus, essential for the optimal activation of Vγ9Vδ2 T cells [76].

5.3.3.2 BTN3A Interactions

Until now, little is known about the receptor–ligand interactions for the BTN3A family or about their precise functions in the tumor microenvironment. Two cytoskeletal proteins have been shown to interact with the intracellular domain of BTN3A1: periplakin and RhoB (Table 5.3). The role of the first one remains unclear; however, periplakin seems to be required for pAg-induced γδ T-cell activation [7]. Concerning the second one, Sebestyen et al. showed in tumor cells that relocalization of RhoB to BTN3A1 induced its immobility on the membrane. Subsequently, a pAg-induced conformational change in BTN3A1 leads to Vγ9Vδ2 T-cell activation [77]. Compte et al. showed that the BTN3A1 ligand is overexpressed in certain T leukemia lines in a leukemia B line and in a myeloerythroid leukemia line, and to a lesser extent in several solid tumor cell lines, and although they did not identify the ligand, they ruled out CD28, Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), inducible T-cell costimulator (ICOS), programmed cell death 1 (PD-1), or B and T lymphocyte attenuator (BTLA) [78]. Interestingly, a recent study demonstrated that BTN3A3 expressed by human breast cancer cell interacts with LSECtin on tumor-associated macrophages (TAM), which enhances tumor stemness and growth [79]. While it is known that BTN3A expressed by tumors cells plays an important role in γδ T-cell activation, the role of BTN3A interactions with other immune cells in tumor microenvironment still needs to be clarified.

Table 5.3 BTN3A interactions in tumors

BTN3A subtype	Ligand	Observations	References
Intracellular ligand			
BTN3A1	pAgs	Human γδ T-cell activation	[73, 74]
BTN3A1	Periplakin	Involved in pAg-induced γδ T-cell activation	[7]
BTN3A1	RhoB GTPase	Induces membrane immobility of BTN3A1 which allows pAg recognition and γδ T-cell activation	[77]
Extracellular ligand			
BTN3A3	LSECTIN	BTN3A3 express by TAM enhances tumor stemness and growth	[79]

5.3.3.3 BTN3A Regulation by the Tumor Microenvironment

Despite BTN3A being widely expressed by immune cells and some tumor cell lines, few studies have analyzed its regulation. Nevertheless, it has been demonstrated that inflammatory stimulus such as IFN-γ and TNF-α treatment increased BTN3A expression on endothelial cells [78]. Moreover, other tumor microenvironmental inflammatory cytokines such as IL-6, CCL3, and hypoxia-associated mediators (IL-10, VEGF, PIGF-1) upregulate BTN3A expression in monocyte-derived human DCs [80]. Since BTN3A plays a crucial role in Vγ9Vδ2 T-cell activation, an exhaustive analysis of the regulation of each isoform in tumor microenvironment is needed.

5.4 Vγ9Vδ2: A New Hope for Fighting Cancer

γδ T cells are considered as good candidates for effective antitumor therapeutic approaches, because they recognize malignant cells, infiltrate tumors, and combine both innate and adaptive response potential with strong cytotoxic and pro-inflammatory activity. Additionally, a deficiency of γδ T cells has been reported in several malignancies, such as breast cancer [81] and hematological [82], liver [81], and gastric tumors [83], which could suggest that these cells may have beneficial effects in controlling tumors. Despite Vδ1+ cells showing promising preclinical results, most studies have focused on Vγ9Vδ2 T

cells, because of their high abundance in the peripheral blood which facilities their ex vivo expansion.

Two main therapeutic strategies based on Vγ9Vδ2 T cells have been proposed for tumor immunotherapy: the in vivo expansion of Vγ9Vδ2 T cells and the adoptive transfer in patients of ex vivo expanded Vγ9Vδ2 T cells (Fig. 5.4). In both cases, activation and amplification of Vγ9Vδ2 T cells can be accomplished through multiple ways with the use of exogenous pAg, aminobisphosphonate drugs, TCR-cross-linking monoclonal antibodies, BTN3A agonist antibodies, or stimulatory tumor cells. Given that BTN3A agonist antibodies might outperform aminobisphosphonate drugs or other metabolic sensitizers in target cells that fail to internalize drugs or which have decreased mevalonate pathway activity, combining approaches should be considered. Moreover, in case of in vivo use of these drugs, it will be important to determine their impact on the other immune cells of the tumor microenvironment.

5.4.1 Exogenous pAg and Aminobisphosphonate Drugs

Most clinical trials focusing on the in vivo or ex vivo stimulation of γδ T cells have used the aminobisphosphonate zolendronate in combination with IL-2. Few studies applying adoptive cell transfer included systemic administration of zoledronate. When used in combination to treat a different type of malignancy, zoledronate showed tolerable toxicity, but revealed inconsistent responses and an overall modest efficacy. Along the same line, BrHPP combined with low doses of IL-2 in several solid tumors was safe, was well tolerated and induced γδ T-cell expansion in patients, but showed weak overall responses [84].

Several hypotheses could explain these disappointing results: first, BrHPP is quickly degraded by plasma phosphatases and zoledronate cannot passively cross the plasma membrane [85]. Furthermore, zoledronate has an unfavorable biodistribution due to covalent binding with calcium in bone.

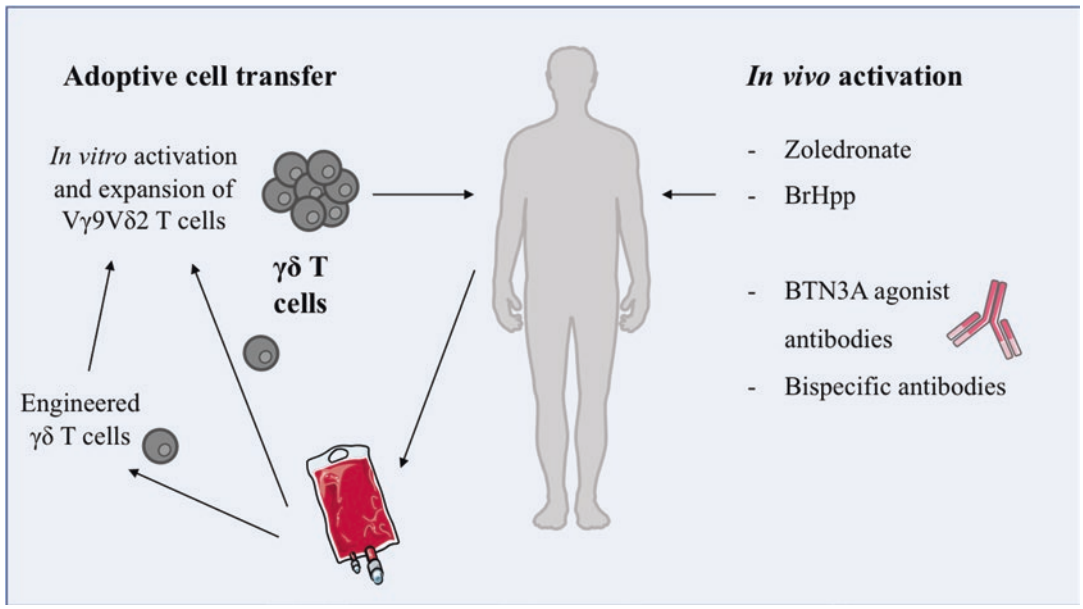

Fig. 5.4 Strategies for γδ T-cell-based immunotherapy

5.4.2 BTN3A Agonist Antibodies

As discussed above, peripheral blood mononuclear cells stimulated with BTN3A agonist antibodies such as 20.1 induced proliferation and expansion of γδ T cells. Moreover, addition of the 20.1 antibody to a panel of human tumor/transformed cell lines induced potent activation of Vγ9Vδ2 T cells [73]. These data support the rational to use BTN3A agonist antibodies in therapeutic approaches. Using the human AML xenograft mouse model, it has been demonstrated that 20.1 associated with Vγ9Vδ2 T-cell immunotherapy exerts a potent antileukemic effect [86]. In PDAC context, 20.1 was also shown to enhance BTN3A-mediated Vγ9Vδ2 T-cell antitumor functions under hypoxic conditions [87]. Because of the widespread expression of BTN3A, additional strategies for the enhancement of selectivity could be used like a bispecific antibody targeting both BTN3A and a tumor antigen. Moreover, antibodies that promote an inactive conformation of BTN3A have also been developed, which could be useful tools to treat autoimmune diseases [88].

5.4.3 Future Perspectives

As discussed above, several signals from the microenvironment, particularly cytokines, can confer some plasticity to γδ T cells and promote their differentiation into γδ T cells with regulatory functions. A therapeutic strategy combining ex vivo or in vivo activation and expansion of Vγ9Vδ2 T cells with systemic approaches to restore antitumor immune response may improve their efficacy. Another approach consists of lentiviral-mediated transduction of T cells with chimeric antigen receptors (CARs), which, thus, enables the CAR-transduced T cells to recognize tumor epitopes independently of their TCR. Until now, most CARs utilize αβ T cells, but due to their potent antitumor effector functions, γδ T cells could be good candidates for this strategy and are currently being tested in clinical trials [89]. Finally, the identification of biomarkers to predict clinical outcome is crucial for patient selection. Interestingly, 10 genes encoding cell surface proteins were identified to be statistically differentially expressed between "gammadelta-susceptible" and "gammadelta-resistant" hematopoietic tumors [90]. Another important issue is the functional status of these cells in the tumor microenvironment and the expression of co-signaling receptors. Our current studies favor their activated state together with their expression of PD1 (Olive et al., manuscript in preparation). Finally, an improvement of γδ T-cell immunotherapy associated with a selection of potential good responders to this treatment could be a promising way for fighting cancer.

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Regulatory T Cells in the Tumor Microenvironment

6

Rebekah E. Dadey, Creg J. Workman, and Dario A. A. Vignali

Abstract

Regulatory T cells (T_{regs}) are an immunosuppressive subpopulation of $CD4^+$ T cells that are endowed with potent suppressive activity and function to limit immune activation and maintain homeostasis. These cells are identified by the hallmark transcription factor FOXP3 and the high-affinity interleukin-2 (IL-2) receptor chain CD25. T_{regs} can be recruited to and persist within the tumor microenvironment (TME), acting as a potent barrier to effective antitumor immunity. This chapter will discuss [i] the history and hallmarks of T_{regs}; [ii] the recruitment, development, and persistence of T_{regs} within the TME; [iii] T_{reg} function within TME; asnd [iv] the therapeutic targeting of T_{regs} in the clinic. This chapter will conclude with a discussion of likely trends and future directions.

Keywords

Regulatory T cells (T_{reg}) · Tumor immunology · Tolerance · Tumor microenvironment (TME) · Foxp3 · CD25 · IL-2 · CTLA4 · NRP1 · LAG3 · CD39 · IL-10 · IL-35 · Cancer immunotherapy · Clinical trials

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6.1 Introduction

Regulatory T cells (T_{regs}) are an immunosuppressive subset of $CD4^+$ T cells that regulate the immune response to maintain homeostasis and limit autoimmunity; however, T_{regs} also play a deleterious role by suppressing antitumor responses [55, 144, 219, 256]. High numbers of

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T_{regs} are found in a variety of human and murine tumors. In human tumors, an increased T_{reg} to $CD8^+$ T cell ratio correlates with worse prognosis in many cancer types [176]. Further, systemic ablation of T_{regs} in mice results in complete tumor clearance, although these mice eventually succumb to lethal autoimmunity [51, 117, 118, 186, 190, 239]. Therefore, targeting T_{regs} specifically in the tumor microenvironment (TME) but not in the periphery may prove efficacious for cancer treatment.

This chapter provides a brief overview of T_{regs} and their hallmarks, subsets, and phenotypes, followed by an in-depth review of T_{regs} in the TME that includes recruitment, persistence, function, and therapeutic potential. We will mainly focus on T_{regs} in the TME, and their interactions with $CD4^+$ and $CD8^+$ T cells, and antigen-presenting cells (APCs); however, T_{regs} can also interact with other cells in the TME. This has been highlighted in several reviews [34, 139, 173].

6.1.1 Discovery of T_{regs}

The concept of a suppressor cell population was initially demonstrated through two seminal studies. The first found that removal of the mouse thymus within 3 days of birth, or neonatal thymectomy, causes immune-mediated destruction of the ovary [178]. The second found that adoptive transfer of thymocytes, most likely a subtype of $CD4^+$ T cells, limits immune response in a model of immune activation [73]. Together, these data suggested there is a subset of T cells that is important to control autoreactive cells.

However, identification and isolation of the "suppressive" subset of T cells proved difficult, so interest in the possibility of such a subset waned. It was not until the discovery of a suppressor-like subset of $CD4^+$ cells with constitutive high expression of the IL-2 receptor component CD25 (*Il2ra*) that interest in T_{regs} was reignited [223, 305]. For the first time, human and murine T_{regs} were isolated and examined based on the cell surface marker CD25. Together with the discovery of the primary hallmark of T_{regs}, the transcription factor *Foxp3*, this provided proof of the existence of a "suppressor"-like cell—and the T_{reg} field was born.

Discovery of the *Foxp3* gene began at the Oak Ridge Laboratory (Oak Ridge, TN, USA) with identification of a spontaneous X-linked mutation in a mouse colony that causes a phenotype of runtiness, scaly tail and skin, and closed eyelids and results in death within 3–4 weeks of age [222]. This mutation causes an increase in infiltration of the immune system into secondary lymphoid organs [76]. It was later discovered that the mutation, coined scurfy, results in truncation of the FOXP3 protein [27]. In addition, further studies found that mutations in the human *FOXP3* gene result in a similar substantive autoimmune phenotype called immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) [20, 21, 264, 288]. FOXP3 was subsequently linked to the $CD25^{high}$ $CD4^+$ "suppressor" cells as the primary transcription factor driving their phenotype [63, 97, 115]. Further, ectopic expression of *Foxp3* in $CD4^+$ T cells was found to confer a suppressive phenotype. Therefore, expression of FOXP3 and CD25 identified and solidified the existence of T_{regs}, leading to a major advancement in immunology.

Despite these exciting discoveries, there are limitations in using CD25 and FOXP3 to mark T_{regs}. For example, in mice and humans, activated T cells upregulate CD25 [277]. Therefore, identifying T_{regs} in an inflamed environment based on CD25 alone is difficult due to the activated phenotype of $CD4^+$ cells [119]. Further, isolation of live T_{regs} based upon FOXP3 expression is difficult because it is an intracellular transcription factor.

Therefore, other markers are continually being investigated to identify T_{regs}. One marker that is minimally expressed on T_{regs} compared to $CD4^+$ effector T cells is CD127, the IL-7 receptor that promotes expansion and survival of T cells [85, 142, 232, 305]. Currently, the combination of $CD25^+$ and $CD127^-$ is one of the best strategies to identify T_{reg}populations in mice and humans.

6.1.2 Hallmarks of T_{regs}

T_{regs} are incredibly unique and have many characteristics that are critical for their function. Expression of FOXP3 and CD25 are two key hallmarks of T_{regs}.

6.1.2.1 FOXP3

FOXP3 is a transcriptional activator and repressor for genes important for T_{reg} function either through direct DNA binding or binding to other transcription factors to alter their interactions with DNA [300]. Some examples of genes regulated by FOXP3 and critical in T_{reg} function are *Il2ra* (encodes CD25), *Tnfrsf18* (encodes GITR/TNFRSF18), and *Nrp1* [encodes Neuropilin-1 (NRP1)] [154, 315]. FOXP3 can also transcriptionally repress genes such as *Ifng* (encodes Interferon-γ) and *Il2* (encodes IL-2), enhancing a suppressive phenotype [145]. While regulation of these genes by FOXP3 plays an important role in T_{reg} development, maintenance, and function, ectopic expression of *Foxp3* will confer a suppressive phenotype but does not confer all signature T_{reg} genes [92, 221, 289, 316]. Consequently, other key molecules may regulate T_{reg} function.

Foxp3 has multiple key regulatory elements, including the promoter, 3′ untranslated region, a super-enhancer, and three intronic conserved noncoding sequences (CNS1–CNS3), that regulate Foxp3 expression and T_{reg} development and function [133, 314]. CNS1 aids in induction of *Foxp3* expression in peripherally derived T_{regs}, while CNS2 maintains FOXP3 expression after cell division [60]. CNS2 is regulated by CpG DNA methylation, which dampens *Foxp3* expression, while demethylation maintains *Foxp3* expression and promotes recruitment of transcription factors that stabilize *Foxp3* expression [172]. CNS3 is critical for de novo expression of *Foxp3* in the thymus, and deletion of this region substantially decreases T_{regs} numbers in the thymus [61, 314].

Other regions of the T_{reg} genome are specifically demethylated that are important to maintain T_{reg} function, including *Ikzf2* (HELIOS), *Ikzf4* (EOS), *Ctla4* (CTLA4), *Il2ra* (CD25), and *Tnfrsf18* (GITR) [62].

6.1.2.2 CD25 and IL-2

In addition to its role in identifying T_{regs}, CD25 also plays a critical role in the development, maintenance, and function of $T_{regs.}$ CD25, or IL-2Rα, is one component of the IL-2 receptor, consisting of CD25, CD122 (IL-2Rβ), and CD132 (common gamma chain, γc). IL-2 binding to its receptor induces a signaling cascade that results in the Janus kinase (JAK)-mediated tyrosine phosphorylation and activation of signal transducer and activator of transcription 5 (STAT5). STAT5 homodimers translocate to the nucleus to facilitate the induction of *Foxp3* expression, which is critical for T_{reg} development and homeostasis [37, 200, 257]. STAT5 also induces expression *of Il2ra* to enforce a positive feedback loop, as well as other key T_{reg} functional genes, such as *Ctla4*, *Tnfrsf18*, and *Icos* [37]. This highlights that the IL-2/STAT5 pathway is not only required for T_{reg} development and maintenance but also necessary for T_{reg} function.

As T_{regs} are unable to make their own IL-2, they rely on other cells as their source of IL-2 [37]. There has been speculation that due to their high CD25 expression, T_{regs} can sequester IL-2 away from other cells as a form of suppression [196]. However, it has been highly contended whether this occurs in vivo. Nonetheless, T_{reg} expression of CD25 and dependence on IL-2 is a key hallmark of T_{regs}.

6.1.3 T_{reg} Subsets

There are two types of T_{regs} commonly identified in vivo: thymically derived T_{regs} (tT_{regs}), and peripherally derived T_{regs} (pT_{regs}) [1]. tT_{regs} or "natural" T_{regs} follow a similar developmental trajectory as other T cells in the thymus. However, pT_{regs} or "induced" or "adaptive" T_{regs} begin as $CD4^+Foxp3^-$ cells in the periphery but are converted into $CD4^+Foxp3^+$ T_{regs} in the periphery by suboptimal T-cell receptor (TCR) stimulation, TGFβ, retinoic acid, and IL-2 [36, 58, 138, 273]. tT_{regs} are thought to be important in maintaining homeostasis due to their higher affinity toward self-peptide compared to $CD4^+$ $FOXP3^-$ cells [98]. pT_{regs} are also specific toward self-antigen;

they can limit autoimmunity, although they are more commonly thought to be important suppressors at mucosal surfaces and environmental barriers [23, 108, 302]. pT_{regs} seem to be less stable than tT_{regs}, which could be due to increased methylation at the CNS2 locus compared to tT_{regs} [180]. However, this finding has been contested [84, 90, 162, 207]. Although pT_{regs} are thought to be less suppressive than tT_{regs}, it is possible that pT_{regs} and tT_{regs} work together to suppress different types of inflammation [84].

The majority of the in vivo pool of $FOXP3^+$ T_{regs} are likely thymically derived, although the absence of specific markers that are unique to either tT_{regs} or pT_{regs} makes it difficult to discern [238]. Two markers, HELIOS and NRP1, have been used to assess T_{reg} origin, although they are not completely faithful. For example, high expression of the Ikaros family member HELIOS is thought to mark tT_{regs} [254], although other studies have demonstrated that pT_{regs} can express HELIOS depending on cell activation status [6, 79]. In addition, although NRP1 was originally thought to be a promising candidate to identify tT_{regs} [286, 301], mice that favor production of only tT_{regs} or pT_{regs} show similar levels of NRP1 expression [249].

While pT_{regs} and tT_{regs} are the key contributing sources of T_{regs}, there are also suppressor cells that lack FOXP3 expression that can also contribute to immune regulation in different settings, namely, Tr1, Th3, and iTr35 cells. The importance of these cells is highlighted in several reviews [22, 34, 42, 227, 303]. To limit our scope, we will not discuss these further.

6.1.4 T_{reg} Phenotypes

In addition to division into pT_{regs} and tT_{regs}, T_{regs} also can be classified by their phenotype. For example, human T_{regs} can be segregated into three populations: resting T_{regs} ($CD45RA^+FOXP3^{lo}$), activated or effector T_{regs} ($CD45RA^-FOXP3^{hi}$), and non-T_{regs} capable of making pro-inflammatory cytokines ($CD45RA^-$ $FOXP3^{lo}$) [161].

T_{regs} are also capable of expressing lineage transcription factors to aid in their suppression of various responses. For example, mouse and human T_{regs} can express the Th1 transcription factor T-BET to suppress Th1 responses [53, 121, 136]. GATA3 expression in T_{regs} is required for general T_{reg} suppression, while $ROR\gamma T^+$ or $STAT3^+$ T_{regs} suppress Th17 inflammation, and $IRF4^+$ T_{regs} suppress Th2 responses [35, 280, 304, 313]. Therefore, T_{regs} can have many phenotypes that contribute to their function.

6.2 T_{REG} Recruitment, Development, and Persistence Within the TME

There are three possible mechanisms behind accumulation of T_{regs} within the TME: (1) T_{regs} are recruited via chemokines, (2) T_{regs} are derived from $CD4^+$ $FOXP3^-$ precursors (pT_{regs}) within the tumor, or (3) T_{regs} are present in the tissue and expand in situ during pathogenesis. This section focuses on recruitment of T_{regs}, development of pT_{regs} within the TME, and persistence of T_{regs} that encompass possible expansion of tissue-resident T_{regs} [152].

6.2.1 Recruitment

CCR4 is the most studied receptor in recruitment of T_{regs} to the TME (Fig. 6.1). The ligands for this receptor, CCL22 and CCL17, are produced by tumor cells and tumor-associated macrophages [48, 101]. CCR4 has been shown to be highly expressed on human ovarian tumor T_{regs} and the CCL22 ligand, but not CCL17, can recruit these cells [48]. However, this discrepancy may depend on the type of tumor and model system. Some murine studies showed that CCL17 rather than CCL22 recruits these cells in a model of breast cancer, while others showed only CCL22 is responsible [151, 183]. Interestingly, $CCR4^+$ T_{regs} in the TME have been identified as effector T_{regs}.

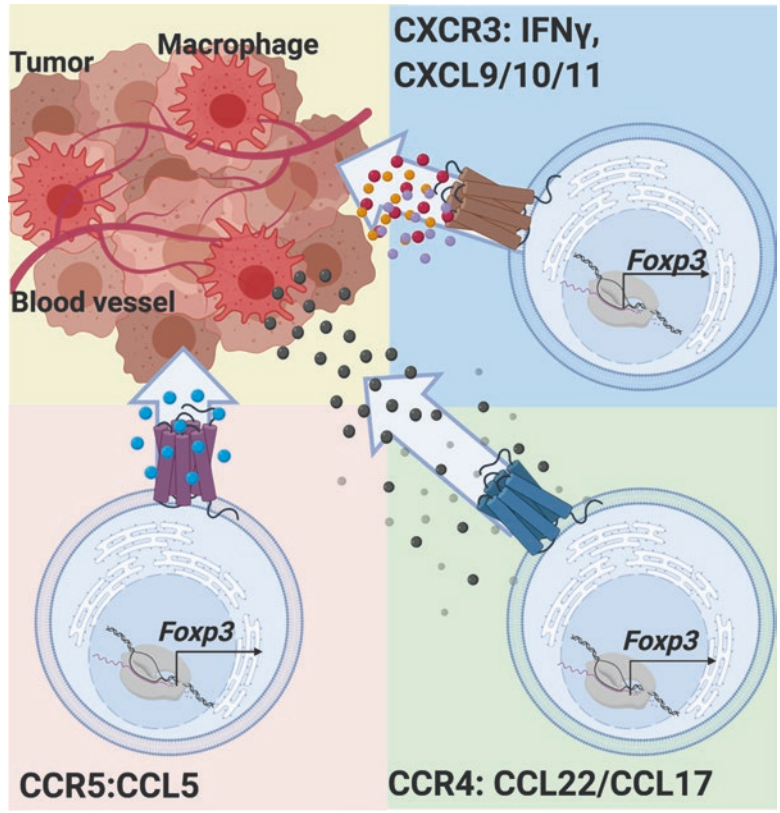

Fig. 6.1 T_{reg}recruitment to the TME

Therefore, T_{regs} recruited by the CCR4:CCL22 and CCR4:CCL17 axis may be more suppressive in the TME [248]. There are efforts to target CCR4 in the clinic, which will be discussed in a later section.

Murine and human models of pancreatic adenocarcinoma show that T_{regs} can express CCR5 and are recruited to tumors through tumor production of CCL5 [251]. Further, myeloid-derived suppressor cells can recruit T_{regs} through CCL5 production [229]. Consequently, reduced tumor-derived CCL5 results in decreased T_{reg} accumulation in the TME.

The TME also produces the pro-inflammatory cytokine IFNγ, which can induce CXCL9, CXCL10, and CXCL11 [168]. These three chemokines bind to the receptor CXCR3. In T_{regs}, IFNγ can also induce CXCR3 expression indirectly through activation and expression of T-BET, which may aid T_{reg} recruitment to the TME [122, 126, 140, 215].

In summary, three key mechanisms of T_{reg} migration to the TME have been identified: CCR4, CCR5, and CXCR3. However, many other axes, such as CCR6:CCL20, CCR7:CCL21, CCR8:CCL1, CCR10:CCL28, and CXCR4:CXCL12, may also aid T_{reg} recruitment to the TME [182, 185].

6.2.2 T_{reg} Development Within the TME

While it is clear that tT_{regs} and pT_{regs} exist in vivo, it is currently debated which cells dominate in limiting the antitumor response. Of course, it is possible that this may vary between different mouse tumor models, human tumor types, and even from patient to patient. In a murine model, adoptive transfer of $CD4^{+}FOXP3^{-}$ cells into mice bearing the TGFβ-producing tumor cell line Panc02 induces FOXP3 in transferred cells [166]. Contrary to these results, TCR sequencing stud-

ies revealed only 3.8% TCR similarity between $CD4^+FOXP3^-$ and T_{regs} in a murine chemical-induced tumor, indicating these T_{regs} did not develop from the $CD4^+FOXP3^-$ precursors [93]. However, other analyses of murine T_{regs} and $CD4^+FOXP3^-$ cells indicates some TCR overlap, ranging from 10% to 20% [59, 98, 193, 194, 293]. Analysis of the human TCR repertoire between these two populations in the blood shows less than 1% overlap, while tumors have a range of 0.5–13.2% overlap [3, 77].

The CNS2 locus of *Foxp3* is demethylated in mouse tumor-infiltrating T cells, indicating a thymic origin [271]. Further, elimination of TGFβ in the 4T1 breast tumor mouse model does not reduce numbers of T_{regs}, further suggesting a thymic origin [271]. However, others have argued that the pool of T_{regs} in tumors is from both thymic and peripheral origins [317].

In human tumors, the source of T_{regs} is also unclear. The CNS2 of *Foxp3* is demethylated in these isolated T_{regs}, which may indicate a thymic origin [271]. However, others have suggested that various human patient cells, such as malignant B cells and tumor cells, can convert $CD4^+$ $FOXP3^-$ to $FOXP3^+$ T_{regs} in vitro [4, 83, 160]. Therefore, more studies are necessary to determine the derivation of T_{regs} in the TME.

6.2.3 T_{reg} Persistence Within the TME

The TME is an extremely unique and metabolically demanding environment that presents many challenges to tumor infiltrating lymphocytes. Here, we discuss T_{reg} antigen specificity and TCR repertoire that contributes to their persistence in the TME, metabolic challenges, and techniques T_{regs} use to maintain stability in the harsh TME (Figs. 6.2 and 6.3).

6.2.3.1 T_{reg} Antigen Specificity Within the TME

T_{regs} in the thymus must undergo TCR rearrangement before migrating to the periphery. This process can create unlimited diversity in TCR repertoire. However, among this diversity, it is commonly understood that T_{regs} generally have a higher affinity for self-antigen [99, 194]. T_{reg} specificity to self-peptide is critical to limit autoreactive immune responses and maintain homeostasis [116, 177]. Interestingly, others have contested that T_{regs} only have slightly higher affinity for self, as they also have the ability to respond to foreign antigens [19, 41, 95, 135, 253].

Within the TME, many self-antigens are presented that are overexpressed, mutated, altered in structure, expressed at the wrong stage of cellular development, and/or expressed due to cellular lineage [270]. Therefore, T_{regs} in the TME may recognize these self-antigens. For example, $T_{regs,}$ in a study of human metastatic melanoma, are specific for cancer germline protein MAGE-A3 [68]. T_{regs} from melanoma can also recognize antigens such as GP100, NYESO-1, and SURVIVIN [2, 3, 9, 265, 275, 276]. Further, T_{regs} from melanoma, gastrointestinal, and ovarian malignancies have high specificity toward mutated neoantigens [3]. These data suggest that T_{regs} in the TME are specific toward self-antigens which may aid in their persistence in the TME.

6.2.3.2 T_{reg} Metabolism Within the TME

The TME is metabolically demanding. The tumor contains rapidly expanding malignant cells that outgrow oxygen availability and induce a hypoxic environment (Fig. 6.2) [105]. Further, the pH of the TME is acidic due to its mechanism of glucose metabolism. Normal cells in the presence of oxygen use oxidative phosphorylation (OXPHOS) to metabolize glucose [107]. Without oxygen, cells convert pyruvate to lactic acid, which is known as anaerobic glycolysis. However, tumor cells are unique in that they convert pyruvate to lactic acid even in the presence of oxygen, which is known as the Warburg effect or aerobic glycolysis [312]. This switch is thought to provide cancer cells with building blocks for nucleotides, amino acids, and lipids [171]. Tumor cells can then use the end product, lactate, as an additional energy source and shuttle excess out into the microenvironment, substantially lowering pH of the TME [33, 104, 247].

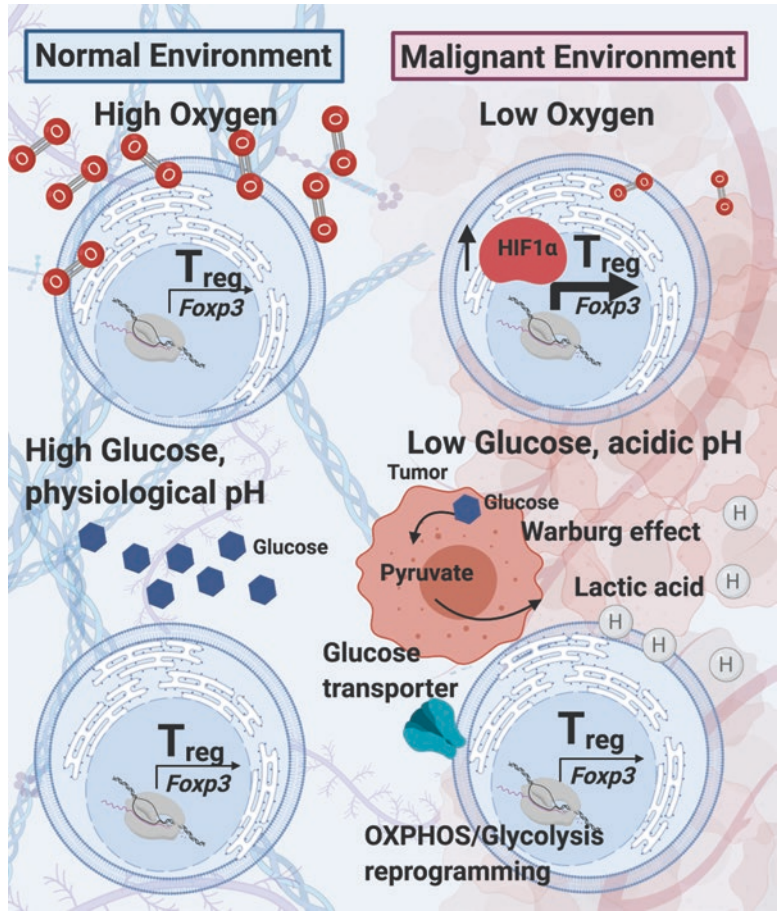

Fig. 6.2 T_{reg}metabolism in the TME

Hypoxia, low glucose, and lactic acid limit effector T-cell function, including decreasing IFNγ production [26]. However, T_{regs} are uniquely capable of living in the high lactate and low glucose TME through metabolic reprogramming to OXPHOS, which allows resistance to the harsh TME [14, 247] (Fig. 6.2). Others showed that T_{regs} in the TME may rely more on glycolysis, with higher levels of glucose transporters and glycolytic flux [192]. This may also aid in their competition for the limited glucose in the TME. In addition, hypoxia upregulates hypoxia inducible factor 1 subunit alpha (HIF1α), which promotes FOXP3 expression in $CD4^+$ $FOXP3^-$ cells, further increasing numbers of T_{regs} in the TME [39, 281]. T_{regs} have developed these characteristics to withstand the harsh metabolic requirements of the TME and thus survive and persist.

6.2.3.3 T_{reg} Stability Within the TME

In addition to changing their metabolism, T_{regs} have other ways to reinforce their stability in the TME (Fig. 6.3). One is the maintenance of FOXP3 expression, as loss of FOXP3 in mice or humans induces an autoimmune response. T_{regs} have multiple mechanisms to sustain FOXP3 expression. First, a defined epigenetic profile focused on CNS2 demethylation at CpG-rich residues is required for the maintenance of FOXP3 expression in the TME [271]. Second, we have discussed previously the importance of CD25 expression on T_{regs} and the fact that STAT5 activation downstream of the IL-2 receptor induces expression of FOXP3. T_{regs} express CD25 in the murine and human TME, thus promoting the expression and maintenance of FOXP3 expression [16]. Third, T_{regs} specifically limit activation of the phosphatidylinositol 3

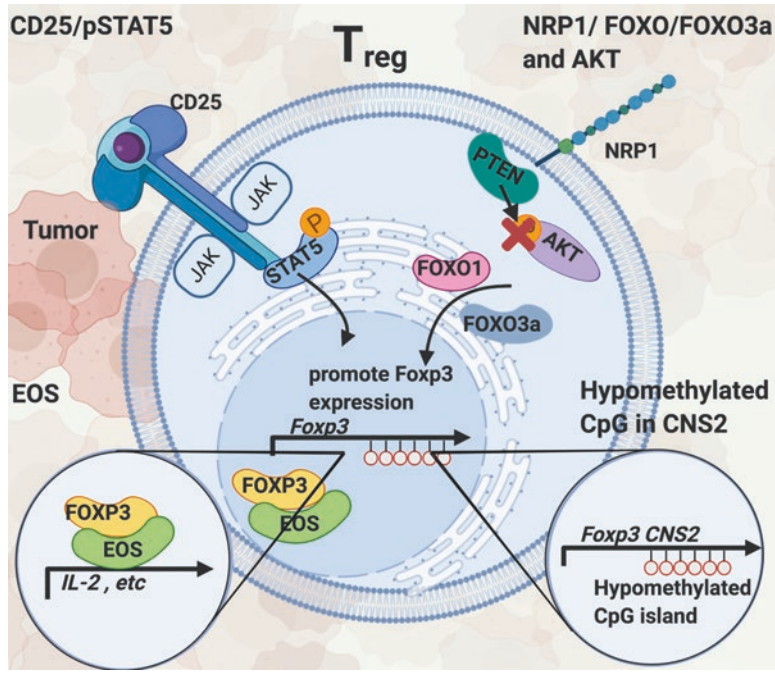

Fig. 6.3 T_{reg}stability in the TME

kinase (PI3K) pathway and protein kinase B (AKT), as their activation limits induction of FOXP3 expression [88, 158, 226]. Limitation of AKT activates FOXO1 and FOXO3a transcription factors to translocate to the nucleus, drive *Foxp3* expression, and bind other loci to induce a suppressive phenotype [114, 181, 188, 189]. However, expression of FOXO proteins in T_{regs} in the TME may have negative consequences as high expression of a constitutively active FOXO1 impedes T_{reg} migration, while low expression of the constitutively active FOXO1 mutant depletes T_{regs} in the tumor [150]. Clearly, more studies are required to determine the importance of the FOXO pathway in tumor-derived T_{regs}.

In addition to FOXP3 expression, the Ikaros transcription factor family member EOS plays an important role in T_{reg} stability. EOS, a transcriptional repressor, binds with FOXP3 to silence genes such as *Il2*, and others key to maintaining a suppressive phenotype [216]. Loss of *Ikzf4* (EOS) through siRNA reduces suppressive capacity of T_{regs} [195]. Further, populations of "EOS-labile" T_{regs} that lose EOS in response to a vaccination strategy are reprogrammed and upregulate inflammatory molecules such as IL-2, IL-17, and CD40L [236]. These cells maintain FOXP3 expression but change their phenotype to help $CD8^+$ T-cell priming [236]. T_{regs} in the human TME express EOS, which is positively correlated with FOXP3 expression [7]. Therefore, expression of EOS is critical for the suppressive phenotype of T_{regs}.

The NRP1-Semaphorin-4a (SEMA4a) axis has been shown to be important for the maintenance of T_{reg} phenotype and function within the TME [51, 189]. NRP1 is expressed on the majority of murine T_{regs}. In contrast, whereas T_{regs} in healthy donor human peripheral blood mononuclear cells (PBMCs) have little to no expression of NRP1, a substantively increased proportion of T_{regs} express NRP1 on T_{regs} in the TME in a subset of patients with melanoma or head and neck cancer [190, 191]. NRP1 expressed on T_{regs} binds phosphatase and tensin homolog (PTEN) at the immunological synapse to limit AKT activation, directing FOXO1 and FOXO3a to the nucleus to further promote T_{reg} stability. Moreover, T_{reg}-restricted deletion of NRP1 leads to changes in intratumoral T_{regs} from a suppressive to a more effector phenotype, secreting IFNγ. This change limits T_{reg} suppression, which enhances the anti-

tumor response [190, 191]. Thus, NRP1-SEMA4a and other key mechanisms are critical to maintain T_{reg} stability in the TME.

T_{regs} have multiple mechanisms to aid their accumulation in the TME. First, T_{regs} can be recruited through multiple chemokine gradients such as CCR4:CCL22/CCL17, CCR5:CCL5, and CXCR3:IFNγ, CXCL9/10/11. Second, T_{regs} can be induced from a $CD4^+$ $FOXP3^-$ T cell. Finally, T_{regs} persist in the TME due to their TCR specificity, altered metabolism, and mechanisms to enforce stability. These three mechanisms contribute to T_{reg} accumulation in the TME.

6.3 T_{REG} Function Within the TME

There are four main mechanisms of T_{reg} suppression: [i] production of inhibitory cytokines, [ii] cytolysis, [iii] targeting dendritic cells (DCs) and inhibitory receptor expression, and [iv] metabolic disruption (Fig. 6.4) [47, 141, 224, 230, 267, 268]. For the purpose of this review, we will only focus on the mechanisms that have been shown to occur within the TME.

6.3.1 Inhibitory Cytokines

6.3.1.1 IL-10

IL-10 is a suppressive cytokine that exerts its suppressive function in many ways. For example, IL-10 inhibits transcription of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, and IL-12 in macrophages [128]. IL-10 can also block T-cell function by targeting APCs, indirectly blocking IL-2, TNFα, and other Th1 cytokines in T cells [167]. IL-10 can also block downstream signaling of the CD28 costimulatory receptor in T cells [252]. IL-10 mediates this suppression through activation of JAK1 and TYK2. These molecules activate STAT3, which can transcriptionally regulate genes or activate other members such as SOCS3, further aiding gene suppression [52, 170].

It is currently thought that T_{regs} make IL-10 in the TME and can be a major source of IL-10 in tumors [228, 246]. T_{reg} production of IL-10 leads to upregulation of inhibitory molecules on $CD8^+$ T cells contributing to their exhaustion. IL-10 is thus an important suppressive molecule that T_{regs} use in tumors, and in vivo, it is currently being investigated in the clinic [279].

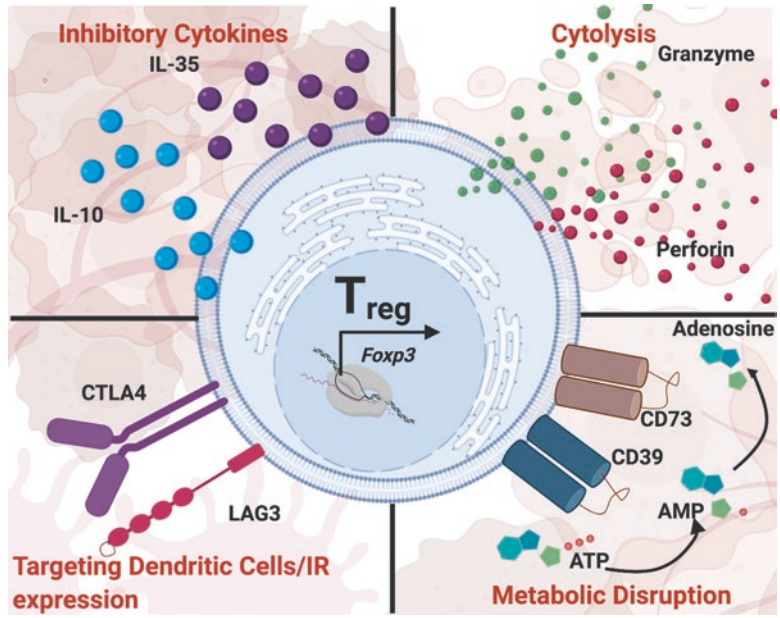

Fig. 6.4 T_{reg}suppression in the TME

6.3.1.2 IL-35

IL-35 is a suppressive cytokine that is composed of p35 (encoded by *IL12a*) and the IL-27β chain, EBI3 (encoded by *Ebi3*) [45]. It is a member of the IL-12 cytokine family, which includes IL-12, IL-23, and IL-27. These cytokines are composed of heterodimers with certain family members sharing the same monomers resulting in diverse functions [269]. For example, IL-12 is a pro-inflammatory cytokine composed of p40 and p35 compared to IL-35, a suppressive cytokine composed of EBI3 and p35 [44]. IL-35 binds to the IL-35 receptor complex, which is made up of GP130 and IL-12Rβ2 [43]. This activates and forms a heterodimer of STAT4 and STAT1, which moves to the nucleus to induce transcription of *IL12a* and *Ebi3*, creating a positive feedback loop. Interestingly, IL-35 can suppress cells in two ways: [i] inhibition of $CD4^+$ and $CD8^+$ effector T-cell proliferation and function, and [ii] induction of an IL-35-induced T_{reg} population. This induced cell population aids immune suppression but does not express FOXP3 [42]. Interestingly, T_{regs} are the main IL-35 producers, and recent studies have shown that T_{regs} in both mice and humans can make IL-35 in the TME. T_{reg}-restricted deletion of *Ebi3*, using the mouse model $Ebi3^{L/L}.Foxp3^{Cre\text{-}YFP}$, led to decreased tumor growth, reduced T-cell exhaustion, and increased pro-inflammatory cytokine production in the TME [261].

T_{regs} have the ability to make various inhibitory cytokines in the TME. There appears to be two separate T_{reg} populations in the TME, an $IL\text{-}35^+$population and an $IL\text{-}10^+$ population, with few double-positive $IL\text{-}10^+IL35^+$ T_{regs} [228, 285]. Interestingly, IL-10 and IL-35 derived from each population directly regulates inhibitory receptor expression on $CD8^+$ T cells, thereby impacting their exhaustion, via the transcriptional repressor BLIMP1 but with slightly different functional outcomes. $IL\text{-}10^+$ T_{regs} seem to limit $CD8^+$ T-cell proliferation and function, whereas $IL\text{-}35^+$ T_{regs} limit $CD8^+$ T-cell memory. These studies demonstrate the important nonredundant role of T_{reg}-produced inhibitory cytokines in the TME.

6.3.2 Cytolysis

Cellular apoptosis is a critical component of immune homeostasis, cellular turnover, and destruction of infected, cancerous, or damaged cells. One way to induce apoptosis is through production of cytolytic molecules such as Perforin (PRF1) or Granzymes (GZM). The pore-forming protein PRF1 forms holes that compromise the integrity of target cell membranes, aiding in passage of molecules that initiate cell death such as GZMs [187]. GZMB induces cell apoptosis by cleaving caspases, which trigger the apoptosis pathway [25]. While GZMs and PRF1 are commonly expressed in $CD8^+$ T cells and NK cells, T_{regs} also use these pathways as a means of suppression. In vitro studies show that mouse T_{regs} use a GZMB-dependent, PRF1-independent pathway, while human T_{regs} use a GZMA- and PRF1-dependent pathway to mediate suppression by cytolysis [78, 81]. Importantly, T_{regs} can express high levels of GZMB and PRF1 to aid in cell death of $CD8^+$ T cells and NK cells in tumors [31]. Therefore, GZM and PRF1 direct cytolysis is a key mechanism of T_{reg} suppression in the TME.

6.3.3 Targeting DCs and Inhibitory Molecule Receptor Expression

DCs are critical for activation of T cells. T_{regs} can inhibit DC function through several molecules, including Cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and Lymphocyte activation-gene 3 (LAG3), which we discuss in this section. T_{regs} also express several inhibitory and activating molecules that can modulate their function and play an important role in immunotherapy and further targeting in the tumor.

6.3.3.1 CTLA4/CD152

T cells require binding of TCR to the peptide-major histocompatibility complex (MHC) for activation and stimulation. However, T cells also require activation of the costimulatory pathway. The costimulatory molecule CD28 is expressed

by T cells and interacts with B7–1 (CD80) or B7–2 (CD86) on APCs. This signal provides T cells with additional signals for activation and survival [28].

CTLA4 is a highly similar molecule to CD28 and also binds CD80 and CD86. CTLA4 does not provide T cells with the same activation signals but rather is a negative regulator, inhibiting CD28 costimulation, IL-2 production, and cell cycle progression [123]. CTLA4 was discovered as a key negative regulator of T-cell activation through administration of a blocking antibody and establishment of *Ctla4* knock-out mice that develop severe lymphoproliferative disease and succumb to the disease within 3–4 weeks of age [130, 255]. T cells upregulate CTLA4 upon activation, and CTLA4 competes with CD28 for binding to CD80 and CD86. Further, T_{regs} constitutively express CTLA4 and use it as a means of suppression [213, 250]. For example, CTLA4 restricts expression of CD80 and CD86 on DCs to limit activation of other cells [32, 291].

In addition to causing downregulation of CD80 and CD86, CTLA4 on T_{regs} can bind to these molecules to induce expression of indoleamine 2,3,-dioxygenase (IDO) [56, 80, 157]. IDO catalyzes breakdown of the amino acid tryptophan to suppressive metabolites including kynurenines. IDO breaking down tryptophan suppresses T cells in two ways: [i] less available tryptophan will limit the ability of T cells to use this amino acid for the cell cycle and other functions [57, 131, 169], and [ii] tryptophan metabolites such as kynurenine, quinolinic acid, and picolinic acid suppress T-cell proliferation and function and can induce apoptosis [56, 205].

T_{regs} in the TME express CTLA4 and utilize this to suppress in the TME. Studies of human head and neck cancer show CTLA4 is highly expressed on T_{regs}, and these cells are more suppressive than CTLA4^{-} T_{regs} [155]. However, studies examining the experimental autoimmune encephalomyelitis (EAE) model suggest that deletion of CTLA4 on T_{regs} may result in better T_{reg} expansion and activation but does not affect their function [201]. This could suggest that CTLA4 acts as a brake on T_{regs}, so removal unleashes T_{reg} activation and expansion. Many studies are still investigating CTLA4 to target T_{regs} and their function in the TME, which is discussed in a later section.

6.3.3.2 LAG3

Lymphocyte activation-gene 3 (LAG3) or CD223 is an inhibitory receptor that is highly homologous to CD4 and is upregulated upon activation [259, 297, 298]. Like CD4, LAG3 binds to MHCII but with a much higher affinity resulting in the negative regulation of T-cell activation [12, 295, 296]. Targeting LAG3 along with Programmed cell death protein 1 (PD-1) has had significant efficacy in limiting tumor growth in murine models [294].

T_{regs} express low levels of LAG3, which is upregulated upon stimulation and required for full T_{reg} suppression [100, 298]. In addition, LAG3 binding to MHC II on DCs limits DC activation to further suppress T-cell activation [137]. T_{regs} from the TME and PBMCs express LAG3, and these LAG3^{+} T_{regs} are fully suppressive [30, 197, 284]. However, these studies are further complicated by evidence that LAG3 expression on T_{regs} in the autoimmune microenvironment inhibits their proliferation and function [310]. The function of LAG3 on T_{regs} may differ in autoimmune versus tumor settings. LAG3 targeting may affect T_{reg} function but also may re-invigorate exhausted CD8^{+} T cells. Further studies are required to understand these differences. LAG3 is currently in clinical trials as a single and combinatory treatment [12, 13].

6.3.3.3 PD-1

Programmed cell death protein 1 (PD-1) is upregulated on activated and exhausted T cells in chronic viral infections, cancers, and other inflammatory responses [211]. Studies have shown that ligation of PD-1 to one of its ligands, programmed death-ligand 1 (PD-L1), delivers an intrinsic signal to dampen immune activation and function, including decreased cytokine production [156, 225] and decreased TCR signaling and stimulation [225, 258]. PD-1 is necessary to limit aberrant T-cell activation, although tumor cells and other cells in the TME can express PD-L1 to dampen T-cell response in the TME [299].

Therefore, it may prove important to limit this pathway for full activation of the T-cell response to tumors.

In a murine model, T-cell ligation of PD-1 to PD-L1 induces FOXP3 expression in $CD4^+$ $Foxp3^-$ T cells, as well as increases the level of suppressive capability [17, 67, 124, 245, 278, 308]. Moreover, T_{regs} require PD-1 expression to suppress activated $CD8^+$ T cells in mice infected with chronic lymphocytic choriomeningitis virus (LCMV) clone 13 [198, 199].

In humans, the role of PD-1 expression on T_{regs} is unclear. PD-1 expression on T_{regs} in hepatitis C virus (HCV) infection is associated with a more effector-like T_{reg} and may contribute to long-term infection, although others have found T_{regs} expressing PD-1 in the liver of HCV-infected patients to have decreased suppressive capability and proliferation [66, 212, 237]. PD-1 is also expressed on T_{regs} in the TME [165, 197], which results in cell dysfunction including decreased suppressive ability and increased production of inflammatory cytokines [146]. In vitro studies show anti-PD-1 blockade enhances T_{reg} suppression and proliferation [110]. This suggests that targeting PD-1 may result in improved T_{reg} function. Therefore, more studies are necessary to elucidate how this could impact anti-PD-1 therapeutic outcome.

6.3.3.4 TIM3

T-cell immunoglobulin and mucin domain 3 (TIM3), also known as hepatitis A virus cellular receptor 2 (HAVCR2), is expressed on T cells, DCs, B cells, macrophages, mast cells, and NK cells [75, 86, 89, 164]. TIM3 binds to four possible ligands: galectin-9, carcinoembryonic antigen cell adhesion molecule 1 (CEACAM-1), high-mobility group protein B1 (HMGB1), and phosphatidylserine (PS) [89]. Ligand binding of GALECTIN-9 and CEACAM-1 may lead to downregulation of TCR signaling molecules [10]. Further, ligand binding of TIM3 on T cells limits pro-inflammatory cytokine expression and cellular proliferation [86].

T_{regs} also express high TIM3 levels that are upregulated upon activation and in the TME [82]. TIM3 and PD-1 are commonly co-expressed, and TIM3 expression is correlated with worse cancer progression in human lung cancer [71]. Moreover, $TIM3^+$ T_{regs} are more suppressive than $TIM3^-$ T_{regs} [10, 72]. Therefore, clinical targeting of TIM3 may reinvigorate $CD8^+$ T cells and limit T_{reg} function. TIM3 is currently being clinically investigated as a single agent therapy and in combination with anti-PD-1 [13, 89].

6.3.4 Metabolic Disruption

T_{regs} are capable of limiting metabolism of other cells through multiple means. We will discuss T_{reg} expression and function of CD39 and CD73.

6.3.4.1 CD39 and CD73

Cells contain a high concentration of intracellular adenosine-tri-phosphate (ATP) that is needed for cellular processes. However, release of extracellular ATP is an indicator that a cell is damaged. These processes activate several immune responses, including production of inflammatory molecules and migration of cells [8]. Two molecules, CD39 and CD73, are ectoenzymes that can use ATP as a substrate to convert into metabolites. CD39 converts ATP to adenosine diphosphate (ADP) and adenosine monophosphate (AMP). CD73 then converts AMP to extracellular adenosine. Adenosine is a suppressive molecule that binds one of its multiple receptors, A1, A2a, A2b, and A3 [134]. This triggers activation of intracellular cAMP, which activates protein kinase A (PKA). PKA then limits TCR, IL-2R signaling, and IL-2 and pro-inflammatory cytokine production to limit T-cell activation [134, 266].

Interestingly, adenosine plays a dual role in T_{regs}. Adenosine can favor T_{reg} stability, but T_{regs} themselves can express high levels of CD39 and CD73 to aid in their suppression [18, 50]. In the TME, mouse T_{regs} express CD39 and CD73 and use them for suppression [64, 125]. However, expression of these markers on human T_{regs} in the TME is less clear. Human tumor studies examin-

ing melanoma and follicular lymphoma have shown expression of CD39⁺ T_{regs} [91, 263]. The latter study demonstrated blockade of adenosine receptors or CD39 catalytic activity results in increased activation and effector cytokine secretion in T cells. CD73 expression on T_{regs} in the TME is contended [8, 91]. Therefore, further studies are needed to clarify the role of CD39 and CD73 in T_{reg}suppression of the antitumor response.

6.4 Therapeutic Targeting of T_{regs} in the Clinic

T_{regs} are key suppressors of the antitumor response, thus targeting their function in tumors could be efficacious. Here, we focus on a few ways T_{regs} are directly and indirectly targeted in the clinic or being tested in preclinical and clinical trials (Table 6.1) [241]. We will only discuss a few of the clinical trials for each target, but a

Table 6.1 Therapies for targeting T_{regs}

Target	Name	Trial phase/FDA approval date	Clinical outcome	Citation(s)
	Denileukin diftitox	FDA approved in but discontinued in 2014	44% ORR in cutaneous T-cell lymphoma Discontinued due to manufacturing and toxicity issues	Dannull et al. (2005) [49] Kaminetzky and Hymes (2008) [111]
CD25	Daclizumab	Phase I/II clinical trials	Range of efficacy in tumor treatments, more studies required. Modifications such as radionuclide may increase efficacy Discontinued in MS treatment due to toxicity issues	Waldmnn (2007) [272] Rech and Vonderheide (2009) [214]
	Lytic anti-CD25	Preclinical trials	Efficacious in initial experiments CRR: 70–100% in mouse studies, upcoming clinical trials to determine efficacy	Arce Vargas et al. (2017) [16] Solomon et al. (2017) [243]
TGFβ	Galunisertib	Phase II clinical trials	May be 10 month increase in survival, along with sorafenib	Qin et al. (2017) [210]
	TGFβR Traps, M7824, RER	Preclinical trials	Inhibit T_{reg} development, may effect suppression	Zwaagstra et al. (2012) [319]
CCR4	Mogamulizumab	FDA approved in 2018	23–34% ORR in clinical trials for relapsed or refractory mycosis fungoides and Sézary disease	Zinzani et al. (2016) [318] Kasamon et al. (2019) [112]
Nrp1	MNRP1685A	Phase 1B clinical trial	Little efficacy alone, toxicity with other agents. Studies discontinued	Patnaik, et al. (2014) [202].
	Anti-Nrp1, (Fc(AAG)-TPP11)	Preclinical trials	50–80% survival rate in mice	Jung et al. (2020) [109]
CTLA4	Ipilimumab	FDA approved in 2011	10.9% ORR alone 40% ORR in combination with anti-PD1	Callahan et al. (2014) [29] Hodi et al. (2010) [94]
TIGIT	BMS-986207, YH29143, Etigilimab, etc.	Phase I/II clinical trials	Drug is well tolerated, may decrease T_{reg} numbers	Anderson et al. (2019) [11] Lee (2019) [132]
GITR	BMS-986156, TRX51, MEDI1873, etc.	Phase I/II clinical trials	May prove efficacy with anti-PD1. More studies necessary	Zappasodi et al. (2019) [307]
CD39/ CD73	TTX-030, KY3475070, MEDI9447, CD73.4, etc	Preclinical trials, Phase I/IB, Phase II	May decrease T_{reg} suppression	Hausler, et al. (2014) [87] Perrot, et al. (2019) [204]

complete list of clinical trials can be found at www.clinicaltrials.gov.

6.4.1 CD25

Treatment of tumor-bearing mice with anti-CD25 leads to depletion of T_{regs}, control of tumor growth, and, in some cases, clearance of the tumor [239]. Therefore, it is of interest to target CD25 expressing cells. Production of an IL-2 diphtheria toxin conjugate (Denileukin diftitox), which depletes all CD25 expressing cells, was approved by the Food and Drug Administration (FDA) after demonstration of a 44% overall response rate (ORR) in recurrent cutaneous T-cell lymphoma [111]. This drug showed efficacy in depleting T_{regs} but was discontinued due to difficulties with manufacturing and toxicity [49]. Other groups are working to make a similar drug that is easier to manufacture [282]. Daclizumab is also an anti-CD25 antibody that was previously approved for patients with multiple sclerosis but has been repurposed for cancer treatment. Treatment with Daclizumab can reduce T_{reg} numbers in metastatic breast cancer, but its efficacy as a therapeutic is unclear [214, 272].

Intriguingly, two modifications to an anti-CD25 antibody have improved its efficacy. First, an altered Fc-region of anti-CD25 mouse antibody increases T_{reg} depletion through antibody-dependent cell-mediated cytotoxicity (ADCC). Treatment of mice with this anti-CD25 antibody in combination of anti-PD-1 resulted in tumor regression in 78.6% of mice [16]. Second, a drug designed to deplete T_{regs} but not block critical IL-2 signaling on other T cells demonstrates tumor regression in 70–100% of mice after one dose [243]. These antibodies must be tested for efficacy in humans. Therefore, CD25 remains a valuable target for T_{reg} targeting in the TME.

6.4.2 TGFβ

We have discussed how TGFβ plays important roles in T_{reg} development in the TME. T_{reg} production of TGFβ may also contribute to functional suppression, although this is contended [102, 274]. Importantly, TGFβ also aids tumor cell survival and function [96]. Higher TGFβ in patient tumors is thought to be associated with worse prognosis [103, 218, 260], although others have refuted this [290]. In any event, it may be a desirable target to inhibit in the TME potentially for its role in T_{reg} development. Primary and preclinical studies demonstrated efficacy of various inhibitors of this pathway by affecting T_{reg} function rather than development or number [143, 206]. For example, the TGFβ receptor I (TGFβRI) small molecule inhibitor Galunisertib can block human T_{reg} suppression and induce tumor regression [96]. Recently, a Phase II study of Galunisertib combined with standard-of-care sorafenib blockade in advanced hepatocellular carcinoma patients improved overall patient survival in responders by approximately 10 months [113]. Further studies are required to examine if this treatment affects T_{reg} number or function. TGFβ receptor traps that bind to TGFβ and inhibit its function are also currently being investigated for cancer treatments [209, 210, 319].

6.4.3 CCR4

T_{regs} can express CCR4, which recruits them to the TME. Strategies to stop this recruitment include the approved drug Mogamulizumab [262, 318]. This drug binds CCR4 and targets T_{regs} for ADCC, reducing T_{reg} numbers in patients [175]. Interestingly, CCR4 expression on T_{regs} is not an indicator of response to the drug. Mogamulizumab is approved for relapsed or refractory mycosis fungoides and Sézary disease, two types of rare cutaneous T-cell lymphoma after clinical trials demonstrated a 23–34% ORR [112, 184]. Mogamulizumab is also currently in clinical trials for patients with advanced and/or metastatic solid tumors and diffuse large B cell lymphoma as a combination study with anti-PD-1 (NCT02281409, NCT03309878).

6.4.4 NRP1

NRP1 is an important molecule that regulates T_{reg} function in the TME [51, 190]. Several studies

have examined the effects of anti-NRP1 blocking antibody on the TME, although these studies have focused on targeting NRP1 expressed on tumor cells [54, 174]. Current studies are investigating the consequence of blocking NRP1 in the TME, with a focus on T_{regs}. A Phase Ib clinical trial showed little efficacy with anti-NRP1 therapy, although the antibody did not block the SEMA-4a-NRP1 axis that is important in T_{regs} [202]. A recent study found that an NRP1 antagonist that downregulates surface NRP1 expression decreases T_{reg} function and increases production of IFNγ in human and mouse tumors ex vivo [109]. Further analysis of disruption of the NRP1 pathway on T_{regs} is warranted for future clinical use.

6.4.5 CTLA4

Anti-CTLA4 was originally designed to block negative regulation of activated T cells. However, T_{regs} also express CTLA4, so anti-CTLA4 therapy can target both activated T cells and T_{regs} [283]. The mechanism of action of anti-CTLA4 remains unclear, as various studies describe that anti-CTLA4 works in different ways. Studies show that anti-CTLA4 enhances activation of $CD4^+$ and $CD8^+$ T cells, while other studies show that it affects T_{regs} [292]. Some studies show that anti-CTLA4 binding to T_{regs} induces ADCC to remove T_{regs} [233, 242], while others show that this does not affect T_{reg} numbers in human cancers [235].

Treating mice with established tumors with anti-CTLA4 results in tumor clearance, which could be due to T_{reg} ADCC [130, 163]. Currently, there are two antibodies that block CTLA4. The first approved was the human monoclonal antibody Ipilimumab. In clinical trials, the antibody improved overall survival in metastatic melanoma with an ORR of 10.9% alone [29, 94]. Due to these trials, Ipilimumab was FDA approved for metastatic melanoma treatment in 2011. Ipilimumab has also been approved for use in combination with anti-PD-1 (Nivolumab) for melanoma, renal cell carcinoma, and metastatic colorectal cancer [129]. However, whether Ipilimumab actually targets T_{regs} remains controversial [234]. A new anti-CTLA4 antihuman antibody with a non-fucosylated Fc region, which increases availability for ADCC, has efficacy in monkeys and is currently in clinical trials (NCT03110107) [208].

The other CTLA4 blocking antibody, Tremelimumab, is also a fully humanized monoclonal antibody but is not currently FDA approved. Tremelimumab failed in Phase III clinical trials [46]. However, this antibody is still in clinical development and may be interesting to examine again for future treatment.

6.4.6 TIGIT

T-cell immunoglobulin and ITIM domain (TIGIT) is upregulated after activation and is expressed on T_{regs}, memory T cells, T-follicular helper cells (T_{FH}), NK cells, and exhausted T cells [24, 153]. TIGIT binds its ligands poliovirus receptor (PVR, CD155) and PVRL2 (CD112) expressed on APCs, other T cells, and tumor cells [203, 244]. Another receptor, CD226 (also known as DNAX accessory molecule 1; DNAM-1) also binds CD155 and CD112 to deliver activating signals, while TIGIT delivers a negative signal [74, 287]. This negative signal arrests important T-cell activation pathways such as PI3K, mitogen-activated protein kinase (MAPK), and T-cell receptor signaling, resulting in decreased effector function [147]. TIGIT binding to CD155 also extrinsically pushes DCs toward a tolerogenic state characterized by production of IL-10 and reduced production of a subunit of the pro-inflammatory cytokine IL-12 [147, 306].

T_{regs} that express TIGIT are thymically derived, more activated, and better suppressors in both humans and mice compared to $TIGIT^-$ T_{regs}. [311]. TIGIT also can be expressed on pT_{regs} [217]. $TIGIT^+$ T_{regs} specifically suppress Th1 and Th17 cells through production of IL-10 [10, 70, 106, 149]. TIGIT expression on T_{regs} in the TME also corresponds with high suppressive capability [65]. Further, deletion of *Tigit* on $CD8^+$ T cells has little effect on tumor growth while deletion of *Tigit* on T_{regs} delays tumor growth, indicating an important role of TIGIT on T_{regs}, specifically in tumors [127]. Therefore, targeting TIGIT may prove efficacious to limit T_{reg} suppression. Anti-

TIGIT therapy showed some promising results in preclinical trials and is currently in clinical trials as a combination therapy along with anti-PD-1 and anti-LAG3 [11, 13, 132].

6.4.7 GITR

Glucocorticoid-induced tumor necrosis factor family-related protein (GITR, or TNFRSF18) is expressed on T cells and upregulated upon their activation [179]. GITR binds to its ligand GITRL, which is expressed on APCs. GITR ligation can activate pathways critical for T-cell activation, including MAPK and extracellular-signal-related kinase (ERK), in effector T cells. GITR is a downstream target of FOXP3 and is highly expressed on naïve T_{regs} and further increased after T_{reg} activation [115, 240]. GITR aids in tT_{reg} and pT_{reg} development, but T_{reg} suppressive function is limited by GITR ligation in the periphery [220, 240]. T_{regs} express the highest levels of GITR in tumors, and $GITR^+$ T_{regs} are functionally suppressive and associated with poor prognosis [69, 231]. Therefore, agonism of GITR in the TME may dually aid activation of effector $CD8^+$ and $CD4^+$ $FOXP3^-$ T cells and limit T_{reg} suppressive capacity. Interestingly, use of an agonist anti-GITR in mice demonstrates specific depletion of T_{regs} in the TME rather than loss of suppressive function [40]. Indeed, high expression of GITR on these cells could depletion of these cells specifically [120]. GITR agonism also showed promising results in mice with combination therapy of anti-PD-1 and others [5, 148]. GITR is in clinical trials, with preliminary results showing a requirement for anti-PD-1 and GITR synergy for efficacy [307].

6.4.8 CD39/CD73

Targeting a main function of T_{reg} suppression, such as CD39 and CD73, is an attractive target for immunotherapy. Knockout of *Entpd1* (CD39) or *Nt5e* (CD73) in mice slows or delays tumor growth, indicating importance of targeting these molecules in the tumor [266]. There are currently several agents targeting both molecules that are being investigated along with combination therapies [87, 204]. For example, multiple monoclonal antibodies are being developed to target the active conformation of CD73 or directly block CD73 activation. CD39 is also currently being investigated through pharmacologic inhibitors and monoclonal antibodies [15]. In vitro analysis shows that limiting CD39 decreases T_{reg} suppression. In mice, in vivo CD39 targeting in combination with chemotherapy resulted in 60% of the mice surviving in a 3-methylcholanthrene 205 tumor challenge [204]. Targeting CD39 and CD73 together has shown to mediate an increase in T-cell proliferation from healthy donor PBMC [204]. Therefore, it will be important to examine the efficacy of targeting CD73 and CD39 in ongoing clinical trials.

Strategies targeting molecules such as CD39/CD73, GITR, TIGIT, CTLA4, NRP1, CCR4, TGFβ, and CD25 could limit T_{reg} number, recruitment, and function in the tumor microenvironment to improve antitumor immunity.

6.5 Summary and Commentary on Likely Trends and Future Directions

Study of T_{regs} has been extremely fast-past paced since their discovery [311]. T_{regs} were initially discovered as suppressor cells that express FOXP3. These cells exhibit multiple mechanisms of tumor recruitment, persist in the TME, and function through production of inhibitory cytokines, targeting dendritic cells, cytolysis, and metabolic disruption. Understanding these important characteristics and abilities of T_{regs} is critical for their targeting in the TME to induce antitumor responses.

There are four questions that highlight future trends in this research. (1) Are there other unique targets specific to T_{regs} in the TME that can be identified and therapeutically targeted? We have discussed how unique T_{regs} are and how there are multiple ways to target their recruitment and function in tumors but given their role within the TME more in-depth analysis is ongoing [38, 159,

309]. (2) Can we change therapies to be more efficacious to T_{regs}? We discussed that changing existing therapies such as anti-CD25 to an Fc-optimized anti-CD25 increases therapeutic potential in synergy with anti-PD-1. Therefore, it may be possible to re-examine our current therapies to better target T_{regs}. (3) Can we identify combinations of therapies that can target multiple cellular functions in the TME? We previously discussed that combination therapies such as anti-CTLA4 and anti-PD-1 can target T_{regs} and exhausted $CD8^+$ T cells. Therefore, future studies should examine the combination potential. (4) How do T_{regs} respond to various therapies compared to other cells in the TME? If we can understand and predict how cells within the TME respond to treatment, we may be able to tailor treatments based on either different immunotherapies or different temporal treatments. For example, T_{regs} could first be targeted for depletion, followed by anti-PD-1 to enhance effector T-cell function. In conclusion, further understanding of T_{reg} recruitment, persistence, and function within the TME may provide answers to these questions, which may ultimately lead to more advanced clinical immunotherapies and more favorable clinical outcomes.

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7 The Hematopoietic Microenvironment in Myeloproliferative Neoplasms: The Interplay Between Nature (Stem Cells) and Nurture (the Niche)

Huichun Zhan and Kenneth Kaushansky

Abstract

Hematopoietic stem cells (HSCs) rely on instructive cues from the marrow microenvironment for their maintenance and function. Accumulating evidence indicates that the survival and proliferation of hematopoietic neoplasms are dependent not only on cell-intrinsic, genetic mutations, and other molecular alterations present within neoplastic stem cells, but also on the ability of the surrounding microenvironmental cells to nurture and promote the malignancy. It is anticipated that a better understanding of the molecular and cellular events responsible for these microenvironmental features of neoplastic hematopoiesis will lead to improved treatment for patients. This review will focus on the myeloproliferative neoplasms (MPNs), polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), in which an acquired signaling kinase mutation (JAK2V617F) plays a central, pathogenetic role in 50–100% of patients with these disorders. Evidence is presented that the development of an MPN requires both an abnormal, mutation-bearing (i.e., neoplastic) HSC and an abnormal, mutation-bearing microenvironment.

Keywords

Myeloproliferative neoplasm · Polycythemia vera · Essential thrombocythemia · Primary myelofibrosis · JAK2V617F · Hematopoiesis · Hematopoietic stem cell · Microenvironment · Vascular niche · Endothelial cell · Megakaryocyte · Clonal expansion · Radioprotection · Thrombopoietin · MPL

7.1 The Hematopoietic Microenvironment

The hematopoietic microenvironment or "stem cell niche" is defined as the site at which HSCs reside and are nurtured, receiving the humoral

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and cell-surface signals that lead to their survival, replication, and/or differentiation into all the mature cells of the blood. Technical breakthroughs in imaging HSCs in the marrow cavity, coupled with a series of elegant functional studies in murine models, have identified a number of HSC niche cells that provide the secreted factors and cell-surface molecules essential for HSC maintenance and function. The cellular components of the marrow HSC niche are derived from both hematopoietic and non-hematopoietic cells. Examples of non-hematopoietic niche cells are perivascular stromal cells and endothelial cells (ECs), and hematopoietic niche cells include large mature megakaryocytes (MKs). All niche cells are located adjacent to sinusoidal blood vessels throughout the marrow, allowing their close juxtaposition or direct contact with HSCs. At these locations, niche cells produce cytokines that affect HSCs, including stem cell factor (SCF), chemokine (C-X-C motif) ligand 12 (CXCL12), thrombopoietin (TPO), each a non-redundant cytokine important for maintaining HSC numbers and enforcing HSC quiescence [1–15]. The vast majority of HSCs reside immediately adjacent to sinusoid blood vessels and close to ECs, perivascular stromal cells, and/or MKs, suggesting the existence of a perivascular niche [6, 7, 13–15]. Other marrow cells, including osteoblasts [16, 17], macrophages [18, 19], and sympathetic nerve fibers [20, 21], indirectly influence HSC function by affecting the HSC supportive capacity of the essential niche cells, through complex crosstalk mechanisms [2, 3, 22]. It has become increasingly evident that the HSC niche consists of a complex assembly of multiple components existing in close proximity to one another, and that the interaction between these cells contributes to the resilience and function of HSCs.

Accumulating evidence also indicates that the hematopoietic niche is altered in patients with hematological neoplasms and that the "neoplastic niche" is an important contributor to the development of hematologic malignancies [23–28]. At least part of mechanism by which a malignancy-promoting niche arises is through the effect of neoplastic cells on adjacent niche cells, although the responsible molecular signals are not well understood. And because the genetic mutations that drive hematological neoplasms can affect both marrow cells and ECs [29–38], a fraction of the niche cells can carry the driver mutation present in the neoplastic stem cell. Once formed by these mechanisms, the "malignant niche" can act to favor progression of the malignancy, both by impairing normal hematopoiesis and by enhancing malignant stem cell expansion [26, 39–43]. Hence, interventions to arrest neoplasia should not be restricted solely to the neoplastic stem cell itself; rather, if neoplasia is fostered by a coordinated corruption of both niche and stem cells, targeting the niche can theoretically provide therapeutic benefit.

7.2 The Marrow Microenvironment in Myeloproliferative Neoplasms

The classic Ph-negative MPNs, which include PV, ET, and PMF, are stem cell disorders characterized by hematopoietic stem/progenitor cell (HSPC) expansion and overproduction of mature blood cells. Patients with MPNs suffer from many debilitating complications including both venous and arterial thrombosis, and, in some, especially if treated with genotoxic agents to control blood cell counts, evolution to acute leukemia. An acquired signaling kinase mutation JAK2V617F is present in virtually all patients with PV, and in ~50% of patients with ET and PMF. The JAK2V617F mutation has a central role in the pathogenesis of MPN, but our understanding of the stem cell expansion that characterizes MPNs remains incomplete, limiting the effectiveness of current treatments. While a variety of therapies can control the abnormal expansion of the progeny of the malignant HSC, the only curative therapy is stem cell transplantation (SCT), a procedure that is toxic and often inadequate, due to relapse of the malignant clone (Fig. 7.1).

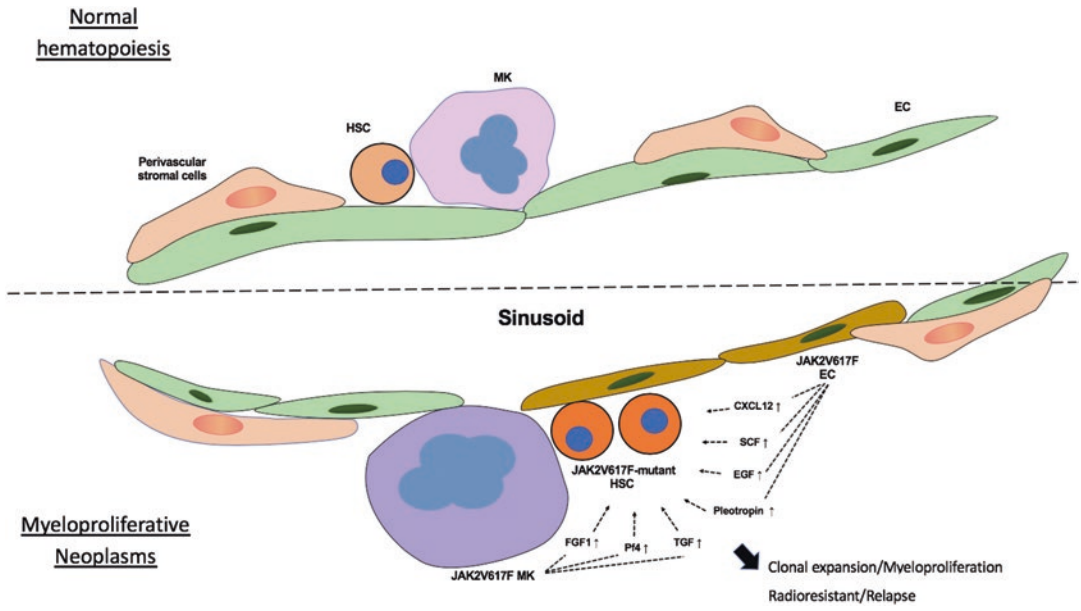

Fig. 7.1 Hematopoietic microenvironment in myeloproliferative neoplasms

Although the etiology of dysregulated hematopoiesis has been mainly attributed to the molecular alterations within the corresponding stem or progenitor cells, recent studies suggest that a diseased hematopoietic microenvironment is a critical element in the development of MPNs [23, 28, 39, 40]. The marrow of patients with MPNs is characterized by increased angiogenesis and MK hyperplasia when compared to normal marrow architecture [44–48]. In addition to mutant blood cells (including MKs), JAK2V617F is also present in isolated liver, spleen, and marrow ECs from patients with MPNs [29–31]. Using both in vitro and in vivo methods, several investigators have shown that the JAK2V617F mutation contributes to these neoplasms by altering the ECs and MKs that participate in the hematopoietic niche, enhancing their production of growth-promoting cytokines and chemokines, ultimately resulting in their differential support of mutant stem cells over their normal counterparts, and imparting relative radiation resistance to neoplastic stem cells. The evidence supporting these conclusions regarding the biological properties of the vascular niche will next be discussed.

7.2.1 JAK2V617F-Bearing Vascular ECs Promote JAK2V617F-Mutant HSC Expansion over Normal HSCs, Both In vitro and In Vivo

The early view that a malignancy arising from a single mutant stem cell can expand and overtake all the (then) existing normal hematopoietic elements because mutant cells "grow faster" than normal cells is far too simplistic. Rather, it is far more likely that mutation-bearing cells have developed multiple molecular and cellular mechanisms, both cell intrinsic and cell extrinsic, which make them more competitive than their normal hematopoietic and microenvironmental counterparts. To explore such microenvironmental influences, studies of the effects of the JAK2V617F mutation on vascular niche function were conducted employing mice that bear a Cre-inducible human JAK2V617F transgene (termed Flip-Flop (FF1) [49]) crossed with a Tie2-Cre transgenic mouse [50] to express JAK2V617F specifically in all hematopoietic cells (including HSCs) and ECs ($Tie2^{+}FF1^{+}$) [51, 52]. As expected, these mice developed a robust MPN

characterized by neutrophilia, thrombocytosis, splenomegaly, and hematopoietic stem and progenitor cell (HSPC) expansion within 2 months of birth.

To assess whether mutant ECs differentially support mutant HSCs, the growth of normal and malignant hematopoietic cells on normal or mutant ECs was assessed by co-culture of normal or malignant Lineage^{neg}cKit^{+} (Lin^{-}cKit^{+}) marrow HSPCs on monolayers of normal or JAK2V617F-mutant ECs. No differences were found between normal and JAK2V617F-mutant HSPC proliferation when cultured on normal ECs. In contrast, JAK2V617F HSPCs displayed a relative growth advantage over normal HSPCs when cultured on JAK2V617F-mutant ECs [53]. These results suggest that the malignant niche differentially supports the proliferation of malignant stem cells over that of normal cells.

Next, to assess whether the enhanced proliferation of JAK2V617F HSPCs on JAK2V617F ECs is also seen in vivo, competitive marrow transplantation experiments were performed in which donor marrow cells from Tie2^{+}FF1^{+} mice were injected intravenously together with wild-type "competitor" marrow cells into lethally irradiated Tie2^{+}FF1^{+} mice (with mutant ECs) or control recipient mice (with only normal ECs). During a 4-month follow-up, Tie2^{+}FF1^{+} recipients displayed a greater level of peripheral blood JAK2V617F-mutant cell expansion than the control recipient mice. By 18 weeks following transplantation, Tie2^{+}FF1^{+} recipients developed a profound MPN phenotype with neutrophilia, thrombocytosis, and moderate splenomegaly. Quantitative evaluation of the marrow HSC compartment revealed significant increases in JAK2V617F CD150^{+}CD48^{-} cells, a population of cells highly enriched in HSCs (~20% display long-term repopulating capacity), in Tie2^{+}FF1^{+} recipients compared with control recipients. In contrast, there was no significant difference in WT HSC cell numbers between Tie2^{+}FF1^{+} recipients and controls. In addition, wild-type donor-derived hematopoietic progenitors were present in 60% of control recipients while none was detected in Tie2/FF1 recipients. Therefore, the JAK2V617F-mutant vascular niche promoted the expansion of JAK2V617F HSCs at the expense of normal hematopoiesis.

Of considerable interest, recipients (with the normal vascular niche) of an equal mixture of Tie2^{+}FF1^{+} and normal cells had mostly normal blood cell counts, and there were no significant differences between the numbers of normal and mutant HSCs in the marrow of the transplanted mice [52]. While this appears to be in conflict with other reports that the JAK2V617F-positive MPN phenotype is transplantable, and usually develops as early as 4 weeks following transplantation, in every such study only JAK2V617F-positive marrow cells were transplanted into WT recipients [51, 54–57]. One possible explanation is that the mutant stem cells have little selective advantage over wild-type cells when transplanted into mice that bear a normal niche, or that the co-transplanted normal marrow cells can "control" expansion of the transplanted mutant-bearing cells, which is also consistent with the clinical observation that in some patients with MPNs, there is a "peaceful coexistence" of the mutant clone and the wild-type clone, with no change in the mutant/wild-type cell ratio over prolonged follow-up [55, 57–62]. But whichever is the actual mechanism, these studies indicate that crosstalk between mutant HSCs and their microenvironment is required to provide the 'selective pressure' for the mutant cells to outcompete normal cells in the development of a MPN.

7.2.2 The JAK2V617F-Mutant ECs Protect JAK2V617F HSCs from Radiation Injury Both In Vitro and In Vivo

Disease relapse is seen in up to 40% of patients with MPNs following allogeneic SCT (especially after reduced intensity conditioning), which contribute to most of the treatment-related morbidity and mortality associated with the only curative treatment for patients with MPNs [63–66]. To investigate the effects of the JAK2V617F-mutant vascular niche on the response of MPN HSCs to radiation, which is frequently used in clinical transplantation, and serves as a reasonable exper-

imental surrogate for many forms of chemotherapy, wild-type marrow cells were transplanted directly into irradiated Tie2^{+}FF1^{+} mice or age-matched normal recipients, using a radiation dose that is 100% myeloablative to normal mice. During a 3-month follow-up, while all normal recipients displayed full donor engraftment, ~ 60% of Tie2^{+}FF1^{+} recipient mice displayed recovery of JAK2V617F-mutant hematopoiesis (mixed donor/recipient chimerism) 10 weeks after irradiation and transplantation. In contrast to the Tie2^{+}FF1^{+} recipients with full wild-type donor engraftment, the mice with mixed chimerism developed neutrophilia, thrombocytosis, splenomegaly, and mutant HSC expansion, similar to what we observed in the primary JAK2V617F-mutant Tie2^{+}FF1^{+} mice [67], indicating that the usually stem-cell lethal dose of irradiation was not uniformly lethal to the HSCs in Tie2^{+}FF1^{+} mice. In essence, the presence of JAK2V617F mutation generated a model of disease refractory to what should have been curative transplantation.

As noted above, Tie2-Cre mice express the recombinase in both ECs and HSCs. To investigate whether the observed radioprotection phenotype was due to the presence of JAK2V617F in Tie2^{+}FF1^{+} HSCs, a chimeric murine model with JAK2V617F-mutant HSCs and a wild-type vascular niche was generated by transplanting Tie2^{+}FF1^{+} marrow cells into wild-type recipients. The transplantation of wild-type marrow cells into wild-type recipients served as a control. Following hematopoietic recovery and full donor cell engraftment, each set of mice were again irradiated with 300 cGy to test the radiation sensitivity of the transplanted wild-type HSCs. In the presence of a wild-type vascular niche, marrow Lin^{-} cell apoptosis was significantly higher in the JAK2V617F-mutant cells compared to normal cells. Thus, radioresistance of mutant HSCs could not account for JAK2V617F-mutant disease relapse following marrow transplantation. To test whether the mutant-bearing niche was responsible for relapse following transplantation, mice with normal HSCs and JAK2V617F ECs were generated by transplanting normal marrow cells into lethally irradiated Tie2^{+}FF1^{+} recipients. The transplantation of normal HSCs into normal recipients again served as a control experiment. Six to ten weeks following transplantation, each set of mice was irradiated with 300 cGy to test the radiation sensitivity of the transplanted HSCs. In this case, normal Lin^{-} cell apoptosis was significantly decreased in mice with JAK2V617F-mutant ECs compared to mice with normal ECs. Taken together, these data indicate that a JAK2V617F-bearing vascular niche contributes directly to HSC radioprotection, which could be responsible for the high incidence of disease relapse in patients undergoing allogeneic stem cell transplantation for MPNs [67].

7.2.3 The JAK2V617F Mutation Alters Vascular Niche Function to Contribute to HSC Expansion and HSC Radioprotection

The mechanism(s) by which the JAK2V617F mutation alters ECs to promote neoplastic hematopoiesis was/were next explored, and revealed several changes. First, JAK2V617F ECs display significantly increased cellular proliferation, cell migration, angiogenesis, and decreased apoptosis (after irradiation) compared with normal ECs in vitro [53], indicating that the mutant kinase could act to expand the vascular niche in JAK2V617F-positive Tie2^{+}FF1^{+} mice, findings that also characterize the marrow vascular density of patients with MPNs [45–47]. Next, the EC expression levels of CXCL12 and SCF, two essential niche factors important for HSC maintenance [1, 3], were increased in freshly isolated marrow ECs from Tie2^{+}FF1^{+} mice compared to those from control ECs. Moreover, the proportion of HSCs expressing the CXCL12 receptor, CXCR4, or the SCF receptor, c-Kit, was significantly increased in JAK2V617F-mutant marrow cells compared with normal marrow cells. Therefore, increased CXCL12 and SCF levels in the JAK2V617F-mutant ECs could contribute to the clonal expansion of JAK2V617F HSCs, via the upregulated CXCR4 and c-Kit receptors in mutant HSCs, compared to wild-type HSCs.

To determine if enhanced cytokine and/or cytokine receptors might also be responsible, at least in part, for the relative radioresistance seen in Tie2^{+}FF1^{+} mice, a number of HSC active cytokines were assessed; the expression levels of CXCL12, epidermal growth factor [68], and pleiotrophin [69] were upregulated in irradiated JAK2V617F ECs compared with that seen in normal ECs. These results suggest that the JAK2V617F-mutant vascular niche contributes to JAK2V617F HSC radioprotection by its expression of HSC active chemokines and cytokines.

7.2.4 Megakaryocytes (MKs) Are an Important Component of the Perivascular Stem Cell Niche in MPNs

MKs are rare, polyploid hematopoietic cells that give rise to blood platelets. MKs are often located adjacent to marrow sinusoids, a critical juxtaposition required for the cells to issue platelets directly into the sinusoidal vascular lumen [70]. A number of investigators have found that MKs are an important component of the hematopoietic vascular niche [6, 7, 10, 12]. In contrast to non-hematopoietic niche cells (e.g., ECs, perivascular stromal cells), niche MKs provide direct feedback to their precursor HSCs, many of which are located adjacent to MKs in vivo [6, 7], and therefore play important roles in malignant HSC clonal expansion during neoplastic hematopoiesis.

MK hyperplasia is a hallmark feature of all three chronic Ph-negative MPNs. To gauge the effects of JAK2V617F-bearing MKs on MPN development, FF1 mice were crossed with Pf4-Cre mice (which bear a Cre recombinase driven by the MK-specific platelet factor 4 promoter) [71] to express JAK2V617F exclusively in the MK lineage (Pf4^{+}FF1^{+}). As expected, the Pf4^{+}FF1^{+} mice developed modest thrombocytosis, splenomegaly, and greatly increased marrow megakaryopoiesis. Somewhat surprisingly, the mice also developed significant increases in HSC numbers [72]. In addition, there were dilated marrow sinusoids in the Pf4^{+}FF1^{+} mice, and MKs were preferentially located near sinusoid vessels. Quantitative analysis revealed increased marrow sinusoid vascular density in Pf4^{+}FF1^{+} mice marrow compared with controls, resembling the marrow of patients with MPNs [44–48]. Further work demonstrated that HSCs from the Pf4^{+}FF1^{+} mice (with JAK2V617F-mutant MK niche) display higher levels of TPO receptor MPL and are more quiescent than those from normal mice (with wild-type MK niche). In addition, using a competitive repopulation assay, marrow cells from the Pf4^{+}FF1^{+} mice were found to display greater engraftment capacity than cells from normal mice [73]. Therefore, the quantitative or qualitative changes (or both) of JAK2V617F-mutant MKs drove HSC expansion in JAK2V617F-positive MPNs.

7.2.5 TPO/MPL Signaling Is Important for HSC Expansion in the Vascular Niche of MPNs

TPO acting through its receptor, the proto-oncogene c-MPL, is a key regulator of megakaryopoiesis and HSC activity [74–80]. MPL is also expressed on several types of ECs, and TPO can stimulate EC growth and angiogenesis [81–83]. Several studies have shown that MPL is essential for the development of an increased neoplastic stem cell pool in MPNs [73, 84–89]. Specifically, in the Tie2^{+}FF1^{+} mice where JAK2V617F is expressed in all hematopoietic cells and ECs, reducing MPL expression attenuated MPN severity with reduced platelet count and HSC numbers, suggesting a gene-dosage effect of MPL levels on the disease process [84]. In the Pf4^{+}FF1^{+} mice where JAK2V617F is expressed exclusively in the MK lineage, ablation of TPO or MPL abolished the phenotype of thrombocytosis or HSC expansion despite the presence of JAK2V617F-bearing MKs [73]. Taken together, these studies demonstrate that TPO/MPL signaling is important for MPN HSC expansion in both Tie2^{+}FF1^{+} and Pf4^{+}FF1^{+} mice, where major components of the hematopoietic vascular niche (ECs and MKs in Tie2^{+}FF1^{+}, and

MKs in $Pf4^{+}FF1^{+}$, respectively) bear the JAK2V617F mutation.

To further determine the role(s) of TPO/MPL signaling in JAK2V617F-bearing vascular niche function, EC MPL mRNA expression was found to be increased in JAK2V617F-bearing lung and marrow ECs, suggesting that TPO/MPL signaling could affect both the general vasculature (e.g., the lung) and the hematopoietic vascular niche (e.g., the marrow) in JAK2V617F-positive MPNs [52, 53]. The effects of TPO on EC function in vitro were also assessed; the cytokine significantly stimulated EC cell migration in a dose-dependent fashion. In addition, compared with untreated ECs, both the EC junction molecules ZO-1[90] (zonula occludens-1, or Tight junction protein-1) and PECAM1 [90] (platelet endothelial cell adhesion molecule, or CD31) were upregulated in TPO-treated ECs, suggesting that TPO/MPL signaling may regulate vascular integrity, important for both hemostasis and tissue stem cell function [91].

Using an in vitro competitive growth assay where both normal $Lin^{-}cKit^{+}$ HSPCs (CD45.1) and JAK2V617F-mutant HSPCs (CD45.2) were cultured together in the presence of conditioned medium collected from either normal or MPL knockout lung ECs, the EC MPL receptor was found to be important for the maintenance/expansion of the JAK2V617F cells over the wild-type cells in vitro [53]. Furthermore, CXCL12 levels are reduced in $MPL^{-/-}$ or $TPO^{-/-}$ marrow ECs compared with normal ECs, in a dose-dependent fashion [52, 73]. Since CXCL12 is important in directing MK migration toward the vascular niche and promoting MK maturation and platelet release [92, 93], the decreased CXCL12 expression in $TPO^{-/-}$ and $MPL^{-/-}$ marrow ECs could impair the interactions between MKs and ECs in the vascular niche. In support of this conclusion, MKs are less likely to be in direct contact with sinusoidal vessels in the $TPO^{-/-}$ and $MPL^{-/-}$ mice compared to normal mice, suggesting that TPO/MPL signaling can affect MK–EC interactions in the vascular niche [73].

Taken together, these results suggest that both secreted factors and cell–cell interactions in the vascular niche contribute to the JAK2V617F HSC expansion in MPNs, and that TPO/MPL signaling is critical for this vascular niche function.

7.2.6 Crosstalk in the Hematopoietic Microenvironment of MPNs

Considering that most HSCs reside close to a marrow sinusoid and MKs are often located adjacent to marrow sinusoids, the interactions between MKs and the vascular ECs in the hematopoietic vascular niche are positioned to play an important role in modulating stem cell function, and, by extrapolation, might be deregulated in disease states. In $Pf4^{+}FF1^{+}$ mice, where JAK2V617F is expressed exclusively in the MK lineage, MK hyperplasia was accompanied by dilated marrow sinusoids and increased sinusoid vascular density in vivo, and JAK2V617F-mutant MKs stimulated EC tube formation (a measure of in vitro angiogenesis) and EC migration in vitro [72]. In $Tie2^{+}FF1^{+}$ mice, where JAK2V617F is expressed in all hematopoietic cells and ECs, mutant ECs directly stimulate mutant MK expansion [52]. Taken together, these data indicate that modulating one vascular niche component can, in turn, impact the function and HSC supportive capacity of the other cell types within the niche, and that the JAK2V617F mutation alters the MK–endothelial "crosstalk" to promote neoplastic hematopoiesis in a murine model of MPN.

Such "crosstalk" also exists between the malignant HSC and its surrounding niche cells. Not only can a diseased hematopoietic niche drive neoplastic hematopoiesis [23–28], but malignant HSCs can also alter the niche to suppress normal hematopoiesis and advance disease [26, 39–43]. These bi-directional interactions indicate a functional symbiosis between malignant HSCs and their microenvironment. Therefore, a better understanding of how the niche is modified in different hematological disease states (including MPNs) could lead to the ability to protect and/or treat the niche, and, hence, provide a new therapeutic approach to hematological malignancies.

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Novel Concepts: Langerhans Cells in the Tumour Microenvironment

8

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Abstract

Langerhans cells (LCs) are immune cells that reside in the stratified epithelium of the skin and mucosal membranes. They play a range of roles in the skin, including antigen presentation and maintenance of peripheral tolerance. Reports of LC numbers have been variable in different cancer types, with the majority of studies indicating a reduction in their number. Changes in the cytokine profile and other secreted molecules, downregulation of surface molecules on cells and hypoxia all contribute to the regulation of LCs in the tumour microenvironment. Functionally, LCs have been reported to regulate immunity and carcinogenesis in different cancer types. An improved understanding of the function and biology of LCs in tumours is essential knowledge that underpins the development of new cancer immunotherapies.

Keywords

Langerhans cell · Tumour microenvironment · Cancer · Cytokines · Surface molecules · Hypoxia · Antigen presentation · Carcinogenesis · Immunity · Immune cells · Angiogenesis · Lymphangiogenesis · Immunotherapy · Human · Mice

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8.1 Langerhans Cells

Langerhans cells (LCs) are unique antigen-presenting cells that reside in the stratified squamous epidermis of cutaneous and mucosal epithelium. LCs were discovered in 1868 by Paul Langerhans, who initially believed that these cells were neurons due to their dendritic morphology [39]. Nearly 100 years later, the antigen-presenting function of these cells was determined.

Langerhans cells can be identified based on the expression of the C-type lectin receptor, langerin (CD207) [81], along with other less-specific markers such as CD1a in humans [21, 66] and major histocompatibility complex (MHC) class II [37]. Langerin is involved in antigen capture and induces the formation of Birbeck granules [8]. Birbeck granules are unique rod or tennis racket-shaped endocytic vesicles that are considered the hallmark of LCs. LCs express the epithelial cell adhesion molecule (EpCAM) in mice [4, 55], which enables LC motility and migration to lymph nodes and modulates responses to epicuta-

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neously applied protein antigen in a mouse model.

8.2 Ontogeny of Langerhans Cells

Cutaneous LCs originate from embryonic macrophages and foetal liver monocytes [29]. LC precursors seed the epidermis during murine embryonic development, but are not able to enter the adult epidermis in the steady state. These precursors differentiate into LCs in the epidermis immediately [29]. These newly differentiated LCs rapidly proliferate to form a radio-resistant cellular network that is capable of self-renewal throughout life [45]. During inflammation, the skin is permissive for the entry of circulating precursors to the epidermis that differentiate locally to LCs. Monocytes are the first bone marrow precursors that differentiate into LCs during inflammation [25, 54]. Unlike the skin, mucosal epithelium has better accessibility to circulating precursors in the steady state. Mucosal LCs arise from adult bone marrow precursors, unlike the embryonic precursor origin of cutaneous LCs [12].

Despite the differing ontogeny in the steady state, skin and mucosal LCs share similarities in anatomic location, phenotype, transcriptomic signature and function [30]. Mouse mucosal LCs can be controlled by the microbiota via regulation of epithelial differentiation signals, which may contribute to their generally less-dendritic appearance [30].

8.3 Langerhans Cell Function

The role of LCs in the skin was initially believed to be primarily one of antigen presentation. Through a number of studies using mice that are selectively depletable of skin antigen presenting cell subsets, langerin-positive dermal dendritic cells (dDCs) have now been identified as the primary antigen-presenting cells in the skin. LCs are not considered necessary for the initiation of an adaptive T-cell response to skin-expressed antigen.

In the steady state, LCs survey the epidermis and migrate to the lymph nodes where they present self or commensal microbial antigens to T cells to induce tolerance and maintain tissue homeostasis (reviewed in [19]). LCs play an important role in maintaining the population of memory T cells in the epidermis [73]. Mucosal LCs express the lipopolysaccharide receptor CD14 and have a high-affinity receptor for IgE [2]. LCs have regulatory roles and may either promote or suppress disease progression depending on the condition (reviewed in [61]).

8.4 LCs in the Tumour Microenvironment

The tumour microenvironment includes neoplastic and non-neoplastic cells along with the extracellular matrix, and cytokines, chemokines and growth factors that may be derived from those cells [68]. LCs have been identified in the tumour microenvironment in a range of cancer types, particularly skin cancers [75]. There is a body of evidence showing the presence of LCs in head and neck [35], gastric [80] and cervical cancers [43], and papillary thyroid carcinoma [70]. Some studies have also identified LCs in breast [79] and prostate cancers [7]. In this chapter, we explore the regulation and function of LCs in cancers, summarised in Fig. 8.1.

8.5 Regulation of LC Numbers and Location in the Tumour Microenvironment

Generally, there is a reduced number of LCs observed in cancer, particularly skin cancer. LC numbers in squamous cell carcinoma (SCC) and

Fig. 8.1 (continued) E-cadherin and BMP7 can affect the retention of LCs in the TME. Hypoxic conditions cause downregulation of langerin and CD1a on LCs, and they become more rounded and less functional. The role of LCs in the TME includes detoxification of toxins in the skin that can result in carcinogenesis via HRAS mutation. LCs regulate immunity in the TME by recruiting immune cells and also mediating anti-tumour T-cell responses. LIGHT is upregulated in LCs during inflammation, promoting lymphangiogenesis in skin. It is currently unclear if this also occurs in tumours

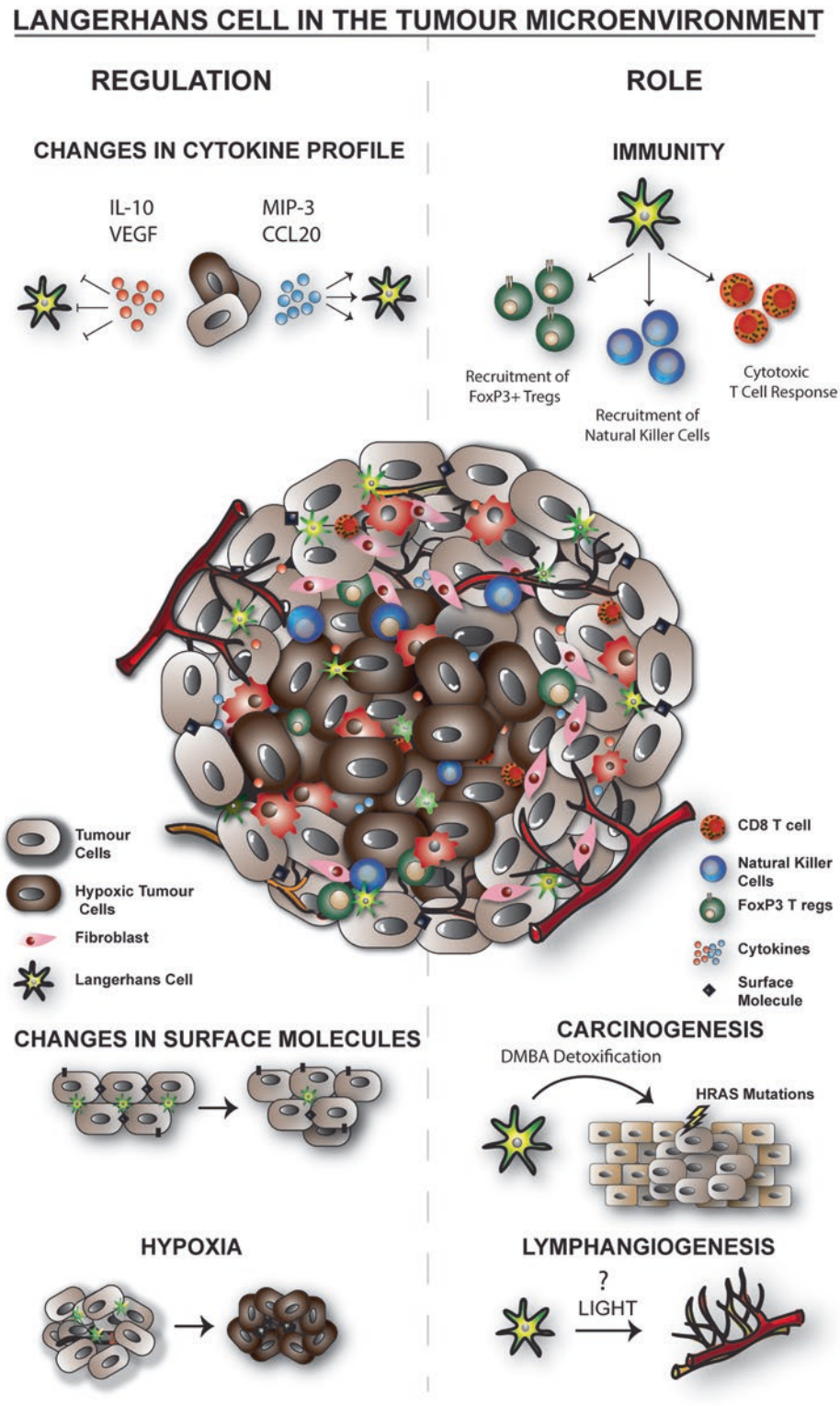

Fig. 8.1 Langerhans cell regulation and role in the tumour microenvironment. Regulation of Langerhans cells (LCs) in the tumour microenvironment (TME) occurs due to several factors. Production of cytokines and other soluble molecules by the tumour cells can either attract or inhibit the migration of LCs into the TME. Changes to surface molecules such as

basal cell carcinoma (BCC) are significantly decreased when compared to normal skin [75]. The LC count is reported to be higher in benign compared with malignant skin tumours, suggesting that carcinogenesis is associated with a reduction in the number of LCs [75]. Similarly, increased LC numbers are associated with less aggressive forms of BCC [69]. Increased numbers of LCs in the normal epidermis at the margin of less aggressive tumours could be indicative of greater immunological resistance, limiting the aggressiveness of the neoplasm [69].

LC numbers in the lesion may have utility as a prognostic marker; however, this may only be the case in certain cancer types. Increased LC numbers in the lesion are correlated with better prognosis in gastric carcinoma [80], thyroid carcinoma, ductal breast cancer [38] and lung carcinoma [14]. Higher LC numbers are associated with increased survival of the patients, particularly with stage III gastric cancer [80]. CD1a and S100 have been used to identify LCs in some studies; however, CD207 (langerin) is considered to be the most robust marker for the identification of LCs [6]. For laryngeal SCC, using S100 as a marker for LCs, numbers were not considered a reliable marker of prognosis in clinical practice [33]. Similarly, using CD1a as a marker for LCs, numbers were increased when compared to normal tissue, but there was no association with the prognosis for laryngeal cancer [20]. However, these differences in results could also arise from the use of antibodies against markers other than langerin for the identification of LCs.

The changes in LC number in the tumour are a consequence of cytokine and chemokine regulation in the microenvironment. Macrophage inflammatory protein-3/C-C motif chemokine ligand 20 produced by tumour cells is selectively chemotactic to LCs [56]. Interleukin (IL)-10, transforming growth factor β (TGFβ) [32], IL-1β [17] and vascular endothelial growth factor (VEGF) [76] may also regulate the recruitment and migration in the tumour microenvironment. IL-10 is a known inhibitor of LC migration [18] that is increased in tumour cells [85]. IL-1β is a critical mediator of chronic inflammation and has been implicated in tumour pathogenesis [3]. When oral SCC cells are treated with IL-1β, they proliferate and their protumorigenic cytokine network is stimulated [40]. Elevated levels of IL-1β, tumour necrosis factor-α and prostaglandin E_2 in chronic periodontitis stimulate dendritic cell (DC) maturation and migration. Environmental factors, such as smoking, could lead to changes in the cytokine profile, which can contribute to a reduction in LC levels or change the phenotype of LCs. There is an increase in LC density in the lateral border of the tongue and lip of patients with oral SCC with a history of smoking [16]. Cytokine profiles compared between tobacco users and non-tobacco users change significantly, with increased VEGF [74]. Further analysis is needed to study the direct relationships between these cytokines and LCs. However, the varied cytokine profiles in different cancers or even in the same cancer present a formidable challenge for the development of immunomodulatory drugs.

CD10 is a zinc dependent metalloproteinase that can be detected in peritumoural fibroblast-like stromal cells within the invasive area of various cancers. CD10 expression is low in precancerous lesions and normal skin tissues [78]. Immunohistochemical analysis indicates increased induction of CD10 in stromal cells in epidermal tumours, especially in SCC, which could be contributing to the tumorigenesis and reduction in LCs [78]. There is a positive correlation between Ki67 levels with LCs and stromal CD10-positive cells but a negative correlation with CD1a-positive cells in the tumour [78], suggesting a potential suppressive role for the CD10-positive cells in the tumour microenvironment on the number of LCs. However, further in vitro analyses are required to confirm the exact relationship.

A pronounced reduction in LCs has been observed in low-grade cervical intraepithelial neoplasia (CIN) [15, 27]. However, LC numbers are increased in cervical cancer, when compared to precancerous CIN lesions [11]. The interaction between LCs and keratinocytes (KCs) is mediated by E-cadherin. Immature LCs adhere to KCs via E-cadherin, which is constitutively expressed by KCs in the basal and suprabasal layers. This interaction is important for both LC localisation and retention. The detachment of LCs from the

surrounding KCs is an essential step in the initiation of their migration from the epidermis. Reduced E-cadherin expression in CIN reduces the retention of LCs, which is proposed to contribute to immune evasion in human papillomavirus (HPV) pre-cancer [44]. Similarly, E-cadherin levels are reduced in oral [34] and cutaneous SCC samples [86], compared with normal skin. More poorly differentiated tumours express less than 40% E-cadherin, which could be leading to the reduced LC levels [83]. There is a loss of cell-to-cell adhesion and gain of cell-to-matrix adhesion when E-cadherin expression is lost, promoting the transformation of pre-malignant to malignant cells. However, in a recent study using a CD11c-specific E-cadherin knockout, it was shown that an absence of E-cadherin-mediated cell adhesion on LCs did not affect their stability in epidermal sheets [10]. The LCs did exhibit altered morphology with fewer dendrites and a more rounded body. However, the lack of E-cadherin on LCs did not affect their proliferation or retention in the skin [10].

HPV type 16 E7 is a cell cycle deregulating protein that contributes to the oncogenesis of HPV16-related cervical cancer [63]. The K14 E7 transgenic mouse expresses HPV16 E7 in the epidermal KCs, which was associated with increased numbers of skin-resident LCs in the skin [1]. The increased LC number was attributed to the chronic inflammatory environment of the skin in this transgenic mouse model. LCs were atypically activated and functionally impaired in this model; however, they were functionally active when extracted from the skin and matured in vitro [1].

Changes to the cell polarity and adhesive properties of cells enable malignant conversion of cells. LCs could contribute to epithelial–mesenchymal transition (EMT) in cutaneous cancers. Many of the cytokines involved in mediating LC migration have also been associated with EMT processes [28], such as TGFβ [26]. BMP7 is important for the maintenance of LCs in the epidermis. Immunohistological analysis of LC niches in early prenatal epidermis and adult basal (KCs) show high levels of BMP7 expression. Mice deficient of BMP7 have diminished levels of LCs, and any remaining LCs are less dendritic [84]. In melanoma, BMP7 can induce mesenchymal–epithelial transition (MET), which can inhibit metastasis in vitro [50].

A common feature of most tumours is the presence of regions that have low levels of oxygen. In increasingly proliferating and expanding tumour tissue, the oxygen demand surpasses the oxygen supply, which creates hypoxic regions [72]. The severity of hypoxia varies in different cancers [49]. Increased hypoxia is associated with poorer prognosis of patients [67].

The hypoxic conditions of cancers could have an effect on the regulation of LCs in tumours. In response to hypoxic conditions, cells rapidly upregulate genes under the control of the transcription factor hypoxia-inducible factor-1α (HIF-1α). HIF-1α can downregulate LC functions in vivo [52]. The phenotypic features and surface expression markers of LC-like cells generated from human monocytes cultured in hypoxic and normoxic conditions have been assessed [60]. The expression of langerin and the activation markers CD86 and CD83 were significantly decreased on cells from the majority of the donors, while CD1a and E-cadherin were reduced in cells from some donors. These results suggest that there could be downregulation of cell surface markers on LCs, creating an apparent loss of the cells rather than actual depletion of LCs from the tumour [60].

Hypoxic conditions also impaired the LCs' ability to stimulate T-cell responses. More LCs in hypoxic regions were shown to be viable, as indicated by the lower percentage of early and late apoptosis, when compared to LCs grown in normoxic cultures [60]. The impairment of LC function in hypoxia could contribute to tumour cell evasion of the immune response.

8.6 LCs Regulate Immunity in the Cancer Microenvironment

Langerhans cells are associated with infiltration of immune cells into the tumour. An increase in FoxP3+ Tregs as a percentage of total CD4+ T cells was observed in melanoma patient samples [71]. To test if there was a direct association between

increased FoxP3+ Tregs in melanoma and LCs, the authors assessed co-localisation of the two cell populations [71]. However, LCs were not co-located with infiltrating Tregs, which led the authors to propose that LCs have a tolerogenic role in melanomas but not by directly effecting Tregs [71]. Melanoma-infiltrating LCs expressed less CD40 and are more likely to express the inhibitory programmed cell death-ligand 1 (PD-L1) marker [71]. Further in vitro studies may help to shed light on the increased Treg accumulation and LCs in melanoma. An analysis of cell infiltrates in radiation therapy demonstrated that a favourable prognosis was associated with LC infiltration [51]. T-cell infiltration into the tumour was associated with the presence of LCs [51], suggesting that they may induce a T-cell-mediated anti-tumour response that can improve the local response in radiation therapy.

Immature LCs express the programmed cell death protein 1 (PD-1) receptor, which helps to maintain tolerance in the skin [59]. As LCs mature, there is a decline in PD-1 receptor expression [59]. Blockade of PD-1 upregulates T-cell responses that can help fight off tumour cells [59]. However, the cells that provide the PD-L1/PD-L2 signal to PD-1 on the LCs are yet to be determined. KCs express high levels of PD-L1/PD-L2 during chronic inflammation [22]. Fujita et al. [24] have shown that LCs from SCC in particular are more mature, which could contribute to a reduced anti-tumour response [24].

LCs do contribute to the anti-tumour response to ovalbumin (OVA)-expressing melanoma cells following epicutaneous immunisation with OVA protein in the mouse, as do dermal dendritic cells [77]. The CD8+ T-cell response that is initiated following the presentation of antigen inhibited growth of the OVA-expressing transplanted melanoma [77]. Depletion of LCs at any point during the process resulted in susceptibility of the mice to the tumour [77].

8.7 Langerhans Cells Regulate Carcinogenesis

The epidermis is exposed to a variety of DNA-damaging chemicals. Cutaneous LCs play an important role in the detoxification of molecules such as polyaromatic hydrocarbons (PAH) in the skin. When toxins such as 2,4-dimethoxybenzaldehyde (DMBA) are detoxified by LCs, a carcinogenic intermediate is produced. The carcinogenic intermediate leads to increased HRAS mutations in the KCs , contributing to their malignant transformation. LC-intact mice are more susceptible to chemical carcinogenesis provoked by DMBA than mice without LCs [47]. The expression of p450 enzyme CYP1B1 is required for the rapid induction of DNA damage within the KCs to enable efficient neoplastic transformation [41]. Depletion of LCs worsened the progression of SCC in a temporarily LC-depletable mouse model. In the absence of LCs, there was reduced recruitment of natural killer (NK) cells into the tumour microenvironment [53]. NK cells are crucial for the elimination of DNA-damaged KCs during the tumour initiation step of chemical carcinogenesis [53]. These results need to be replicated in the same mouse model to make conclusive statements regarding the contribution of LCs in carcinogenesis.

8.8 LCs Regulate Lymphangiogenesis and Angiogenesis in the Tumour Microenvironment

Tumour growth and metastasis depend on angiogenesis and lymphangiogenesis triggered by chemical signals produced by tumour cells in a rapid growth phase [57]. In the absence of vascular support, tumours may become apoptotic or necrotic [58]. A role for LCs in tumour lymphatic development has not been defined; however, LCs do contribute to lymphatic vessel formation in the skin [54, 82]. LIGHT (an acronym for homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for herpesvirus entry mediator, a receptor expressed by T lymphocytes) is an important ligand that is required for lymphoid tissue development and homeostasis [23, 87]. LIGHT expression is significantly upregulated in skin LCs during inflammation, and LC signals play a dominant role in lymph endothelial cell activation

[82]. A direct role for LCs in tumour lymphangiogenesis is still to be confirmed.

Lymphangiogenesis occurs following angiogenesis and relies on angiogenic factors in order for it to occur [42]. Pericytes contribute to angiogenesis in the tumour microenvironment [9], by producing pericyte-derived milk fat globulin E8 (MFG-E8) [48]. MFG-E8 is also produced by other immune cells, especially LCs [46], also implicating them in angiogenesis. Further investigation of their role in angiogenesis is warranted.

8.9 Langerhans Cells in Tumour Immunotherapy

Through translational studies it has been shown that DC-based immunisation is safe and feasible for patients with cancer. Most DC-based vaccines have used monocyte-derived DCs, but LCs derived from CD34+ haematopoietic cells are superior at activating a cytotoxic T-cell response [62]. Peptide-loaded LC vaccinations against melanoma elicited tumour responses that were comparable to monocyte-derived DCs in vivo [65]. A Phase I study of LCs electroporated with tyrosinase-related protein-2 (TRP-2) mRNA, a melanosomal differentiation antigen, in patients with melanoma was conducted [13]. The vaccines induced greater T-cell activation and diversity against the TRP-2 antigen, which correlated with clinical benefits [13]. Apart from mild delayed-type hypersensitivity reactions, no major toxicities were observed post vaccination [13]. LCs electroporated with Wilms Tumour 1 (WT1) induced sufficiently strong WT1-specific cytotoxic T lymphocytes in vitro [64]. These studies along with other clinical study data [5] highlight the feasibility and safety of LC immunisation, and the use of vaccination in combination with other immune therapies could further improve clinical outcomes for cancer patients.

8.10 Future Directions

The potential for LCs to amplify immune function in an antigen-specific manner makes them ideal candidates for cancer immunotherapy, which attempts to eradicate tumours through the manipulation of host immunity. The superior ability of LCs over other skin DCs to induce cytotoxic T-cell responses in vitro [62, 77] makes them ideal to be exploited for therapy. Protein antigen applied onto barrier-disrupted skin induces a long-lasting cytotoxic T-cell response that is potent enough to control and inhibit tumour growth [77]. In order for immunotherapies to be maximally effective, a thorough understanding of LC biology and function is required.

The identification of the distinct DC subset – langerin+ dermal DCs, has revealed that many of the functions attributed to LCs are in fact being carried out by dermal DCs. Many of these studies need to be revisited to separate the role of langerin-positive DCs from LCs. The inducible LC depletion mouse model, such as the Langerin-diphtheria toxin receptor (DTR) mouse, [36] depletes both the populations of langerin-positive cells (LCs and dDCs). Using the langerin-diphtheria toxin subunit A (DTA) model [31], or the generation of a specific mouse model that enables the inducible-targeted depletion of LCs over the DCs, would be highly useful to confirm the roles of the two langerin-positive populations in cancer. Single-cell sequencing would be highly beneficial to further define the roles of the different types of langerin-positive cells in cancer. This technology might help to uncover any potential subsets of LCs that could play a role in tumorigenesis and cancer. This may also help to clarify the controversy over the roles that have been attributed to LCs that may instead be a function of DCs, further paving the way for the targeting of antigen presentation for immune therapy against cancer.

8.11 Conclusion

Although there are varied levels of LCs reported in different cancers, the general trend is for numbers to be reduced. This could be an immune evasion mechanism that occurs in the neoplastic environment. The regulation of LCs in cancer could be mediated by changes in the cytokine milieu, downregulation of cell surface adhesion molecules, such as E-cadherin, or a result of the

infiltration of other immune cells. Studies involving a LC-only depletable mouse model, single sequencing and standardised immunohistochemical protocols are necessary to further elucidate the function of LCs in cancers.

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Mast Cells in the Tumor Microenvironment

9

Angélica Aponte-López and Samira Muñoz-Cruz

Abstract

Mast cells are tissue-resident, innate immune cells that play a key role in the inflammatory response and tissue homeostasis. Mast cells accumulate in the tumor stroma of different human cancer types, and increased mast cell density has been associated to either good or poor prognosis, depending on the tumor type and stage. Mast cells play a multifaceted role in the tumor microenvironment by modulating various events of tumor biology, such as cell proliferation and survival, angiogenesis, invasiveness, and metastasis. Moreover, tumor-associated mast cells have the potential to shape the tumor microenvironment by establishing crosstalk with other tumor-infiltrating cells. This chapter reviews the current understanding of the role of mast cells in the tumor microenvironment. These cells have received much less attention than other tumor-associated immune cells but are now recognized as critical components of the tumor microenvironment and could hold promise as a potential target to improve cancer immunotherapy.

Keywords

Tumor microenvironment · Mast cells · Tumor-associated mast cells · Breast cancer · Lung cancer · Prostate cancer · Colorectal cancer · Gastric cancer · Pancreatic cancer · Anti-tumoral · Pro-tumoral · Histamine · Tryptase · Angiogenesis · Lymphangiogenesis

9.1 Introduction

The mast cell (MC) is a tissue-resident, innate immune cell that plays a key role in the host defense and homeostatic response but also contributes to several immune-mediated disorders, such as allergic reactions, autoimmune diseases, and cancer.

Although MC accumulation at tumor sites has been reported for many years, there is still controversy about the contribution of this cell to

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tumor development. This chapter provides a review of the literature focusing on the MC role in the tumor microenvironment of different human solid cancers. After briefly reviewing new insights into MC biology, with a major focus on MC phenotype and function, as a direct consequence of the local microenvironment, we analyze the described mechanisms for MC recruitment into the tumor microenvironment. We also discuss evidence reporting MCs antitumoral role, as well as evidence supporting a pro-tumoral role, and the mechanisms implicated in each response. The goal of this chapter is to give insight into the multifaceted role played by MCs in the tumor microenvironment and puts forward some perspectives for future studies.

9.2 Biology of the Mast Cell

MCs are tissue-resident cells extensively distributed throughout the body, especially prominent in protective tissue barriers, such as the skin, airways, and gut mucosa. MCs differentiation into one of their distinct phenotypes and their functions are strongly determined by growth factors and cytokines present in the tissue microenvironment [1]. In rodents, MC subtypes are classified based on their tissue location in two major populations: connective tissue mast cells (CTMCs), which reside constitutively in most connective tissues, and mucosal mast cells (MMCs), which reside in the intestinal and respiratory mucosa. MMCs arise from bone marrow-derived MC progenitors that are recruited and undergo maturation in a T cell-dependent manner. Unlike MMCs, CTMCs are seeded during embryogenesis, by MC "primitive" progenitors (derived from yolk sac) and progenitors derived from "definitive" fetal hematopoietic stem cells, and their maintenance in adult tissues occurs independently of bone marrow progenitors [2, 3]. Their human counterparts are classified based on the proteases they contain, tryptase alone (MC_T), chymase alone (MC_C), or both (MC_{TC}). These subsets differ in their tissue localization and function [4]. The origin of human MCs and the factors that influence each subtype are yet to be completely understood. It is worth noting that both human and rodent MCs are highly heterogeneous and moldable, and intraspecies as well as interspecies heterogeneity has been reported [5]. MC phenotype and function are profoundly shaped by the microenvironment where they originate, mature, and reside. Indeed, MCs are endowed with a high degree of site-specific plasticity, and tissue-specific MCs display differences in granule content, cytokine expression patterns, and receptors, which provide context-related functions to these cells [4]. Even within the same tissue and under basal conditions, MC populations are phenotypically different and can generate further specific subpopulations [6, 7]. MCs also display specific activation-associated transcriptional signatures, for example, interleukin (IL)-33 activated MCs are transcriptionally and most likely functionally distinct than MCs activated via cross-linking of the high-affinity receptor for IgE (FcεRI) [8]. Therefore, the traditional classification based on the produced proteases is too simplistic and a further classification, that takes into consideration the variety of tissue-specific MC subtypes, has been proposed [4]. Supporting this idea, recent studies identified that CTMCs from distinct anatomical locations or with a different fetal origin, had considerable heterogeneity in gene profiles revealing different CTMC subsets [2, 3, 9]. Also, these studies found evidence for previously unappreciated CTMC turnover, in the absence of tissue inflammation and with tissue-specific kinetics [2, 9]. The existence of distinct CTMC subsets confirms that MC identity and function are strongly influenced by their developmental origin and microenvironment.

MCs are characterized by a cytoplasm packed with secretory granules, filled with a broad array of immunomodulatory and vasoactive mediators such as histamine, heparin, cytokines such as tumor necrosis factor-alpha (TNF-α), and different proteases. Indeed, half of the content in secretory granules of mature MCs consists of proteases, tryptase being the predominant protease in human MC [10].

MCs are notable for their extraordinary ability to respond rapidly to stimuli (Fig. 9.1). Upon activation and depending on the type of stimuli

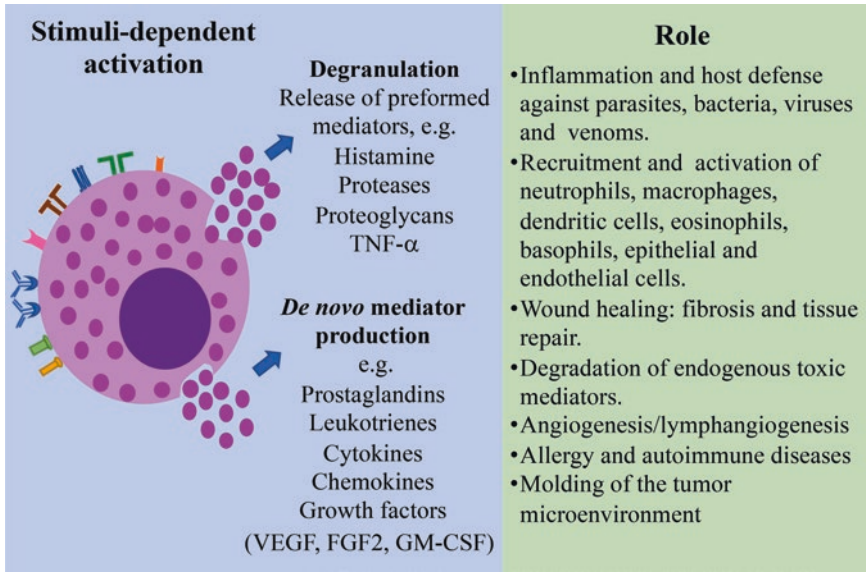

Fig. 9.1 Mast cells (MCs) express several receptors on their surface, which give them the ability to recognize a wide range of endogenous/exogenous ligands. Upon activation by diverse mechanisms, MCs can release a broad array of biologically active mediators that can be divided into two major categories: preformed mediators, stored within the MC granules and released immediately after activation (a process called degranulation) and de novo synthesized mediators, produced following activation. These mediators are directly or indirectly involved in several physiological and pathophysiological processes

and receptor involved, MCs can release three distinct classes of bioactive molecules: granule-stored preformed mediators that are released within seconds to minutes (degranulation); de novo-synthesized lipid mediators, prostaglandins, and leukotrienes, produced within minutes; and a variety of cytokines, chemokines, and growth and angiogenic factors that are produced, following their transcription and translation, within hours [11]. MC activation is mediated by a variety of receptors expressed on their surface, the most well-known pathway of activation is mediated by the cross-linkage of their high-affinity IgE receptor (FcεRI), but MC activation can also be triggered by other receptors, such as Toll-like receptors (TLRs), complement receptors, adenosine receptor, and cytokine and chemokine receptors [5]. The nature of the MC response is dependent on the stimulating ligand.

MCs are multifunctional cells implicated in several physiologic and disease responses. They contribute to tissue homeostasis by promoting inflammation, angiogenesis, and wound healing [12]. They also accumulate in injured and inflamed tissue, where they can amplify or suppress inflammation. MCs also play a key role in the host defense, acting as sentinels, sensing their environment via multiple cell surface receptors to orchestrate the immune response through the fine-tuned release of their biologically active mediators [11]. MC-derived mediators can influence migration, maturation, and function of different cell types, including dendritic cells (DCs), macrophages, eosinophils, natural killer cells (NK), T cells, B cells, fibroblasts, endothelial, and epithelial cells [13]. For example, MC-derived TNF-α is required for efficient DCs and cytotoxic T cells responses, since it promotes DC maturation and migration, and boost the T-cell-priming efficiency [14]. This mediator is also a critical factor for neutrophil recruitment. Similarly, MC-derived prostaglandins and leukotrienes act mainly as proinflammatory factors [15], whereas histamine has pleiotropic effects dependent upon the receptor subtype it is bound to. Therefore, histamine not only enhances the proliferation and

activation of different immune and nonimmune cells but also inhibits cell proliferation and stimulates immune cell suppressor activity. One of the most important modulatory effects of histamine is its influence on T lymphocyte function and differentiation. Specifically, through its H1 receptor, histamine influences T-cell development into Th1 and leads to a decrease of T cell suppressor activity, but through its H2 receptor, histamine stimulates T lymphocyte suppressor activity and inhibits cytolytic activity [15]. It is worth to note that the effector functions of MCs could be different depending on the tissue in which the response occurs, and then the same MC-derived mediator can induce different effects.

Under physiological conditions, MCs can induce and enhance angiogenesis through the production of a variety of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), tryptase, and other proteases [16]. These molecules also contribute to cancer progression. Indeed, one of the main MC effects on tumor growth is related to their pro-angiogenic function.

9.3 Tumor-Associated Mast Cells (TAMCs)

Increased understanding of the relationship between cancer cells and their microenvironment has shed light on how tumors evolve as complex systems, involving dynamic interactions between tumor cells and different cell types, including infiltrating immune cells. The interaction of tumor cells with the immune cells in their microenvironment is essential for determining the tumor fate [17].

MC infiltration is commonly found in different human cancer types, and their accumulation, either at the peri-tumoral or intra-tumoral level, has been associated with both promotion and suppression of tumor growth [16].

TAMCs may arise in tumor microenvironment either by recruitment of neighboring tissue-resident MCs and/or MC progenitors (MCP) via healthy vasculature close to the tumor site or by the proliferation of both mature tissue-resident MCs and MCPs. MCs could be recruited by various inflammatory stimuli within the tumor microenvironment, including hypoxia, cellular injury, and tissue ischemia. MCs could also be recruited by soluble factors secreted from the tumor cells and noncancerous stromal cells. So far, the precise molecular mechanisms involved in the MC accumulation in tumors remain poorly studied.

In healthy tissue, stem cell factor (SCF) is the most characterized chemotactic factor for MC recruitment. Similarly, MC infiltration in tumors is mainly mediated by tumor-derived SCF and its receptor c-kit in MC [18–20]. Experiments using cancer cells from clear cell renal cell carcinoma (ccRCC), the most common histological subtype of renal cell carcinoma (RCC), identified SCF as a key mediator of MC recruitment. Additionally, assays using 3D coculture models showed that hypoxia inducible factor (HIF-2α) expression in these cancer cells was responsible for inducing SCF secretion and subsequently MC recruitment [20]. In vitro evidence showed that colon cancer cells recruited MCs by releasing SCF, and this effect was mediated via bidirectional crosstalk [21]. This study also demonstrated that MC recruitment, rather than local proliferation, was the determinant factor for the increased mast cell density (MCD) observed in colorectal cancer (CRC). Besides SCF, other growth factors produced by noncancerous stromal cells, such as FGF-2, VEGF, and platelet-derived endothelial cell growth factor (PD-ECGF) mediated MC recruitment both in vivo and in vitro [22, 23].

Recently, chemokines were described as important factors for MC recruitment to the tumor microenvironment. For instance, experiments using cell culture supernatants from gastric cancer (GC) tissues, demonstrated the importance of CXCL12 and its receptor CXCR4 in MC recruitment [24]. These experiments indicated that the CXCL12-CXCR4 chemotactic axis could be one of the mechanisms for MC recruitment to the tumor microenvironment, in gastric cancer in vivo, since high MC infiltration correlated with high CXCL12 levels in tumor tissues. Moreover, TAMCs were preferably

located intratumorally, and their numbers increased with tumor progression, showing a positive correlation between MCD and increased advanced lymphatic invasion, tumor size, and tumor stage, in tissues from patients with this disease [24, 25]. Another study reported that CCL15, a chemokine constitutively secreted by colon cancer cells, was as an important chemotactic factor for MCs in vitro, eventually promoting MCs migration [21].

9.4 Mast Cells' Functions in the Tumor Microenvironment

It is clear that mast cells infiltrate tumors of different types of cancer, but what is the role of tumor-associated mast cells?

Although MCs have been long recognized as early and persistent tumor-infiltrating cells, they remain less studied than other components of the tumor microenvironment [26]. Undeniably, MCs can influence directly or indirectly the tumor biology and fate, but their functions in the tumor microenvironment are complex and still poorly understood.

MCs are a rich source of diverse biologically active mediators (cytokines, chemokines, growth factors, matrix metalloproteinases, and proteases), with pro-inflammatory, immunoregulatory, and angiogenic properties. Similar to its physiological functions, MCs can exert diverse functions during cancer development and progression (Fig. 9.2). Depending largely on the microenvironmental stimuli, MCs can inhibit or promote several processes of tumor biology, such as proliferation and survival, angiogenesis, lymphangiogenesis, tissue remodeling, disruption of the extracellular matrix, invasion, and metastatic spread [16]. Therefore, TAMCs could be either pro-tumorigenic, anti-tumorigenic, or innocent bystander cells.

In this chapter, we discuss the role of MCs as a potential prognostic marker in cancer, as well as some anti- and pro-tumor mechanisms by which MCs potentially modulate the tumor microenvironment.

9.4.1 Anti-Tumoral Role of Mast Cells

9.4.1.1 Tumor-Associated Mast Cells as a Good Prognostic Marker

The prognostic value of TAMCs in human solid tumors is still unclear and controversial. Some clinical studies have considered TAMCs as indicators of better prognosis in certain human cancers, such as breast, prostate, and lung cancer (Table 9.1). In this context, MC infiltration in breast cancer has been considered a favorable prognostic factor, and in some cases, this good prognosis was independent of age, tumor grade, and molecular cancer subtype [27–32]. Similarly, in nonsmall-cell lung cancer (NSCLC), high MC infiltration was considered an indicator of good prognosis, independently of tumor stage [33, 34]. In another study, high MCD was linked to a better prognosis in stage I NSCLC but not in stage II [35]. Interestingly, in prostate cancer, an experimental study in mice found that TAMCs exerted different functions according to tumor stage and that MC inactivation promoted the occurrence of highly malignant neuroendocrine cancers [36]. Clinical studies in humans have shown that in prostate cancer, the prognostic role of TAMCs depends not only on the tumor stage but also on the MC location within tumor tissue. So far, the results obtained indicate that intra-tumoral and peri-tumoral MCs have opposing effects on prostate cancer outcome. Therefore, high intratumoral but not peri-tumoral MC numbers were associated with a favorable prognosis [37–40].

Few studies have investigated the role of specific MC subtypes in cancer. From this perspective, a recent study found that both MC_T and MC_{TC} phenotypes were associated with less aggressive breast cancer and that increased numbers of any of the MC subtypes correlated with a better prognosis [32]. This is consistent with another study reporting that both MC_T and MC_{TC} phenotypes correlated with improved survival in NSCLC [34]. In colon cancer, only one study has associated high MCD to longer overall survival in patients [41]. Together, these data indicate that MCs may contribute to the anti-tumor response in these cancer types; however, more high-quality

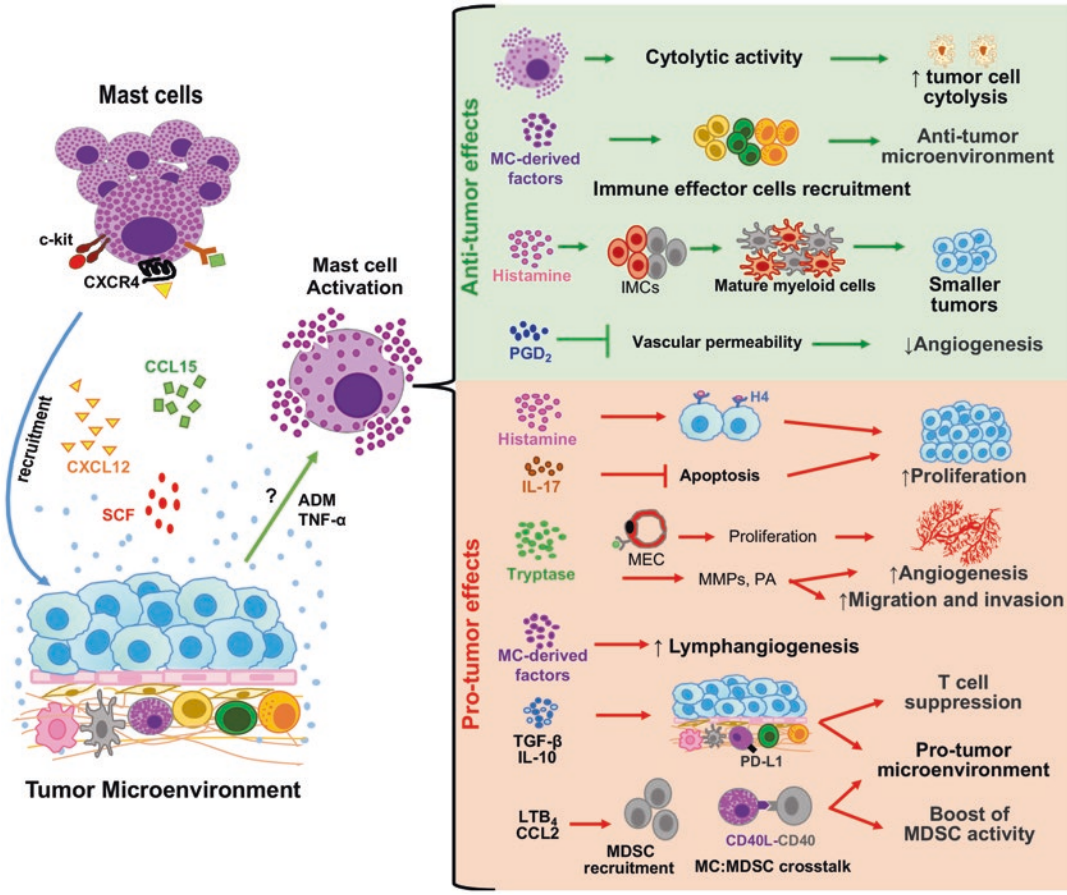

Fig. 9.2 Multifaceted effects of mast cells in the tumor microenvironment. Mast cells (MCs) accumulate in the tumor microenvironment either by the proliferation of local tissue-resident MCs or via recruitment of neighboring tissue-resident MCs and/or MC progenitors, by tumor-derived factors such as SCF, CXCL12, and CCL15. MCs could also be recruited and activated by various inflammatory stimuli within the tumor microenvironment. Activated MCs can exert antitumor effects through direct tumor cell lysis, or indirectly through the release of mediators that promote recruitment and maturation of immune effector cells in the tumor microenvironment. MCs can also contribute to the antitumor microenvironment by decreasing tumor angiogenesis through the inhibition of vascular permeability via MC production of prostaglandin D2 (PGD_2). On the other side, activated MCs can also exert pro-tumor effects. In particular, MC-derived histamine can enhance tumor cell proliferation through binding to its H4 receptor. Tumor derived-adrenomedullin (ADM) stimulates MCs to produce IL-17, which in turn suppresses cancer cell apoptosis contributing to cancer growth. Besides, MCs contribute to angiogenesis in the tumor microenvironment by secreting several angiogenesis-promoting factors. Tryptase, a MC-specific protease, enhances angiogenesis directly by stimulating endothelial cell proliferation and vascular tube formation, or indirectly by activating matrix-metalloproteases (MMPs) and plasminogen activator (PA), which in turn degrade extracellular-matrix components to provide space for neovascular growth. MC-derived factors are also potent promoters of lymphangiogenesis. Moreover, MCs also contribute to the development of tumor-favoring microenvironment by suppressing T-lymphocytes function, via MC secretion of IL-10 and TGF-β or by recruitment of myeloid-derived suppressor cells (MDSC) via secretion of CCL-2 and leukotriene B4 (LTB_4). Tumor-derived TNF-α upregulates PD-L1 expression in the MC, which represents a mechanism of immune suppression via direct interaction between MCs and T lymphocytes in a PDL1-dependent manner. MCs can also exacerbate the immunosuppressive tumor microenvironment by establishing crosstalk with MDSC through CD40:CD40L axis. IMCs immature myeloid cells, MEC microvascular endothelial cells, PD-L1 programmed death-ligand 1

Table 9.1 Correlation of tumor-infiltrating MCs with prognosis in different human solid cancers

Cancer type	Good prognosis (clinicopathological observation)	Poor prognosis (clinicopathological observation)
Breast cancer	Correlation with better OS [27] Correlation with low-grade tumors, no correlation with OS [28] Correlation with HHR cancer [29] Correlation with better OS, independently of grade, LN and ER status [30] Correlation with less aggressive molecular subtypes [31, 32]	Correlation with increased MVD [61] Correlation with high-grade tumors [62] Correlation with LVI, PI, and LNM [63] Specific correlation of itMC with aggressive molecular subtypes [64] Correlation with poor response to chemotherapy in the inflammatory type [65]
Lung cancer	Correlation with better OS independently of NSCLC stage [33, 34] Correlation of ptMC with better 5 years survival in stage-I NSCLC, but not in stage-II NSCLC [35][a]	Correlation of itMC with MVD in stage-I NSCLC [35, 66] and worse OS in stage-I LAC but not in LSCC [66] Correlation with MVD and worse OS [67]
Prostate cancer	Correlation of itMC with low-grade tumors and better DFS [37, 39]; less metastasis and better OS [38][a] Correlation with improved DMFS [40]	Correlation with high-stage cancer and worse PFS [68] Correlation of ptMC with high-stage cancer and worse OS [38][a]
Colo-rectal cancer	Correlation with better OS [41]	Correlation with MVD [46, 49], LVI, LNM, and worse OS [46] Correlation with worse OS [47, 48, 51, 52] and worse DFS [48, 51] Correlation with worse OS after resection for CRLM [50]
Gastric cancer	Correlation with better OS and DFS in stage I cancer [80]	Correlation with angiogenesis [53–55] and high-grade tumors [55] Correlation with increased MVD and LNM [56] Correlation with worse OS [24, 54]
Pancreatic cancer		Correlation with high-grade tumors [57] and worse OS [57–59] Correlation with MVI, LVI, and LNM, in the intra-tumoral border zone, but not in the peri-tumoral or in the intra-tumoral center zone in PDAC [59][b]

CRLM colorectal liver metastases, *DFS* disease-free survival, *DMFS* distant metastasis-free survival, *ER* estrogen receptor, *HHR* high hormone-receptive, *itMC* intra-tumoral mast cell, *LAC* lung adenocarcinoma, *LN* lymph node, *LNM* lymph node metastasis, *LSCC* lung squamous cell carcinoma, *LVI* lymphovascular invasion, *MVD* microvascular density, *MVI* microvascular invasion, *NSCLC* nonsmall-cell lung carcinoma, *OS* overall survival, *PDAC* pancreatic ductal adenocarcinoma, *PFS* progression-free survival, *PI* peri-neural invasion, *ptMC* peri-tumoral mast cell

[a]These studies demonstrated that intra-tumoral and peri-tumoral mast cells had opposite functions (anti-tumor or pro-tumor)

[b]This study underlies the relevance of zone-specific distribution of mast cells in the prognosis of patients with pancreatic ductal adenocarcinoma

clinical studies and standardization of methods are needed before MCD can be considered a biomarker of prognosis for routine use in clinical practice.

9.4.1.2 Mast Cells' Potential to Exert Anti-tumor Effects

Little has been studied about the mechanisms implicated in the anti-tumor activity of MCs (Fig. 9.2). Through microscopic analysis of breast cancer tissues, one study reported that peri-tumoral MCs showed cytolytic activity against tumor cells [29]. Another study, using a mouse model of lung carcinoma, demonstrated that MCs decreased angiogenesis and vascular permeability in the tumor microenvironment, through the production of prostaglandin D2 (PGD_2). Additionally, this study showed that MC-derived PGD_2 reduced TNF-α synthesis, and then limited the pro-tumor response in the tumor

microenvironment [42]. This work identified MC-derived PGD_2 as an anti-angiogenic factor in lung carcinoma. Besides, analysis in an experimental model of chemical skin carcinogenesis, in MC-deficient $Kit^{W}/Kit^{W\text{-}v}$ mice, demonstrated that the absence of MCs led to an increased tumor incidence and growth. This observation was associated with reduced infiltration of Gr-1+ granulocytes, F4/80+ macrophages, B220+ B cells, and CD8+ T cells in sites of skin carcinogenesis. The authors suggested that MCs contributed to the anti-tumor response indirectly by promoting the recruitment of immune cells and immunosurveillance in the tumor microenvironment. This finding was supported by demonstrating that local adoptive transfer of MCs restored cell infiltration, leading to an active immune response that did not allow tumor establishment [43]. The potential of MCs to stimulate the anti-tumor immune response was also demonstrated using a model of murine melanoma. TLR2-activated MCs were able to inhibit tumor growth in vivo, by recruitment of NK and T cells to tumor sites and reduction of angiogenesis. MC-derived IL-6 but not TNF-α was required for tumor growth inhibition after TLR2-mediated MC activation [44]. Comparably to the effect observed in melanoma, TLR2-activated MCs also inhibited the growth of lung cancer in vivo, and this effect was also associated with mononuclear cell infiltration and decreased angiogenesis. In vitro experiments also showed that tumor cell proliferation decreased in the presence of TLR2-activated MCs supernatants, indicating direct MCs anti-tumor effects. Furthermore, in vitro chemotaxis experiments using $CCL3^{-/-}$ murine-derived MCs demonstrated a clear role for CCL3 in mediating MC-dependent recruitment of immune effector cells [44]. Similarly, another study using a model of murine melanoma demonstrated that TLR7-activated dermal MCs secreted CCL2, resulting in skin inflammation and recruitment of plasmacytoid DCs to tumor sites, which after transformation into a subset of killer DCs directly eliminated tumor cells [45]. Overall, these studies indicated that TLR-mediated activation could harness MCs to exert tumor inhibitory functions, such as the recruitment of immune effector cells to tumor sites and the boost of cancer immunosurveillance.

9.4.2 Pro-tumoral Role of Mast Cells

9.4.2.1 Tumor-Associated Mast Cells as a Poor Prognostic Marker

Several scientific studies support MCs pro-tumoral function and association of TAMCs with a poor clinical prognosis of various solid tumors (Table 9.1). Mostly in colon [46–52], gastric [53–56], and pancreatic cancer [57–60], a growing number of clinical studies have associated high TAMC numbers with tumor progression and worse prognosis in patients. A similar association, though controversial, have been reported for breast [26, 61–65], lung [35, 66, 67], and prostate cancer [38, 68].

The mechanisms that potentially mediate MCs pro-tumoral functions include stimulation of tumor cells growth, induction of an immunosuppressive tumor microenvironment, promotion of angiogenesis and lymphangiogenesis, and facilitation of invasion and metastasis (Fig. 9.2).

9.4.2.2 Mast Cells as Promoters of Tumor Cell Proliferation and Survival

MCs can induce proliferation of tumor cells either by direct cell–cell contact or through the release of mediators that directly or indirectly stimulate proliferation and survival. Studies using MC-deficient mice demonstrated that MCs were an essential hematopoietic component for the development and growth of preneoplastic polyps [69]. Moreover, MC-secreted mediators promoted tumor growth by stimulation of colon cancer cell proliferation, through bidirectional communication between MCs and cancer cells, without the need of cell–cell contact [21]. A recent study showed that adrenomedullin, an acid peptide amide in supernatants from cancerous gastric tissue, stimulated MC production of IL-17A, which in turn promoted proliferation by suppressing apoptosis in GC cells, contributing to cancer growth and progression [25]. These data were supported by histopathological results

showing that increased numbers of intra-tumoral MCs correlated with tumor progression and poor survival in GC patients [25]. An in vitro study showed that MCs, in coculture with lung cancer cells, released high levels of histamine, β-hexosaminidase, and tryptase. Interestingly, histamine was the only MC-derived mediator capable of inducing cancer cell proliferation through its H4 receptor [70]. Although some studies have demonstrated that tryptase can induce proteinase-activated receptor 2 (PAR-2)-mediated proliferation of some types of cancer cells, here it was demonstrated that this protease did not directly affect lung cancer cell proliferation.

9.4.2.3 Mast Cells' Contribution to Tumor Angiogenesis and Lymphangiogenesis

MCs can be potent inductors of angiogenesis because of their ability to synthesize and release several common angiogenic components, such as FGF, IL-8, transforming growth factor (TGF-β), TNF-α, and VEGF, as well as noncommon angiogenic components, such as tryptase. Currently, a great amount of evidence has shown that mast cell density (MCD) is strongly correlated to angiogenesis in different human cancers, and among the proangiogenic factors released by MCs tryptase, it is one of the most powerful [23]. A relationship between microvascular density (MVD) and MCD has been shown in various human tumors. A recent paper showed a positive correlation between the MCD and MVD in oral squamous cell carcinoma (OSCC) [71]. Also, in gastric cancer, MCD correlates with angiogenesis, growth, and cancer progression [54]. In patients with breast cancer, high levels of tryptase in serum correlated with high TAMCs numbers and strongly with MVD, supporting the involvement of MC-derived tryptase in tumor angiogenesis [61]. Moreover, experimental data using MC-deficient mice have also provided strong evidence for a positive correlation between the MCD in mammary tumors and angiogenesis. Histological examination of tumors in MC-deficient mice that spontaneously develop breast cancer revealed a marked decrease in angiogenesis compared to control mice, thus supporting the fact that MCs contribution to angiogenesis was strongly due to their ability to promote tumor vascularization [72].

Recent evidence showed a positive relationship between TAMCs and lymphatic vessels (LV), suggesting that MCs may also contribute to the formation of LV in the tumor microenvironment [63, 73]. Analysis of the association between TAMCs and lymphangiogenesis in different molecular subtypes of breast cancer, showed a significant correlation between a high number of peri-tumoral MCs and newly formed LV, in the luminal A-type and the basal-like subtypes [73]. Interestingly, the basal-like subtype exhibited a particular behavior concerning TAMCs and LV density (LVD). This subtype was the only one that showed a significant correlation between the overall MCD (peri-tumoral and intra-tumoral MC count) and LVD. These findings indicated that TAMCs response was specific for each molecular subtype of breast cancer, and this could influence lymphovascular invasion dependent on each molecular tumor subtype [73].

Although MC association with cancer angiogenesis and lymphangiogenesis has been widely demonstrated, the specific mechanisms and factors implicated are only partially characterized, and the MC role in these events seems to be tumor type-dependent.

9.4.2.4 Mast Cells as Promoters of Invasion and Metastasis

In addition to releasing their specific proteases tryptase and chymase, MCs can release different matrix metalloproteases, which degrade components of the extracellular matrix (ECM), and have a key role in cancer progression [16]. Some studies have demonstrated the potential role of MCs in favoring cancer invasion and metastasis. In a cohort of patients with breast cancer, MCs were detected in all metastatic lymph nodes, but not in reactive lymph nodes, which indicated a specific MC role in nodal metastasis of breast cancer [63]. Another study showed that increased tryptase expression, in tumor tissues of breast cancer, was associated with a higher tumor grade and greater lymph node metastasis [74]. Supporting

this, in vitro assays showed that tryptase promoted the invasion and migration of the breast cancer cell line MDA-MB-231, along with activation of matrix metalloproteinase-2, which could facilitate vascular invasion and accelerate metastatic spread [74]. Recently, a meta-analysis of cohort studies evaluating the prognostic role of MC, in different human solid tumors, found that increased tryptase + MC infiltration was significantly associated with lymph node metastasis in NSCLC, hepatocellular, and colorectal cancer [75].

9.4.2.5 Mast Cells Contribute to an Immunosuppressive Tumor Microenvironment

MCs can contribute to the generation of a tumor-favoring microenvironment by disrupting the anti-tumor immunity (Fig. 9.2). A recent study found that intra-tumoral MCs, in samples from patients with gastric cancer, expressed a significantly higher level of immunosuppressive molecule PD-L1. Since crosstalk between PD-L1 and PD-1 is one of the main mechanisms leading to immunosuppression of T cells, this result suggested that MCs may play a role to directly modulate effector function in the tumor microenvironment [24]. This study also demonstrated that TNF-α produced by tumor cell cultures significantly upregulated PD-L1 expression in MCs by activating the nuclear factor-κB (NF-κB) signaling pathway. Through experimental evidence, the authors showed that TAMC inhibited the normal T-cell function in a PD-L1-dependent manner. The results also indicated a significant negative correlation between MC numbers and CD8+ T cells. Additionally, the percentage of MC was significantly increased in patients with advanced stages of GC, suggesting that MC contributes to tumor growth and GC progression via PD-L1 [24]. Besides, in colorectal cancer, CD8+ T-cell infiltration was negatively correlated with MC infiltration. Likewise, Th1-type chemokines CXCL9 and CXCL10, which recruit and promote cytotoxicity of T and natural killer (NK) cells, were highly upregulated in low MCD tumors, suggesting that tumors with less MCs infiltration had a more intense immune response, which could explain the better prognosis in such patients. Importantly, the authors showed that patients with a lower MCD had better survival rates after receiving adjuvant chemotherapy [52]. Using gene set enrichment analysis, it was reported that tumor-infiltrating MC in ccRCC promoted an immunosuppressive environment through suppression of CD8+ T-cell function, via secretion of IL-10 and TGF-β. Furthermore, the expression of characteristic genes of the adaptive immune system and cytotoxic functions [interferon-γ (IFN-γ) and granzyme B (GZMB)] were markedly downregulated in tumors with a high MCD [20].

MCs also contribute to immune suppression through the recruitment of myeloid-derived suppressor cells (MDSCs) and boost their suppressor activity (Fig. 9.2). In a murine model of hepatocarcinoma, activated MCs modified the tumor microenvironment by upregulating CCL2, and the Th2 cytokines IL-10 and IL-13. Moreover, MCs induced IL-17 expression in MDSCs, which in turn regulated the infiltration and enhanced the suppressive function of Treg cells in the tumor microenvironment [76]. In a mouse model of colon cancer, MCs were required to enhance MDSC-mediated immune suppression, through a mechanism involving IFN-γ and nitric oxide production. In the same study, in vitro migration assays showed that activated MCs induced the migration of MDSCs, partly through MC-derived leukotrienes [77]. Also, crosstalk between MCs and MDSCs through the CD40:CD40L axis was responsible for shaping the MC-derived proinflammatory microenvironment (CCL2, IL-6, and TNF-α), that could further support MDSC activation, resulting in a tumor-promoting microenvironment [77]. The fact that MDSCs selectively increased the production of proinflammatory mediators by MCs when cocultured, highlighted the existence of a bidirectional modulation between these two cell populations in the tumor microenvironment.

9.4.2.6 Mast Cells' Role in Cancer Therapeutic Resistance

In addition to the pro-tumoral functions described above, MCs may also modulate the response of

cancer cells to therapy. In vitro assays demonstrated that MC culture supernatants blocked gemcitabine (GEM)/nabpaclitaxel (NAB)-induced apoptosis in pancreatic cancer cell lines, through the activation of TGF-β1 signaling. Furthermore, these MC-derived supernatants reduced the anti-invasive activity of GEM/NAB. These data showed a functional interplay between MCs and pancreatic cancer cells, which induced resistance to GEM/NAB. This observation was supported by the finding that unresponsiveness to GEM/NAB correlated with increased levels of tryptase and TGF-β1 in the blood of pancreatic ductal adenocarcinoma patients. Thus, MCs seem to play a crucial role in tumor resistance to GEM/NAB [60]. Analysis of tumor tissue of inflammatory breast cancer (IBC), an aggressive form of breast cancer characterized by the clinical appearance of inflammation, showed that the MCD was significantly associated with poor response to neoadjuvant chemotherapy in all disease stages and molecular subtypes of IBC. Moreover, MCs were located within range for direct or paracrine interactions with $CD8^+$ T cells, as well as $CD163^+$ macrophages and tumor cells. The authors suggested that interaction of MCs with these immune cells might be exerting an inhibitory effect in IBC, through suppressing $CD8^+$ T cells, enhancing immunosuppressive $CD163^+$ macrophages, and directly promoting tumor cell growth [65]. This study indicated that MCs could represent a possible therapeutic target to enhance the response to chemotherapy.

9.5 Concluding Remarks

Despite recent advances in understanding the mast cell role in tumor biology, we still have limited knowledge of the molecular mechanisms driving mast cells functions in the tumor microenvironment. Most studies agree that mast cells are tumor-infiltrating cells of different human cancers; however, conflicting data exist about the role played by these cells. Also, in specific cancer types, there is a discrepancy in the correlation between the mast cell density and cancer prognosis.

The role of mast cells in the tumor microenvironment might be more complex than suggested by the studies reviewed here, and mast cells functions could rely heavily on the poorly described role of tumor microenvironment in shaping the mast cell response.

Similar to other cell constituents of the tumor microenvironment, such as macrophages, mast cells are extremely moldable and can change their phenotype and functions in response to a changing microenvironment. While tumor-associated macrophages (TAMs) have been extensively studied, and distinct specialized TAMs subpopulations have been well described [78], mast cells have received much less attention, and mast cell diversity and function in distinct tumor microenvironments have not been described up to now. Elucidation of how mast cell plasticity impacts on mechanisms orchestrating a pro-tumor or anti-tumor milieu could explain the contradictory findings regarding the mast cell function in the tumor microenvironment.

In general, cancer is a highly heterogeneous disease with a great variety of genetic and histological clinical subtypes, with each subtype also exhibiting a high heterogeneity within itself. The landscape is made even more complex by the intrinsic mast cell heterogeneity and plasticity, making it possible that within each cancer subtype a variety of tumor associated-mast cell subpopulations exists. Consequently, mast cell contribution, either positive or negative, could be specific to certain cancer subtypes or tumor microenvironments, which could dictate context-dependent functions to the mast cell.

The interaction of mast cells with other tumor-associated stromal cell types, in addition to immune cells, such as fibroblasts, pericytes, endothelial cells, and adipocytes, should be investigated. These stromal cells are key components of the tumor microenvironment, which support tumor growth. Specifically, endothelial cells and pericytes participate in tumor angiogenesis [79]. Therefore, crosstalk between these stromal cells and mast cells may occur within the tumor microenvironment.

More work is needed to understand the intricate crosstalk between mast cells and the tumor microenvironment. Uncovering the mechanisms orchestrating this reciprocal communication will allow mast cell inclusion in future therapeutic approaches.

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10 B Cells in the Gastrointestinal Tumor Microenvironment with a Focus on Pancreatic Cancer: Opportunities for Precision Medicine?

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Abstract

We review state-of-the-art in translational and clinical studies focusing on the tumor microenvironment (TME) with a focus on tumor-infiltrating B cells (TIBs). The TME is a dynamic matrix of mutations, immune-regulatory networks, and distinct cell-to-cell interactions which collectively impact on disease progress. We discuss relevant findings concerning B cells in pancreatic cancer, the concepts of "bystander" B cells, the role of antigen-specific B cells contributing to augmenting anticancer-directed immune responses, the role of B cells as prognostic markers for response to checkpoint inhibitors (ICBs), and the potential use in adoptive cell tumor-infiltrating lymphocyte (TIL) products.

Keywords

Immunosuppression · Pancreatic cancer · Immune responses · Inflammation · Breg · TIL · B cells · Antibody · Complement · Immune checkpoint inhibition · ICB · Cytokines · Prognostic markers · TIB · RNA profiling · CDR3 · Therapy · Adjuvant therapy · Chemotherapy

10.1 Introductory Note

Gastrointestinal malignancies accounted for over 30% of cancer-related deaths globally in 2018 [1]. Pancreatic cancer – most importantly pancreatic ductal adenocarcinoma (PDAC) – is a deadly gastrointestinal disease and the seventh leading cause of cancer-related deaths worldwide, with a meagre 5–7% of patients surviving up to 5 years post diagnosis and at least 80–85% of them presenting with metastatic disease [2] at the time of

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diagnosis. Novel chemotherapy-based treatment regimens such FOLFIRINOX [3, 4] and gemcitabine-nab paclitaxel [5, 6] have improved clinical outcomes in certain groups of patients with PDAC. Nevertheless, the immune landscape in pancreatic tumors plays a major role in mediating its refractory nature to productive host responses, including receptiveness to precision medicine strategies such as immune checkpoint blockade and cellular immunotherapy [7].

We explore in this chapter the state-of-the-art in translational and clinical studies focusing on the tumor microenvironment (TME) with an emphasis on the role of B cells therein – also referred to as tumor-infiltrating B cells (TIBs). The TME is a dynamic matrix of mutations, immune-regulatory networks, and distinct cell-to-cell interactions which collectively impact on disease progress. Among lymphocytic cells, conventional and unconventional T-cell subsets (tumor-infiltrating lymphocytes, TILs) as well as natural killer (NK) have been extensively described, while the knowledge base for B cells remains rather scarce. The sections in this chapter cover concepts and findings related to B cells in the context of gastrointestinal cancers with a special focus on pancreatic cancer where relevant examples are available. Novel concepts such as "bystander" B cells, strategizing immune checkpoint inhibition to also include B-cell activity in patients with cancer as well as their potential use in adoptive cell transfer protocols for the treatment of gastrointestinal malignancies including pancreatic cancer, are also discussed. We also discuss current clinical drug trials which may augment B-cell activity or target their presence in the TME which may show a disease-modifying effect in cancer.

10.2 The Tumor Microenvironment in Gastrointestinal Cancers

The unique nature of the tumor microenvironment (TME) in solid tumors comprises an independent area of intense research due to the intricacies surrounding its sustainability, impact on drug resistance, metastasis, and immune surveillance and immunological control of cancer spread [7, 8]. Cellularity of the TME comprises a variety of immune-cell infiltrates, including but not limited to lymphocytes (T, B, and NK cells), myeloid cells (macrophages, dendritic cells, suppressor cells), granulocytes (mainly neutrophils), fibroblasts, and other newly described cell types such as innate lymphoid cells [9]. Some of these cells, that is, myeloid-derived suppressor cells (MDSCs), cancer-associated fibroblasts (CAFs), and mesenchymal stromal cells (MSCs), also occupy the tumor stroma – the cardinal support structure for the tumor's scaffold and survival [10–12]. The cells produce several cardinal biological mediators which help sustain tumor growth and survival, including in pancreatic cancer, that is, transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and CCL5 as well as export of exosomes containing nucleic acids and/or proteins which help sustain tumor progression [10–12]. Tumor-infiltrating macrophages (TAMs) also have an equal responsibility in impeding productive immune responses in pancreatic tumor lesions, thereby promoting cancer progression [13]. Regulatory T cells (Tregs) contribute significantly to the inhibition of productive cellular immune responses in cancer [14]. The uptake, processing, and presentation of immune-tolerizing antigens, that is, sialic acid-containing proteins by professional antigen-presenting cells (APCs) in the TME, can result in Treg generation while hampering tumor-directed T helper 1 (Th1)-cell development [15]. Previous findings show that sialic acid-containing surface antigens on pancreatic cancer cells contribute to tumor formation as well as cell adhesion and migration [16, 17]. Thus, the nature of the antigens in addition to growth factors, chemoattractants, and selected subsets of APCs present in the TME can directly impose local immunosuppression.

Additionally to tumor initiation, progression of disease is also mediated by the major driver mutations associated with pancreatic cancer in the *KRAS*, *SMAD4*, *CDKN2A,* and *TP53* genes [12, 18–20]. Mutations in *KRAS*, *TP53,* and

SMAD4 are also implicated in the pathogenesis of colon/colorectal cancer [21], which, although presents a better prognosis than pancreatic cancer, was nonetheless responsible for over 850,000 deaths in 2018 [1]. Overt inflammation is a noteworthy cause of driver mutations; thus, hyperactivation of innate immune pathways, that is, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), interleukin (IL)-6 overproduction, and adaptive immune responses, that is, enhanced IL-17 and tumor necrosis factor (TNF)-α production in addition to expression of pro-tumor IL-13 as well as IL-35 in tissue, can also lead to suppression of productive and targeted antitumor responses and, conversely, support tumor survival and spread of transformed cells [22–26]. There is also evidence in preclinical modeling of pancreatic cancer, where p53 silencing or mutant KRAS introduction in otherwise healthy organisms or cells perpetrates tumor initiation and progression [19, 27–30]. Furthermore, the upregulation of cell surface molecules such as mucins (MUC) and ICAM-1 in pancreatic cancer cells by oncogenic KRAS contributes to enhanced disease invasiveness [31, 32]. Mutation in the major histocompatibility complex (MHC) class I pathway is a critical component of tumor resistance to cell-mediated immune attack, dampening or even eliminating tumor-directed immune responses afforded by CD8+ T cells, including the recognition of cancer-associated mutations [33–38]. Loss of or disruptions in the MHC-I pathway have also been reported in patients with pancreatic cancer [39–41], adding to the mechanism of immune suppression and tolerance in the TME.

10.3 A Role for B Cells in Gastrointestinal and Pancreatic Cancer Pathophysiology

Further to T lymphocytes, B cells are an integral component of the immune-cell infiltrate in tumors including pancreatic cancer [42–44]. B-cell (CD20+) infiltrates in the TME have been linked to positive prognosis in several solid cancers, including but not limited to epithelial ovarian cancer [45], non–small-cell lung cancer [46, 47], head and neck cancer [48], and cutaneous primary melanoma [49]. The presence of TIBs (CD20+ B cells) in tumor-associated tertiary lymphoid structures (TLSs) in colorectal cancer and PDAC has been associated with improved survival of patients [50–52]. Occurrence of CD20+ B cells and Th1 cells at the tumor margin in gastric cancer has also been observed to reflect better survival [53]. Similarly, CD20+ B-cell and CD8+ T-cell infiltrates in gastric cancer TLS have been shown to associate with an improved prognosis for patients [54].

The recognition of mutant cancer epitopes, that is, KRAS by antibodies in the TME, is possible [43] – a possible role of B cells as APCs in amplifying the local T-cell responses cannot be dismissed [55]. This supports the notion that there are specific B-cell receptors (BCRs) in the TME equipped with the capacity to recognize cancer mutations. B-cell infiltration into PDAC tumors has been controversial [56], although a recent study using a mouse model, genetically predisposed to contract pancreatic cancer, demonstrated that the activation of B-cell subsets in the TME is associated with a favorable prognosis [57]. Furthermore, the production of pro-inflammatory cytokines, that is, IFN-γ, TNF-α [58], and IL-17, by some B-cell subsets (as seen in rheumatoid arthritis, which is representative of a relevant inflammatory condition [59]) inevitably promotes the activation of potent effector T-cell phenotypes, which could either kill or help tumor proliferation in the context of cancer. IL-35-producing B cells have been observed in the peripheral blood of patients with advanced gastric cancer, concomitant with high frequencies of Tregs, MDSCs, and IL-10-producing B cells [60]. However, the presence of this B-cell subset in the TME remains to be demonstrated. Follicular T-helper cell (TFH)-derived IL-21 itself is a strong inducer of B-cell differentiation into antibody-secreting plasma cells [61], the effect of which is enhanced in the presence of IL-6 [62], suggesting a possible engagement of antibody-dependent cellular cytotoxicity (ADCC) in the B-cell rich TME. IL-6 and IL-21

are also necessary for augmenting T-cell activation and antigen-specific responses [63–66]. Nevertheless, IL-6 has been implicated in the pathogenesis of pancreatic immunopathology and tumorigenesis [67] and must, therefore, be handled with caution concerning immune stimulation.

Extracellular cleavage of adenosine molecules from ATP/ADP/AMP released by cancer cells in the TME is chiefly facilitated by the surface expression of CD73, a 5′-ectonucleotidase and CD39, which is a nucleoside triphosphate dephosphorylase [68]. The cell-free adenosine then binds to its receptors, that is, A1, A2A/B, and A3, on several cell types, that is, macrophages, dendritic cells, lymphocytes, and epithelial cells, to activate intracellular pathways which include response to tissue injury by modulating superfluous local inflammation. This activity is beneficial for tissue protection during chronic infection [69] as well as in autoimmune disease [70]. It has also been shown in zebrafish and mice harboring type 1 diabetes mellitus that activation of the adenosine signaling pathway is crucial for resolution of inflammation leading to increased tissue regeneration of pancreatic β cells [71], while another study using a mouse model of high-fat diet-induced obesity evidenced that the loss of adenosine signaling was concomitant with pancreatic dysfunction and reduced insulin secretion [72]. However, the anti-inflammatory repercussions of adenosine signaling, that is, reduced production of IFN-γ, TNF-α, IL-2, and IL-1 to name a few, would dampen cellular immune responses (effector/memory T cells) to tumor cells, thus promoting a favorable environment for their survival and proliferation [73].

Regulatory B cells (Bregs), defined as CD19+ CD39+ CD73+ cells, represent the highest frequency of CD39/CD73 expression among immune cells, and have been shown to actively produce immunosuppressive adenosine [74]. Later, platinum-based chemotherapy was shown to lead to the decrease of CD39+ Bregs in patients with head and neck squamous cell carcinoma (HNSCC), concomitant with reduced adenosine in peripheral circulation [75]. Thus, in addition to the conventional myeloid cell which expresses CD39 and CD73, TME-associated Bregs present yet another immunosuppressive cell type involved in the pathogenesis of solid cancers. Indeed, IL-18-induced PD-1 expression on B cells in pancreatic cancer was recently shown to impede ADCC and NK-cell activity, further to IL-10 production in the TME [76]. Whether CD39+ CD73+ B cells responsive to IL-18 signaling occur in the pancreatic TME and their role in disease progression remain to be studied. Another subset of Bregs expressing high amounts of the immune checkpoint molecules programmed cell death (PD-1) as well as CD24, CD27, CD5, CD38, and CD69 has been described in the TME, but not in blood, from patients with hepatocellular carcinoma (HCC), a gastrointestinal cancer which claimed almost 800,000 lives in 2018 [1, 77]. Intriguingly, culture supernatants from HCC cell lines were able to induce PD-1 upregulation in healthy peripheral blood B cells in addition to production of pro-inflammatory (IL-1β, IL-6, IFN-γ) and anti-inflammatory (IL-4, IL-10) cytokines. Finally, yet importantly, the authors showed – using in vitro and mouse experiments – that PD-1^{hi} Bregs hamper productive antitumor responses by T cells. Thus, Bregs appear to be susceptible to their immediate environment and can be crucial immunomodulators of antitumor immune responses in the TME.

Tregs also release exosomes which contain microRNAs, one of which is known as Let-7d and has inhibitory effects on Th1 responses, thus effector T-cell activation and IFN-γ production against the tumor [78]. Let-7d has also been shown to negatively affect antibody production by B cells by interfering with glucose and glutamine metabolism [79]. Removing this component by means of drug treatment with gemcitabine [80], fludarabine [81], or cyclophosphamide [82] can potentiate the efficacy of immunotherapies, such as that shown in the context of peptide-based vaccination of patients with renal cell carcinoma [83].

An interesting new observation demonstrates that the use of MEK inhibitors such as trametinib in colorectal cancer may promote cancer-cell proliferation by increasing stem-cell plasticity [84]. A previous study showed that MEK inhibi-

tion in metastatic melanoma cells is partly mediated by insulin-like growth factor 1 (IGF-1)-expressing CD20+ TIB also positive for the transcription factor paired box protein 5 (Pax5), which is reversible with IGF-1 neutralization in vitro [85]. Furthermore, treatment of patients with metastatic melanoma with ofatumumab (anti-CD20 monoclonal antibody to deplete B cells) resulted in significant reduction of CD20+ Pax5+ TIB along with tumor regression. Since IGF-1 has been discussed as a potential therapeutic target in pancreatic cancer [86], TIB and circulating B cells as a source of this factor cannot be dismissed and may be useful in diagnosis as well as treatment monitoring. Figure 10.1 is a schematic representation of the possible roles played by infiltrating B cells in the TME in line with the points discussed above.

10.3.1 The Concept of "Bystander B Cells" in the TME

B cells infiltrating cancer or chronic inflammatory lesions may comprise up to 40% of immune cells in some tumor histologies [87], often residing in tertiary lymphoid structures (TLSs) [44]. Although the beneficial or detrimental role of B cells in antitumor immune responses is debated, an increased number of B cells infiltrating into melanoma lesions has been correlated with improved survival [49]. Some of these tumor-infiltrating B cells (TIBs) have reported to recognize driver mutations, for example, mutant p53 in colorectal cancer [88] or mutant KRAS in lesions from patients with pancreatic cancer [43]. Bystander T cells directed against unrelated target epitopes are implicated in diverse diseases [89, 90], a situation similar to B-cell responses associated with autoimmunity as the "price-tag" for successful cancer-directed immune responses, with the prototype of the neoplastic syndrome associated with humoral autoimmunity in lung cancer [91]. Similarly to bystander T cells, the collateral immune activation of B cells could also be utilized to augment clinically relevant B- and T-cell responses in patients with cancer, for example, by active transfer of IL-21 and CD40-activated B cells [92], that may serve as "cytokine producers," antigen-presenting cells, or – not mutually exclusively – producers of tumor-reactive Ig leading to complement activation or Fc receptor-augmented antigen uptake. We recently described that TIBs in human pancreatic cancer lesions or glioblastoma recognize a broad repertoire of CMV and EBV-specific target epitopes [93], complementing the earlier finding that CMV-specific T cells infiltrate melanoma lesions while retaining their function despite upregulated PD1 expression [94]. This suggests that targeting bystander B cells for the immunological therapy of patients with cancer may be achieved via (i) adoptive transfer [92], (ii) checkpoint inhibition [95], and/or iii) augmenting the combined axis of intratumoral anti-CMV [94] or anti-EBV T-cell and B-cell responses associated with an anticancer pro-inflammatory microenvironment.

Another concept to study further is the potential presentation of tolerogenic cancer-associated antigens to Tregs by B-cell subsets in the TME via the MHC-II pathway, given that Treg-reactive neoepitopes were recently described [96]. As cancer-derived private neoepitopes can also induce IL-13 production by some T-cell subsets [97], whether B cells have a part to play herein pertaining to antigen presentation in the TME is worth investigating. Conversely, the presence of neutralizing antibodies against tolerogenic (or potentially pathogenic) cancer neoantigens would also be useful to decipher the role of certain B/plasma-cell populations the TME. Figure 10.2 shows histological observations of B-cell infiltration into human PDAC lesion in addition to a schematic which describes the possible effects of bystander B cells in the TME.

10.3.2 Immune Checkpoint Blockade and Anticancer Immune Responses: B-Cell Involvement

Immune checkpoint molecules such as programmed cell death 1 (PD-1) and its ligands PD-L1/2, cytotoxic T-lymphocyte-associated

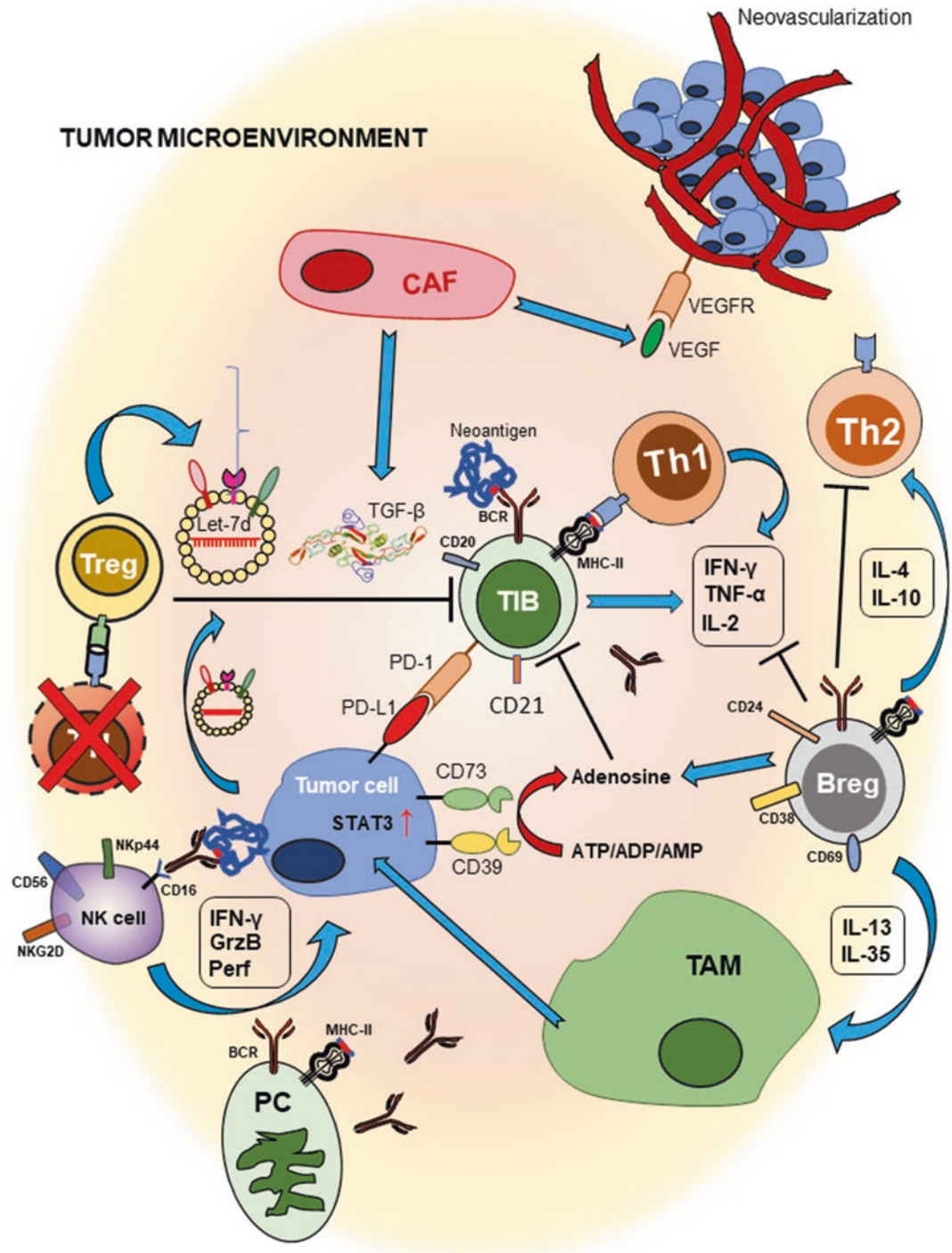

Fig. 10.1 Possible roles of TIB in the gastrointestinal/pancreatic TME. As shown in the schematic, TIB recognizing cancer neoantigens could activate productive antitumor responses in the TME by promoting Th1 polarization as well as inflammatory antibody production by TIB-derived plasma cells. Bindings of IgG to the Fcγ receptor (CD16) on NK cells also engage antitumor activity, releasing cytotoxic molecules as well as IFN-γ. However, production of adenosine by tumor cells as well as regulatory B cells (Bregs) can impede productive TIL and TIB responses

antigen 4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), although evolutionarily preserved to carefully tailor immune responses to provide optimal host protection without excessive tissue damage, their premature overexpression in tumors and T-cell infiltrates functions in favor of tumor survival and dissemination [98]. Thus, immune checkpoint expression in human cancer is a clinically and biologically relevant form of immune suppression. Along these lines, therapeutic targeting of PD-1 and CTLA-4 has been the greatest breakthrough in clinical oncology as of recent times [99], a turning point in immuno-oncology paving the way for future immune-based interventions.

A role for memory B cells in promoting immune activation in tumor of patients with cancer who response positively to anti-PD-1/anti-CTLA-4 therapy was recently appreciated [100], suggesting that local B-cell responses – in addition to T cells – constitute an integral arm of antitumor responses mobilized by ICB. Successful anti-PD-1 therapy may also involve B-cell infiltration of metastatic lesions [95], where an early increase in CD21lo B cells is likely to predict occurrence of immune-related adverse events in patients with cancer received combination ICB therapy [101]. In addition to several other immune-cell types in the TME, B cells could also augment MHC-II-dependent CD4+ T-cell responses to private mutations in PDAC where MHC-I-restricted CD8+ T-cell reactivity may fail [43, 44]. This consolidates the role of B cells in augmenting and sustaining inflammatory responses in cancer, jointly (with T cells and NK cells) contributing to tumor rejection and not only unproductive and detrimental pathology [102]. Also, as discussed earlier in this review, PD-1 expressed by B cells in the TME – induced by nonproductive inflammation – is also a target of ICB, as shown in hematological malignancies [103] and warrants investigation in pancreatic cancer. This notion has been corroborated in cancers of different histology. Soft-tissue sarcomas can be grouped into distinct phenotypes, that is, immune absent/low (groups A and B), vascularized (group C), and immune positive (groups D and E) [104]. B cells may be associated with pericytes [105, 106], for instance, for group B, or group E, rich in TLS and dendritic cells. B cells turned out to be the strongest prognostic indicator, independent of T cells [104]. This has also been observed for patients with melanoma, although with a different angle: Clonal expansion of B cells, Ig switch, and the quality of immune effector functions are associated with better responses to ICB [107]: B-cell-associated immature TLS may show inhibitor effects, whereas well-structured, "mature" TLSs are associated with improved antitumor responses [107], changes that may be influenced by standard (neoadjuvant) chemotherapy of cancer that induces complement (C3) products binding to the complement receptor 2 on (antitumor directed) activated (ICOSL+ IL-10^{low}) B cells [108].

A molecule with immune checkpoint properties in pancreatic cancer is the focal adhesion kinase (FAK). Although with important roles in neovascularization and the migratory capacity of cells [109], FAK promotes fibrosis and poor T-cell infiltration into PDAC tissue, thus worsen-

Fig. 10.1 (continued) in the TME via subsequent production of anti-inflammatory factors, that is, TGF-β, IL-13, IL-35, IL-4, and IL-10, also involving Treg participation. The latter are cytokines associated with Th2 skewing of T cells, while IL-13 and IL-35 may support tumor-cell growth as well as M2 polarization of TAMs. Also, some activated B cells may express PD-1 which, by binding to PD-L1 on the surface of tumor cells, can abrogate tumor recognition and killing. Tregs also contribute to dampening of B-cell (and T-cell) responses in the TME via miRNA-dependent mechanisms, that is, Let-7d in exosomes. CAFs also produce TGF-β as well as VEGF, which directly enables neovascularization in the TME and plays an important role in establishment of hypoxia. Activation of B cells via C3 components binding to CD21 (the complement receptor 2) may shape the B-cell phenotype and promote anticancer-directed responses. B cells in well-organized TLS, in close vicinity to T cells, are associated with better survival, by serving as antigen-presenting cells and producers of anticancer-directed antibodies. CAF cancer-associated fibroblasts, Treg regulatory T cells, MHC-II major histocompatibility complex class II, TAM tumor-associated macrophage, PC plasma cell, VEGF vascular endothelial growth factor, ATP adenosine triphosphate, ADP adenosine diphosphate, AMP adenosine monophosphate

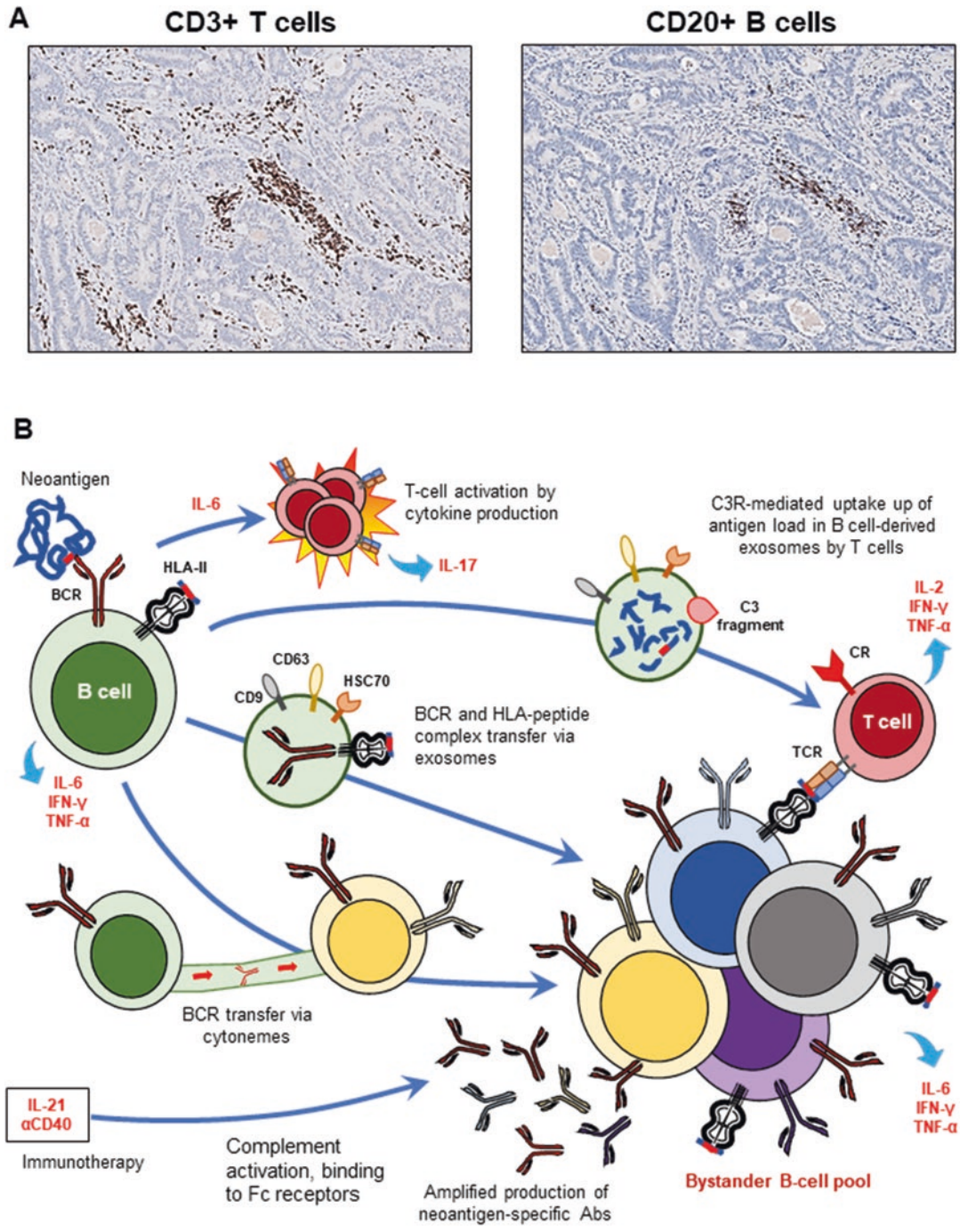

Fig. 10.2 Bystander B cells in patients with cancer. (**a**) Immunohistology showing infiltrating of CD20+ B cells into human pancreatic cancer lesions in the same loci as CD3+ T cells (brown patches, top panel). (**b**) Activation of bystander B cells during antigen-specific immune recognition. Direct uptake of an antigen (represented by the neoantigen) triggers its own activation and ability to produce antibodies, following differentiation into a plasma cell, and cytokines. This initial immune response then triggers the transactivation of bystander B cells in its vicinity by several mechanisms. Exosomes containing HLA-peptide complexes displaying neoantigen-derived epitopes or BCRs specific for the aforementioned neoantigen can be transferred to the bystander B cells not in

ing the immunosuppressive nature of the TME [28]. Therapeutic targeting of FAK using small molecules has shown promise in preclinical mouse models of PDAC [28, 110] as well as in a human organoid system [111], based on its reversal of local immunosuppression by limiting MDSC and Treg infiltration, increased sensitivity to PD-1/CTLA-4 blockade, and killing of PDAC cells in vitro, further to providing evidence that FAK inhibition can overturn disease outcome in PDAC in tumors with a mesenchymal phenotype [110]. Safety and efficacy clinical trials of FAK inhibitors in patients with solid tumors including PDAC as well as hematological malignancies are currently underway [112]. It has been shown in mice that FAK expression in progenitor B cells is important for their spatial organization and homeostasis in the bone marrow, especially under inflammatory conditions [113]. As such, it would be worthwhile to investigate whether FAK inhibition in patients with PDAC also affects B-cell infiltration into the TME as well as their numbers in blood, thus having an influence on egress from the bone marrow to traffic to sites of disease.

The gut microbiome was recently shown to regulate clinically beneficial antitumor immune responses following ICB in patients with metastatic melanoma [114–116]. A recent study showed quite the contrary in the context of PDAC, where ablation of the host gut microbiota in fact promoted antitumor functions in addition to improving the efficacy of PD-1 blockade [117]. Interestingly, patients with PDAC are also likely to have changes in their oral microbiota, that is, increases in numbers of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, in associated with poor dental health and greater risk of developing pancreatic cancer [118, 119]. More recently, the role of complement has been reappreciated in pancreatic cancer, showing that *Malassezia furfur* activation of C3 and interaction with mannan-binding protein induce tumor progression associated with chronic inflammation [120], whereas distinct bacterial species, among them *Pseudoxanthomonas–Streptomyces–Saccharopolyspora–Bacillus clausii*, were associated with longer survival [121]. Whether biologically and clinically, humoral relevant antitumor immune responses are targeting cancer-specific antigens can be mediated by cross-reactive antibodies, elicited by human gut commensals, as shown for autoimmune responses [122] has to be demonstrated. This would not be unexpected, since anticancer immune responses can be conceptualized as very focused autoimmune responses. Nevertheless, it would be worthwhile to investigate whether changes in the oral, pancreatic, and gut microbiota of patients with PDAC who undergo ICB are concomitant clinical responses. In other words, blood and tissue samples from these patients may help better understand alterations in immunological mediators as well as immune-cell frequencies, that is, B and T cells which will aid immunotherapy development.

10.3.3 B-Cell Metabolism in the TME: Knowns and Unknowns

Unlike the metabolic profiles of T-cell subsets in the TME, B-cell metabolism and its effect on immune responses are not as well studied. Prior research has shown that the activity of glycogen synthase kinase B (GSK3) in murine B cells is

Fig. 10.2 (continued) direct contact with the neoantigen. In addition to an increased population of B cells expressing neoantigen-specific BCRs and associated antibody production, these transactivated bystander B cells may also produce T-cell-activating cytokines, including IL-6, IFN-γ, and TNF-α to amplify the cellular immune response. B-cell-derived IL-6 can induce T-cell activation and subsequent IL-17 production which, if directed only against mutated epitopes, may be beneficial in the early stages of antitumor inflammation. The deposition of a fragment of complement C3 on the surface of B-cell-derived, antigen-loaded exosomes can also bind to the complement receptor (CR) expressed on the surface of T cells for antigen acquisition and activation, resulting in production of pro-inflammatory cytokines by the latter. BCR transfer between B cells has also shown to be possible via cytonemes – molecular nanotubes which form an intercellular passageway for material transfer. IL-21 and anti-CD40, as shown in preclinical studies, can be excellent immunotherapy candidates to promote plasma cell differentiation and induce antibody production in vivo. Thus, B cells may amplify the ensuing immune response in tissue compartments also when antigen load is limiting. Abs antibodies, BCR B-cell receptor, TCR T-cell receptor, HLA-II human leukocyte antigen class II, C3R complement C3 receptor

necessary for glycolysis, mitochondrial biogenesis, CD40-mediated activation, and antibody production as well as reactive oxygen species generation [123]. Importantly, this enzyme was found to be essential for the proliferation of germinal center B cells, particularly for survival and growth in hypoxic environments (pertinent to the TME), which necessitates high glucose consumption and glycolytic breakdown. Driven by Pax5 and Ikaros activity, B cells in lymphoma are noted to express high levels of AMPK, which is also the target of metformin – a highly effective antidiabetic drug that concomitantly promotes CD8+ T-cell activity [124, 125]. The mammalian target of rapamycin (mTOR), which is also upregulated in lymphoma B cells and is necessary for NF-κB induction, antibody production, and mitochondrial output [126], is druggable by metformin, although its effects have been shown in the context of T-cell physiology [127]. Pharmacological inhibition of mTOR/PI3K signaling in human lung cells infected with influenza virus has been shown to arrest pathogen replication, reduce myelocytomatosis virus oncogene cellular homolog (Myc) induction, and protect mice from lethal influenza virus infection [128]. Myc overexpression is also a common molecular marker in B-cell-associated hematological malignancies and is negatively regulated by the aforementioned GSK3 [123, 126]. Glucose transporter 1 (Glut1), a cell membrane-bound nutrient channel necessary for glucose homeostasis, was shown to be crucial for B-cell proliferation and antibody production following BCR stimulation [129]. Myc/PI3K/Akt axis is also necessary for Glut1 upregulation in B cells [130], thus presenting an important molecular network incorporating GSK3 and mTOR which controls B-cell metabolism in tissue – and the TME. Another important factor pertinent to the TME is hypoxia, often involving the expression of the hypoxia-inducible factor 1 alpha (HIF-1α). Low oxygen tension in tissue has been demonstrated, using germinal centers in mice, to affect antibody class switching, increase the rate of B-cell death, and reduce their proliferation [131]. HIF-1α is necessary for engaging the glyoxylate shunt pathway in the absence of oxygen, where glucose is catabolized to lactate instead of pyruvate [132]. Importantly, mTOR is required for the subsequent expression of HIF-1α protein [132].

Whether these pathways are also activated in B cells in gastrointestinal TMEs requires thorough assessment. Considering that TIBs can produce antibodies in vitro following several days to weeks of culture [43, 93], it is likely that like TIL, their functionality in the TME is also subdued owing to local immunosuppression but is nevertheless restorable under suitable conditions ex vivo.

10.4 The Clinical Utility of B Cells in Precision Oncology

Gaining a deeper understanding of the significance of B cells in solid cancers not only helps strengthen the knowledge base for the disease process but also helps the development of next-generation therapies. The classical role of B cells as APCs and producers of antibodies – following their differentiation into plasma cells – offers broad clinical utility in developing next-generation cancer immunotherapy drugs. Co-activation of the humoral and cellular arms of the immune system, for example, antibody/B-cell and T-cell responses to the same antigen, that is, NY-ESO-1 [133, 134], mesothelin [135], and mucins [136], is an important and relevant biological phenomenon requiring further dissection. Adoptive transfer of memory B cells, unlike T cells, has not been clinically attempted but may be pursuable given that cultivating them in large numbers in the laboratory is possible [137]. Based on immunoglobulin heavy chain CDR3 spectratyping (the antigen-binding region of the BCR akin to that of the TCR), in vitro expansion of TIL with IL-2, IL-15, and IL-21 can also give rise to various flavors of BCR-IgM and BCR-IgG expressing TIB populations (Fig. 10.3), certain subsets of which may be useful in orchestrating a desired antigen-specific response. Personalized vaccination strategies may also benefit by administering neoantigen-loaded B cells to patients not only to induce strong T-cell responses in their capacity as APCs but also to locally produce

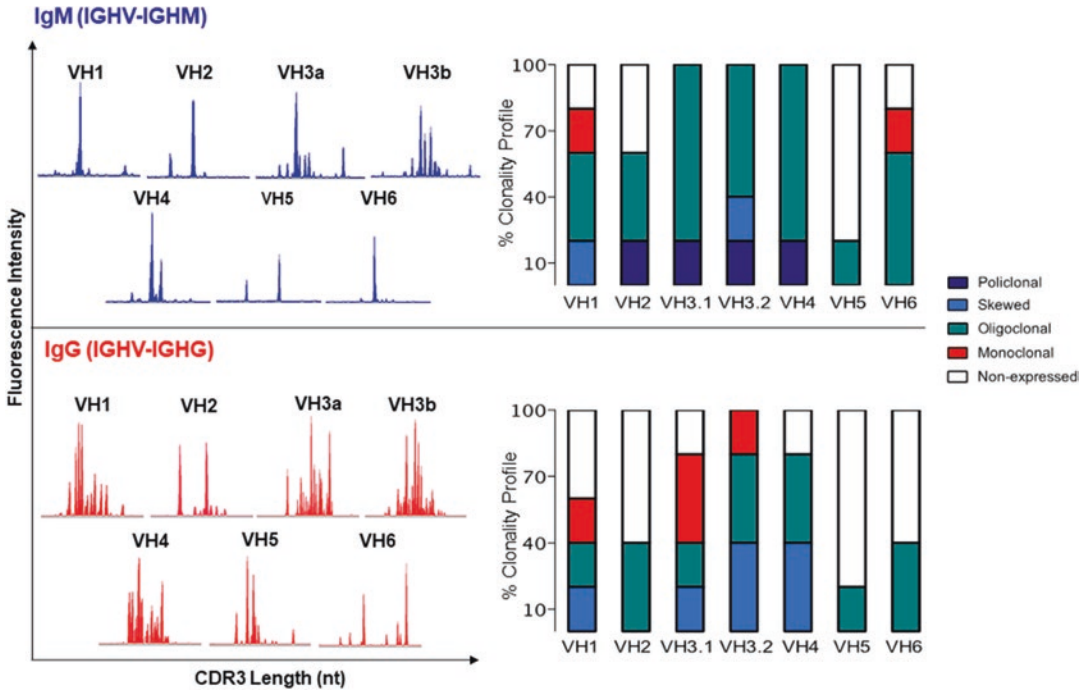

Fig. 10.3 IGH CDR3 spectratyping. Immunoglobulin heavy chain (IGH) variable segment expression and CDR3 length distribution for IgM (panel A) and IgG (panel B) transcripts (left panels) and TIB clonality profiles (right panels). Total RNA from TIL fractions from a patient with PDAC was isolated for first strand cDNA synthesis with the Superscript III system (Invitrogen). Diversity of immunoglobulin heavy chain (IGH) transcripts in TIB was evaluated with a CDR3 spectratyping assay for heavy chain variable segments (IGHV) IGHV1-6 families and common primers specific for IGHM and IGHG gene segments. Shown are a representative IGH-M and G spectratype for an individual TIL culture and mean clonality profiles for five different TIL cultures. Switched memory B cells and clonal expansion appear to be associated with better response to ICB

mutation-specific antibodies as well as pro-inflammatory, anticancer-directed cytokines [138]. B cells close to tumor-infiltrating T cells [139] appear to be antigen driven; the adoptive transfer of TIL, containing both T and B cells in a preclinical cancer model, showed that both components of the cellular immune system provide a better anticancer response as compared to T or B cells alone, a concept that has not yet been translated into clinical cell therapy trials [140].

Chimeric antigen receptor-bearing T cells (CAR-T) represent a breakthrough in cellular immunotherapy, with two pharmaceutical products currently licensed for clinical use in patients with B-cell malignancies with further candidate in clinical trials for solid tumors [141, 142]. CARs are a fusion of an exterior, antigen-binding, antibody-derived single-chain variable fragment (scFv) with a CDR3 sequence of interest and the intracellular molecules necessary for triggering a pro-inflammatory T-cell response. Thus, screening of antibody responses to a variety of cancer-associated targets – including mutations – may be used for CAR-T development provided the molecules are surface expressed. This may also apply to KRAS and p53 mutations, where simultaneous activation of T-cell and humoral immune responses to mutated epitopes has been observed [43, 143].

Antibodies reactive to protein targets derived from EBV and CMV produced by tumor-infiltrating B cells as well as those in the periphery are of great significance due to the role of these viral pathogens in modulating antitumor responses in patients with brain or pancreatic cancer [93, 144]. Serum analysis of IgG molecules in blood, based on their recognition of EBV/CMV epitopes, may be useful in informing

of the clinical status of the patient from an immunological perspective and their amenability to immunotherapy, thus serving as a biomarker. This hypothesis, however, requires formal testing using suitable patient-derived cancer samples.

Therapeutic interventions targeting growth factors in the pancreatic TME, in addition to specific receptor-targeted drugs, that is, anti-EGFR antibodies/small molecules and anti-KRAS drugs, may not only kill the tumor cells directly but also induce immunomodulation in the TME, which can simultaneously activate the immune system and elicit productive antigen-specific responses. Along these lines, antibodies targeting mutant KRAS may be able to specifically target the aberrantly expressed protein in cancer cells [43, 145, 146]. Phosphorylation of the Bruton tyrosine kinase (BTK), which is activated following B-cell receptor (BCR) engagement with FcγR on macrophages via phosphoinositide-3-kinase gamma (PI3Kγ) in the latter, can lead to M2 polarization of TAMs as well as Th2 skewing of cellular immune responses – with IL-10 and IL-4 involvement [147]. BTK can be inhibited by ibrutinib (PCI-32765) [148], while P13Kγ is susceptible to treatment with TG100-115, an experimental drug with anticancer properties [149]. Using both drugs, Gunderson and colleagues demonstrated improved pro-inflammatory responses in BTK+/P13Kγ+ TAMs in a mouse model of PDAC [147]. As such, BTK inhibition presents a B-cell-dependent therapeutic strategy in PDAC, which can be better visualized using patient samples from the ongoing clinical trial (NCT02562898).

In $KRAS_{G12D}$-driven PDAC model established in mice lacking HIF-1α, B-cell depletion using an anti-CD20 monoclonal antibody inhibited the progression of pancreatic intraepithelial neoplasia (PanIN) to PDAC [150]. Primarily, HIF-1α expression and stabilization in these animals were observed to occur early during tumor development, the absence of which promoted disease exacerbation. The authors also observed that the latter scenario entailed the production of CXC13 in the TME, a strong B-cell chemoattractant, which lead to infiltration of CD20+ B cells that were eventually responsible for PDAC development. This observation needs to be repeated in patients with PDAC or those diagnosed with PanIN (as well as individuals presenting with other gastrointestinal disease) to see whether HIF-1α destabilization can be observed in cancer tissue and coinciding with CD20+ enrichment in the TME. Indeed, CD20+ B cells in the TME per se and not the TLS appear to not reflect a good prognosis for survival [52].

Tregs have been shown to promote the survival of IgA+ B cells which recognize a gut microbiota-associated antigen in mice [151], indicating that the former may also assisting in modulating B-cell immune responses during cancer pathogenesis. Gut bacteria-specific IgA in mice was previously demonstrated to necessitate PD-1 expression on plasma cells for optimal regulation of the microbiota composition [152]. As such, PD-1+ plasma cells – unlike B cells – may have an important role in gut homeostasis and, therefore, tissue protection and reduced chances of contracting cancer. Also, the possibility of cross-reactivity between commensal bacteria-derived targets and host-associated epitopes leading to productive anticancer antibody responses cannot be dismissed [153]. These hypotheses, however, remain to be formally tested in patients with pancreatic cancer (as well as other gastrointestinal malignancies) in relation to those showing clinical responses to surgery, chemotherapy, and immunological treatment, that is, cancer vaccines, cellular therapy, and changes in the gut microbiota using high-end molecular techniques couples with immunoassays, that is, bacterial species enriched in stool samples, IgA/IgG abundance in serum and TME, as well as their respective specificities using peptide-based identification [93]. However, it may be necessary to bear in mind the influence of the time of day when samples are collected due to differences in leukocyte populations and properties in blood and, possibly, in tissue owing to the effect on their migratory patterns [154].

Table 10.1 summarizes clinically pursued therapeutic strategies at present which involve modulation of B-cell responses (mostly at the clinical stage, others still in the preclinical phase) to improve treatment outcomes in pancreatic cancer.

Table 10.1

Target	Therapeutic agents	Description	Status
Adenosine signaling	Anti-CD73 (+/− PD-1)	Cell surface-bound 5′-ectonucleotidase which converts ATP/ADP/AMP to adenosine. Inhibiting CD73 blocks the conversion of ATP/ADP/AMP to adenosine	Phase I clinical study undertaken by Corvus Pharmaceuticals Inc. to test CPI-006, an anti-CD73 monoclonal antibody tested as a single agent or in combination with pembrolizumab or CPI444 (A2A receptor antagonist) in patients with various solid tumors including pancreatic cancer (ClinicalTrials.gov identifier: NCT03454451)
	CD73 + PD-L1		Phase I clinical study by MedImmune/Astra Zeneca to investigate the efficacy of MEDI9447 (oleclumab), a therapeutic monoclonal antibody against CD73 given either as a single agent or in combination with MEDI4736 (durvalumab, anti-PD-L1) in patients with solid tumors including pancreatic cancer (ClinicalTrials.gov identifier: NCT02503774)
	Anti-CD39	Cell surface-bound nucleoside dephosphorylase which converts ATP/ADP/AMP to adenosine. Inhibiting CD39 blocks the conversion of ATP/ADP/AMP to adenosine	Preclinical development of IPH52, a first-in-class therapeutic anti-CD39 monoclonal antibody at Innate Pharma, France
	SCH-58261	Adenosine receptor antagonist, with very high selectivity for the A2A receptor. Blocks the uptake of adenosine molecules into the cell to activate a variety of anti-inflammatory pathways	Delivery of CAR-T cells harboring liposomal SCH-58261 improved anticancer immunotoxicity in vivo (mouse model) (Siriwon et al., 2018); SCH-58261 administration blocked A2A receptor activity and lead to delay in tumor progression in a mouse model of HNSCC (Ma et al., 2017). No clinical trials in patients with pancreatic cancer have been reported as of 2018
	CPI-444	Adenosine receptor antagonist, with very high selectivity for the A2A receptor. Blocks the uptake of adenosine molecules into the cell to activate a variety of anti-inflammatory pathways	Phase I clinical study undertaken by Corvus Pharmaceuticals Inc. to test CPI-444 alongside CPI-006 (anti-CD73) in patients with solid tumors including pancreatic cancer (ClinicalTrials.gov identifier: NCT03454451)
Bruton tyrosine kinase (BTK)	Ibrutinib	Blocks BTK activity and downstream anti-inflammatory effects in tumor-associated macrophages as well as pro-tumor B-cell populations to promote effective antitumor T-cell responses in the TME	Phase I/II clinical study currently underway, investigating ibrutinib alongside gemcitabine and nab-paclitaxel in patients with pancreatic ductal adenocarcinoma at UCSF (ClinicalTrials.gov identifier: NCT02562898)

(continued)

Table 10.1 (continued)

Target	Therapeutic agents	Description	Status
			Phase II study currently underway in patients with pancreatic neuroendocrine tumors (pNETs) by H. Lee Moffitt Cancer Center and Research Institute Collaborator and Pharmacyclics LLC. Pharmacyclics is also carrying out a phase II/III study using the same drug combinations in patients with metastatic PDAC (study name: RESOLVE) (ClinicalTrials.gov identifier: NCT02436668)
BTK + PD-L1			A phase I/II study sponsored by Pharmacyclics is currently underway investigating the clinical efficacy of ibrutinib with or without MEDI4736 (durvalumab, anti-PD-L1) in patients with refractory solid tumors including pancreatic cancer (ClinicalTrials.gov identifier: NCT02403271)
Phosphoinositide-3-kinase gamma (PI3Kγ)	TG100-115	Blocks the activity of PI3Kγ which reverses the anti-inflammatory properties of tumor-associate macrophages	Preclinical studies in a mouse model of PDAC showed improved survival (Gunderson et al., 2016), while antitumor activity of the drug has also been validated in breast cancer cells (Song et al., 2017). No clinical trials in patients with pancreatic cancer have been reported as of 2018
PI3Kδ	INCB050465	Blocks the activity of PI3Kδ which reverses the anti-inflammatory properties of tumor-associate macrophages	Phase I clinical trial undertaken by Incyte Corp. to assess the efficacy of INCB050465 in combination with pembrolizumab in patients with advanced solid tumors including pancreatic cancer for inducing potent antitumor immune responses in the TME (ClinicalTrials.gov Identifier: NCT02646748)
Cancer vaccine	GVAX (with cyclophosphamide +/− anti-PD-1)	Tumor cell heterologously expressing GM-CSF which has undergone clinical testing in patients with pancreatic cancer	Phase I/II clinical trial at Sidney Kimmel Comprehensive Cancer Centre (Johns Hopkins University) to assess the combination of cyclophosphamide with GVAX in the presence or absence of nivolumab to induce immunological changes in the TME and to achieve improved clinical efficacy in patients with pancreatic cancer (ClinicalTrials.gov Identifier: NCT02451982)

(continued)

Table 10.1 (continued)

Target	Therapeutic agents	Description	Status
Chemokine antagonist	Olaptesed pegol (+/− anti-PD-1)	Olaptesed pegol blocks the activity of CXCL12, which is implicated in EMT egress (and metastasis) in the TME of pancreatic cancer. CXCL12 has also been shown to be important for B-cell development and colonization in the bone marrow [156]	Phase I/II clinical study by NOXXON Pharma AG (Germany) currently recruiting participants with pancreatic cancer to test for the activity of olaptesed pegol with and without pembrolizumab for improving antitumor responses in the TME (ClinicalTrials.gov Identifier: NCT03168139)
Focal adhesion kinase inhibitor	Defactinib	Focal adhesion kinase (FAK) or protein tyrosine kinase 2 is necessary for pancreatic cancer cell proliferation and metastasis due to its role is activating cell adhesion signaling cascades	Phase I study at Washington University Medical School involving patients with pancreatic cancer assessing the activity of defactinib with gemcitabine and pembrolizumab to induce immunomodulation in the TME as well as cancer cell death (ClinicalTrials.gov Identifier: NCT02546531)
JAK1 inhibitor	Itacitinib (INCB039110)	Selective inhibitor of the JAK1 molecules which is imperative for IFN-γ signaling, but hyperactivation of this pathway also leads to immune exhaustion and cancer progression in solid tumors. May pose a risk of uncontrolled B-cell growth and cancerous transformation [157]	Phase I clinical trial undertaken by Incyte Corp. to assess the efficacy of itacitinib in combination with pembrolizumab in patients with advanced solid tumors including pancreatic cancer for inducing potent antitumor immune responses in the TME (ClinicalTrials.gov Identifier: NCT02646748)

10.5 Concluding Note

Given that a sizeable component of current research in pancreatic cancer therapy focusses on modulating immune responses in the TME, learning from other disease modalities, therefore, becomes very useful. This also applies to expanding our current knowledge on B cells and their role in orchestrating clinically relevant immune responses in the TME. A very recent article describes how CRISPR technology can be used to manipulate tumor cells to start recognizing other cancerous cells in the primary and metastasis TME as a threat and kills them [155]. Thus, it is even possible to turn cancer cells against themselves at the genetic level, which opens up additional possibilities to treat advanced malignancies.

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Microglia in the Brain Tumor Microenvironment

11

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Abstract

Microglia are the brain resident phagocytes that act as the primary form of the immune defense in the central nervous system. These cells originate from primitive macrophages that arise from the yolk sac. Advances in imaging and single-cell RNA-seq technologies provided new insights into the complexity of microglia biology.

Microglia play an essential role in the brain development and maintenance of brain homeostasis. They are also crucial in injury repair in the central nervous system. The tumor microenvironment is complex and includes neoplastic cells as well as varieties of host and infiltrating immune cells. Microglia are part of the glioma microenvironment and play a critical part in initiating and maintaining tumor growth and spread. Microglia can also act as effector cells in treatments against gliomas. In this chapter, we summarize the current knowledge of how and where microglia are generated. We also discuss their functions during brain development, injury repair, and homeostasis. Moreover, we discuss the role of microglia in the tumor microenvironment of gliomas and highlight their therapeutic implications.

Keywords

Microglia · Brain tumor · Glioma · High-grade glioma · Glioblastoma · Gliomagenesis · Macrophage · Development · Pericyte · M1 · M2 · Microenvironment extracellular matrix · Immune therapy · CD47

11.1 Introduction

Gliomas are a heterogeneous group of tumors (WHO Grade I–IV), including astrocytoma, oligodendroglioma, and ependymoma [40]. They

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represent the most common brain tumor in adults, making up 30% of all Central Nervous System (CNS) neoplasms and more than 80% of malignant CNS tumors [24]. The most common glioma in adults is Glioblastoma Multiforme (GBM), which is a highly aggressive tumor with a dismal prognosis. The current standard-of-care for adults with malignant glioma is maximal surgical resection, followed by radiation and occasionally chemotherapy. With few available therapy options and little improvement in survival over the past several decades, this disease is in dire need of a new treatment paradigm.

In recent years, immunotherapy in the treatment of CNS neoplasms has become a subject of great interest. The CNS is home to microglia, a resident intracerebral phagocyte critically involved in brain development, homeostasis, and response to injury and disease. Our understanding of the origin, function, and phenotype of microglia has dramatically advanced in the past three decades. Because of these advances, it is now possible to study microglia in the context of brain tumor pathophysiology. In this chapter, we will provide a brief introduction to the origin and functions of microglia and subsequently discuss the role of microglia within the brain tumor microenvironment. Finally, we will examine the therapeutic potential of microglia as a target or effector cell in the treatment of glioma.

11.2 Embryological Origin, Development, and Function of Microglia

11.2.1 Origin and Development

The exact origin of microglia has remained a subject of debate since their discovery in 1919 by del Río Hortega. Microglia were initially thought to derive from the neuroectoderm, along with neurons and other resident CNS cells that make up the brain parenchyma. When microglia were found to express distinctive macrophage antigens on their surface, an extracerebral hematopoietic origin was suggested [56]. In the 1990s, researchers posited that microglial progenitors arise from the mesodermal tissue in the yolk sac and migrate into the brain rudiment in rodents and humans [2, 3]. In 2010, Ginhoux et al. provided conclusive evidence that these "microglial progenitors" are, in fact, primitive macrophages [23].

By E9.5, around the start of angiogenesis, these primitive macrophages surround the developing neuroepithelium and begin migrating into the neuroectoderm by E10.5. At this stage, immature, amoeboid microglia begin populating the cerebral cortex and white matter before migrating into the telencephalon [49]. These amoeboid microglia are highly active and proliferative, expressing Ki67 and Runx1, a transcription factor involved in myeloid lineage differentiation [67]. Mature, ramified microglia can be observed throughout the fetal brain parenchyma by E28, earlier than any other glial cell [51].

Though the origin of microglia from a singular source is widely accepted, the heterogeneity of microglia has led to further investigation into their ontogeny. It has been suggested that a Hoxb8+ subset of microglia are derived from the bone marrow of adult mice or during a "second wave" of hematopoiesis during development [14].

11.2.2 Function of Microglia Within the Developing Fetal and Postnatal Brain

The development and organization of the cerebral cortex during prenatal development is dependent upon the proper balance between proliferation and inhibition of growth: too many neural precursor cells and resulting cortical neurons can have devastating effects. Microglia serve a vital role in this balance by regulating the number of precursor cells within the neural proliferative zones through phagocytosis. Importantly, evidence suggests that microglia not only phagocytose abnormal or apoptotic cells, but also viable precursor cells [16].

Microglia also play a critical role in normal postnatal brain development. Microglia are actively involved in the formation of neuronal circuitry, primarily through pruning of extraneous presynaptic material [71]. During a specific window of time in which synaptic creation and elimination are peaking, the complement cascade

proteins C1q and C3b are expressed on the surface of developing synapses. As the only known CNS-resident cell with a receptor for activated C3 (C3b), microglia are the most likely phagocytes responding to opsonized C3b [64].

During the third postnatal week (P21), microglia numbers begin to decline gradually and reach a population steady state by postnatal week six [52]. Using a multicolor "Microfetti" fate-mapping mouse model, Tay et al. concluded that every mature microglial cell is plastic and can divide to give rise to a clone [66]. Thus, the population of microglia within the developed brain is self-sustaining and maintained by a precise balance of apoptosis and proliferation in the absence of injury or disease.

11.3 Characterization of Microglia

11.3.1 Homeostasis

In addition to the controversy surrounding the ontogeny of microglia, the phenotypic characterization of microglia has been a source of debate and conjecture. In order to study their unique physiological role, it is necessary to distinguish microglia from other antigen-presenting or CNS-resident cells. Early studies of microglia within the glioma microenvironment relied on surface proteins, such as CD45, to eliminate non-immune cells and CD11b to further isolate dendritic cells. Other early studies relied on $CD45^{low}$ expression and cell morphology to distinguish microglia from other cells. However, these two characteristics are subject to change with disease and injury. Further, it is known that microglia within the tumor microenvironment (TME) can upregulate CD45, further complicating its use in this context [6].

The first functional surface protein with reported expression on the surface of mature microglia was the fractalkine CX_3CR1, which is necessary for neuron–microglia crosstalk [30]. Jung et al. subsequently established a transgenic mouse model for the study of microglia in which CX_3CR1 was replaced with a reporter GFP gene [35]. The specificity of CX_3CR1 expression in microglia was confirmed in a gene expression profile analysis across various phagocyte populations [21]. TMEM119 is another promising microglia-specific surface marker. TMEM119 may be particularly useful in studying microglia throughout development, as its expression is evident from early development through maturation [7]. A third microglia-specific surface marker, P2RY12, was identified through RNAseq and proteomics [11]. The presence of both TMEM119 and P2RY12 has been confirmed in other transcriptomic profiling studies [78].

Recent advances in single-cell RNA-sequencing has allowed for regional and temporal transcriptional-level characterization of microglia across both the human and mouse brain [44]. This technology has generated a tremendously comprehensive profile of microglial regional heterogeneity in both healthy and diseased human brain. In the healthy, homeostatic brain, *TMEM119*, *P2RY13*, *CX3CR1*, *SLC2A5*, and *P2RY12* are the most enriched genes. The expression of these "core" genes changes in the setting of demyelination and neurodegeneration. Additional genes are often enriched depending on disease state, which may establish a disease-specific genetic signature.

11.3.2 Polarization

Peripheral blood-derived macrophages are often characterized by their state of polarization. M1macrophages are considered "pro-inflammatory," while M2macrophages are considered anti-inflammatory and are involved in tissue healing [48]. This dichotomy may be an oversimplification of actual macrophage activity in vivo, which likely exists as a complex spectrum. However, this M1/M2 nomenclature is widely utilized to describe macrophage behavior [75].

The existence of a similar polarization state or activity spectrum in microglia in vivo is controversial [60]. Based upon this a priori definition of activated macrophage classification, multiple studies have similarly attempted to categorize microglia into this dichotomous relationship. However, in-depth transcriptomic analyses of microglia have failed to prove this relationship in vivo [72]. Such investigations have revealed that microglia are incredibly diverse. This con-

clusion should come as no surprise given their complex range of functions within an ever-changing microenvironment across the lifespan.

11.4 Function of Microglia

11.4.1 Homeostasis

In 2005, two landmark studies provided conclusive proof of active microglia within the homeostatic adult brain [17, 53]. Thanks to technological advances in microscopy allowing for intravital imaging, interactions between microglia and other CNS-resident cells within their native environment could be visualized for the first time. This imaging modality revealed highly active "resting" microglia, perpetually surveying their environment by extending their long processes. Microglia can detect minute changes within their microenvironment due to the presence of diverse signaling receptors for both endogenous and exogenous insults. After a threat is detected, microglia react through phagocytosis or production of inflammatory and trophic factors [19]. This response, while necessary for the protection of the CNS, can also lead to aberrant effects.

The adult brain has two sites of continued neurogenesis: the subgranular zone (SGZ) of the dentate nucleus found in the hippocampus and in the olfactory bulb. Within the SGZ, the majority of quiescent neural progenitor cells (NPCs) that become activated die as neuroblasts before becoming immature neurons. Apoptotic cells and debris are phagocytosed by unchallenged microglia within this niche. In this setting, microglia do not need to be "activated," as has been reported in other conditions. These microglia maintain their high phagocytic efficiency in the presence of inflammatory conditions and in spite of decreased neurogenesis due to age [63].

11.4.2 Injury

An in-depth discussion of microglia's role in diseases outside of brain tumors is beyond the scope of this chapter; however, the inclusion of some background information is warranted. In the setting of brain tumors, injury occurs during tumor growth and radiation therapy-induced inflammation. Both processes elicit changes within the brain that promote activation and chemotaxis of microglia.

Microglia are primarily directed to sites of injury to the blood-brain barrier (BBB) by stimulation of P2Y G-protein coupled receptors. Upon P2Y receptor stimulation by extracellular ATP, ADP, or UTP, microglia rapidly converge at the site of injury and begin proliferating [17]. In addition to stimulating the motility of microglia, ATP can induce microglial production of IL1β, TNF-α, and plasminogen [31]. Clopidogrel was administered in a mouse model to inhibit P2YR12. As a result, chemotaxis of microglia to sites of BBB injury and subsequent closure of the defect were greatly impaired [39].

Neuroinflammation is a significant complication resulting from radiation therapy for glioma [26]. In response to irradiation, it has been reported that microglia begin secreting cytotoxic and proinflammatory factors, including IL-6, TNF-α, and PGE_2 [33]. PGE_2 release by microglia has been suggested to significantly contribute to inflammation and reactive gliosis following irradiation. Irradiation-induced changes to the BBB further attract microglia, which bolster the proinflammatory milieu. While this inflammation can be pharmacologically mitigated by the use of COX-2 inhibitors, potentially positive neuroprotective functions of microglia are also stifled.

11.5 Microglia Within the Tumor Microenvironment

In 1925, Wilder Penfield published the first description of microglia in the context of glioma. Penfield, who studied the development and behavior of microglia under del Rio-Hortega, suggested that microglia within the TME play a significant role in extracellular matrix (ECM) remodeling and the destruction of by-products from this process [55]. It was not until much later that this ECM-remodeling role of microglia was suggested to be pro-tumorigenic and not merely a

reaction to tissue injury. In 2002, Bettinger et al. published a study in which Boyden chambers were used to study the effect of microglia on glioma cell migration in vitro [8]. They found that in the presence of microglia or microglia-conditioned media, glioma cells exhibited up to a threefold increase in motility (Fig. 11.1).

11.5.1 Immune-Suppression and Evasion

Increased prostaglandin synthases and upstream enzyme cyclooxygenase-2 (COX-2) have been reported in a variety of malignancies, including glioma [47]. COX-2 is an inducible enzyme involved in the conversion of arachidonic acid to prostaglandins (PGE_1 and PGE_2). COX-2 upregulation in gliomas is associated with more aggressive tumors and a worse prognosis [62]. PGE_2 blocks the activation of T cells by inhibiting T cell–dendritic cell interactions [73]. Glioma cells are known to release PGE_2. However, when glioma-released soluble factors are present, microglia may release a more significant quantity of PGE_2 [50]. In addition to inhibition of T-cell activation, PGE_2 production is associated with TNF downregulation, which is a potential mechanism of decreased TRAIL-mediated apoptosis in glioma cells [29].

Fas-Fas ligand (FasL) may also play a role in the microglia-induced immunosuppressive environment of gliomas [5]. Some level of FasL expression is seen in neurons, astrocytes, and microglia in homeostatic and pathological conditions, perhaps as a protective mechanism against inflammation. Upregulation of FasL on the surface of tumor-associated microglia leads to apoptosis of FasL-expressing T cells. Supporting this hypothesis, when FasL expression was inhibited in a murine model of glioma, leukocyte infiltration into tumors increased up to threefold.

In addition to its involvement in ECM remodeling, discussed below, TLR2 plays a role in glioma immune evasion. In a murine model of glioma, TLR2 was reported to cause downregulation of MHC Class II expression on microglia [59]. This decreases the antigen-presenting capabilities of microglia, limiting the role of CD4+ T cells in antitumor immunity.

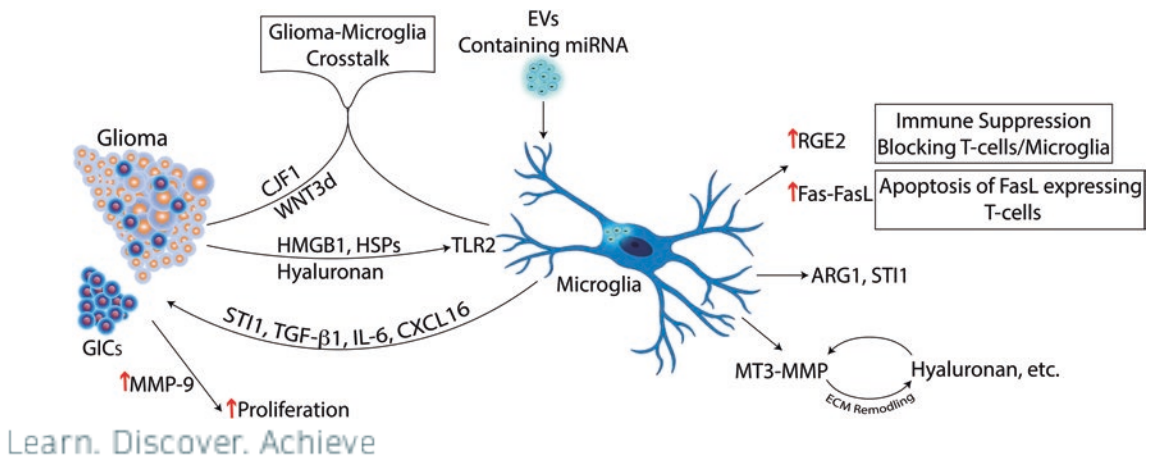

Fig. 11.1 The complex interplay between brain-resident microglia and tumor cells is highlighted by this very simplified schematic. Brain microglia and infiltrating peripheral macrophages are reprogrammed or re-educated to produce growth factors that increase glioma cell proliferation, attenuate glioma cell apoptosis, and promote tumor cell migration. Chemokines produced by glioma cells actively recruit resident microglia from the brain, as well as macrophages from the blood, through binding to their cognate receptors. In addition, glioma cells either intrinsically produce proteins that increase cytokine release and induce extensive ECM remodeling or can co-opt glioma-associated microglia to do the same. Extensive remodeling of the ECM is not restricted to physical alteration of the tissue microenvironment but also induce immune suppression by blocking the infiltration of T cells and inducing apoptosis of Fas-L-expressing T cells

11.5.2 ECM Remodeling

Microglia secrete a variety of factors to increase their motility, which is necessary to their role as resident surveyors of the CNS. In the setting of glioma, this increase in motility comes at a cost. Glioma cells exploit ECM remodeling, which is necessary to allow microglia to survey their environment and migrate toward threats.

TGF-β1, which is necessary for microglial development and activation, also promotes glioma invasiveness and progression. In a syngeneic mouse glioma model, high levels of TGF-β1 production by TAMs caused an increase in MMP-9 production by CD133+ glioma "stem-like cells" (GSLCs). This paracrine stimulation via the TGFBR2 pathway is thought to be the direct cause of glioma invasiveness into surrounding parenchyma [76]. Another modulator of MMP-9, STI1 (stress inducible protein 1), is also upregulated in the TME and its increase is associated with disease progression [13].

Another proinflammatory cytokine, IL-6, has been implicated as a protumorigenic signal in the TME of gliomas. IL-6 secretion by microglia/TAMs is induced by interaction with CD133+ GSLCs and acts as a mitogen for GSLCs [18]. The exact pathway by which this occurs is the subject of debate, with in vitro evidence suggesting TLR4 or CCR2 as the key mediator of this pathway [18, 77].

TLR2 has also been implicated as a mediator of microglia–glioma interactions [68]. TLR2 stimulation ex vivo leads to MT1-MMP upregulation in microglia, presumably leading to remodeling of the ECM and further invasion of glioma cells in vivo. The importance of TLR2 in glioma pathobiology was further confirmed in a TLR2-KO mouse model in which TLR2-KO mice showed smaller tumors and better survival than those in WT mice. A vicious cycle of TLR–glioma interactions has been posited in which microglia expressing TLR2 are stimulated by glioma-released factors (e.g. HMGB1, HSPs, hyaluronan) and microglia increase ECM remodeling by TLR2-induced MT1-MMP upregulation. This ECM remodeling further amplifies TLR2 signaling, thereby advancing glioma invasion and expansion [68]. When the MT1-MMP pathway is inhibited in vivo, glioma cell growth and invasion are attenuated [43]. This MT1-MMP upregulation in mouse microglia was not observed in human microglia; instead, MT3-MMP serves an analogous role in the human glioma microenvironment [54].

11.5.3 Microglia and Pericytes

Pericytes are multifunctional cells that wrap around endothelial cells lining the microvasculature of tumors, as well as normal capillaries and venules. These cells serve a multitude of essential blood vessel functions, including regulation of flow, clearance of cellular debris via phagocytosis, and help in the maturation and stabilization of the endothelial cells. Pericytes interact with blood vessel-associated cells via paracrine signaling and direct cell-to-cell membrane interactions. Pericytes play an essential role in maintaining the blood–brain barrier (BBB) during homeostasis and disease, and have unique functions within the CNS [4].

Pericytes have recently been classified into two subtypes [9]. Both subtypes (Type-1 and Type-2) express the classical pericyte markers CD146/PDGFRβ/NG2, but only Type-2 express Nestin. Using a syngeneic model, Birbrair et al. found that only Type-2 pericytes participate in normal and tumor angiogenesis both in vivo and in vitro [10]. Additionally, cerebral host pericytes are actively recruited to the tumor site and participate in vascular formation in a murine syngeneic orthotopic model of glioma [65].

Depending upon disease states (e.g., tumor progression or radiation-induced injury) pericytes can alter the activation state of microglia. In the setting of neuroinflammation, pericytes are stimulated by TNF-α and release proinflammatory factors, causing upregulation of iNOS and IL-1β in microglia [46]. Microglia in this setting are phagocytic and likely to be actively involved in tissue remodeling, which may also contribute to tumor invasion. Conversely, microglia can influence pericytes to promote tumor growth. Wallman et al. recently demon-

strated that M2-polarized microglia induced high expression of PDGFRβ expression in glioma cells and stimulated their migratory capacity [70]. They proposed that microglia–glioma cell-to-cell contact regulates PDGFRβ transcription, affecting the known transcription factors promoting PDGFRβ transcription. The microglial PDGFRβ expression results in a feed-forward cycle of tissue remodeling, PDGF release, and chemotaxis of angiogenic pericytes and migratory glioma cells. In a mouse model of PDGFβ-drivenhigh-grade glioma, the vast majority of tumor pericytes were found in close association with microglia rather than in conjugation with perfused vessels [70]. Thus, microglia act to actively recruit pericytes to the tumor microvasculature.

Interestingly, glioblastoma-associated pericytes have also been observed to express signals associated with immune suppression, such as IL-10 and TGF-β [61]. These signals are associated with inactive microglia, thus reducing their ability to act as phagocytes. Recent evidence suggests that targeting pericytes in glioma improves response to therapy, either by increasing drug permeability or by decreasing immune suppression [25]. Another study used an Ang-2/VEGF bi-specific antibody to target tumor angiogenesis in GBM. The authors observed increased survival and reprogramming of microglia and macrophages to an antitumor phenotype [36]. Collectively, these results suggest that reversing the immune-suppressive environment supported by pericytes is a promising treatment strategy.

11.5.4 Microglia–Glioma Crosstalk

Glioma cells are thought to secrete a variety of factors that attract microglia/macrophages to the tumor site, among other downstream effects. One such factor released by glioma cells is CSF-1 (M-CSF), a ligand of CSF-1R [37]. Glioma cells constitutively release CSF-1, which attracts TAMs to the tumor site and enhances TAM–glioma crosstalk. CSF-1R is also necessary for maintenance of the steady-state microglia population in adult mice [20]. In a preclinical murine model, inhibition of CSF-1R with the small molecule PLX3397 stopped glioma cells from invading the surrounding parenchyma, perhaps due to inhibition of microglia activation and proliferation [15].

The WNT/β-catenin pathway is important for gliomagenesis, and its expression correlates with disease progression [58]. Microglia express a variety of WNT receptors, including multiple FZDs and LRP6 [28]. Expression of WNT3a by glioma cells is thought to increase glioma–microglia interactions. Matias et al. have demonstrated that WNT3a stimulation of microglia induces their expression of ARG-1 and STI1 and pushes them toward an "M2-like" phenotype [45].

CXCL16, released by glioma cells, polarizes microglia and TAMs toward an "anti-inflammatory" phenotype in vitro. The importance of the CXCL16/CXCR6 axis for tumor growth was confirmed in a syngeneic mouse model. GL261, a mouse glioma cell line, expresses both CXCL16 and CXCR6, with the highest expression on CD133+ cells. When GL261 tumor cells with a knockdown of *CXCR6* were engrafted in WT mice, a lower tumor volume was observed compared to mice engrafted with parental GL261 cells. Additionally, stimulation of tumors with CXCL16 causes increased proliferation and invasion into surrounding parenchyma [38].

Within the tumor microenvironment, microglia readily take up extracellular vesicles (EVs) released by glioma cells. These extracellular vesicles contain proteins, lipids, and nucleic acids, which, upon uptake into microglia, can serve as intercellular messengers [41]. Recent evidence suggests that microRNA (miRNA) contained in these vesicles may affect tumor-associated microglial transcription. Specifically, miR-21 is thought to downregulate *Btg2*, which is involved in the regulation of cell proliferation [1]. *Btg2* downregulation by miR-21 leads to increased microglia proliferation, which in turn increases glioma invasiveness.

11.6 Therapeutic Implications

11.6.1 Microglia as a Therapeutic Target

The preclinical success of CSFR-1 inhibitor, PLX3397, set the stage for a clinical trial for the treatment of recurrent GBM. Although the treatment was well tolerated and effectively crossed the BBB, no clinical response was seen (NCT01349036) [12]. In another Phase II trial (NCT01790503), PLX3397 was combined with temozolomide and radiation for the treatment of primary GBM. The complete results of this study have not been published, but the available preliminary results do not suggest this treatment approach was effective.

Microglia suppression by minocycline was effective in reducing glioma growth and progression in preclinical murine models of glioma [43]. Minocycline may block expression of MT1-MMP, slowing ECM remodeling by microglia. However, this success was not replicated when minocycline was used as an adjuvant treatment in human clinical trials (NCT01580969, NCT02272270, and NCT02770378) [27]. This lack of translation is likely due to the involvement of MT3-MMP expression, rather than MT1-MMP, in human gliomas, which was discussed earlier in this chapter.

THIK-1, a constitutively active K^+ channel on the surface of microglia, has recently been reported to regulate microglial motility, surveillance, and IL-1β release [42]. IL-1β is known to be necessary for glioma angiogenesis and invasion [69]. Pharmacological inhibition of THIK-1 as a therapy in the treatment of glioma has been suggested [57]. Assessment of the efficacy of THIK-1 inhibition as a treatment strategy against glioma has thus far been limited by a lack of drugs targeting THIK-1 and the need for a better understanding of this pathway.

11.6.2 Microglia as an Effector Cell

One common mechanism of tumor cell immune evasion is the upregulation of CD47, which, upon binding to SIRPα on the phagocyte's surface, acts as an antiphagocytic "don't eat me" signal [34]. This protective mechanism has been successfully abrogated in multiple in vivo studies by the treatment of tumors with an anti-CD47 mAb [22, 32, 74]. Until recently, peripheral $CCR2^+$macrophages were the only known effector cell of this treatment. Using a transgenic mouse model constitutively expressing RFP^+ $CCR2^+$ peripheral macrophages and GFP^+ CX_3CR1^+ microglia, Hutter et al. showed for the first time that microglia are also potent mediators of the anti-CD47 response against an adult GBM xenograft [32]. Perhaps the most striking finding of this study was the strength of phagocytic response to anti-CD47 treatment even in mice with absent $CCR2^+$peripheral macrophages. With a lower inflammatory signature than activated peripheral macrophages, microglia have promising therapeutic potential in the treatment of glioma.

11.7 Conclusion and Future Directions

Despite recent advances in understanding the ontogeny and physiology of microglia, many questions remain. By necessity, microglia promote ECM remodeling, a function that has been implicated as pro-tumorigenic. However, efforts to target this process to reduce tumor invasion have been unsuccessful in clinical trials, perhaps due to inter-species differences or heterogeneity of microglia. A recent in vivo study has shown microglia to be a promising effector cell in checkpoint inhibitor therapy against GBM [32]. Future studies should aim to further characterize these microglia in order to fully exploit this potential.

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