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Alexander Birbrair *Editor*

Tumor Microenvironment

Hematopoietic Cells – Part A

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Hematopoietic Cells – Part A

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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 A* 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-the-art techniques. These advantages facilitated the 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. Eight chapters written by experts in the field summarize the present knowledge about distinct hematopoietic components during tumor development.

Rakesh K. Singh and colleagues from the University of Nebraska Medical Center discuss the role of neutrophils in the tumor microenvironment. Gilda Varricchi and colleagues from the University of Naples Federico II describe basophils in the tumor microenvironment and its surroundings. Wan L. Lam and colleagues from British Columbia Cancer Research Centre compile our understanding of the many faces of T helper cells in human tumors. Kota Iwahori from Osaka University updates us with what we know about tumoral cytotoxic CD8+ lymphocytes. Nicholas A. Zumwalde and Jenny E. Gumperz from the University of Wisconsin School of Medicine and Public Health summarize current knowledge on mucosal-associated invariant T cells in tumors of epithelial origin. Richard H. Gomer and colleagues from Texas A&M University

address the importance of fibrocytes in the tumor microenvironment. Mark R. Gillrie and colleagues from the University of Calgary focus on monocytic cells in tumors. Finally, Matthew Dysthe and Robin Parihar from Baylor College of Medicine give an overview of myeloid-derived suppressor cells in the tumor microenvironment.

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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Neutrophils in the Tumor Microenvironment

1

Lingyun Wu, Sugandha Saxena, and Rakesh K. Singh

Abstract

Neutrophils are the first responders to inflammation, infection, and injury. As one of the most abundant leukocytes in the immune system, neutrophils play an essential role in cancer progression, through multiple mechanisms, including promoting angiogenesis, immunosuppression, and cancer metastasis. Recent studies demonstrating elevated neutrophil to lymphocyte ratios suggest neutrophil as a potential therapeutic target and biomarker for disease status in cancer. This chapter will discuss the phenotypic and functional changes in the neutrophil in the tumor microenvironment, the underlying mechanism(s) of neutrophil facilitated cancer metastasis, and clinical potential of neutrophils as a prognostic/diagnostic marker and therapeutic target.

Keywords

Tumor microenvironment · Neutrophil · CXCR2 ligands · IL17 · Pro-tumor chemokines · Pro-tumor cytokines · Neutrophil-released proteases · Metastasis · Angiogenesis · NETs · NLR

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1.1 Introduction

Neutrophils or polymorphonuclear (PMN) leukocytes originate from the myeloid lineage and are the most abundant white blood cell types. Every day, nearly 10^{11} neutrophils are produced in the bone marrow and represent the most active cell type for the innate immune system [1, 2]. The name neutrophil is derived from the positive staining of both hematoxylin and eosin dyes. Neutrophils are first responders of acute inflammation and capture invading microorganisms through different mechanisms such as phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs) [2]. Until recently, host defense, immune modulation, and tissue injury were considered the only function of neutrophils [3]. However, it has been observed that other than simply killing the microbe, neutrophils function in a more complicated mechanism(s). Thus, neutrophils play a pivotal role in chronic inflammatory diseases such as cancer. Accumulating evidence suggests that neutrophils display phenotypic heterogeneity and functional versatility and are transcriptionally active cells as they respond to multiple signals by producing several inflammatory cytokines and factors that regulate the immune system [4, 5].

Current literature suggests an important role of neutrophils in the tumor microenvironment [1]. However, the pro- or antitumor nature of neutrophils in different cancer types is still

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inconclusive [6, 7]. The tumor microenvironment plays a crucial role in cancer metastasis [8] and significantly affects the therapeutic response and the overall outcome of cancer patients. This chapter will discuss the phenotypic and functional changes in the neutrophil in the tumor microenvironment, the underlying mechanism(s) of neutrophil facilitated cancer metastasis, and clinical potential of neutrophils as a prognostic/diagnostic marker and therapeutic target.

1.2 Neutrophil Life Cycle

Neutrophils compose a significant part of granulocytes and play pivotal roles during inflammation, infection, and cancer progression [9, 10]. Additionally, the neutrophils are the most abundant leukocytes in multiple species, including human. In whole blood, the proportion of neutrophils in healthy adults ranges from 30% to 70%; meanwhile, the neutrophil numbers may fluctuate under disease conditions [11]. The neutrophils are commonly short-lived cell types compared to other immune cell types (less than 24 h). Meanwhile, the half-disappeared time in the circulation of neutrophils was around 8 h [12]. However, in vivo labeling in humans with the use of $^{2}H_{2}O$ under homeostatic conditions demonstrated the neutrophil lifetime could be as long as 5.4 days [13].

1.2.1 Granulopoiesis

Neutrophils are derived from the common myeloid progenitor cells, which are the precursor of the cells in the innate immune system [14]. The common myeloid progenitor cells (Lin^{-}, $Sca\text{-}1^{-}$, $c\text{-}kit^{+}$, $IL\text{-}7R^{-}$, $Fc\gamma R^{lo}$ cell population) further differentiated into granulocyte–monocyte progenitor cells (Lin^{-}, $Sca\text{-}1^{-}$, $c\text{-}kit^{+}$, $IL\text{-}7R^{-}$, $Fc\gamma R^{hi}$ cell population), and this process requires the expression of C/EBP-α [15]. The granulocyte–monocyte progenitor cells then further differentiate into monocytes or granulocyte precursor cells [16]. The granulocyte precursor cells give rise to neutrophils by the transition from promyelocyte, myelocyte, metamyelocyte, band cells, then to neutrophils [4, 17]. The commitment to neutrophils during this stage requires the expressions of regulators such as C/EBP-ε [16, 18]. Mice without C/EBP-ε expression developed usually but failed to generate functional neutrophils and eosinophils [18].

The differentiation of neutrophils requires the gradual replacement of proliferation by differentiation in the myeloid progenitor cells [19] and also requires the neutrophil granulopoiesis. The granulopoiesis is divided into three processes: firstly, the formation of primary granules; secondly, the beginning of nuclear segmentation, the appearance of secondary granules, and exiting from the cell cycle; and thirdly, the final segmented nuclei together with tertiary and secretory granules [19]. Additionally, the neutrophil primary granules formed at myeloblast to promyelocyte stage; the secondary granules can be found at the myelocyte to metamyelocyte stages; the tertiary granules are detected at the band cell stage; meanwhile, only mature neutrophils are with secretory vesicles [4].

1.2.2 Neutrophil Dynamics: From Bone Marrow to the Circulation

As the first responder in inflammation or infection, neutrophils react quickly and mobilize out of the bone marrow reserve by crossing the sinusoidal endothelium and in an abluminal to the luminal direction [20, 21]. The mobilization of neutrophils from bone marrow to circulation is delicately regulated by factors such as granulocyte colony-stimulating factor (G-CSF), CXCR2, and CXCR2 ligands, together with CXCR4 and CXCR4 ligands [22]. The mobilization of neutrophils requires the upregulation of G-CSF and CXCR2 signaling, together with the downregulation of CXCR4 signaling [20]. The liver and spleen are the primary organs for the neutrophil clearance in the circulation [23]. However, recent studies showed that bone marrow also functions as the sites of neutrophil construction. According to the radiolabel of the senescent neutrophils in mice model, the senescent neutrophils were 32% in

bone marrow, 29% in the liver, and 31% in the spleen [24]. The homing of neutrophils to bone marrow requires the upregulation of CXCR4 in neutrophils [25], and the neutrophils backing to the bone marrow will be under apoptosis and digested by bone marrow macrophages [23, 26]. During inflammation, the neutrophils can also be taken up by the macrophages at the sites of inflammation [25]. Under disease conditions such as inflammation or cancer, the half-life of neutrophils are very different, which varies from shorter life spans to longer life spans [26, 27], and also accompanied with dysregulated neutrophil numbers, morphologies, and differentiation states in the circulation system [10, 15, 28]. In cancer cases, the existence of subpopulations of neutrophils made the situation even more complicated [4].

The neutrophils mobilized to the sites where they are required; once the neutrophils arrived, they phagocyte and release chemokines, cytokines, and proteases, and then they are cleaned up by other immune cells, including the macrophages. The dynamic of neutrophils sounds like a straightforward story. However, the population of neutrophils in the human body is not simple. The neutrophils may behave differently according to various stimuli [4]. More research is required to reveal the heterogeneity of neutrophils in a complex disease like cancer.

1.3 Neutrophil Population in Health and Disease

As the most abundant leukocyte in the innate immune system, neutrophils can compose 70% of the leukocyte population [4]. Mature neutrophils are stored in large numbers in the bone marrow. The pool of mature neutrophils is termed as the bone marrow reserve. Typically, individual mice usually have a total number of 120 million neutrophils: meanwhile, in humans, the neutrophil numbers in bone marrow can reach up to 5×10^{10} to 10×10^{10} neutrophils/day, with a total blood granulocyte pool of 65×10^7 cells/kg [26]. In humans, the overall numbers of neutrophil fluctuation depend not only on the blood volume of each individual but also on the ethnic groups, age, health stage, and smoking status. For instance, African American participants possessed significantly lower neutrophil counts in the blood (mean differences, 0.83×10^9 cells/L; $P < 0.001$) relative to white participants, whereas relative to the white participants, Mexican-American participants had higher neutrophil numbers (mean differences, 0.11×10^9 cells/L; $P = 0.026$). Smoking status is positively linked with neutrophil numbers in all three ethnic groups [29].

As discussed previously, the homeostasis of neutrophil numbers requires the sophisticated counterbalance of both positive and negative feedback signaling. The activation of positive neutrophil mobilization pathways spontaneously stimulates the regulation of negative neutrophil mobilization pathways to strike the delicate balance of neutrophil numbers in the human body, for example, feedback inhibition of SOCS3 to STAT3-mediated G-CSF-induced neutrophil granulopoiesis [30]. In disease conditions such as infection, inflammation, congenital disease, and cancer, the homeostasis of neutrophils is disturbed temporarily, or even for a long term, which leads to the variation of neutrophil numbers.

Neutrophils are the crucial regulators during microbe infection. The neutrophils can clear up the microbe by mechanisms including phagocytosis, ROS/RNS production, and NET formation [31]. The number of neutrophils increased dramatically (around 5×10^6 to 10×10^6 increase in neutrophil numbers in 1.5 h) in the peripheral blood once activated by LPS of *Escherichia coli*. Additionally, neutrophils in response to LPS challenges quickly altered their expression profiles such as initialization of the expressions of cytokines such as TNFα and downregulation of surface receptors such as FcγRII and TLR4 [32].

Similar to infection, increased neutrophil numbers in the blood are a commonly accepted clinical feature in inflammatory diseases. The acute inflammatory response induced by a thioglycolate injection resulted in the 4.5-fold increases of neutrophil numbers in blood within hours (original numbers of neutrophils in 6–8-week BALB/cJ mouse circulation: 1.5×10^9/L) [23]. The number of neutrophils also fluctuates during different disease

conditions, such as neutropenia seen in patients with solid tumor malignancies filtrated in the bone marrow or patients with lymphoproliferative malignancies such as natural killer cell lymphomas [33]. Radiation therapy used on multiple sites of cancer patients' bone marrow can also result in neutropenia [33]. Additionally, neutropenia observed in cancer patients is mostly due to the administration of chemotherapy drugs [33]. However, higher levels of neutrophils are found in the blood of patients with advanced cancer, and this might be due to the upregulation of G-CSF in multiple cancer types [34]. Moreover, the association study in 5782 tumors and 25 types of cancers showed higher PMN numbers indicated lower survival rates in cancer patients [35], implying the neutrophils are not favorable immune cell type to the majority of the cancer patients.

1.3.1 Neutrophil Frequency and Phenotype in Cancer

The life cycle of neutrophils begins with production in the bone marrow, followed by entry into the circulation and migration into the site of infection or inflammation, and finally being cleared by tissue-resident macrophages [25]. During this life cycle, neutrophils can undergo different phenotypic as well as functional changes in the frequency of circulatory neutrophils during tumor progression [4].

One such change is a well-established observation that peripheral neutrophils in the blood are increased in cancer patients [1]. However, this increase in the peripheral neutrophil count is not limited to the cancer condition but is observed under other conditions as described previously. Scientists have tried to use this observation in the form of neutrophil to lymphocyte ratio (NLR) and correlated it with cancer patient outcomes. A meta-analysis study, by Templeton et al. in 2014, compiled observations from 100 such studies with different types and stages of cancer, which revealed that NLR > 4 is associated with lower overall survival rates [36]. A limitation of measuring NLR is that it does not give us any mechanistic insight into the condition.

1.3.2 Low-Density Neutrophils (LDNs)

LDNs are a group of immature cells with banded or segmented nuclei and myelocyte-like cells [4], which represents another subpopulation of neutrophils found in low-density fraction by the Ficoll density gradient [37]. Unlike neutrophils, which are found in the high-density fraction at the bottom of the tube, LDN was associated with many pathological disorders [4] such as asthma or AIDS. However, the LDNs gained attention because of their association with cancer [38, 39]. Specific molecular markers, immunosuppressive characteristics, and functions have not been defined for LDNs, thus leading to different schools of thought about their origin. One possibility is that these immature cells are released from bone marrow during chronic inflammation or cancer or that LDNs are activated neutrophils that have undergone degranulation and, therefore, have a reduced density [4].

1.3.3 Myeloid-Derived Suppressor Cells (MDSCs)

Apart from the increase in the number of neutrophils in cancer patients, there is also an increase in immature myeloid cell populations [38]. These morphologically immature cells with a band or myelocyte-like nuclei [40] are named MDSC because of their immunosuppressive nature and pro-tumor behavior. MDSC has been found to play a critical role during tumor progression [40]. MDSCs are heterogeneous populations which represent cells in different differentiated stages and can be divided into two categories: the granulocytic (G-MDSC) whose morphology and phenotype are similar to neutrophils and represent 80% of the whole MDSC population, and the monocytic (M-MDSC) whose morphology and phenotype are similar to the monocytes and represent around 20% of the whole MDSC population [41]. Other than malignant tumors, MDSC could also appear in infections, autoimmune diseases, diabetes [42], and tuberculosis [43, 44]. The conventional role

of MDSCs is usually involved in immunosuppression, and the T cell functions are the main target [15, 44].

In mice, both neutrophils and G-MDSC are defined by $CD11b^{+}Ly6G^{+}$, whereas monocytic MDSCs are defined by CD11b+Ly6C+ [4, 45]. Even though human MDSCs are more complicated with six different markers used to define G-MDSC ($CD11b^{+}CD14^{-}CD15^{+}CD33^{+}CD66b^{+}HLA\text{-}DR^{-}$), unfortunately, still there is no clear distinction between neutrophils and G-MDSC [4]; further investigations are warranted to define whether neutrophils and G-MDSC are subpopulations or separate cell types. A possible way to isolate neutrophils from MDSCs is through centrifugation using a standard Ficoll gradient, as neutrophils are high-density cells in comparison with G-MDSCs (enriched in the low-density fraction) [15].

1.3.4 Tumor-Associated Neutrophils (TANs)

The changes in circulatory neutrophils are also reflected in the infiltration of neutrophils inside the tumor [4]. Neutrophils inside the tumor are called TANs. TANs can play dual roles in cancer progression, and according to the pro- or antitumor properties of these cells, we can classify TAN into N1 and N2 types.

Similar to the classification of tumor-associated macrophages in the tumor microenvironment (M1 as antitumor macrophage and M2 as pro-tumor macrophage), Fridlender et al. proposed the concept of polarization of TANs as N1 with antitumor and N2 with pro-tumor properties. Fridlender presented N1 TANs by blocking transforming growth factor-beta (TGF-β2) in tumor-bearing mice, which were functionally and morphologically different from N2 TANs [7]. N1 TANs were toxic to cancer cells by using the oxygen radical-dependent mechanism, with increased expression of tumor necrosis factor-alpha (TNF-α), intercellular adhesion molecule 1 (ICAM-1), and FAS. Additionally, N1 were morphologically different from N2 TANs by having hyper-segmented nuclei [7]. On the other hand, N2 TANs with characteristic circular nuclei had pro-tumor characteristics as they suppressed T cell immunity by expressing increased levels of arginase as well as other pro-tumor factors such as CCL2, CCL5, neutrophil elastase (NE), and cathepsin G (CG). Differences in the nuclei of N1 and N2 neutrophils also indicate a possibility that they represent different maturation stages rather than phenotypic subtypes [5].

Various stimuli present in the tumor microenvironment can activate neutrophils to different phenotypes. Thus the primary binary classification of neutrophils is an oversimplification. Neutrophils can have different levels of plasticity with N1 and N2 as extreme phenotypes in complex diseases such as cancer. At present, there are no suitable markers, which define N1 and N2 in humans. Another significant limitation of this system is that the work, which leads to the emergence of the N1 and N2 TANs concept, has only been performed in murine models and is yet to be replicated in humans [46].

An interesting question is whether TANs can also be associated with survival outcomes. Recently, tumor transcriptomics-based computational study partially answered this question by revealing that TANs are the most adverse prognostic cell population in over 3000 solid tumors, comprised of 14 different cancer types [35]. On the other hand, chemotherapy decreased cancer patients' neutrophils in peripheral blood (neutropenia) numbers, which is a sign of effective chemotherapy treatment. However, to overcome neutropenia, patients are often treated with G-CSF, which has been shown to promote breast cancer metastasis [47]. Thus, it is an interesting question of whether the administration of G-CSF post-chemotherapy is beneficial or detrimental to final clinical outcomes [6].

1.4 Functions of Neutrophils in the Tumor Microenvironment

Generally, TANs represent a pro-tumor factor in different tumor types [1, 10, 48] and are associated with the least favorable overall survival for solid tumor patients in comparison with other leukocytes

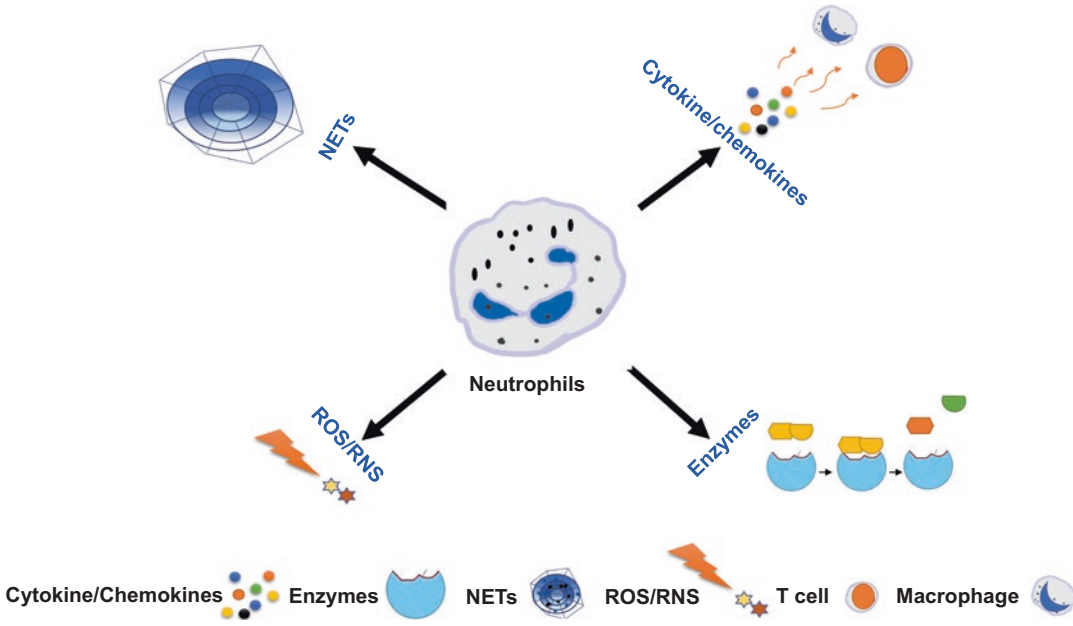

Fig. 1.1 Neutrophil-released factors in the tumor microenvironment. The neutrophils in the tumor microenvironment may facilitate cancer progression through secretion of proteases such as NE and CG, chemokines, and cytokines (which leads to the recruitment of other pro-tumor immune cells or T cell immunosuppression), together with the release of RNS/ROS. Neutrophils also facilitate cancer progression through formations of NETs

present in the tumor [35]. In this section, we will discuss different functions associated with neutrophil biology in the light of the tumor microenvironment (Fig. 1.1). The majority of cases discussed in this section reported neutrophils played pro-tumor roles through multiple mechanisms; nevertheless, there are few reports that indicate the antitumor role of neutrophils in cancer [10].

1.4.1 Neutrophil-Released Reactive Oxygen Species (ROS)/Reactive Nitrogen Species (RNS)

As discussed in previous sections, one of the primary functions of neutrophils is to eliminate infection at the inflammatory site during an immune response [3], with phagocytosis being one of the essential killing mechanisms [49]. Neutrophils engulf the pathogen and form a phagosome which later fuses with a lysosome [50]. For killing the pathogen, NADPH oxidase present in neutrophils' granules changes the pH of the fused phagosome and lysosome structure, which is now termed as phagolysosome [51] and results in the production of reactive oxygen species (ROS) through the respiratory burst [52]. However, the released ROS by neutrophils in the tumor microenvironment usually play a pro-tumor role by damaging the DNA bases [53], which results in mutations [53, 54]. In general, the tumor microenvironment has a high level of ROS, which can not only initiate cancer but also lead to epithelial damage and inflammation inside the tumor [1], increasing cellular proliferation, suppressing immune cell [34, 55], chemoresistance [56], and EMT, which leads to an invasive phenotype in multiple cancer types [57]. Hydrogen peroxide, one of the ROS, can regulate different cell signaling pathways, which are important in cellular biology, such as the PI3K/Akt, IKK/NF-κB, and MAPK/Erk1/2 pathway, by acting as secondary messengers. However, hydrogen peroxide production by neutrophils is also considered as one of the mechanisms of eliminating tumor cells [58]. For instance, neutrophils, after physical contact the with cancer cells, can secrete hydrogen peroxide,

resulting in tumor cell death by Ca^{2+} influx through the TRPM2 Ca^{2+} channel [59]. Similarly, in TANs, interaction between the Met receptor and its ligand, the hepatocyte growth factor (HGF), triggered the release of nitric oxide to eliminate the tumor cells [60]. Therefore, the level of ROS/RNS production by neutrophils will dictate their pro- or antitumor behavior in the tumor microenvironment [10].

1.4.2 Neutrophil-Secreted Cytokines and Chemokines

Neutrophils respond to different stimuli present in the tumor microenvironment by releasing various cytokines and chemokines [61–63]. These neutrophil-secreted cytokines and chemokines will not only determine the pro- or antitumor response on other tumor-associated stromal cells, but the neutrophil will also educate itself for a pro- or antitumor behavior [7, 10, 48, 64]. For instance, neutrophil-secreted factors, such as oncostatin M (OSM) or TGF-β into the tumor microenvironment, have been shown to polarize the macrophage towards a pro-tumor phenotype (M2 type) [64, 65]. Similarly, nitric oxide secreted by neutrophils has been shown to suppress T cell cytotoxicity [66].

Many recent studies have tipped the balance of neutrophil-secreted chemokines and cytokines towards a pro-tumor behavior. For example, Queen et al. have shown that co-culture of neutrophils with human breast cancer cell lines triggered the release of oncostatin M (OSM) by neutrophils, thereby facilitating angiogenesis through the induction of vascular endothelial growth factor (VEGF) [67]. In another breast cancer study [68], neutrophil-released TGF-β has also been shown to promote tumor cell resistance to gemcitabine by inducing epithelial to mesenchymal changes in tumor cells [69].

TANs have also been shown to secrete pro-inflammatory cytokines into the tumor microenvironment, such as IL17, CXC, and CC chemokines [10, 70–76]. These pro-inflammatory cytokines, such as IL17, can promote tumor progression by acting directly on pancreatic cancer cells and inducing them with stem cell-like features [77] or indirectly promoting cancer progression by facilitating neutrophil mobilization through upregulation of CXCR2 ligand expression (Fig. 1.2) [76]. Other pro-inflammatory factors, such as CXC chemokines, are well-known for the recruitment of neutrophils to the tumor site [75]. De Oliveria et al. demonstrated higher levels of CXCL8 during an inflammatory response in a zebra fish model, which resulted in higher numbers of neutrophil recruitment [78]. Thus neutrophil-secreted CXCL8 in the head and neck cancer suggests a feedforward loop for neutrophil recruitment in the tumor microenvironment [70]. Apart from CXC chemokines, a number of cancer studies report that neutrophils secrete a significant amount of CC ligands [72, 73], which are chemoattractants for monocyte, regulatory T cells, and other immune cell populations [79]. There are reports suggesting a correlation between the higher levels of CC ligands with lower survival rates for cancer patients [72, 74]. However, it is important to consider that neutrophils are not exclusive in the tumor microenvironment for the secretion of tumor-promoting factors. Other immune cell population present in the tumor microenvironment, such as macrophages [80], lymphocytes [80] (including Th17 cells [81] and γδ T cells [66]), B cells [82], are also known to secrete tumor-promoting factors. As discussed previously, the proliferation and maturation of neutrophils in bone marrow require cytokines and chemokines such as G-CSF [83], CXCR2 chemokines, and IL17. Multiple cell types in the tumor microenvironment contribute to the pool of G-CSF, CXCR2 ligands, and IL17. In the tumor microenvironment, the primary source of G-CSF includes cancer cells [84], fibroblasts [85], macrophages, and lymphocytes [86], while the significant contributors of IL17 include Th17 cells [87] and γδ T cells [88].

1.4.3 Neutrophil-Released Enzymes

The versatile functions of neutrophils are dedicated to the different cytoplasmic granules present inside a mature neutrophil. These cytoplasmic

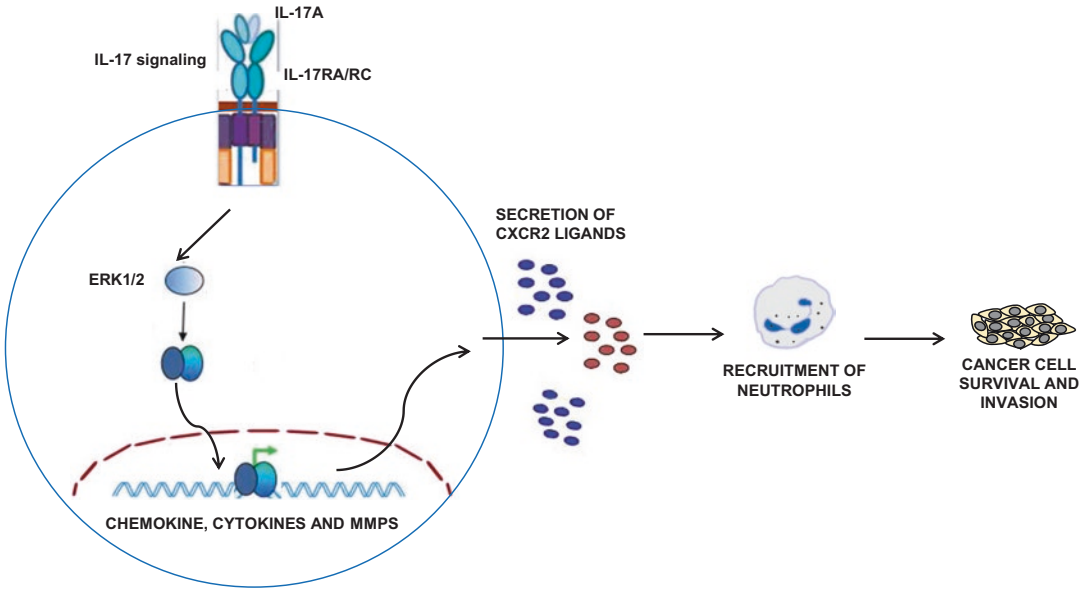

Fig. 1.2 The potential mechanism regarding IL17-induced chemokine/cytokine secretion. IL17 enhanced expression of ERK signaling in multiple cell types, including cancer cells, which results in upregulation of cytokines and chemokines such as CXCR2 ligands. The upregulation of CXCR2 ligands results in positive neutrophils mobilization to the tumor sites

granules are releasable membrane-bound organelle with three major types present in neutrophils: the primary or azurophil, secondary or specific, and tertiary or gelatinase granules [89]. Primary granules are associated with microbicidal functions, whereas secondary and tertiary are associated with extracellular matrix interaction and modification. Various proteases derived from neutrophil granules such as CG, NE, and matrix metalloprotease 9 (MMP-9) play a pro-tumor role through mechanisms [10], including epithelial to mesenchymal transition and extracellular matrix (ECM) remodeling [90], which lead to enhanced metastasis.

NE and CG are serine proteases, which are pre-synthesized in promyelocytes in the bone marrow and then stored in neutrophil primary granules. Both NE and CG are found to be entrapped in negatively charged NETs because of their high isoelectric points [91]. Recent studies suggest that NE can upregulate EGFR/MEK/ERK signaling [92], and phosphatidylinositol 3-kinase (PI3K) signaling [93], and have also been shown to promote cancer cell proliferation and therapy resistance [94, 95]. Also, higher levels of NE in metastatic breast cancer patients are associated with a poor response to tamoxifen therapy [96]. Similarly, inhibition of NE prevents the release of pro-cancer factor TGF-α, thereby suppressing the growth of gastric carcinoma cells [97], as well as suppressing tumor progression in breast and prostate cancer [95, 98].

Interestingly, cancer cell lacking endogenous NE expression can uptake NE through the neuropilin-1 receptor [99]. CG has been reported to facilitate the E-cadherin-dependent aggregation of MCF-7 mammary carcinoma cells [100], by using insulin-like growth factor-1 signaling [101]. Also, Akizuki et al. showed that higher levels of NE correlated with lower survival rates in breast cancer patients, thereby demonstrating the potential of NE as an independent prognostic marker [102]. Additionally, NE can be utilized as a therapeutic target for colorectal cancer [103], whereas CG can serve as a potential therapeutic target for breast cancer patients [104].

Unlike serine proteases such as CG and NE, MMP-9 is stored in neutrophil tertiary granules [105] and requires zinc as a cofactor for its catalytic activity [106]. An active MMP-9 can remodel

the extracellular matrix by the degradation of extracellular proteins [106], facilitating membrane cleavage [107], and activate pro-tumor factors such as TGF-β [108]. TNF, TGF-β, and VEGF [105, 109, 110] are known to regulate the release of MMP-9 by neutrophils. MMP-9 is a pro-angiogenic factor, which promotes resistance to sunitinib (a common chemotherapy drug for multiple cancer types, in renal cell carcinoma patients) [111]. MMP-9 has been explored extensively in breast cancer. MMP-9 has high expression levels in breast cancer tissue in comparison with the healthy tissue [112] and has higher levels present in metastatic breast tumors [113], which suggests an association of MMP-9 with breast cancer development and tumor progression. MMP-9 significantly promotes angiogenesis and metastasis in triple-negative breast cancer [114] and predicts poor survival in hormone-responsive small mammary tumors [115]. All these studies strengthen the potential of MMP-9 as a prognostic biomarker for breast cancer patients.

Neutrophils also release MMP-8 (collagenase-2), which generates chemotactic Pro-Gly-Pro (PGP) tripeptide and is important for neutrophil mobilization [108]; however, unlike other proteases, the role of MMP-8 in tumor progression is controversial. A study in breast cancer has shown an inverse correlation between MMP-8 expression and lymph node metastasis [116]; however, a recent study by Thirkettle et al. demonstrated that MMP-8 can upregulate pro-tumor cytokines, IL-6 and IL-8, thus suggesting pro-cancer behavior [117]. In other cancer types, such as melanoma and the lung carcinoma, the antimetastatic role of MMP-8 has been shown through enhanced adhesion to type I collagen and laminin-1 present in the extracellular matrix [117]; on the contrary, higher levels of MMP-8 in the serum of colorectal cancer patients predict lower patient survival [118].

1.4.4 Neutrophil Extracellular Traps (NETs)

NET can be defined as a network of extracellular fibers composed of a DNA scaffold decorated with granule-derived proteins such as NE, CG, MMP-9, and others. For the first time, Brinkmann et al. reported the formation of NET cell death or NETosis, as a new killing mechanism used by neutrophils apart from traditional phagocytosis or degranulation [119]. Initially, neutrophils were reported to form NETs for eliminating the pathogen through rupture of the cytoplasmic membrane, on activation by stimuli such as CXCL8 or lipopolysaccharide (LPS) [120], which also leads to the generation of ROS by NADPH oxidase [121]. Neutrophils have also been demonstrated to form NETs, without undergoing lytic death, through the release of mitochondrial DNA [121–123].

Similar to other pathological diseases, there are reports suggesting that neutrophils' NET formation in the tumor microenvironment plays an active pro-tumor role during disease progression [10, 124, 125]. There is an increase in the level of NETs in plasma of cancer patients (pancreatic cancer, colorectal cancer, lung cancer, and bladder cancer) in comparison with healthy controls [126, 127]; similarly, Ewing's sarcoma patients with metastasis have higher levels of NETs [128], suggesting that NETs could be considered a potential diagnostic marker target. A recent study has demonstrated that NETs can directly function on tumors cells by enhancing their proliferation through activating NF-κB signaling pathways [129].

1.5 Role of Neutrophil in Tumor Initiation, Growth, and Metastasis

Neutrophils, an active player in the tumor microenvironment, have been found to play a prominent role in tumor development, growth, and metastasis [130, 131]. Before discussing the different mechanisms through which neutrophils participate in the process of metastasis (Fig. 1.3), we will introduce the metastatic cascade. Metastasis is defined as the migration of cancer cells from the primary tumor site of origin to nearby or distant sites, which lead to the formation of secondary growth of tumor cells. Despite improvements in the treatment of a resectable tumor, metastasis is the driver of mortality. Metastasis is not a random process [132], but a

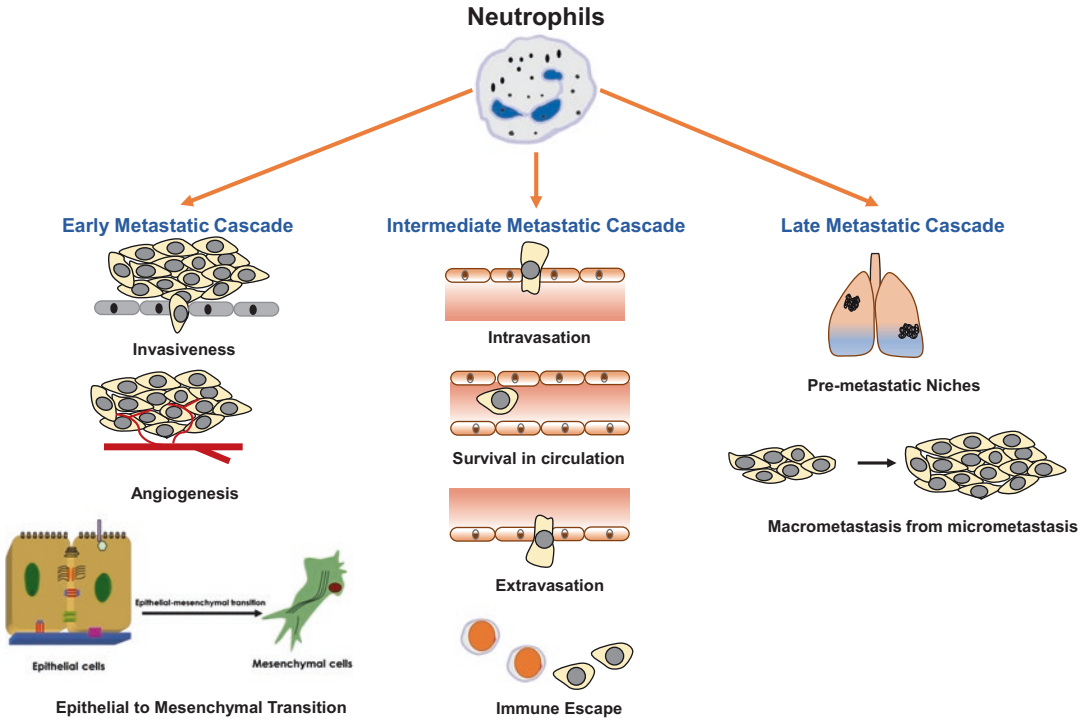

Fig. 1.3 Putative mechanism(s) regarding neutrophils facilitated cancer progression. The neutrophils can facilitate cancer progression by multiple mechanisms, including metastasis

result of the successful completion of multistep biological events, known as the invasion–metastasis cascade [133, 134]. This cascade involves local invasion, entry of cancer cells from a well-defined tumor boundary into the surrounding tumor stroma, followed by a second step intravasation, and the entry of invasive cancer cells into the lumen of lymphatic or blood vessels. After intravasation, the survival ability of tumor cells in the circulation is tested [133]. After surviving this part of their journey, the tumor cells are arrested at a distant organ site. The tumor cells must then extravasate by either involving microcolony growth, which ruptures the wall of the surrounding vessels, or by penetrating the vessel through the endothelial cells and pericytes. Additionally, the tumor cells must survive at the distant site to form micrometastases. After the successful survival of cancer cells in the foreign microenvironment, reinitiation of cancer cell proliferation is necessary for the formation of macrometastasis. Evidence suggests that one or more of the steps of the invasion–metastasis cascade are rarely completed successfully, thereby making the process of metastasis a highly inefficient one.

1.5.1 The Role of Neutrophils in the Early Metastatic Cascade

Neutrophils are well-known to support the early metastatic cascade. However, there is a growing body of literature, which suggests that neutrophils play important roles in all steps of the metastatic cascade [1, 135, 136]. One of the fundamental properties of tumor progression and the beginning of metastatic cascade is the gain of invasive behavior in tumor cells. Neutrophils aid the invasive properties of tumor cells by secreting a wide variety of proteases such as MMP-8, MMP-9, CG, and others. These proteases are well-known to degrade a variety of structural proteins present in the extracellular environment

[136–138]. Serine proteases are also known to trigger angiogenesis [139, 140] by releasing factors such as VEGF [141]. Also, an orthotopic breast cancer mouse model suggests that neutrophils induce tumor cells with the production of MMP-12 and MMP-13 [142].

Moreover, neutrophils use myeloperoxidase to produce hypochlorous acid, which can also activate the secreted "inactivate" form of proteases [143]. Recently, TGF-β derived from neutrophils were shown to induce epithelial to mesenchymal transition, a process known to increase the invasiveness of cells in pulmonary adenocarcinoma cells [144]. Until now, we have discussed neutrophil contact-independent mechanisms inducing invasiveness in the tumor cells; however, there are reports which suggest that contact-dependent signaling between TLR4 receptors on neutrophils and hyaluronan on hepatocarcinoma cancer cells promote cellular migration [136, 145]. Similarly, the interaction of neutrophils with gastric cancer cells promotes cellular migration and invasion by inducing epithelial to mesenchymal transition [146].

1.5.2 Role of Neutrophils in Intermediate Metastatic Cascade

In this section, we will discuss how neutrophils support the intermediate steps of the metastatic cascades, such as intravasation, the survival of tumor cells, and extravasation. With the invasive property, tumor cells face a new set of challenges, such as the absence of cell to extracellular matrix interactions, increase in shear forces, and escaping immune cell surveillance to successfully survive the intermediate steps of metastatic cascade [136]. Formation of cell aggregates enhances tumor cell survival [147, 148] and neutrophils aid this process with the help of cathepsin G [149] or cellular markers like CD11a and CD11b [150].

Neutrophils play a role in helping tumor cells escape immune surveillance by contributing to tumor acidosis through the mobilization of H+-pump ATPase, which can hamper the antitumor activity of natural killer (NK) cells and T cells [5]. Recent studies suggest that the presence of neutrophils blunt NK cell [151] or leukocyte activation [146], thus promoting intravascular survival. Lastly, neutrophils can both directly and indirectly aid tumor cell crossing the endothelium lining [136]. Numerous studies have shown the co-localization of neutrophils with tumor cells by expression of selectin molecules present on the neutrophil cell surface, thus facilitating adhesion of tumor cells and neutrophils to the endothelium [135, 152, 153]. Not only the expression of selectins and integrins but also NETs promote metastasis through endothelium and tumor cell adhesion [125, 154–156]. All these studies suggest neutrophil as an important mediator between tumor cells and endothelium lining. However, it remains undetermined whether neutrophils act as a direct bridge between tumor cells and endothelium or neutrophils secrete endothelium activating factors which increase adherence of tumor cells to activated endothelium [136].

1.5.3 Role of Neutrophils in the Late Metastatic Cascade

Successful macrometastasis formation in a new environment is the endgame for a tumor cell. Neutrophils play a central role in the formation of premetastatic niches by arriving at the metastatic site before the arrival of tumor cells and favoring tumor cell survival and proliferation [8, 136, 157]. Neutrophils accumulate in premetastatic niches either through CXCR2-dependent [158, 159] or CXCR4-dependent mechanism [160]. Neutrophil-derived factors such as oncostatin M [67], elastase [161], and S100A8 and S100A9 [157] trigger tumor cell proliferation. Apart from providing tumor growth-promoting factors, neutrophils can also drive the formation of macrometastasis from micrometastasis by inducing angiogenesis [136, 162, 163], which is similar to the need for vascular supply in primary tumor growth. In multiple cancer types,

neutrophil can also support the final establishment of metastasis through immunosuppression of T cells [1, 158, 159, 164].

1.6 The Clinical Significance of Neutrophils

The role of neutrophils in tumor biology is now widely recognized, and its potential as a biomarker or therapeutic agent is being explored. Based on the above discussion, neutrophils function in the tumor microenvironment through the release of ROS, the formation of NET, and the secretion of cytokines. Moreover, neutrophils are not considered neutral towards cancer progression anymore [1, 10]; they encompass plastic phenotype with two extreme polarization state and possess functional heterogeneity [4, 6]. This opens up the potential for therapeutic intervention, but only after overcoming the limitations of our current research tools.

1.6.1 Neutrophils as a Potential Biomarker for Cancer Patients

Most of these studies indicate that higher NLR in cancer patients is correlated with poor clinical outcomes in cancer patients [10, 35, 165–172]. The detection of NLR is easy and inexpensive, as the detection of NLR can be performed using blood analyses [172]. NLR has been proposed as an attractive indicator for treatment decision and risk for cancer patients. However, there are several limitations for NLR application into clinics. It is challenging to translate NLR for personalized prognosis and treatment decision for the individual patient, as cutoff NLR varies for high-risk or low-risk classification in different cancer cases; meanwhile, neutrophil numbers vary between different individuals [1]. One approach to deriving maximum information from NLR is to perform analysis on a regular basis over time, and these results may be combined with other neutrophil-activating and neutrophil-polarizing factors such as IL-1β and IL-17 in serum [102, 103, 173]. Increased NLR value over time may indicate reoccurrence or progression of the disease.

Neutrophils inside the solid tumors emerged as the least favorable cell populations regarding cancer patient survival [35]. Additionally, compared to healthy tissue, there is a significant increase in the number of TAN in the tumor, which indicates the possibility of using TAN for prognostic tools [110]. However, similar to NLR, an association of TAN with different tumor progression is variable. Markers used to identify TAN (cell morphology, myeloperoxidase, and others) are not expressed uniformly in all tumor types. Thus TAN isolation method is complicated. Thus, an advanced technique is required for TAN use in the clinic.

1.6.2 Neutrophils as Therapeutic Targets in Cancer Patients

Neutrophils and neutrophil-released factors could be considered as a potential prognostic marker for cancer patients. There are several possibilities to target neutrophils, such as preventing neutrophil expansion in the bone marrow, inhibiting neutrophil trafficking to the tumor, preventing polarization of neutrophils towards N2 type, and lastly targeting neutrophil-associated mediators [1].

Clinically, one of the most appropriate ways of targeting neutrophils is by utilizing agents treating autoimmune and inflammatory diseases. CXCR2 inhibitor, AZD5069, reduced absolute neutrophil counts in bronchiectasis patients [174], but AZD5069 as a therapeutic agent for cancer patients is still under investigation. Similarly, clinical trials with reparixin (CXCR1 and CXCR2 inhibitor) [175] are ongoing in cancer patients [1]. Other molecules that stimulate the expansion of neutrophils are IL-23 and IL-17 [176]. There are approved antagonists for IL-23 and IL-17, which are tested in psoriasis [176]. More preclinical studies are warranted to move these drugs in cancer patients [176].

An example of targeting neutrophil-associated factors comes from the application of NE inhibitor to cancer patients. The NE inhibitor, sivelestat

sodium hydrate, has been used in patients suffering from thoracic esophagus carcinoma [177]. Another example is the elimination of NET by DNase I digestion, and this method is being tested in several ongoing clinical trials but not in cancer [178]. More clinical studies are needed to evaluate the therapeutic effects of targeting neutrophils in cancer patients. Another concern which still needs to be addressed is the use of G-CSF or GM-CSF to resolve chemotherapy-induced neutropenia, as G-CSF polarizes neutrophils towards pro-tumor behavior. In the future, neutrophil targeting approaches can be combined with anticancer therapies such as chemotherapy and immunotherapy, such as T cell checkpoint inhibitor [1]. Combinational therapy may be more beneficial to cancer patients rather than targeting neutrophil alone.

1.7 Concluding Remarks

Neutrophils are emerging as an important player in the tumor microenvironment, together with a new realization of their role, which extends beyond just microbial elimination during an immune response. The fact that host-related factors are more accessible to target than genetically unstable cancer cells is also bringing new excitement to this field. A plethora of literature now eliminates the myth of neutrophil neutrality and short life span in tumor biology, with evidence accumulated to show that neutrophils are not only important in different stages of tumorigenesis but also in the metastatic cascade. The remarkable ability of neutrophils showing phenotype plasticity, which results in a heterogeneous population, necessitates the urgency to understand the concert between different possible factors, such as metabolite availability or hypoxia in the tumor microenvironment; meanwhile, governing neutrophil maturity and polarization may lead to pro- or antitumor behavior.

With the possibility of such diverse neutrophil phenotypes, simple depletion of neutrophils is not an answer for therapeutic intervention. Thus, we need to fill in the knowledge gap by identifying differentiable markers for various neutrophil populations. Advanced techniques like single-cell sequencing and single-cell fate mapping may provide us with an answer to identify the polarization state of neutrophils in the future. Moreover, neutrophils and the partner in crime interact with cancer cells and may disguise cancer cells from other immune cells by immunosuppression and provide advantages to overcome metastatic cascade. Neutrophils cytoplasmic content and degranulation process plays an important role in introducing new membrane proteins on the surface of neutrophils and dictates interaction between neutrophils and cancer cells, together with other cell populations in the tumor microenvironment.

Thus, understanding these processes on the molecular level will open the potential therapeutic avenues. Additionally, there exists crosstalk between neutrophils and other immune cells in the tumor microenvironment, similar to the conditions in other inflammatory diseases. Thus, neutrophil inhibitors used in inflammatory diseases may find a role in cancer biology as well. Very importantly, much of our understanding about neutrophil biology in tumor microenvironment comes from mouse models, because of the limitation of short survival period of neutrophils in ex vivo culture. Before extrapolating these mouse model-based findings in clinics, we should be critical about the species-based differences in neutrophils, including tumor evolution and immunity. Our current literature has not merely increased our understanding and excitement about neutrophil biology in the tumor microenvironment but also promoted more research to find a cure for cancer patients in the future. Still, an extensive research effort is needed to completely delineate the neutrophil-facilitated tumor progression and metastasis and translate experiment data into clinical use for cancer patients.

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2 Basophils in Tumor Microenvironment and Surroundings

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Abstract

Basophils represent approximately 1% of human peripheral blood leukocytes. Their effector functions were initially appreciated in the 1970s when basophils were shown to express the high-affinity receptor (FcεRI) for IgE and to release proinflammatory mediators (histamine and cysteinyl leukotriene C_4) and immunoregulatory cytokines (i.e., IL-4 and IL-13). Basophils in the mouse were subsequently identified and immunologically characterized. There are many similarities but also several differences between human and mouse basophils. Basophil-deficient mice have enabled to examine the in vivo roles of basophils in several immune disorders and, more recently, in tumor immunity. Activated human basophils release several proangiogenic molecules such as vascular endothelial growth factor-A (VEGF-A), vascular endothelial growth factor-B (VEGF-B), CXCL8, angiopoietin 1 (ANGPT1), and hepatocyte growth factor (HGF). On the other side, basophils can exert anti-tumorigenic effects by releasing granzyme B, TNF-α, and histamine. Circulating basophils have been associated with certain human hematologic (i.e., chronic myeloid leukemia) and solid tumors. Basophils have been found in tumor microenvironment (TME) of human lung adenocarcinoma and pancreatic cancer. Basophils played a role in melanoma rejection in basophil-deficient mouse model. By contrast, basophils appear to play a pro-tumorigenic role in experimental and human pancreatic cancer. In conclusion, the roles of basophils in experimental and human cancers have been little investigated and remain largely unknown. The elucidation of the roles of basophils in tumor immunity will demand studies on increasing complexity beyond those assessing basophil density and their microlocalization in TME. There are several fundamental questions to be addressed in experimental models and clinical studies before we understand whether basophils are an ally, adversary, or even innocent bystanders in cancers.

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Abbreviations

ANGPTs	Angiopoietins
APCs	Antigen-presenting cells
BAFF	B-cell-activating factor
BSA	Bovine serum albumin
CAFs	Cancer-associated fibroblasts
CML	Chronic myeloid leukemia
DCs	Dendritic cells
DMBA	7,12-Dimethylbenz(a)athracene
DT	Diphtheria toxin
FcεRI	High-affinity receptor
LTC_4	Cysteinyl leukotriene C_4
PAF	Platelet-activating factor
PD-1	Programmed cell death-1
PDAC	Pancreatic ductal adenocarcinoma
PD-L1	Programmed death-ligand 1
PGD_2	Prostaglandin D_2
TDLNs	Tumor-draining lymph nodes
Th2	T helper 2
TME	Tumor microenvironment
T_{reg}	T regulatory cell
TSLP	Thymic stromal lymphopoietin
uPA	Urokinase plasminogen activator
VEGF-A	Vascular endothelial growth factor-A

2.1 General Aspects

Basophils, first described by Paul Ehrlich in 1879 [1], represent less than 1% of human peripheral blood leukocytes. Their effector functions were not appreciated until the 1970s when basophils were shown to express the high-affinity IgE receptor (FcεRI) for IgE and release of histamine [2–4]. The difficulties in purifying sufficient numbers of human basophils and the absence of basophil-deficient animals hampered the advance of basophil research. Basophils share some characteristics with mast cells, including the presence of similar but distinct basophilic granules in the cytoplasm [5], surface expression of FcεRI, and release of proinflammatory mediators, such as histamine and cysteinyl leukotrienes [6, 7]. Basophils circulate in the peripheral blood and are rarely present in peripheral tissues unless inflammation occurs in mice [8] and in humans [9–13]. The life span of basophils is relatively short (≅2.5 d in mice) [14], and therefore newly generated basophils are constantly supplied from the bone marrow to the peripheral blood [15]. Mouse basophils were clearly characterized by Dvorak et al. as a granular cell population in murine bone marrow with some ultrastructural characteristics similar to mammalian basophils [16]. Recent development of basophil-deficient mice [17–19] has enabled us to examine the in vivo roles of basophils in a variety of immune settings.

In the past, basophils were regarded erroneously as blood-circulating mast cell precursors that could migrate to peripheral tissues and mature into tissue-resident mast cells. There is compelling evidence that basophils and mast cells are distinct cell lineages differentiated from hematopoietic stem cells in the bone marrow [7, 20, 21]. Like other myeloid lineages, basophils develop from hematopoietic stem cells in the bone marrow [15]. It has been suggested that human basophils develop from common basophil-eosinophil progenitors [22, 23]. IL-3 is the most important growth and activating cytokine for human and mouse basophils [24]. Murine basophils can be generated in vitro by culturing bone marrow cells in the presence of IL-3 or thymic stromal lymphopoietin (TSLP) [25]. IL-3-elicited and TSLP-elicited murine basophils differ in terms of gene expression, phenotype, and functions, suggesting heterogeneity among the basophil population [26]. Basophils can be detected in mice deficient for both IL-3 and TSLP signaling, indicating that neither is essential for

basophil development. It has been suggested that approximately 10% of human basophils express the TSLP receptor [7] and the TSLP increases histamine release from basophils [27]. By contrast, a collaborative study demonstrated that human basophils do not express the IL-7Rα [28] and do not respond to TSLP [28, 29]. The above findings emphasize some of the differences between human and mouse basophils [7, 30, 31].

2.2 Basophils as a Source of Cytokines, Chemokines, Angiogenic Molecules, and Granzyme B

Human basophils, differently from mast cells, produce a restricted profile of cytokines [7, 21]. A variety of immunologic stimuli induce the release of substantial amounts of IL-4 [32–36]. Activated human basophils also produce IL-13 [37–39]. IL-4 and IL-13 are potent mediators for type 2 immunity with both overlapping and distinct functions [40]. Schroeder and collaborators first demonstrated that human basophils secrete IL-3 exerting strong autocrine priming effects on these cells [24]. Activation of human basophils induces the release of several proangiogenic molecules. For instance, immunologically activated human basophils release VEGF-A, the most potent proangiogenic molecule [41, 42]. Angiopoietins (ANGPTs) are a family of growth factors that play a role in angiogenesis and lymphangiogenesis [43]. Human basophils constitutively express ANGPT1 and ANGPT2 mRNAs [44]. ANGPTs were detected in cytoplasmic vesicles of basophils and their activation induced the release of ANGPT1. Human basophils can also release hepatocyte growth factor (HGF) [45]. The latter findings suggest that human basophils can modulate angiogenesis and lymphangiogenesis [42, 46, 47]. Basophils also produce CXCL8 [48] which can contribute to epithelial-to-mesenchymal transition in tumors [49]. Interestingly, human [50] and mouse (Schiavoni and Mattei, unpublished observations) basophils release granzyme B which possesses cytotoxic effects on cancer cells [51, 52]. Mouse, but not human [53], basophils represent an important source of TNF-α [18]. Mouse [54, 55], but not human, basophils produce IL-6 [48]. These findings highlight some of the similarities and differences between human and mouse basophils as a source of cytokines.

2.3 Are Mouse and Human Basophils Antigen-Presenting Cells (APCs)?

Activated human [32, 33] and mouse basophils [25, 53] produce large quantities of IL-4. In mice it has been shown that, under certain experimental conditions, basophils migrate to lymph nodes and secrete IL-4, promoting the differentiation of naive $CD4^+$ T cells toward Th2 cells [56]. Three independent groups reported that murine basophils express MHC class II (MHC-II) and costimulatory molecules (i.e., CD80, CD86, and CD40), which are necessary for antigen presentation to naive T cells [57–59]. These studies suggested that mouse basophils can function dually as antigen-presenting cells (APCs) and IL-4-producing cells, driving Th2 cell differentiation, even in the absence of classical APCs [i.e., dendritic cells (DCs)]. By contrast, subsequent studies demonstrated the critical role of DCs, but not basophils, in Th2 differentiation [60–62]. Thus, the functional significance of basophils as APCs remained highly controversial [63]. The group of Karasuyama recently reported an unexpected mechanism of MHC-II acquisition by mouse basophils [64]. These cells express little or no MHC-II by themselves, but they can capture peptide-MHC-II complexes from DCs through a mechanism called trogocytosis, in a cell contact-dependent manner. Thus, MHC-II-dressed mouse basophils can provide peptide-MHC-II complexes and IL-4 to naive $CD4^+$ T cells that in turn differentiate to Th2 cells. This finding tends to reconcile, at least in part, some of the discrepancies observed in previous studies.

Resting human peripheral blood basophils express little or no HLA-DR, but they can be induced to express it when activated in vitro with

stimuli, such as cytokines [59, 65–67]. Nevertheless, human basophils did not induce antigen-specific T-cell proliferation [67–69]. Human peripheral blood basophils do not express HLA-DR and co-stimulatory molecules (CD80 and CD86) [68, 70, 71]. It would be interesting to investigate whether human basophils can acquire peptide-HLA-DR complexes from DCs through trogocytosis and function as APCs, as observed with murine cells.

2.4 Basophil-Deficient Mice

For decades the absence of basophil-deficient mouse hampered the advance of basophil research. During the last years several models of basophil-deficient mice have been developed. Initial experimental studies employed in vivo administration of antibodies that deplete basophils in mice to study the role of these cells. These antibodies recognize either the FcεRI (clone MAR-1) [72] or the activating receptor CD200 receptor 3 (CD200R3) (clone Ba103), which are both expressed by basophils and mast cells. Although both antibodies can efficiently deplete basophils in vivo, they can also activate mast cells and can cause anaphylaxis [62, 73]. Furthermore, the depletion of basophils by Ba103 is FcR dependent and might therefore activate myeloid cells and natural killer (NK) cells [74]. MAR-1 also depletes a subset of FcεRI-expressing DCs [60]. Several functions have been attributed to basophils based on studies using these depleting antibodies [59, 75]. For example, this experimental approach has led to the conclusion that basophils have a role as APCs during Th2 cell polarization [58, 59]. Similarly, it has been suggested that basophils can cause IgG_1-mediated anaphylaxis [76] and that they contribute to protective immunity against *Trichuris muris* [57]. More recently, several new mouse strains with constitutive or diphtheria toxin (DT)-inducible depletion of basophils have been generated [77]. Genetically engineered basophil-deficient mouse models include $Mcpt8^{DTR}$ [8], Mcpt8Cre [62], Basoph8 [78], BAS-TRECK [79], and $Runx1^{P1N/P1N}$ mice [80]. These new genetically engineered basophil-deficient mice allowed to deepen our knowledge on the in vivo role of these cells in different pathophysiological conditions.

2.5 Peripheral Blood Basophils and Human Cancer

Basophilia is frequently observed during the accelerated phase of chronic myeloid leukemia (CML) [81]. The transcription factor IKAROS is absent or reduced in bone marrow blasts from most patients with advanced CML [82]. Forced expression of the dominant-negative isoform of IKAROS in $CD34^+$ cells from patients with chronic CML resulted in disrupted IKAROS activity and enhanced ability to differentiate into basophils [82]. The latter findings suggest that a loss of IKAROS contributes to myeloid disease progression in CML with basophilia. It has been reported that basophils from patients with CML specifically express abundant HGF, which promotes CML cell expansion in an autocrine fashion [45]. A study using a mouse model of CML demonstrated that basophil-like leukemia cells contribute to CML development by providing the chemokine CCL3 [83]. In this model CML development induced a marked accumulation of basophil-like leukemia cells that produced CCL3 in the bone marrow. Basophil-derived CCL3 negatively regulated the proliferation of normal hematopoietic stem/progenitor cells and supported the predominant expansion of leukemia cells [84]. Indeed, basophil depletion prevented the development of CML. Basophilia appears to be an independent risk factor for evolution of myelodysplastic syndrome to acute myeloid leukemia [85, 86].

Circulating basophils have also been associated with certain solid tumors [87]. For instance, basopenia appears to be associated with worse prognosis of colorectal cancer [88]. By contrast, peripheral blood basophils have no predictive role in breast cancer [89] and oral squamous cell carcinoma [90]. In a mouse model of breast cancer, circulating basophils appeared to exert a protective role in the formation of metastases [91].

2.6 Basophils in Tumor Microenvironment of Human Lung Adenocarcinoma

There is compelling evidence that basophils can migrate into the sites of inflammation in mice [8] and in humans [9–12, 92]. Basophils can also be recruited into TMEs by several chemotactic molecules produced by tumor and immune cells [6, 41, 93–97] (Fig. 2.1). Lavin and collaborators compared the immune landscape in peripheral blood and in TME of patients with early (stage I) lung adenocarcinoma by single-cell analysis [13]. Basophils were present in both TME and noninvolved lung parenchyma as early as in stage I adenocarcinoma. They found quantitative and qualitative differences in basophils present in peripheral blood when compared to cells in TME and noninvolved lung tissue. Interestingly, a small percentage of basophils in TME and in noninvolved lung parenchyma expressed PD-L1. This study elegantly demonstrated, as early as in stage I disease, that lung adenocarcinoma lesions were accompanied by marked alteration of immune cells, including basophils, in TME.

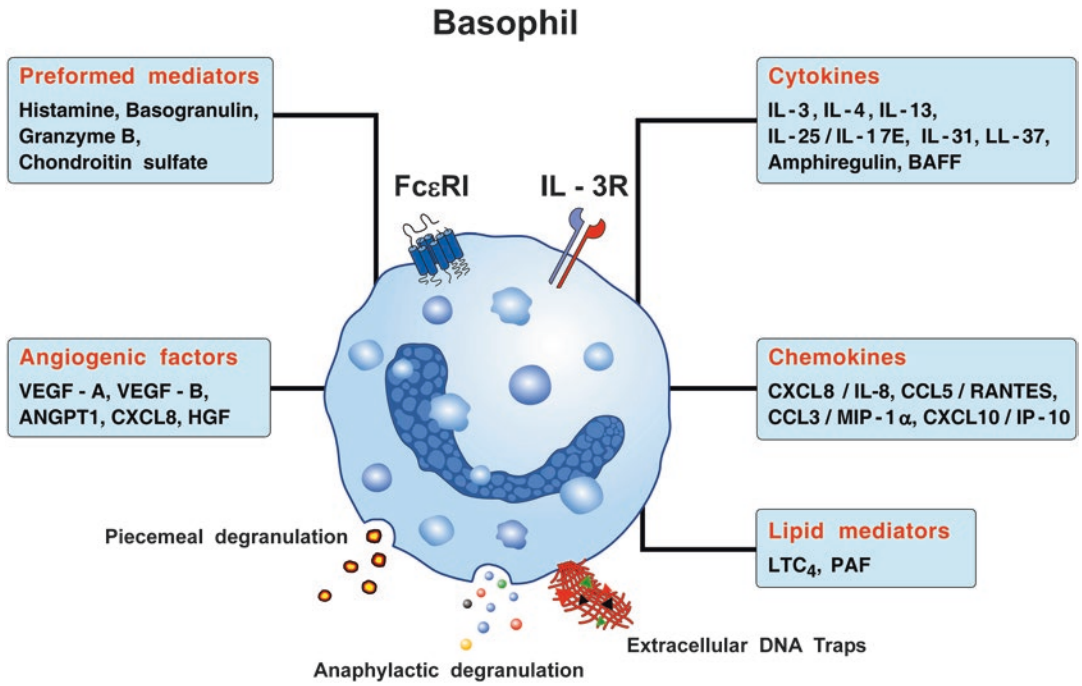

Fig. 2.1 Proinflammatory and immunoregulatory mediators released from human basophils. These cells express a variety of receptors that regulate their development, homeostasis, and effector functions on the cytoplasmic surface. Basophils express the high-affinity receptors for IgE (FcεRI) which bind IgE with high affinity. These cells also express the α-chain (IL-3Rα/CD123) and a common βc (CD131) that bind IL-3, which plays a major role in basophil development [137, 138]. Secretory granules of basophils contain histamine complexed with chondroitin sulfate, basogranulin [139], granzyme B [50], and tryptase at levels of less than 1% of human mast cells. Immunologic activation of basophils leads to the release of histamine, basogranulin, and granzyme B and the production of IL-4 [32, 33, 35, 36, 140], IL-13 [37–39], IL-3 [24], VEGF-A and VEGF-B [41], ANGPT1 [44], and HGF [45]. Basophil activation induces the de novo synthesis of cysteinyl leukotriene C_4 (LTC_4) [141] and platelet-activating factor (PAF) [142]. Human basophils produce several chemokines [48] and, under specific conditions, can release IL-25/IL-17E, IL-31, LL-37, amphiregulin, and B-cell-activating factor (BAFF) [7, 143–145]. Human basophils activated by a variety of IgE- and non-IgE-mediated stimuli rapidly release membrane-free granules to the external microenvironment (anaphylactic degranulation). Basophils infiltrating the sites of inflammation can release packets of granule contents (piecemeal degranulation) [5]. Human basophils are also able to form extracellular DNA traps upon IL-3 priming and subsequent immunologic activation [146, 147]

A recent elegant study found that during lung development basophils acquire a unique phenotype, due to local exposure of specific signals (i.e., IL-33, GM-CSF), which regulates alveolar macrophage maturation and function [55]. The authors found that basophils represented a significant proportion of immune cellular composition during lung development. These cells broadly interacted with immune (e.g., monocytes, macrophages, neutrophils, ILCs) and nonimmune cells (e.g., endothelial cells, epithelial cells, fibroblasts) through the production of several cytokines (e.g., IL-4, IL-6, IL-13, TNF-α). Interestingly, the gene expression profile of lung basophils differed from that of blood-circulating basophils and was characterized by a unique gene signature including *IL6, IL13, Cxcl2, Tnf, Osm, and Ccl4*. The authors attributed the modulation of phenotype of lung basophils mainly to IL-33 and with minor contribution of GM-CSF. Moreover, lung basophils promoted M2 polarization of lung macrophages. Finally, the authors reported that basophils isolated from both the lung and the TME of mice implanted with B16 melanoma cells expressed several cytokines (e.g., *IL4, IL6, Osm, IL13*). This important study demonstrates that lung basophils acquire the expression of several cytokines and growth factors, critical for immune and nonimmune cell functions due to the exposure to lung-specific signals. Collectively, the results of these two important studies indicate that tissue-resident basophils can acquire distinct features from peripheral blood basophils and can play important roles in lung development and presumably in human lung cancer.

Schroeder and collaborators recently demonstrated that highly purified human basophils release histamine and secrete IL-4/IL-13 when co-cultured with the epithelial cell line, A549, an adenocarcinoma of lung origin [29]. This study further determined that an IgE-binding lectin (expressed on the A549 cells) was likely responsible for this activation of basophils, with all indicators pointing to galectin-3. Indeed, a follow-up study from the same group showed that A549 clones generated to be deficient in galectin-3 protein no longer activated basophils for these responses [98]. In addition, basophils co-cultured with microspheres coated with galectin-3 protein [but not bovine serum albumin (BSA) or galectin-9] likewise secreted IL-4/IL-13. However, when added exogenously as a soluble protein, galectin-3 only marginally activated basophils and only at relatively high concentrations, suggesting that the lectin may better facilitate cellular activation when immobilized on a matrix, whether epithelial cells (A549) or microspheres. While more studies are needed, the significance of these findings currently points to the fact that galectin-3 is now implicated as a biomarker and/or factor contributing to the pathogenesis of a wide range of conditions, particularly in cancer and cardiovascular disease, but also in autoimmunity (lupus erythematosus), wound healing, and asthma [99]. Evidence that galectin-3 modulates the immune responsiveness of basophils (and potentially other IgE-bearing cells) could offer novel insight into how these cells might be activated in the absence of specific IgE/allergen interactions. Indeed, this mechanism of activation could prove relevant to the recent findings showing IL-4-producing basophils in lupus erythematosus [100] and cancer [101].

2.7 Basophils in Experimental Melanoma

The role of basophils has been evaluated in a mouse model of melanoma [102]. A model of T_{reg} depletion was associated with increased production of IL-3, which caused basophil infiltration in the TME. This model was associated with complete rejection of tumors, which was found to be dependent on chemokines (i.e., CCL3 and CCL4) produced by infiltrating basophils. These chemokines caused tumor infiltration of $CD8^+$ T cells, which presumably exerted cytotoxic effect. Administration of MAR-1 (i.e., anti-FcεRI) to deplete basophils prevented the rejection of tumors. The authors

concluded that basophils were required for tumor eradication. As previously mentioned, MAR-1 can partially deplete also mast cells and DCs that express FcεRI. Thus, the role of basophils in melanoma rejection will need to be confirmed using genetically engineered basophil-deficient mice.

In a series of ongoing experiments, we have investigated the direct antitumor activities of bone marrow-derived murine basophils following activation with IL-33, an alarmin known to activate the tumoricidal functions in eosinophils [103]. We observed that activation of basophils with IL-33 results in upregulation of granzyme B transcripts (Fig. 2.2a) and surface expression of the degranulation marker CD63 (Fig. 2.2b). In addition, when IL-33-activated basophils were co-cultured with B16.F10 murine metastatic melanoma cells, we found substantial restriction of tumor cell growth, compared to melanoma cells cultured with resting basophils (Fig. 2.2c). These preliminary observations suggest that under proper stimulation basophils can acquire tumoricidal properties and indicate that basophils may orchestrate antitumor immune responses at multiple levels. These interesting findings deserve further investigations in vitro and in vivo.

2.8 Basophils in Experimental and Human Pancreatic Cancer

Ann Dvorak demonstrated the presence of basophils in the stroma of pancreatic cancer showing distinctive ultrastructural morphological features of piecemeal degranulation [5]. The role of basophils and their mediators in experimental and human pancreatic cancer has been elegantly investigated by Protti and collaborators [101]. In a large cohort of pancreatic ductal adenocarcinoma (PDAC), they found basophils expressing *IL4* in tumor-draining lymph nodes (TDLNs) of PDAC patients. Basophils in TDLNs served as an independent prognostic biomarker of patient survival after surgery. The authors confirmed the recruitment of basophils in TDLNs in a mouse model of pancreatic cancer. In this model activated cancer-associated fibroblasts (CAFs) released TSLP which activated DCs. These cells induced IL-3 release from $CD4^+$ T cells. IL-3 activated basophils to produce IL-4. CCL7, produced by DCs and $CD14^+$ monocytes, was, at least in part, responsible for basophils migration from arterial blood into TDLNs. In this setting, basophils were the major source of IL-4 presum-

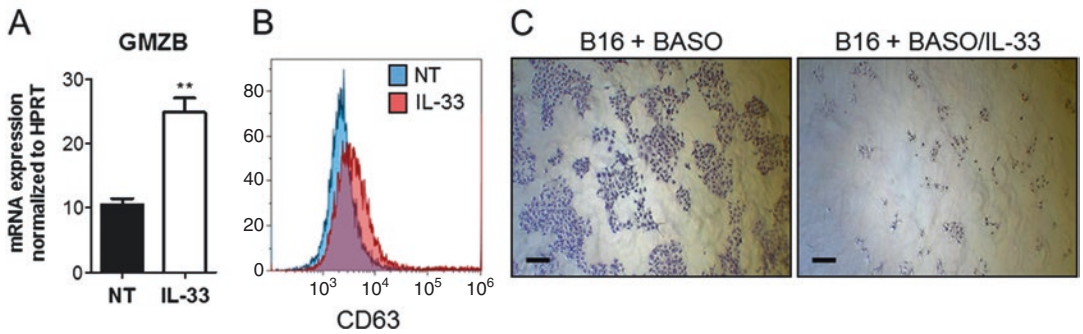

Fig. 2.2 Activation of basophils with the alarmin IL-33 promotes tumoricidal functions. Basophils were generated by culture of murine bone marrow cells in medium containing IL-3 (2 ng/mL) for 10 days. Basophils were then harvested and cultured in medium alone or with added IL-33 (100 ng/mL) for 18 h. (**a**) qRT-PCR analysis of expression of granzyme B. Mean expression values in triplicate samples ± SD are shown. $^{**}P < 0.01$, Wilcoxon's *t* test. (**b**) Flow cytometry analysis of surface CD63 expression. (**c**) Growth of B16.F10 melanoma cells after 24 h co-culture with basophils alone or with added IL-33 (100 ng/mL). At the end of the co-culture, adherent tumor cells were stained with crystal violet to visualize tumor-covered area. Scale bar, 150 μm

ably contributing to both Th2 and M2 polarization in pancreatic cancer. The authors concluded that basophils and their mediator (i.e., IL-4) play a relevant pro-tumorigenic role in PDAC progression.

2.9 Conclusions and Outstanding Questions

Although peripheral blood basophils represent less than 1% of human leukocytes, there is compelling evidence that they can infiltrate the site of inflammation [9, 10, 18, 92, 104]. Importantly, basophils can be found in TME in human gastric cancer [11, 12] in early lung adenocarcinoma [13] and in PDAC [101]. Moreover, basophils can be identified in experimental melanoma [102] and in TDLNs in a model of pancreatic cancer [101]. The mechanisms regulating the trafficking of basophils into TDLNs, and their contributions to the evolving microenvironment of the metastatic niche, remain poorly understood. Single-cell RNA-seq will be necessary to characterize the basophils in TDLNs.

Human basophils release several angiogenic factors such as VEGF-A and VEGF-B [41], CXCL8 [49], ANGPT1 [44], and HGF [45]. CXCL8 and TNF-α can induce epithelial-to-mesenchymal transition [49, 105]. IL-4 and IL-13 can favor M2 polarization of tumor-associated macrophages [106, 107]. On the other side, basophils can exert anti-tumorigenic effects by releasing granzyme B [51, 52] and TNF-α [18] that possess cytotoxic effects on cancer cells. Moreover, histamine promotes DC maturation and can inhibit experimental tumor growth [108–110]. These findings suggest that basophils have the potential to play an anti-tumorigenic or a pro-tumorigenic role in tumor immunity (Fig. 2.3).

There is increasing evidence that basophils in peripheral blood differ from those found in TME [13]. This is not surprising because peripheral blood basophils circulate at physiological pH and normoxia, whereas peritumoral and intratumoral basophils are embedded in a hostile microenvironment characterized by increased levels of lactate, PGE_2, adenosine, IFN-α, and a low pH [111–114], which can profoundly influence basophil phenotype [115, 116]. Studies on basophil biology are usually performed at physiological pH and normoxia. It will be important to investigate how the tumor milieu activates/modulates the production of mediators and the expression of receptors in tumor-infiltrating basophils. Analyses of basophils in TDLNs have only recently began [101]. High-dimensional analysis, particularly single-cell RNA-seq, will be necessary to characterize basophils in TDLNs and in TME.

There is increasing evidence that immune cells in TME can play different roles in early and late stages of tumorigenesis [115, 117–120]. Basophils have been identified in the immune landscape of tumor and noninvolved lung tissue in early lung adenocarcinoma [13]. The hypothesis that basophils and their mediators play diverse roles in different phases of tumor initiation and growth deserves investigation.

Several models of basophil-deficient mice have been described. Initial studies were conducted using administration of antibodies (i.e., MAR-1 and Ba103) that transiently deplete basophils [72, 121]. However, these models can interfere with other immune cells [60, 74]. Recently, several mouse strains with constitutive or inducible depletion of basophils have been described. Studies using antibody-depleted basophils [102] and genetically engineered models [101] yielded apparently discordant findings on the role of basophils in cancer. Results obtained with basophil-deficient mouse models should be interpreted with caution because even new mouse mutants showed some hematological abnormalities. Perhaps, future studies attempting to evaluate the basophil role in a complex and heterogeneous disorder, such as cancer, should be performed using more than one model of basophil deficiency.

IgE is an ancient and highly conserved immunoglobulin isotype found in mammals. There is evidence that IgE has evolved to provide protection against infections and environmental toxins [6, 18, 122, 123]. Basophils express FcεRI which binds IgE [2, 4]. IgE has been suggested to play a protective role in tumor growth [124, 125]. In a

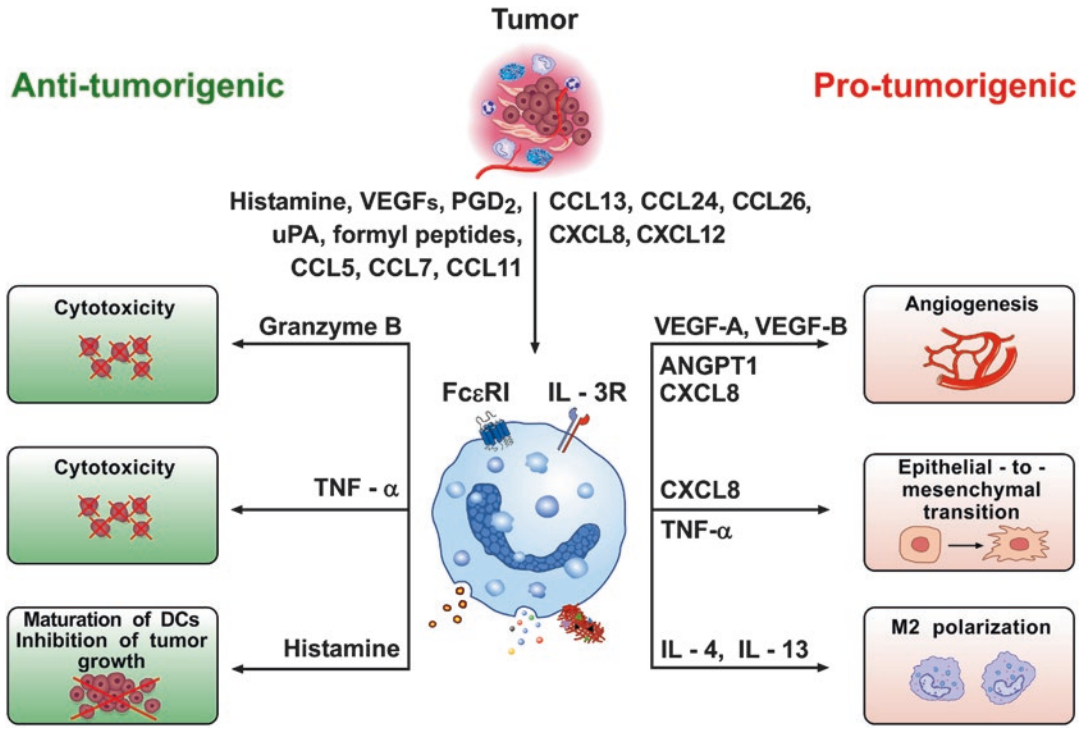

Fig. 2.3 Basophils can be recruited into tumor microenvironments (TMEs) by several chemotactic molecules [e.g., VEGFs, histamine, prostaglandin D_2 (PGD_2), urokinase plasminogen activator (uPA), formyl peptides, CCL5, CCL7, CCL11, CCL13, CCL24, CCL26,CXCL8, CXCL12] produced by tumor or immune cells [6, 41, 93–97]. Basophils in the TMEs can exert anti-tumorigenic and/or pro-tumorigenic roles. Basophils can exert direct tumor cytotoxic effects via granzyme B [50] and TNF-α [18]. Histamine promotes dendritic cell (DC) maturation and inhibits tumor growth [108–110]. On the other side, basophils represent a potentially major source of several angiogenic molecules (VEGF-A, VEGF-B, ANGPT1, CXCL8, and HGF) [44, 45, 48]. CXCL8 and TNF-α can induce epithelial-to-mesenchymal transition [49, 105]. IL-4 and IL-13 can favor M2 polarization of tumor-associated macrophages [106]

mouse model of skin tumorigenesis, topical exposure to a common xenobiotic and carcinogen (i.e., 7,12-dimethylbenzatracene: DMBA) caused a potent IgE response that provided protection against carcinogenesis [126]. Although the mechanism by which IgE inhibited tumor growth in this model remains to be determined, the authors speculated that it "might involve soluble factors and/or cytotoxicity mediated by basophils." Further studies should investigate the role, if any, of IgE-mediated activation of basophils in experimental and human tumors.

Tumor cells evade host immune attack by expressing several checkpoints, such as programmed cell death-1 (PD-1) and PD-1 ligands (PD-L1 and PD-L2) [127, 128]. Monoclonal antibodies targeting the PD-1/PD-L1 pathway unleash antitumor immunity and have revolutionized the treatment of cancer [129, 130]. PD-L1 is also expressed on the surfaces of various immune cells such as macrophages and DCs [13, 131–133], mast cells [13, 134, 135], and basophils in TME [13]. Recent evidence indicates that PD-L1 expressed in immune cells within TME, rather than on tumor cells, plays an essential role in immune checkpoint blockade therapy [132, 133]. Moreover, secreted PD-L1 can interfere with immune checkpoint therapy in cancer [136]. An interesting task will be to investigate the role of PD-L1$^+$ basophils in TME in the context of immune checkpoint blockade.

In conclusion, the roles of basophils in experimental and human cancer have been little investigated and are currently largely unknown.

The elucidation of basophils in tumor immunity will demand studies on increasing complexity beyond those assessing basophil density and their microlocalization in TME. There are several unanswered fundamental questions to be addressed in experimental models and clinical studies before we understand whether basophils are an ally, adversary, or even innocent bystanders in cancers.

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3 Janus or Hydra: The Many Faces of T Helper Cells in the Human Tumour Microenvironment

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Abstract

CD4+ T helper (T_H) cells are key regulators in the tumour immune microenvironment (TIME), mediating the adaptive immunological response towards cancer, mainly through the activation of cytotoxic CD8+ T cells. After antigen recognition and proper co-stimulation, naïve T_H cells are activated, undergo clonal expansion, and release cytokines that will define the differentiation of a specific effector T_H cell subtype. These different subtypes have different functions, which can mediate both anti- and pro-tumour immunological responses. Here, we present the dual role of T_H cells restraining or promoting the tumour, the factors controlling their homing and differentiation in the TIME, their influence on immunotherapy, and their use as prognostic indicators.

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Keywords

Tumour immune microenvironment · Neoplasia · Effector cells · Lineage · Differentiation · Cytokines · Chemokines · T helper cells · Regulatory T cells · T follicular helper cells · Dual function · Immune evasion · Immunotherapy

3.1 Introduction

Although most of our knowledge on the adaptive immunological response against cancer relies on cytotoxic CD8+ T cells, T helper (T_H) cells are also key regulators of the tumour immune microenvironment (TIME) [1]. T_H cells each possess the cell cluster of differentiation surface marker CD4 and thus are also known as CD4+ T cells. T_H cells assist other lymphocytes through the activation of other immune cells such as cytotoxic T cells and macrophages. Specific subsets of T_H are also known to contribute to the maturation of B cells into plasma cells and memory B cells. To perform specialized functions such as these, a naïve T_H cell must be activated. For activation to occur, an antigen-presenting cell (APC) presents an antigen on its major histocompatibility complex (MHC) class II molecule and binds with the T-cell receptor (TCR) of the T_H cell. Upon recognition of the antigen-MHC molecule and proper co-stimulation, the naïve T_H cell becomes activated, undergoes clonal expansion, and releases cytokines that programme the cells to differentiate into a specific effector cell type, which have different roles (Fig. 3.1, Table 3.1). T_H1 cells produce interferon-γ (IFN-γ), interleukin 2 (IL-2), and tumour necrosis factor-α (TNF-α) and are mostly involved in immune responses against bacteria and viruses [2]. T_H2 cells are characterized by the expression of IL-4, IL-5, and IL-13 and play a significant role in the immune response against extracellular pathogens, such as parasites [2]. T_H17 cells express IL-17A, IL-17F, and IL-22 and are critical for antifungal and antibacterial responses [3]. Another subset of cells, T follicular helper (T_{FH}) cells, contributes to humoral immunity within germinal centres and characteristically present with CXCR5-positive expression [4]. These cells produce IL-21 and IL-4, which are important for B-cell stimulation, immunoglobulin class switching, and homing of B cells to B cell-rich germinal centre of secondary/tertiary lymphoid organs [5]. Another type of CD4+ cell, the T regulatory (T_{Reg}) cell, expresses CD25; they secrete the cytokine IL-10 and have been shown to carry an immunosuppressive role [6]. The contribution of T_{Regs} in immune evasion observed in cancer is an area of active research, and these cell subsets are targets for cancer immunotherapeutics [7, 8]. Many other T helper cell subsets with well-described functions have been defined, including TH3, TR1, TH9, and TH22. In this chapter, we focus on the major subsets of T_H cells and discuss their roles in the TIME.

In addition to their traditional roles in the immune response against pathogenic microorganisms, accumulating evidence has emerged on the importance of CD4+ T cells and their role in mediating anti-tumour responses [9, 10]. Accumulating evidence suggests that select CD4+ T_H cell subsets may have a more "direct" role in inhibiting tumour growth and progression that are independent of their more "indirect" helper activities [11]. However, recent studies have revealed additional CD4+ T_H cell functions that can not only influence tumour immunity and inhibit growth but, paradoxically, can also promote tumour growth and progression [12, 13].

It is generally accepted that human tumours are immunogenic, meaning that they may provoke an immune response. Tumour immunogenicity varies greatly between types of cancer and between different individuals with the same type of cancer [14]. These responses are mostly mediated by T cells, and their presence is often associated with a more favourable outcome [15]. Immune checkpoint inhibitors are derived from advanced melanoma squamous non-small cell lung cancer [16]. These solid tumour cancers, which can be hard to treat, have shown favourable responses when treated with immunotherapy [17, 18]. The immune cell population is a major factor that influences prevention or encourages initiation, metastasis and invasion, and

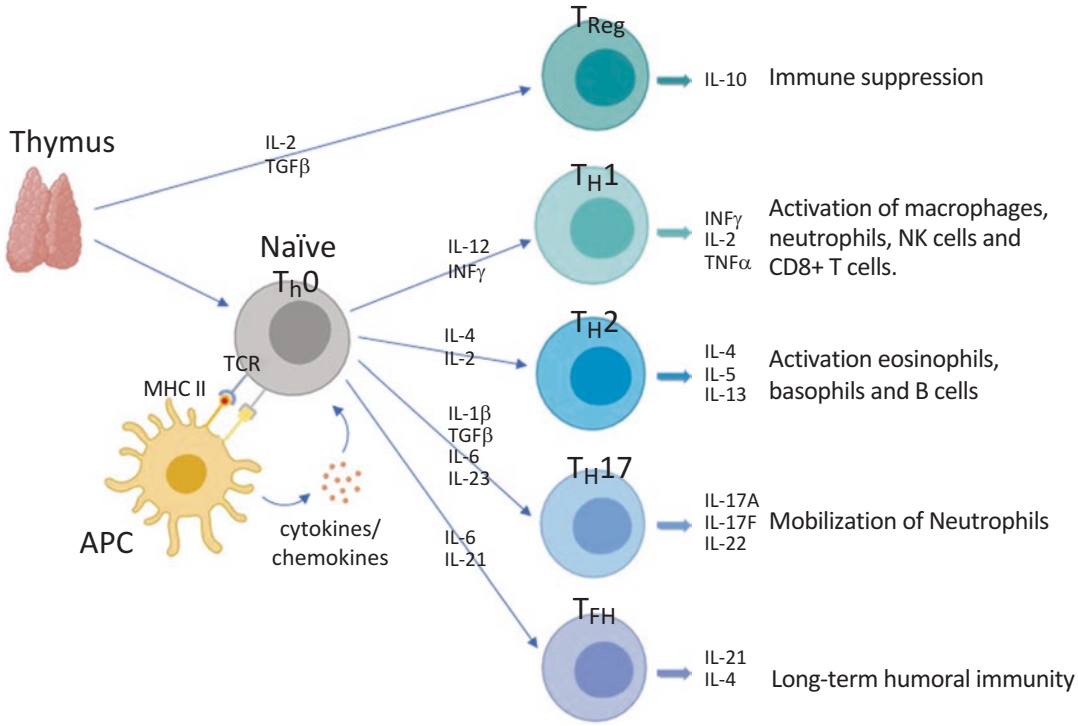

Fig. 3.1 Canonical lineage and differentiation of CD4+ immune cells

Table 3.1 Factors regulating T helper cell differentiation

Cytokines produced by APC programme naïve CD4+ T_H cell differentiation	IL-12 IFN**γ**	IL-4 IL-2	IL-6 IL-21	IL-1β TGFβ IL-6 IL-23	IL-2 TGFβ
CD4+ T helper cells	**T_H1**	**T_H2**	**T_{FH}**	**T_H17**	**T_{Reg}**
Cytokines produced	INF-γ IL-2 TNFα	IL-4 IL-5 IL-13	IL-21 IL-4	IL-17A IL-17F IL-22	IL-10
Key transcription factors	Tbet	GATA-3	BCL6	RORyt	FOXP3
Role in immune defence	Antiviral, antibacterial immunities	Extracellular pathogens	Humoral immunity within germinal centres	Antifungal and host defence against intra- and extracellular bacterial infection	Immunosuppression
Signal transducer	STAT4	STAT5 STAT6	STAT3	STAT3	STAT5

angiogenesis. The composition and characteristics of the TIME differ between different types of cancer or between patients that have the same type of cancer. The TIME is composed of resident stromal cells and non-resident components. It can be classified according to the composition of the immune infiltrate and the nature of the inflammatory response. Currently, three broad classes exist (Fig. 3.2): (1) poorly immunogenic, or "cold", where immune cells (mainly cytotoxic T lymphocytes) are only present along the tumour periphery; (2) infiltrated, inflamed, or immuno-

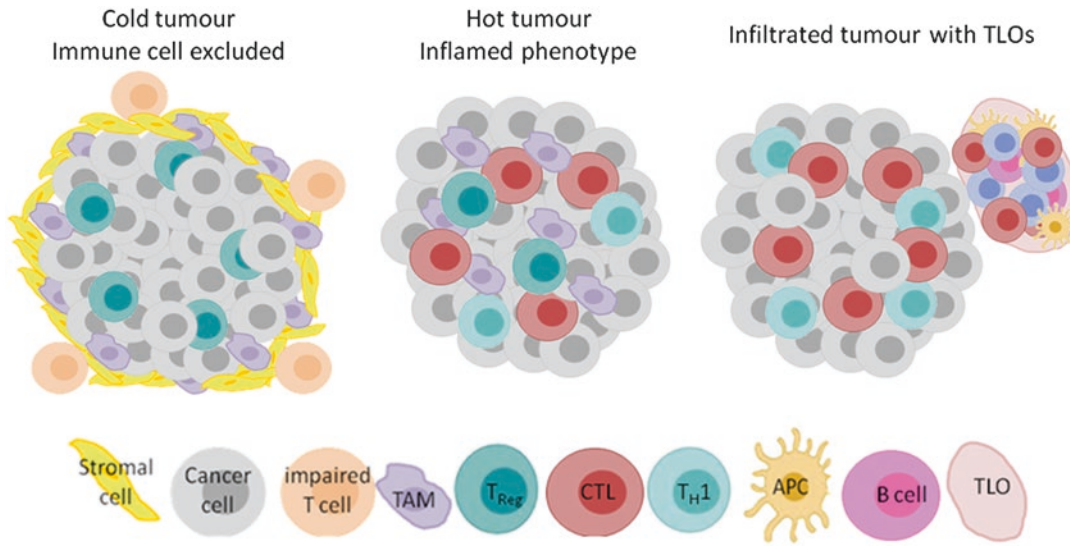

Fig. 3.2 Three phenotypes of the tumour immune microenvironment. T^{Reg} regulatory T cell, *TH* T helper cell, *TFH* T follicular helper cell, *EMT* endothelio-mesecnhymal transition, *NK* natural killer cell, *IFN* interferon, *DC* dendritic cell, *TAM* tumour-associated macrophage, *CTL* cytotoxic T lymphocyte, *APC* antigen-presenting cell, *TLO* tertiary lymphoid organ

logically "hot", with an abundance of programmed death ligand 1 (PD-L1) expression and highly activated cytotoxic T cells; and (3) those with groups of immune cells with constituents similar to those in lymph nodes, including B cells, dendritic cells, and T_{Reg} cells [19]. This later categorization undoubtedly misses key subclasses that require higher-resolution techniques to uncover and characterize heterogeneity in immune cell composition.

Here, we review the dual pro- and anti-tumour functions of T_H cells as well as factors influencing their homing and differentiation in the TIME. Finally, we provide an overview of the interactions between cancer immunotherapy and T_H cells and the prognostic role of T_H cell infiltration.

3.2 Dual Role of T Helper Cells in Tumour Development and Progression

According to the cytokine context of the TIME, naïve CD4+ T cells can differentiate into specific T_H cell subtypes, and other already differentiated subsets are recruited to the area [1]. While CD8+ cytotoxic and interferon-gamma-producing CD4+ T_H1 helper cells are the main players against tumours, other types of CD4+ cells can act in favour of cancer in combination with other cell types, such as myeloid-derived suppressive cells (MDSC) and tumour-associated macrophages (TAM). Pro-tumour functions driven by these cells and their secreted factors are able to inhibit anti-tumour innate and adaptive immune responses [1].

Further, the recruitment of specific T-cell subset to the TIME has been shown to correlate with prognosis and immunotherapeutic efficacy, underlying the importance of tumour infiltration and the role of T-cell homing to and within the tumour [20, 21]. In normal conditions, naïve T cells are produced in the thymus and cycle through complex networks of blood vessels, lymphatic vessels, and lymph nodes until they are signalled to home into specific tissues [22]. Homing of T cells is a very complex and tissue-specific process requiring activation of various patterns of receptors on the surface of the T cells specific to the tissue they will infiltrate [23]. Depending on the specific activation of receptors,

the T cell will express specific chemokines and integrins. Integrin activation causes firm adherence to the vessel, so the T cell can begin transmigration through the endothelial surface of the vessel and into the tissue they are homing toward. This process is the same for T cells destined to home into tumours, but homing T cells to tumours is not always successful because tumours may possess a number of deficits to prevent the process of proper T-cell homing and infiltration [23].

As each major T-cell subset is regulated by different mechanisms of differentiation and recruitment, we discuss the dichotomous roles of each type of CD4+ T cells and their potential use as prognostic markers below.

3.2.1 T Helper Type 1

T helper 1 lymphocytes are important players in modulating immune response against cancer, linking innate and adaptive immunity, since IFN-γ also induces anti-tumour activity from tumour-infiltrating macrophages [24]. It was previously demonstrated that IFN-γ and TNF-α produced specifically by T_H1 cells are necessary for inducing senescence in cancer cells and to turn macrophages cytotoxic to tumour cells [24, 25]. In fact, increased circulating levels of IFN-γ and TNF-α were described as a protective factor for prostate cancer [26]. In addition, many studies reports good patient outcome related to T_H1 cell and related cytokines in the TIME and in the blood of patients in a variety of cancer types [27–31]. Therefore, T_H1 is known to consistently promote immune responses against tumour cells.

3.2.2 T Helper Type 2

T helper type 2 lymphocytes are known to have less effective anti-tumour response than T_H1, presenting dual functions depending on the context [32, 33]. The shift from T_H1 to T_H2 in the TIME has been reported in a variety of cancer types [29, 34–38]. A pan-cancer analysis from The Cancer Genome Atlas consortium (more than 10,000 tumours from 33 cancer types) revealed 6 immune subtypes defined by genetic and immunological features, including the T_H1:T_H2 ratio [39]. Tumours characterized by a T_H2 immune infiltrate bias, the *wound healing* subtype T_H ratio, is enriched in colorectal, lung squamous cell, breast cancer (luminal A molecular subtype), head and neck (classical molecular subtype), and chromosomally unstable gastrointestinal cancer. This immunogenomic subtype was associated with shortened survival [39], agreeing with previous reports of high T_H2/T_H1 ratio as a poor prognostic indicator [29, 37, 40]. The trafficking of T_H1 or T_H2 cells into the TIME is influenced by many factors secreted by tumour cells [41]. In addition, the switch from T_H1 to T_H2 immune response was shown to be influenced by T_{Reg} cells in hepatocellular carcinoma after transarterial chemoembolization treatment [29]. Nonetheless, T_H2 have been described to modulate anti-tumour activity in many cancer types and conditions [32, 42–45], relying on the attraction of innate immune cells to the tumour [46, 47]. Specific T_H2 adoptive cell therapy in mice models has been shown to eliminate myeloma and lymphoma cells, in a process independently of CD8+, natural killer, B cells, and IFN-γ and dependent of M2-type macrophages [45].

3.2.3 Regulatory T Cells

Tumour escape strategies comprise primarily the recruitment of immunosuppressive cells to the TIME [48, 49]. One important inhibitory cell subset thought to contribute to the suppressive immunity associated with cancers is the regulatory T cell (T_{Reg}) [50, 51]. While the existence of "suppressor" T cells was discussed as early as the 1970s, discovery and definition of what is now referred to as T_{Regs} began in earnest in 1995 when autoimmunity was rescued in a mouse model with CD25+ T cells, leading to the first description of a highly immune inhibitory T cell [6, 52]. Since that time, T_{Reg} cell subsets have been further defined to include naturally occurring CD4+CD25^{high} T_{Reg} cells, inducible T_{Reg} cell subsets such as T_r1 and T_H3 cells, and those derived

from the induced expression of CD25 in CD4+CD25− subsets in the periphery, all capable of immunosuppression [53–56]. In addition, the transcription factor forkhead box P3 (FoxP3) has been further identified as a common marker for T_{Reg} cells [57].

In general, CD4+CD25^{high}FoxP3+ T_{Reg} cells are antigen-experienced memory T cells capable of inhibiting a variety of immune cell subsets including CD4+CD25− T cells, CD8+ T cells [58], dendritic cells [59], natural killer cells, natural killer T cells [60], and B cells [61]. T_{Reg} cells represent only between 5 and 10% of the T-cell populations in healthy human conditions [62]. They are present at higher levels in a wide range of human neoplasias [7, 63–68] and support tumour development and progression [69]. Suppression of effector T cells and NK cells by T_{Reg} was found to be cell-cell contact dependent [58], but other demonstrated that the function of T_{Reg} is dependent on the cytokines IL-10, IL-35, and TGFβ [70]. T_{Reg} also depletes immune-inducing cytokines, such as IL-2 [71, 72].

3.2.4 T Helper Type 17

The dual role of T_H17 cells in inflammatory disease and cancer has been widely reported [73, 74]. T_H17 cells were demonstrated to have anti-tumour functions, by inducing the recruitment of dendritic cells in the tumour and in the adjacent lymph nodes promoting tumour-specific cytotoxic T cell responses [75]. In ovarian cancer, it was demonstrated that the presence of T_H17 cells in the TME was correlated with the infiltration of effector T cells in the tumour [76].

Nevertheless, T_H17 cells can also release potent immunosuppressive signals into the TIME, supporting their dichotomous nature [77]. Moreover, IL-17 can result in pro-tumour responses through effects in the tumour cells, myeloid-derived suppressor cells, and other components of the stroma [78]. It was previously demonstrated that IL-17 derived from T_H17 cells promotes migration and invasion and induces stem cell-like features in lung cancer using the STAT3-NF-κB-Notch1 signalling [79]. In colorectal cancer, infiltrating IL-17-producing cells are associated with poor prognosis [30].

3.2.5 T Follicular Helper

Although T_{FH} cells are reported to have pro-tumour functions in haematologic types of cancer [80], in solid tumours, they are generally associated with anti-tumour functions [81–83]. In lung [81] and breast cancer [82], T_{FH} cells are enriched in the germinal centre of tumour-adjacent tertiary lymphoid organs. Moreover, the T_{FH} density positively correlates with the lung cancer mutation burden, indicating their role in the immune response against cancer neoantigens [81].

Conversely, differentiated T_{FH} cells also express high levels of programmed cell death protein 1 (PD-1), suggesting that they can also decrease the activation of T cells [4]. However, PD-1/PD-L1 blockage results in deficient germinal centre formation and cytokine production by the T_{FH} cells [84].

3.2.6 T Helper Type 9

T_H9 cells are thought to have a strong anti-tumour effect in the presence of TGF-β and IL-4 [85, 86]. This anti-tumour property is related to their effects on mast cells, dendritic cell recruitment, and promoting cytotoxic function by CD8+ T cells [86, 87]. Additionally, IL-9 was shown to directly inhibit the proliferation of melanoma cells in mice models, and IL-9 blocking is able to enhance both melanoma and lung cancer growth [86]. Conversely, pro-tumourigenic roles of T_H9/IL-9 in lymphoma and gastric cancer have been described [88–90]. In fact, IL-9 can also induce immunosuppressive responses from T_{Reg} [91]. Thus, the context of activity may dictate the pro- or anti-tumour effect of T_H9 cells.

3.2.7 T Helper Type 22

T_H22 cells and IL-22 have been shown to be tumour-promoting in the TIME and have

therefore been suggested as potential immunotherapy targets [92–95]. When IL-22 is overexpressed in a mouse model of liver cancer, increased proliferation of the cancer cells was observed [92, 93]. Similarly, reduced proliferation in IL-22-deficient mice was demonstrated. In colon cancer, IL-22 can promote proliferation and stem cell-like features through the activation of STAT3 signalling and consequent epigenetic modification on development-related genes [96]. In breast and lung cancer models, it was demonstrated that IL-22 secretion from CD4+ memory T cells to support the tumour cell proliferation can be induced through NLRP3 inflammasome activation and release of IL-1β from immune cells [94]. In patients, T_H22 cells and IL-22 have been found in the primary tumour, serum, and malignant pleural effusion of these patients [97, 98].

3.3 Regulation of T Helper Cell Homing and Differentiation in the TIME

The process of T-cell maturation from a naïve T_H in the thymus involves the destruction of self-reactive lymphocytes, but the existence of autoimmunity demonstrates that while T cells do undergo education to promote central tolerance, self-reactive T cells can escape this process [99]. Indeed, self-reactivity is required for the detection and destruction of cancer cells, a crucial immunological function requiring recognition of self-antigen [100]. A delicate balance is therefore required to ensure that the immune system responds appropriately to altered self-like state observed in cancer but also kept under careful control to prevent self-destruction and autoimmunity. This important function is achieved through the co-recruitment of suppressor cells and inhibitory molecules in addition to effector cells during an immune response.

3.3.1 Homing of Regulatory T Cells

An increase of regulatory T cells has been observed in both the peripheral blood and the tumour microenvironment from a wide variety of both solid tumours and haematological cancers such as breast, prostate, lung, and pancreatic cancers, lymphomas, and leukaemias [65, 68, 101–106]. T_{Regs} are recruited to the TIME upon signals delivered by the tumour, mainly CCL22 [1, 69]. Another source of T_{Regs} in the TIME is the conversion of T effector cells into T_{Regs}, through interaction with DCs in the context of high TGFβ and IDO, which are secreted by tumour cells [107].

3.3.2 Dendritic Cells as Key Regulators of TIME Composition

Interactions between specific DC subsets and immature T cells are crucial for generating and maintaining both effector T cells and T_{Regs} [108, 109]. This duality highlights the versatility and variability of DCs and their capacity to shape the immune response [110]. Infiltrating DCs should activate anti-tumour responses, but tumour cells are capable of suppressing DC function and promoting their activity to induce T_{Regs} [111]. Detailed descriptions of the numerous types of DCs are beyond the scope of this chapter, but the capacity to overcome the suppressive tumour microenvironment, recruit T cells into the tumour bed, and activate effector T cell responses appears to be dependent on the chemokines CXCL9 and CXCL10 produced by CD103+DCs [108]. On the contrary, development of T cells into a regulatory phenotype in the tumour microenvironment appears to be mediated by plasmacytoid DCs and dependent on expression of inducible co-stimulatory ligand (ICOS-L) [112]. Naturally occurring T_{Regs} are similarly induced by DCs in the thymus through interactions with CD80 and CD86 controlled by Hassall's corpuscles [113]. Interestingly, CD8+ T_{Regs} also exist in humans and can be induced through interaction with CD40 ligand on plasmacytoid DCs [114]. While targeting T_{Regs} could improve immunotherapy outcomes, the function and activity of T cells are tightly controlled by DCs making them an attractive target for immunotherapeutic approaches [115, 116].

3.4 T Helper Cells in the Context of Immunotherapy

Immunotherapy using antibodies directed against immune checkpoint inhibitors such as PD-1, PD-L1, and CTLA-4, has emerged as a major treatment modality for metastatic cancer in various malignancies, including melanoma and lung cancer.

In addition to release of CD8+ T-cell inhibition, PD-1 blockade also alters T_H cell function. Since PD-1 signalling induces T_H1 cells to transdifferentiate in T_{Regs}, it was supposed that PD-1 blockade would help in reducing the immunosuppressive role of T_{Regs} [117]. Nevertheless, depending on TIME context, it has been shown to stimulate T_{Reg} suppressive signals [118, 119], to impair germinal centre formation and cytokine production by T_{FH} cells [84], and to promote hyperprogression of cancer [119], a pattern of progression that exists in approximately 10% of patients treated with anti-PD-1/PD-L1 [120].

Disrupting CTLA-4 interaction with CD80 induces T_H cell infiltration into tumours [121, 122], particularly a subset of T_H cells with high expression of ICOS and secretion of IFN-gamma [123].

It has been shown in mice studies that anti-CTLA-4 antibodies induce tumour rejection by selective depletion of T_{Regs} in the tumours [124]. Nevertheless, results from human studies are controversial, and it is not yet clear if the depletion of T_{Regs} plays a major role in the clinical setting [122].

Major predictive factors of treatment outcome in anti-CTLA-4 and anti-PD-1/PD-L1 blockade are cancer type, tumour mutational burden, CD8+ T-cell infiltration, and TCR repertoire diversity [125]. Nonetheless, T_H cells have also been studied as biomarkers to predict response to immune checkpoint inhibition. In metastatic melanoma, a high level of pretreatment infiltrating T_{Regs} and tumour infiltration by $ICOS^{high}$ T_H cells during treatment was associated with response to anti-CTLA-4 therapy [126, 127].

3.5 Prognostic Role of T Helper Cells

The density and cell type characterization of tumour-resident T cells have been described in the prognostication of cancer patients [128, 129]. Improved survival was correlated with the presence of these cells in the TIME [129–131]. The integration of CD4+ and CD8+ quantification can improve the prediction of the patient outcome, and the estimation of CD8+/CD4+ ratios is frequently suggested [132, 133]. However, the dual role of some CD4+ lymphocyte subtypes adds more complexity in the immunology response against cancer and should be considered [130]. In general, the presence of interferon-γ-producing CD4+ T_H1 lymphocytes are related to a favourable prognosis, while the presence of other CD4+ subtypes is cancer type-dependent (Table 3.2) [130, 134]. A high T_H2:T_H1 ratio, representing a T_H2 prevalence trend in the TIME, can predict shortened survival in many cancer types [29, 34–38]. In resected colorectal cancer, a high level of T_H1 infiltration and a low level of T_H17 infiltration are associated with prolonged disease-free survival [30]. Interestingly, the integration of the density and location of these cells (tumour core or invasive margin) resulted in better predictions. In oesophageal squamous carcinoma and non-small cell lung cancer, T_H1 infiltration correlates with a better prognosis [135, 136]. Combining T_H1 infiltration assessment to numeration of CTL resulted in added predictability.

High level of T_{Reg} infiltration is a reliable indicator of more aggressive disease in many cancers [7, 137], such as breast [138], gastric [139], head and neck [63], liver [140], lung [141], pancreas [142], and ovary [68]. The prevalence of T_{Regs} in the lymph nodes of patients with non-small cell lung cancer has prognostic value, with 5-year survival rates significantly lower in patients with higher proportions of T_{Regs} present [143]. Another study found increasing prevalence of T_{Regs} in non-small cell lung cancer patients that correlated with disease stage and tumour burden [144]. A

Table 3.2 T helper cells as prognostic factors in tumours

High abundance	Association with clinical outcome	References
T_H1	Favourable prognosis in RCC, CRC, oesophageal squamous carcinoma	[30, 135, 136, 163]
	Response to neoadjuvant chemotherapy in HER2+ and triple-negative breast cancer	[164]
T_{Reg}	Favourable prognosis in CRC	[147–149]
	Poor prognosis in NSCLC, HCC, HNSCC, RCC, MPM, melanoma, and bladder, gastric, pancreatic, breast, and ovarian cancer	[139, 142, 144, 145, 157, 165–175]
	Response to anti-CTLA-4 in melanoma	[127]
T_{FH}	Favourable prognosis in CRC, NSCLC, and breast cancer	[81–83, 151]
T_H17	Poor prognosis in HNSCC and CRC	[30, 176]
T_H22	Poor prognosis in GI tumours	[92, 156, 177]
PD-1^+LAG3^+TIM3^+	Favourable prognosis in MPM	[157]
$ICOS^{high}$	Response to anti-CTLA-4 in melanoma	[126]
TLO	Favourable prognosis in NSCLC and melanoma	[151, 152]

RCC renal cell carcinoma, *CRC* colorectal carcinoma, *NSCLC* non-small cell carcinoma, *HCC* hepatocellular carcinoma, *MPM* malignant pleural mesothelioma, *HNSCC* head and neck squamous cell carcinoma, *GI* gastrointestinal, *TLO* tertiary lymphoid organ.

meta-analysis comprising over 86,000 lung cancer patients showed that the presence of tumour-infiltrating CD4+ cells is associated with better prognosis; however, FOXP3+ cells are a poor prognostic marker [145]. In addition, the enrichment of migrated CD4+ T and CD8+ T lymphocytes in the pleural effusions from lung adenocarcinoma was reported as a good prognosis indicator [146].

Although enrichment of T_{Reg} cells within tumours of various origins can signify poor prognosis, the opposite may be true for colon cancer. For example, T_{Reg} infiltration is frequently described as a good prognostic marker in colorectal cancer [147–149]. However, colorectal carcinoma can be infiltrated in variable ratios by two different types of T_{Regs}, one suppression-competent and other non-suppressive [150]. In fact, the infiltration by predominantly non-suppressive T_{Regs} is related to improved survival in colorectal cancer patients [150].

T_{FH} cell infiltration, measured through the expression of CXCL13, CXCR5, and IL-21, is associated with longer disease-free survival in CRC and breast cancer [82, 83]. Moreover, deletion or dysfunction of CXCL13 correlates with shorter DFS in CRC. The presence of tertiary lymphoid organs (TLOs), which formation is dependent on T_{FH}, is a good outcome predictor in various malignancies [151, 152].

Although T_H9 has been shown to induce potent anti-tumour responses [86, 153, 154], it delivers survival and proliferation signals in lymphoma cells, where high IL-9 is considered a poor prognostic marker [88, 89]. Increase of T_H22 cells was also reported as related with advanced tumour stages, and higher IL-22 expression in the TIME is associated with shorter survival of hepatocellular carcinoma and gastric cancer [155, 156].

Recently, it was shown that tumour-infiltrating CD4+ lymphocytes upregulating molecules responsible for CD8+ T-cell exhaustion (PD-1, LAG-3, and TIM-3) were associated with shorter overall survival in malignant pleural mesothelioma [157]. Similarly, the enrichment of ineffective CD4+ memory T cells in the TIME of follicular lymphoma, due to the lack of co-stimulatory receptors, was correlated with a shorter survival [158].

In addition to the T-cell subtype, the T-cell receptor (TCR) repertoire is related to the outcome of cancer patients [159–162]. Higher intratumour T-cell receptor (TCR) heterogeneity, which is positively correlated with the neoantigen heterogeneity, is associated with an increased lung cancer recurrence risk [159]. TCR heterogeneity from both CD4+ and CD8+ was associ-

ated with a higher recurrence risk in lung adenocarcinoma [160]. Interestingly, TCR diversity was higher in CD4+ compared to CD8+ cells [160].

3.6 Conclusions

T helper cells are key players of the immune system and modulate the efficiency of anti-tumour immune response. Of these, T_{Regs} are an intensively studied subset since their immunosuppressive role has been well documented in other clinical settings. In the cancer setting, they have been extensively studied in the context of their use as therapeutic targets and prognostic biomarkers in various tumours, including lung cancer. In comparison, there is a relative paucity of data on other T_H cell subsets; however, T_{FH} cells have recently attracted interest as regulators of tertiary lymphoid organ organization and B-cell function. As such they may be leveraged to obtain long-term immune response, an elusive goal for most patients even when treated with combined immunotherapy.

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Cytotoxic CD8+ Lymphocytes in the Tumor Microenvironment

4

Kota Iwahori

Abstract

In the tumor microenvironment, CD8+ T cells play a major role in tumor immunity. CD8+ T cells differentiate to cytotoxic T cells, traffic into the tumor microenvironment, and exhibit cytotoxicity against tumor cells. These processes have both positive and negative effects. Enhancements in the cytotoxic activity of tumor antigen-specific cytotoxic T cells in the tumor microenvironment are crucial for the development of cancer immunotherapy. To achieve this, several immunotherapies, including cancer vaccines, T cells engineered to express chimeric antigen receptors (CAR T cells), and bispecific T-cell engagers (BiTEs), have been developed. In contrast to cancer vaccines, CAR T cells, and BiTEs, immune checkpoint inhibitors enhance the activity of cytotoxic T cells by inhibiting the negative regulators of T cells.

The total number, type, and activity of tumor antigen-specific cytotoxic T cells in the tumor microenvironment need to be clarified, particularly for the development of companion diagnostics to identify patients for whom these therapies are effective. Therefore, technologies including TCR repertoire, single-cell, and T-cell cytotoxicity analyses using BiTEs have been developed.

Based on these and future innovations, the generation of effective cancer immunotherapies is anticipated.

Keywords

Cytotoxic T cells · CD8+ T cells · Tumor microenvironment · T-cell receptor · T-cell cytotoxicity · Tumor-specific antigen · Neoantigen · T-cell exhaustion · T-cell metabolism · Immune checkpoint inhibitor · PD-1 · PD-L1 · CTLA-4 · Tumor mutation burden · Bispecific T-cell engager

4.1 Introduction

Cancer immunotherapy, including immune checkpoint inhibitors, exerts beneficial effects for cancer patients. However, immune checkpoint inhibitors are only advantageous for a limited population of cancer patients [1]. Therefore, companion diagnostics are needed to identify patients for whom these therapies are effective.

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One of the main effectors of cancer immunotherapy is tumor antigen-specific cytotoxic T cells in the tumor microenvironment. $CD8^+$ T cells differentiate to cytotoxic T cells, traffic into the tumor microenvironment, and exhibit cytotoxicity against tumor cells. Positive and negative effects are associated with these processes. By enhancing the cytotoxic activity of these T cells against tumor cells, cancer immunotherapy exhibits efficacy in cancer patients. Therefore, it is crucial to evaluate cytotoxic T cells in the tumor microenvironment. One of the main focuses of cancer immunology and immunotherapy is to identify the total number, type, and activity of tumor antigen-specific cytotoxic T cells in the tumor microenvironment. In this chapter, the focuses of discussion are how to enhance the ability of cytotoxic T cells for cancer immunotherapy based on positive and negative regulators of cytotoxic T cells and evaluate the activity of cytotoxic T cells as a step toward the development of companion diagnostics for cancer immunotherapy.

4.2 $CD8^+$ T Cells in the Tumor Microenvironment

In the tumor microenvironment, $CD8^+$ T cells play a major role in tumor immunity. $CD8^+$ T cells differentiate to cytotoxic T cells, traffic into the tumor microenvironment, and exhibit cytotoxicity against tumor cells. Positive and negative effects are associated with these processes.

Regarding the differentiation of $CD8^+$ T cells to cytotoxic T cells, naïve $CD8^+$ T cells interact with the peptide-major histocompatibility complex (MHC) on antigen-presenting cells (APC) through T-cell receptors (TCR), and this is followed by a co-stimulatory signal and stimulation from extracellular cytokines. These activated $CD8^+$ T cells differentiate into effector $CD8^+$ T cells. Terminally differentiated effector $CD8^+$ T cells are IL-2 dependent and highly cytotoxic [2, 3]. On the other hand, tissue-resident memory T cells have been identified among memory $CD8^+$ T cells based on their local tissue residency without recirculating into the blood [4, 5]. These T cells are characterized by core markers, including CD103, which bind its ligand, the epithelial cell marker E-cadherin, thereby favoring the location and retention of these T cells in epithelial tumor regions [6]. An analysis of tissue-resident memory T cells in non-small cell lung cancer revealed that these T cells expressed transcripts encoding products linked to the cytotoxic function of $CD8^+$ T cells [7]. Co-stimulatory signals are induced by interactions between co-stimulatory receptors, such as CD28 on T cells and their ligands CD80/CD86 on APC. On the other hand, $CD8^+$ T cells express inhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA-4), which interacts with CD80/CD86. CTLA-4 binds CD80/CD86 with higher affinity than CD28 [8–10]. Ipilimumab, a CTLA-4 blocking antibody, was the first immune checkpoint inhibitor for cancer immunotherapy [11].

A cytotoxic T cell (CTL) recognizes, through its TCR, an antigen-MHC complex on a tumor cell, forming the immunological synapse. Upon activation of the CTL, granules within the CTL open into the immunological synapse, into which they release their contents. Granules within the CTL contain the FAS ligand, perforin, and granzymes. There are two distinct pathways for CTL-mediated cytotoxicity to the target cell. In one pathway, FAS ligands interact with FAS on the target cell, leading to apoptosis of the target cell through the activation of caspase-8 and caspase-3. In the other pathway, perforin opens a channel in the target cell membrane through which granzyme B enters the cytosol, leading to apoptosis of the target cell through the activation of caspase-3 [12].

$CD8^+$ T-cell function is influenced by metabolic changes in the tumor microenvironment. Hypoxic conditions in the tumor microenvironment exert different effects on the functions of each T-cell subset [13]. While naïve and central memory T cells are suppressed under hypoxic conditions, effector memory T cells show enhanced proliferation, survival, and cytotoxic activity. Under hypoxic conditions, effector memory T cells exert their metabolic functions partially through glycolysis. In addition to glucose, $CD8^+$ T cells are able to use amino acids as

alternative sources of energy. Arginine and tryptophan are essential for T cells and cannot be produced by T-cell metabolism. Arginase expressed in tumor cells inhibits T-cell function via the deprivation of arginine. Indoleamine 2,3-dioxygenase (IDO) converts tryptophan into its metabolite kynurenine. CD8+ T cells also use fatty acids as a source of energy under a hypoxic and hypoglycemic environment [14]. When simultaneously subjected to hypoglycemia and hypoxia, CD8+ T cells enhance PPAR-α signaling and the catabolism of fatty acids.

In the tumor microenvironment in which an antigen persists, CD8+ T cells develop a loss of function in a process termed "exhaustion." Exhausted T cells are characterized by deficits in their ability to proliferate and elicit effector functions (cytotoxicity and cytokine production) upon a stimulation through the TCR [15]. During activation through the TCR with an antigen, CD8+ T cells express PD-1, which interacts with PD-L1 on tumor cells and immune cells. Upon activation by PD-L1, PD-1 is considered to suppress signaling through the TCR. Recent studies reported that CD28 is a major target of PD-1 signaling [16, 17]. Other than PD-1, CD8+ T cells express co-inhibitory receptors, including LAG-3, TIM-3, and TIGIT, which leads to inhibitory signals in T-cell exhaustion [18].

4.3 Tumor Antigen-Specific T Cells for Cancer Immunotherapy

The identification of MAGE-1 as a tumor-specific antigen for melanoma opened a new era for studies on tumor antigen-specific T cells. MAGE-1 was identified for the first time as a tumor-specific antigen targeted by cytotoxic T cells [19]. After this breakthrough, various types of tumor-specific antigens were identified. These findings led to the development of cancer immunology and immunotherapy.

Tumor antigens are mainly divided into two populations, non-mutated proteins and mutated proteins. Non-mutated proteins include cancer/testis antigens and virally expressed proteins. In contrast, tumor antigens derived from mutated proteins in tumor cells are neoantigens.

Tumor-specific antigens derived from tumor cells are captured by antigen-presenting cells. Antigen-presenting cells present these antigens on their surface using MHC molecules and this is followed by the activation of T cells via TCR and a co-stimulation. After these processes, cytotoxic T cells have the ability to attack tumor cells via perforin-granzyme and FAS pathways [12]. Various types of immunotherapeutic strategies have been developed based on these mechanisms.

Cancer vaccines consist of peptides and proteins derived from tumor-specific antigens, which were immunized to produce tumor antigen-specific cytotoxic T cells. T cells engineered to express chimeric antigen receptors (CAR T cells) have been genetically engineered to express a single-chain variable fragment (scFv) of a tumor antigen-specific antibody and the signaling domains of T-cell receptors (TCR) with co-stimulatory molecules (Fig. 4.1). CAR T cells are a type of artificial cytotoxic T cell. In contrast to the success of CAR T-cell therapy for hematological malignancies, difficulties have been associated with CAR T-cell therapy for solid tumors and have been attributed to the tumor microenvironment around T cells. Therefore, by overviewing attempts to improve CAR T-cell therapies, the critical factors of cytotoxic T cells in the tumor environment may be clarified.

The breakthrough for CAR T-cell therapies began with the development of co-stimulatory molecules for CAR T cells [20, 21]. In the tumor microenvironment, TCR-stimulated T cells without a co-stimulation become anergy. Tumor antigen-specific T cells stimulated via both TCR and co-stimulatory receptors become cytotoxic T cells exhibiting cytotoxic activity against tumor cells. The prototype of co-stimulatory receptors is CD28 [22]. CD28 or 41BB co-stimulatory endodomains are the most frequently utilized to generate CAR T cells, leading to successful clinical efficacy for hematological malignancies [23, 24].

Another challenge to improve CAR T-cell therapies is the trafficking of T cells to tumor

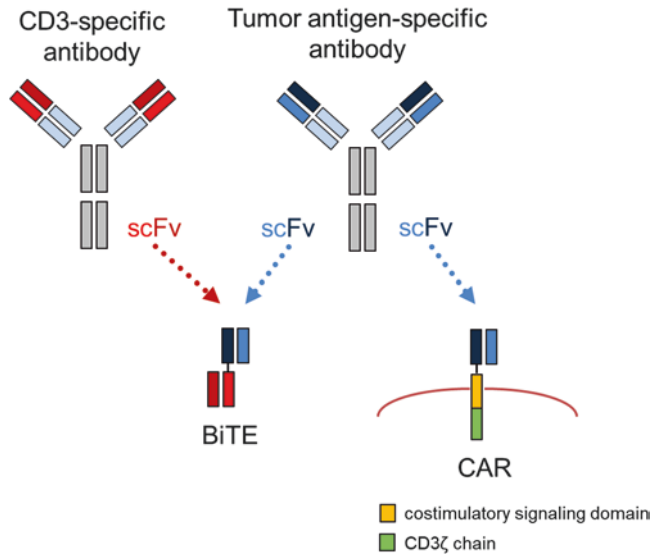

Fig. 4.1 Chimeric antigen receptor (CAR) and bispecific T-cell engager (BiTE). CAR consists of a single-chain variable fragment specific to a tumor antigen and a co-stimulatory signaling domain in tandem with the CD3ζ chain. In contrast, BiTE consists of two single-chain variable fragments coupled with a linker; one binds to CD3, and the other binds to a tumor antigen. T cells are activated and redirected to tumor cell lysis by these two domains

sites. The ability of cytotoxic T cells to migrate into tumors is regulated by multiple mechanisms including chemokines and chemokine receptors [25]. Due to the interaction between chemokines secreted from tumors and chemokine receptors on T cells, T cells traffic into tumors. Attempts to develop CAR T cells co-expressing chemokine receptors have been made based on these mechanisms [26].

Negative regulators including immunosuppressive cytokines, such as transforming growth factor β (TGFβ) or IL-10 secreted from tumor sites [27, 28]; immunosuppressive cells, including regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs) [29, 30]; PD-L1 expression on the cell surface [31]; and an immunosuppressive metabolic environment, such as low tryptophan induced by indoleamine 2,3-dioxygenase (IDO), have been detected in the tumor microenvironment [32]. To overcome these immunosuppressive factors, improvements in CAR T cells have been attempted.

Similar to CAR T cells, bispecific T-cell engagers (BiTEs) have been developed using scFv technology (Fig. 4.1). BiTEs consist of two scFvs connected by a short linker, which are specific for CD3 expressed on T cells and an antigen expressed on the surface of tumor cells. The pattern of T-cell cytotoxicity induced by BiTE shows some similarities to tumor cell killing by endogenous tumor antigen-specific T cells [33]. Blinatumomab, one of the BiTEs specific to CD19, has been approved for the treatment of B-cell acute lymphoblastic leukemia (B-ALL) [34].

There are several limitations for BiTEs. They have a short half-life that necessitates a continuous systemic infusion that may be associated with toxicities, a lack of active biodistribution, and the absence of self-amplification [35]. To overcome these limitations, genetically modified T cells secreting BiTE (engager T cells) have been developed in preclinical studies [36–38]. These engager T cells expanded in vivo and secreted more BiTEs upon activation, obviating the need for the continuous infusion of BiTEs.

In contrast to cancer vaccines, CAR T cells, and BiTEs, immune checkpoint inhibitors enhance the activity of cytotoxic T cells by inhibiting the negative regulators of T cells. Activated cytotoxic T cells express immune checkpoint

molecules on their surface and become exhausted. Among immune checkpoint molecules on T cells, PD-1 is expressed on activated cytotoxic T cells, which receive inhibitory signals by interacting with PD-L1 on tumor or immune cells. Anti-PD-1/PD-L1 therapies have shown great success for cancer immunotherapy [39, 40]. There are various types of immune checkpoint inhibitors that are used in clinical practice and trials.

The most widely used ICIs are anti-PD-1 antibodies. To develop companion diagnostics for anti-PD-1 antibodies, the tumor microenvironment has been extensively investigated from the aspect of cancer immunology. Several biomarkers have been reportedly associated with responses to the anti-PD-1 antibody, including the proportion of CD8-positive T cells, the PD-L1 expression, and the tumor mutation burden (TMB) [41, 42]. TMB is defined as the number of non-synonymous mutations (somatic, coding, base substitutions, and short indels) per megabase (mut/MB) of genome examined. A high TMB may increase the possibility of generating immunogenic neoantigens, which facilitate the recognition of a tumor as foreign, leading to the generation of neoantigen-specific T cells [43–45]. The concept of neoantigen-specific T cells produced the therapeutic strategies of neoantigen vaccines and genetically engineered neoantigen-specific T cells [46, 47].

Differences exist in cytotoxic T cells in the tumor microenvironment among various types of cancers. Since a high TMB is estimated to lead to the generation of neoantigen-specific T cells, TMB may be one of the factors associated with the antitumor cytotoxicity of T cells in the tumor microenvironment. An analysis of 27 cancer types revealed that the median frequency of non-synonymous mutations varied by more than 1000-fold across cancer types [48]. Pediatric cancers showed frequencies as low as 0.1/Mb, whereas melanoma and lung cancer exceeded 100/Mb [48]. The high mutation rate in melanoma is largely explained by the mutagenic effects of ultraviolet radiation [49]. Lung cancers share a mutational spectrum, consistent with their exposure to the polycyclic aromatic hydrocarbons in tobacco smoke [50].

4.4 Detection of Cytotoxic T Cells in the Tumor Microenvironment

To evaluate cytotoxic T cells in the tumor microenvironment, it is critical to identify the types and activities of each tumor antigen-specific cytotoxic T cell. A tetramer assay may be used to analyze the types of tumor antigen-specific T cells. The tetramer assay identifies T cells that target specific antigens.

Attempts have been made to identify phenotypic markers of tumor antigen-specific T cells. For example, the CD103 integrin is one of the promising markers for tumor antigen-specific T cells. CD103 on CD8+ T cells binds to the epithelial cell marker E-cadherin, which induces the retention of T cells in epithelial tumors and maturation of immunological synapses, resulting in TCR-dependent cytotoxicity against tumor cells [51].

The TCR repertoire analysis provides useful information for understanding T-cell populations. The TCR repertoire analysis revealed the types of TCR and their frequencies. Furthermore, a single-cell analysis added detailed T-cell profiling to the information provided by the TCR repertoire analysis based on the gene expression data of each T cell [52].

Although the single-cell analysis reveals the gene expression of T cells, gene expression is not necessarily equal to the function of the cell. Based on gene expression, synthesized proteins exhibit functions in cells. Therefore, functional assays are needed to evaluate the activities of cytotoxic T cells. Several methods are employed to analyze T-cell activities. The main principle of these methods is to detect cytokines (mainly IFNγ, TNFα, and IL-2) from stimulated T cells. The methods used to stimulate T cells are antigen peptides, CD3/CD28, or PMA/ionomycin stimulations. Cytokines produced by T cells are analyzed by flow cytometry, ELISA, or ELISpot. For example, in the analysis of IFNγ, TNFα, and IL-2 production from CD8 T cells in breast and melanoma tumors, CD8+ T cells from breast tumors retain a polyfunctional effector cytokine production capacity for cytokines [53].

The crucial function of cytotoxic T cells is cytotoxicity against tumor cells. BiTE is useful for evaluating T-cell cytotoxicity. BiTE binds both T cells (via CD3) and tumor cells (via tumor antigen), leading to T-cell cytotoxicity against tumor cells. A single-cell suspension of a tumor digest may be evaluated for T-cell cytotoxicity using a BiTE assay. The linkage of tumor cells and T cells by BiTE leads to the formation of similar immunological synapses to those formed by endogenous cytotoxic T cells and tumor cells [54–56]. The mechanisms underlying T-cell cytotoxicity against tumor cells induced by BiTE are dependent on the perforin and granzyme B pathway [54].

In contrast to CAR T cells, BiTEs do not contain molecules for a co-stimulation to activate naïve T cells. The lack of a T-cell co-stimulation of BiTEs via CD28 will prevent the differentiation of naïve T-cell clones and explains the predominant engagement for the redirected lysis of effector T cells, which are no longer in need of a CD28 co-stimulation. Several antigens have been used for the BiTE assay (Fig. 4.2). Lung, ovarian, and renal tumor tissues were analyzed using folate receptor 1 (FolR1) as a target antigen and the FolR1-positive ovarian cancer cell line Skov3 [33]. In this study, TILs with a high frequency of PD-1^{hi} showed significantly impaired tumor cell killing and did not respond to the PD-1 blockade. BiTE specific to CD19 was also utilized with the CD19^{+} B cell line C1R:A2 to evaluate TILs in breast and melanoma tumor tissues [53]. This analysis revealed that PD-1^{+} CD8^{+} TILs from breast tumors retained cytotoxic activity better than TILs from melanoma tumors. Regarding non-small cell lung cancer (NSCLC), different types of BiTE have been used to evaluate TILs in lung tumor tissues [57, 58]. A previous study developed a BiTE assay system to evaluate the cytotoxicity of TILs by using BiTE specific to EphA2 and the EphA2-positive cell line U251. This assay revealed that the cytotoxicity of TILs in lung tumors was related to T-cell immune profiling, cytokine production, and smoking status. Another study utilized BiTEs specific to mesothelin and the mesothelin-positive cell line EMMESO and found that the cytotoxicity of TILs in lung tumors was related to cytokine production by CD8^{+} TILs [58].

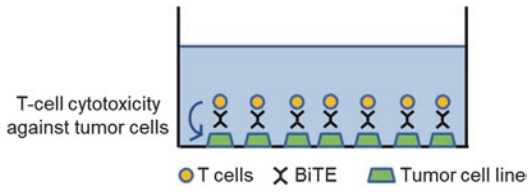

Fig. 4.2 BiTE assay. Tumor cell lines were co-cultured with T cells and BiTE specific to CD3 and the tumor antigen. After the co-culture, T-cell cytotoxicity was measured by evaluating the viability of tumor cell lines

One of the main purposes of these BiTE assays is to evaluate total T-cell cytotoxicity in the tumor microenvironment, thereby clarifying the real strength of antitumor immune responses. In order to achieve this, the number of TILs in a co-culture assay with BiTE is a critical issue. Among previously reported BiTE assays, the numbers of TILs differed. The number of T cells was set based on CD45^{+}CD3^{+} cells with bulk tumor-infiltrating cells (TICs)[33], positively isolated CD8^{+} T cells [53], CD8^{+} T cells with bulk TICs [58], or bulk TICs [57] in each assay. The BiTE assay with isolated CD8^{+} T cells is considered to be ideal for evaluating the cytotoxicity of CD8^{+} T cells from tumor tissue. However, in the tumor microenvironment, the ratios of each cell population are also a critical factor and different in each patient.

Another critical issue for these assays is non-specificity to tumor antigens. In the tumor microenvironment, cytotoxic T cells are not necessarily tumor antigen-specific. One potential issue for evaluating T-cell cytotoxicity is distinguishing between tumor antigen-specific and tumor-unrelated T-cell cytotoxicities. Previous studies reported that in human lung and colorectal cancer tissues, CD8+ T cells were not only specific for tumor antigens but also recognized a wide range of epitopes unrelated to cancer (such as those from Epstein–Barr virus, human cytomegalovirus, or influenza virus) [59, 60]. However, these virus-specific T cells in the tumor microenvironment may contribute to antitumor immunity [61]. Virus-specific memory T cells may be repurposed for antitumor immunotherapy.

To evaluate the accuracy of the various methodologies described above, it is critical to understand the real strength of antitumor immune responses in the tumor microenvironment. Therefore, the efficacy of immunotherapy in cancer patients is considered to be one of the best evaluation methods. However, due to limited tumor volumes, difficulties are associated with the analysis of cytotoxic T cells for advanced-stage patients eligible for ICIs. Therefore, the analysis of peripheral blood in evaluations of cytotoxic T cells will be the focus of future research.

4.5 Relationship Between Cytotoxic T Cells in Tumors and Peripheral Blood

Previous studies reported a correlation between T cells in tumors and peripheral blood. These findings led to liquid biopsy for cancer immunotherapy. By using the tetramer assay, tumor-associated antigen-specific T cells were identified in peripheral blood. For example, peripheral $CD8^+$ T cell populations specific to the tumor-associated antigen MART-1 or tyrosinase were identified in patients with metastatic melanoma using peptide/HLA-A∗0201 tetramers [62].

Moreover, specific TCR clonotypes were found in tumors and peripheral blood [63, 64]. Although tetramer and TCR repertoire analyses of peripheral blood are potential biomarkers for monitoring the tumor immune microenvironment, the total activity of cytotoxic T cells in the tumor microenvironment remains difficult to evaluate. Recent studies examined peripheral T-cell cytotoxicity. In a co-culture of peripheral T cells and tumor organoids, tumor-reactive T cells exhibited cytotoxicity against autologous tumor organoids, not autologous healthy organoids [65]. A simpler method was developed using BiTE. Peripheral T-cell cytotoxicity was easily measured in a co-culture of PBMC and a tumor cell line with BiTEs. The peripheral T-cell cytotoxicity of early-stage lung cancer patients correlated with T-cell cytotoxicity in the lung tumor tissue of the same patients [57]. This correlation was supported by T-cell immune profiles, cytokine production, and the findings of the TCR repertoire analysis from peripheral blood and lung tumor tissues. Moreover, peripheral T-cell cytotoxicity has the potential to predict the efficacy of anti-PD-1 therapy.

4.6 Future Directions

In the future development of effective cancer immunotherapy and companion diagnostics of precision medicine for cancer immunotherapy, the main focus will continue to be cytotoxic T cells in the tumor microenvironment. To clarify the total number, type, and activity of tumor antigen-specific cytotoxic T cells in the tumor microenvironment, the development of innovative technologies is eagerly desired. Although a single-cell analysis of T cells is a promising approach, this analysis is based on gene expression, and, thus, the development of technologies for a functional analysis of T cells is also important. A T-cell cytotoxicity analysis using BiTE technology provides a useful option. In the future, to clarify the function of each T cell, a single-cell imaging analysis of T cells in the tumor microenvironment may open a new era in this field [66]. Attempts to identify positive and negative regulators of cytotoxic T cells will also be conducted. Due to future innovations, the generation of effective cancer immunotherapies is anticipated.

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Mucosal-Associated Invariant T Cells in Tumors of Epithelial Origin

5

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Abstract

Mucosal-associated invariant T (MAIT) cells are innate T lymphocytes that circulate in blood and also reside in mucosal tissues. Blood MAIT cells are typically highly Th1-polarized, while those in mucosal tissues include both Th1- and Th17-polarized subsets. MAIT cells mount cytokine and cytolytic responses as a result of T cell receptor (TCR)-mediated recognition of microbially derived metabolites of riboflavin (vitamin B_2) presented by the MR1 antigen-presenting molecule. Additionally, MAIT cells can be activated by inflammatory cytokines produced by antigen-presenting cells (APCs) that have been exposed to pathogen-associated molecular patterns (PAMPs). Since the antigenic metabolites of riboflavin recognized by MAIT cells are produced by many microorganisms, including pathogens as well as non-pathogenic colonists, the inflammatory state of the tissue may be a key feature that determines the nature of MAIT cell responses. Under normal conditions where inflammatory cytokines are not produced, MAIT cell responses to microbial metabolites may simply serve to help maintain a healthy balance between epithelial cells and microbial colonists. In contrast, in situations where inflammatory cytokines are produced (e.g., pathogenic infection or damage to epithelial tissue), MAIT cell responses may be more potently pro-inflammatory. Since chronic inflammation and microbial drivers are associated with tumorigenesis and also trigger MAIT cell responses, the nexus of MAIT cells, local microbiomes, and epithelial cells may play an important role in epithelial carcinogenesis. This chapter reviews current information about MAIT cells and epithelial tumors, where the balance of evidence suggests that enrichment of Th17-polarized MAIT cells at tumor sites associates with poor patient prognosis. Studying the role of MAIT cells and their interactions with resident microbes offers a novel view of the biology of epithelial tumor progression and may ultimately lead to new approaches to target MAIT cells clinically.

Keywords

MAIT cells · Microbiome · Epithelial tumors · IL-17A · IFN-γ · Inflammation · Dysbiosis · Riboflavin · Cytotoxicity · MR1 · Exhaustion · Th1 · Th17 · Tissue residency · Tumor-infiltrating lymphocytes

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5.1 Introduction

Mucosal-associated invariant T (MAIT) cells are a conserved population of innate T lymphocytes that are enriched at mucosal sites and express a semi-invariant T cell receptor (TCR), in which there is a canonical TCRα chain rearrangement (TRAV1-2 recombined with TRAJ33) that is paired with diversely rearranged TCRβ chains that preferentially use particular Vβ gene segments (e.g., TRBV20 or TRBV6) [1, 2]. Unlike conventional T cells that recognize peptides displayed on major histocompatibility complex (MHC) class I and II molecules, MAIT cells recognize antigens displayed by the MHC-related protein 1 (MR1) [2]. The antigens recognized by MAIT cells are small-molecule metabolites produced by microbes. The best characterized of these are metabolic intermediates produced along the riboflavin (vitamin B_2) biosynthetic pathway. While multiple riboflavin pathway metabolites stimulate MAIT cells to varying degrees, the most highly potent is 5-(2-oxoprophylideneamino)-6-D-ribitylaminouracil (5-RU) [3, 4]. Metabolites of folic acid (vitamin B9) derived from microbes, diet, or supplements also bind to MR1 molecules, but these have been found to inhibit MAIT cells [3, 5]. Thus, MAIT cell activation is both positively and negatively regulated by antigens derived from microbes.

Based on their highly restricted TCR usage, their activation by non-peptidic molecules that resemble molecular patterns, and their expression of a key transcription factor (promyelocytic leukemia zinc finger or "PLZF") that is characteristically used by innate lymphocytes [6–8], MAIT cells are considered to be innate T lymphocytes. However, like conventional MHC-restricted T cells, MAIT cells go through development and selection in the thymus. Whereas conventional T cells undergo positive and negative selection by MHC molecules expressed on thymic epithelial cells and dendritic cells, respectively, MAIT cells are selected on double-positive ($CD4^+CD8^+$) cortical thymocytes in an MR1-dependent manner [9]. Unlike conventional T cells, developing MAIT cells in the murine thymus already show evidence of preprogramming for eventual lymphoid or nonlymphoid tissue localization [10]. Moreover, murine MAIT cells acquire expression in the thymus of either T-bet or RORγt, transcription factors that drive Th1- or Th17-like functions, respectively, and that may help to direct their subsequent tissue localization [11]. Notably, this delineation is not as clear in humans, as MAIT cells from human thymus co-express T-bet and RORγt [12]. Nevertheless, in both humans and mice, MAIT cells appear to undergo greater functional specialization during development in the thymus than conventional T cells.

Once they exit the thymus, exposure to microbes appears to drive MAIT cell expansion [11, 13]. In support of this hypothesis, the frequency of human MAIT cells in the blood increases with age, starting with very few MAIT cells detectable in umbilical cord blood and increasing until about 30 years of age [12]. Compared to adult humans, laboratory mice have very low frequencies of MAIT cells in circulation, presumably as a result of their lower microbial exposure [14]. Germ-free mice (lacking microbial colonists) have extremely low frequencies of MAIT cells, and specific-pathogen-free mice (which have commensal microbes, but lack specific pathogens) have higher MAIT cell frequencies [11]. When germ-free mice are reconstituted with human microbiota, they acquire similar levels of MAIT cells in the colon as are found in specific-pathogen-free mice [15, 16]. These findings from murine models suggest that MAIT cells expand in the periphery in response to nonpathogenic microbial colonists. A recent study of adult human volunteers infected with *Salmonella* Paratyphi A found that MAIT cells expanded during the infection in a manner that was biased according to TCR clonotype [17], suggesting that microbial infections may also shape the peripheral MAIT cell repertoire. Together, these observations underscore the importance of microbes in driving clonal expansion of MAIT cells in the periphery and illustrate that MAIT cells are responsive both to nonpathogenic microbial colonists and to infections by pathogenic microbes.

MAIT cells typically comprise 1–10% of $CD3^+$ T cells in human blood [18, 19], and frequencies are significantly higher in the liver where they can make up as much as 50% of the T cell population [20]. As suggested by their name, MAIT cells have also been detected in mucosal tissues, including lung, kidney, intestine, female genital tract, prostate, ovary, and breast [21–28]. Recently, it was shown that MAIT cells are present in human lymph, suggesting that tissue egress and lymphatic recirculation occur under steady-state conditions [29]. However, MAIT cells circulating in peripheral blood may represent a distinct compartment from those in mucosal tissues, since MAIT cells from blood and tissues show differences in their cytokine production profiles. Blood MAIT cells typically produce Th1 cytokines (IFN-γ and TNF-α) with little or no evidence of IL-17 production [1, 30], while MAIT cells from tissues show a much greater proportion of IL-17 producers [25, 28]. Furthermore, parabiosis experiments in mice suggest that most MAIT cells found in tissues are resident cells that do not recirculate [10]. Thus, MAIT cells appear to comprise two distinct populations: one that is tissue-resident and is highly enriched for IL-17 producers and a population that circulates in the blood and is almost exclusively Th1-like.

In recent years, it has become clear that complex microbiomes reside on essentially all tissues that are contiguous with body surfaces, including on areas that were once thought to be largely sterile like breast epithelial ducts and lungs [31–34]. Moreover, the microbial makeup of tissue microbiomes varies according to tissue type [35]. A number of studies have suggested that MAIT cell populations respond in a nonuniform way to different microbial species, with certain TCR clonotypes being favored for specific microbial species [1, 36]. Thus, it seems quite possible that the makeup of the local microbiome is involved in MAIT cell tissue localization and retention—in other words, that MAIT cells may "choose" their tissue residence based on how well their specific semi-invariant TCRs fit with the local supply of microbially derived Ags.

Under normal circumstances, MAIT cells in tissues likely carry out functions that contribute to the maintenance of a healthy equilibrium between host epithelia and resident microbes. However, dysbiosis (changes in the microbial populations colonizing tissues) has been shown to contribute to the development of cancers of epithelial origin, such as colorectal cancer [37], gastric adenocarcinoma [38], and breast cancer [33]. Since MAIT cells are resident in mucosal tissues and specifically activated by microbial metabolites, and the nature of the local microbial colonists may have an important impact on cancers of epithelial origin, this chapter will focus on the tripartite nexus of epithelial tumors, microbes, and MAIT cells.

5.2 Microbes and Cancer

Microbial infection, or in some cases simply the presence of microbial components that have breached epithelial barriers, is inextricably linked to inflammation. Exposure to microbial ligands initially induces an acute inflammatory response by the host that involves the permeabilization of blood vessels, local accumulation of fluid (edema), and recruitment of leukocytes. Mediators associated with acute inflammation facilitate lymphocyte activation, expansion, and recruitment to sites of infection. Acute inflammation is self-resolving upon elimination of the pathogen and its corresponding molecular components from the system. However, when clearance of a pathogen is not successful or in cases where there is continuing insult or irritation from toxins or other damaging factors, chronic inflammation can occur. Chronic inflammation was first hypothesized to play a role in cancer in 1863 [39]; since then, it has been appreciated that prolonged exposure of host cells to inflammatory factors (e.g., cytokines, lipid mediators, reactive oxygen species) can drive tumorigenesis by creating genomic instability, genomic mutations, and epigenetic modifications [40]. Examples of this include chronic inflammatory disorders such as inflammatory bowel disease that are associ-

ated with an increased risk for certain tissue-related cancers [41]. However, in addition to their role in chronic inflammation, it is now clear that microbes can promote tumorigenesis in a variety of other ways, as detailed below.

5.2.1 Colorectal Cancer

Colorectal cancer (CRC) is the third most lethal neoplasia in males and females in the United States [37]. Healthy epithelial cells lining the lumen of the colon are disrupted during the initiation phase by processes associated with the environment, diet, or presence of certain bacterial species [37]. For example, *Bacteroides fragilis* produces a metalloprotease enterotoxin that can disrupt the intestinal barrier by increasing cell proliferation and cleaving adhesion molecules [37]. After epithelial barrier layers are compromised, microbial factors can begin to drive tumorigenesis. Several major microbial drivers of CRC have been defined. *Enterococcus faecalis* produces superoxides that can cause DNA-protein cross-linking, modifications of DNA bases, and breaks in the genomic DNA of epithelial cells [37]. *Escherichia coli* of the subgroup B2 can facilitate epithelial cell genome instability by producing colibactin, a toxin that can initiate DNA double-stranded breaks and subsequently increase gene mutations [37]. *Fusobacterium nucleatum* appears to promote both tumorigenesis and tumor immunosuppression by invading epithelial cells, facilitating tumor cell proliferation by Toll-like receptor (TLR) ligation, and inhibiting leukocyte activity [42].

5.2.2 Gastric Cancer

Gastric cancer is the fifth most common cancer in the world and has been associated with the presence of *Helicobacter pylori*, which colonizes the gastric mucosal tissue of roughly 50% of the human population [43]. Certain strains of *H. pylori* contribute to the development of gastric adenocarcinoma by releasing compounds ("virulence factors") that cause oxidative stress and DNA damage. Furthermore, over time as *H. pylori* continues to colonize the stomach, the gastric mucosa begins to atrophy, affecting the uptake of nutrients and gastric acidity, and an outgrowth of other microbes can occur because these alterations create more favorable growth conditions. The presence of *H. pylori*, as well as the newly expanded microbiota, facilitates an immune response that can lead to tissue damage and chronic inflammation that can subsequently promote carcinogenesis [44].

5.2.3 Breast Cancer

Breast cancer most often arises from the basal and luminal epithelial cells lining the ducts and lobules that make up the breast ductal network. The human breast has recently been recognized to harbor a microbiome of its own [31–33, 45]. Several studies have demonstrated that bacteria from the phylum *Proteobacteria* are the most abundant in healthy breast tissue [31, 33]. However, in cancerous breast tissue, the taxa *Enterobacteriaceae*, *Bacillus*, and *Staphylococcus* were found to be in higher abundance compared to healthy tissue [32]. Breast isolates from some of these taxa (*E. coli* and *S. epidermidis*) were able to facilitate DNA double-stranded breaks, while *Bacillus* did not [32]. A third study revealed that *Methylobacterium radiotolerans* was also relatively enriched in breast tumor tissue [33]. Thus, together these studies suggest that breast cancer is associated with dysbiosis of the breast microbiome.

5.2.4 Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer. In 75% of HCC cases, the pathogenic driver is either hepatitis B virus (HBV) or hepatitis C virus (HCV) [46]. HBV is thought to drive tumorigenesis by induction of host genome instability after viral genome integration and by insertional activation of oncogenes [46]. HCV does not integrate into

the host genome; however, it is thought to promote tumorigenesis by modulating cell signaling, cell metabolism, and host immunity. In addition to these viral components, there is mounting evidence that alteration in the gut microbiota is also a critical factor in the progression of chronic liver diseases to HCC [47].

5.3 MAIT Cells and Microbes

5.3.1 Activation Pathways

MAIT cell activation can be triggered in either a TCR-dependent or in a cytokine-driven (TCR-independent) manner or by a combination of both of these signals. The functional responses of MAIT cells include production of effector cytokines (IFN-γ, TNF-α, and IL-17), granzyme B production and degranulation, and proliferation. However, different routes of activation give rise to distinct patterns of response. TCR triggering alone drives short-term IFN-γ and TNF-α responses, but not sustained effector function in human blood MAIT cells [48]. Similarly, when blood MAIT cells are activated by cytokines alone, they produced a partial response profile consisting of IFN-γ and granzyme B production, but little or no TNF-α [19, 48, 49]. In order to produce their full spectrum of functional responses, MAIT cells require some combination of pro-inflammatory cytokines (IL-12, IL-15, IL-18, and/or IFN-α) in concert with TCR triggering [19, 49, 50]. In vivo, TCR-dependent stimulation requires that the MAIT TCR recognizes MR1 molecules displaying an appropriate antigen at the surface of an antigen-presenting cell (APC) [3]. MR1 molecules are widely expressed by many cell types, including by immune cells that patrol epithelial tissues as well as by epithelial cells themselves [12]. However, in most cases, the APC either must be infected by a riboflavin-producing microbe or must have been directly exposed to the microbe in order for relevant antigens to be taken up and loaded into MR1 molecules. Thus, the proximity of APCs to metabolically active microbes, as well as APC responses to microbial exposure (e.g., production of inflammatory cytokines), likely also plays an important role in MAIT cell activation.

5.3.2 Role of Inflammation

The requirement for both TCR and pro-inflammatory cytokine signals in order for MAIT cells to produce a full spectrum of functional responses suggests that inflammation may differentiate MAIT cell responses to a microbial infection from their responses to non-damaging commensal microbes. Since both pathogenic and nonpathogenic microbes produce antigenic compounds that activate MAIT cells, TCR triggering in the absence of inflammatory cytokines (as might result from the presence of a riboflavin producing commensal microbe) may induce a functional, but unsustained, response that is oriented toward maintaining homeostasis. On the other hand, recognition of microbial antigens in the context of an inflammatory response (as would likely be the case during a microbial infection) might cue MAIT cells to produce a strong defensive response. This idea that TCR signals combine with the state of inflammation to dictate the response of MAIT cells was recently underscored by Prlic and colleagues [19, 48].

5.3.3 Differing Antigenicity of Bacterial Species

The major phyla of bacteria found in the human gut are typically *Firmicutes* and *Bacteroidetes*, although there is variability in the ratio of these phyla from one individual to another [51]. Regarding MAIT cell activation and these phyla, Tastan et al. showed that *Bacteroidetes* had a much higher MAIT TCR stimulation capacity compared to *Firmicutes* [52]. In addition, *Firmicute* species tended to have low MAIT TCR stimulation capacity even though their genomes encoded the riboflavin biosynthetic pathway; suggesting that low levels of riboflavin were being produced [52]. These data suggest that variation in microbial riboflavin production levels may impact MAIT cell activation in vivo.

An additional consideration is the role of microbially derived folate metabolites in regulating MAIT cell responses. Folate metabolites have been shown to function as inhibitory TCR ligands when presented by MR1, whereas riboflavin metabolites are activating [3, 4]. Thus, microbial diversity in regard to folate- versus riboflavin-producing species may also play an important role in dictating MAIT cell responses. While both folate and riboflavin metabolites are likely produced by the complex microbiomes populating many of our tissues, it is not yet clear whether the presence of folate metabolites overrides MAIT activation by riboflavin metabolites or vice versa.

Chronic inflammatory pathologies have also been associated with a reduction in bacterial diversity and changes in the abundance of certain bacteria [53], which could in turn lead to altered TCR stimulation of MAIT cells. For example, inflammatory bowel disease (IBD) is associated with both dysbiosis and inflammation in the gut. The frequency of colonic MAIT cells in IBD patients was found to be positively correlated with disease activity [54], suggesting that they play a pro-pathogenic role in this context. However, it is unclear whether the recruitment, activation, or functional profile of MAIT cells in IBD is principally related to changes in microbial representation (with accompanying changes in the relative levels of folate and/or riboflavin metabolites) or whether this is mainly dependent on co-stimulation from host-derived inflammatory factors. Thus, understanding epithelial MAIT cell activation in vivo may require determining how the makeup of the local microbiome affects the availability of activating versus inhibitory antigens and how the resulting TCR signals are integrated with inflammatory cues.

5.4 MAIT Cells and Cancer

While it has been clear for over a decade that MAIT cells are present in tumor environments [23], there is not yet a clear understanding of the biological functions or clinical significance of MAIT cells in epithelial cancers. As a general paradigm, it is thought that Th1 responses are optimal for fighting cancers, while Th17 responses tend to be pro-tumorigenic. Since MAIT cells can have either Th1-like or Th17-like properties, they could provide either protective or deleterious effects in cancer. Moreover, the impact of MAIT cells on epithelial tumors may depend on whether they infiltrate from the blood MAIT compartment, which is normally almost completely Th1-biased, or whether they derive from the mucosal compartment, since this is much more enriched for Th17-like cells [1, 25, 28, 30]. Here we provide a brief review of recent findings relating to MAIT cells at sites of epithelial carcinogenesis and within cancerous tissues, as well as in the blood circulation of cancer patients (Fig. 5.1).

5.4.1 Colorectal Cancer (CRC)

Studies of human CRC patients have shown increased percentages of MAIT cells at tumor sites compared to unaffected tissue from the

Fig. 5.1 (continued) MAIT cells include Th1-polarized subset that may carry out defensive functions, and are also markedly enriched for Th17-polarized cells that produce IL-17A, which can function in promoting epithelium barrier integrity. (**b**) Pre-cancer and microbial dysbiosis trigger TCR and inflammation. In the presence of inflammatory triggers (e.g., dysbiotic microbiome and/or environmental irritants), pathogen- and/or danger-associated molecular patterns (PAMPs/DAMPs) can enter tissues and drive the production of inflammatory cytokines from tissue-resident monocytes. When MAIT cells are activated by antigen in the presence of such inflammatory cytokines, the MAIT cells exhibit strong effector functions. This effector function can be either a Th1 or Th17 response. Chronic inflammation is associated with pre-cancerous changes to epithelial cells. IL-17 produced by MAIT cells may drive inflammation that plays pro-tumorigenic roles in this context, whereas a Th1 response might be associated with removal of nascently neoplastic cells by cytolysis. (**c**) Tumor environment. Once a tumor is established, tumor infiltrating MAIT cells may skew towards tissue resident subsets that are IL-17 producers. Th1-polarized MAIT cells from blood or tissues may become suppressed (grey cell) if the enter the tumor. Dysbiosis of the local microbiome may be common at sites of epithelial tumors, and this may be associated with selective recruitment of Th17-polarized MAIT cells and/or with MAIT cell exhaustion/suppression. Accumulation of IL-17-producing MAIT cells in tumors is correlated with poor patient prognosis

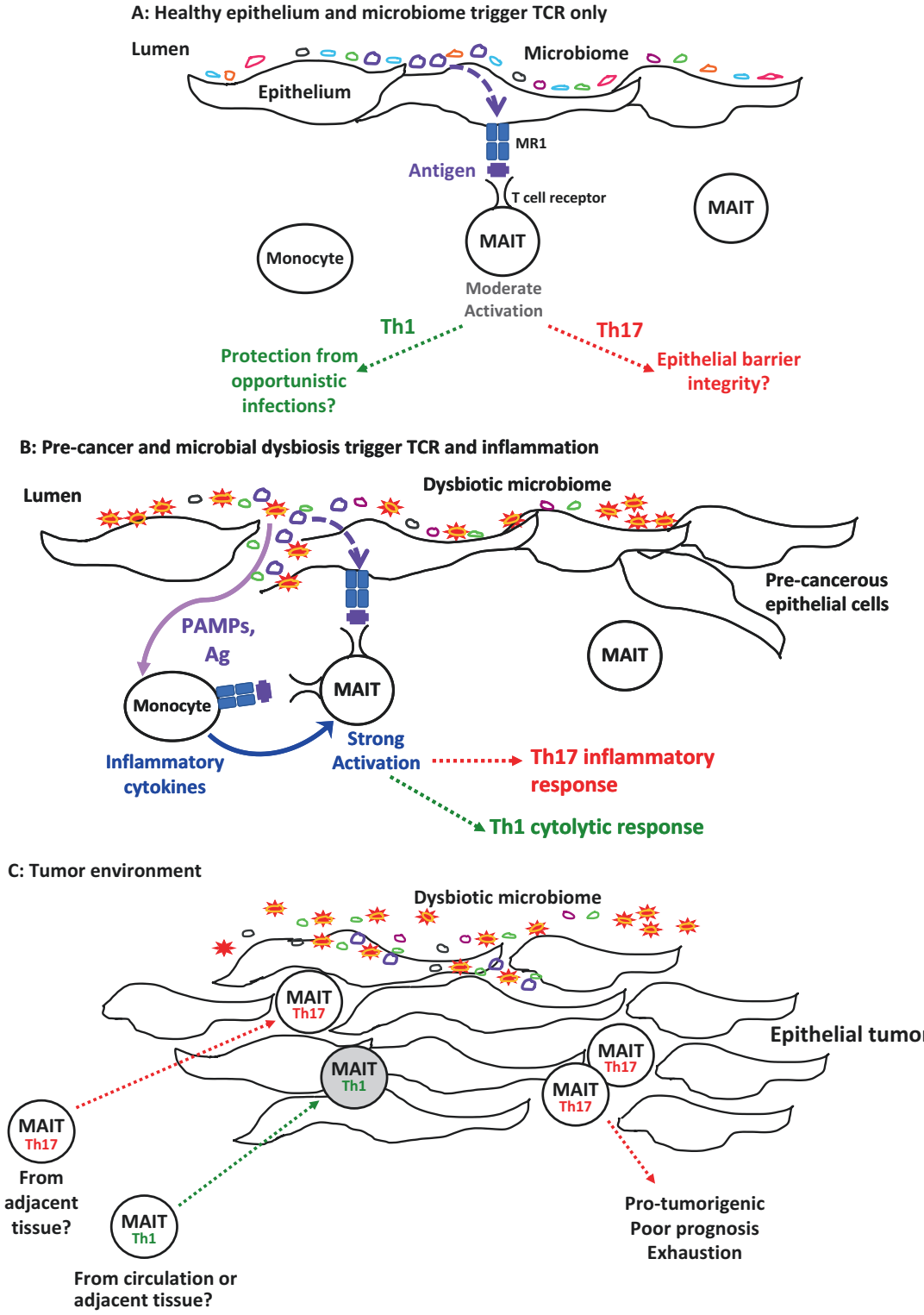

Fig. 5.1 (**a**) Healthy epithelium and microbiome trigger TCR only. Normal epithelial surfaces harbor a healthy microbiome, some of which produce riboflavin metabolites (antigen), which when taken up by epithelial cells are presented on MR1 molecules and can activate MAIT cells. If TCR is the only stimulatory signal, the MAIT cells exhibit only moderate effector functions characterized by unsustained cytokine responses. Tissue resident

same individual [55–58], suggesting that MAIT cells are selectively recruited to CRC tumor tissue. It is not clear whether MAIT cells present in CRC tumor tissue derive from the mucosal MAIT compartment or are recruited from the MAIT subset that circulates in the blood. Several studies observed that the frequency of MAIT cells in the blood of CRC patients appeared diminished compared to healthy control subjects [18, 57, 59], which might indicate recruitment from the blood to the tumor site, although another study found no evidence of a decrease in circulating MAIT cells [55]. In support of the possibility that blood MAIT cells are recruited to tumors, Won et al. showed that circulating MAIT cells in CRC patients expressed chemokine receptors (CCR6 and CXCR6) that are specific for chemokines strongly expressed by CRCs [59], while Ling et al. detected high frequencies of CCR6 expressing MAIT cells from both CRC patients and non-tumor controls [57]. Nonetheless, this suggests a potential mechanism of recruitment for MAIT cells to CRCs.

A functional analysis of MAIT cells from CRC tumors showed that they had partially diminished IFN-γ production compared to MAIT cells from unaffected tissue of the same individual [55], whereas the tumor MAIT cells did not appear compromised in production of IL-17A or IL-2 or cytolytic capacity [55, 56]. Another analysis found a positive correlation between the level of transcript for MAIT TCR and both IL-17A (to a greater extent) and IFN-γ (to a lesser extent) in CRC tissue [57]. Effector function in circulating MAIT cells from cancer patients was also variable as Ling et al. determined that both IFN-γ and TNF-α were decreased and IL-17A was increased in circulating MAIT cells from CRC patients compared to controls [57], while Won et al. showed collectively no changes in IFN-γ, TNF-α, and IL-17A expression from blood MAIT cells from ten mucosal-associated tumor patients (four gastric, three colon, three lung) compared to healthy controls [59]. MAIT cells from colorectal liver metastases (CRLM) had reduced production of IFN-γ compared to healthy hepatic tissue [18, 60].

Overall, these analyses are consistent with MAIT cells tending to have detrimental effects on CRC, since those found within the tumor tissue appear more biased toward a Th17 profile than a Th1 profile. Consistent with this, Won et al. showed an inverse correlation between blood MAIT cells and N staging, suggesting that decreases in blood MAIT cells could reflect cancer progression in mucosal-associated cancers including CRC [59]. Moreover, Zabijak et al. showed that an increase in MAIT cell accumulation at the tumor led to worse patient outcome [58]. Finally, MAIT cell infiltration was pronounced in patients with either early or advanced CRC [57].

5.4.2 Hepatocellular Carcinoma (HCC)

HCC is a cancer that is largely driven by a pre-existing viral infection, such as by hepatitis B virus (HBV) or hepatitis C virus (HCV) [46]. Although HBV and HCV do not produce riboflavin metabolite antigens, MAIT cells have been shown to respond to hepatitis infection via cytokine-driven activation that is due mainly to IL-18 and IFN-α [49]. Since the liver is the tissue that houses the highest percentages of MAIT cells as a proportion of total T cells [20], it seems highly likely that MAIT cells may play important roles in cancers of the liver.

MAIT cell infiltration into HCC tumor tissue appeared decreased when compared to peritumor (the tissue around the tumor) or samples of healthy tissue from the same subjects [61, 62]. One study observed decreased MAIT cell frequencies in the circulation of HCC patients compared to healthy controls [62], while a different study showed no significant differences in MAIT cell frequencies in the circulation of six liver cancer patients compared to healthy controls [59]. MAIT cells from HCC tumor tissue displayed significantly decreased ability to produce the effector cytokines IFN-γ and IL-17A and also produced minimal granzyme B and perforin after stimulation [62]. Instead of an antitumor response, the HCC-derived MAIT cells produced

the pro-tumorigenic cytokine IL-8 and displayed higher percentages of PD-1$^+$, CTLA-4$^+$, and TIM-3$^+$ MAIT cells relative to healthy tissue [62]. Contrary to CRC, chemokine receptors (CCR6, CXCR6, and CCR9) were downregulated by both circulating MAIT cells and tumor-derived MAIT cells from HCC patients compared to controls [62]. However, like CRC, MAIT cell tumor infiltration correlated with poor outcomes in HCC patients [62].

5.4.3 Gastric Cancer

While there has as yet been little direct analysis of MAIT cells within human gastric cancer tumors, there are some interesting indications that MAIT cells may be activated by *H. pylori* infection (a pre-disposing factor for gastric cancer). MAIT cells residing within the gastric lamina propria were found to produce IFN-γ and TNF-α and showed evidence of degranulation in response to *H. pylori*-infected macrophages [38]. In this analysis *H. pylori* infection correlated with a decrease in blood MAIT cells, but no change in gastric lamina propria MAIT cells [38]. However, in a different study, there was a trend toward an increase in MAIT cells in both the circulation and in the gastric mucosae after *H. pylori* infection [63]. Thus, while not completely consistent, perhaps due to differences in patient populations or timing of the analysis in relation to the course of the infection, these studies nevertheless suggest that MAIT cells are activated by *H. pylori* infection.

Analysis of circulating MAIT cell frequencies in 15 gastric cancer patients revealed a significant decrease compared to blood MAIT cells of healthy control subjects [59]. However, when circulating MAIT cells from ten mucosal-associated cancer patients (four gastric, three colon, and three lung) were studied collectively, there was no difference in the ability of the patients' MAIT cells to produce IFN-γ, TNF-α, and IL-17A compared to those of healthy controls [59]. Thus, while the frequency of blood MAIT cells may be reduced in gastric cancer, their functional responses may not be compromised.

5.4.4 Cervical Cancer

MAIT cells have been detected in female genital tract mucosal tissue at frequencies similar to those of blood, but MAIT cells from the vaginal mucosa are preferentially biased toward IL-17A production compared to blood MAIT cells [25]. An analysis of MAIT cells in the blood of cervical cancer patients revealed reduced frequencies compared to healthy control subjects [64]. Moreover, patients with advanced-stage cervical cancer appeared to have lower frequencies of circulating MAIT cells compared to patients with early-stage cancer [64].

5.4.5 Breast Cancer

Until recently, breast ducts (where most breast cancers arise) were not considered to be a mucosal site. However, a recent study showed that murine mammary glands are temporal mucosal tissues that follow strong mucosal immune programs during lactation and involution [65]. Like gastric cancer, there has as yet been little direct analysis of MAIT cells in breast cancer, although Won et al. found no difference in blood MAIT cell frequencies in 13 breast cancer patients compared to healthy control subjects [59]. We recently showed that MAIT cells from healthy human breast epithelial ducts produced a Th17-skewed response to breast carcinoma cells that had been exposed to *E. coli*, whereas they produced both IFN-γ and IL-17A in response to mitogen activation [28]. Notably, *E. coli* is present at higher relative abundance in the microbiome of human breast tumors compared to healthy breast tissue [31, 32]. Moreover, *E. coli* induced stronger MAIT cell TCR stimulation, while *S. epidermidis* and *B. subtilis*, two species from taxa found to be in higher abundance in breast cancer compared to healthy tissue [32], produced intermediate-to-low MAIT TCR stimulation [52]. Based on these results, it is intriguing to speculate that the presence of certain types of bacteria may play a role in the Th17 bias of MAIT cells observed in many epithelial cancers.

5.5 Conclusion

Given their enrichment in mucosal tissues, their ability to produce either Th1 or Th17 responses to specific microbial antigens, and the emerging understanding that epithelial tumors often contain altered microbiomes, it is of great interest to understand the role of MAIT cells in epithelial tumorigenesis. MAIT cells from healthy mucosal tissues are highly enriched for IL-17A production [25, 28] relative to blood MAIT cells, which tend to show strong IFN-γ responses [1, 30]. Analyses of certain types of epithelial tumors (particularly CRC) suggest that MAIT cells are recruited into tumor tissue, resulting in their selective enrichment within the tumor microenvironment. Overall, the available data suggest that MAIT cells within established tumors often show decreased IFN-γ production and may instead favor production of IL-17 depending on tumor type (Table 5.1). Circulating MAIT cells from patients with established epithelial cancers show variable effector cytokine potential, with IFN-γ and TNF-α either decreased or unaffected compared to controls and IL-17A either unaffected or increased compared to controls (Table 5.1). These possible trends toward elevated Th17-like functions of MAIT cells in certain cancers suggest they may contribute to pro-tumorigenic pathways by promoting neutrophilic inflammation, facilitating angiogenesis [66], and promoting epithelial cell metaplasias such as mucus hyper-secretion [67].

Despite the likelihood that MAIT cells have pro-pathogenic effects in established tumors, it is nevertheless possible that they have anti-neoplastic effects prior to tumorigenesis, since IL-17 can also play anti-tumorigenic roles such as increasing epithelial barrier integrity, which in turn reduces exposure to damaging compounds that can drive neoplastic changes. Additionally, MAIT cells with Th1-like functional profiles, or even cytolytic Th17-like MAIT cells, may contribute to immunosurveillance in mucosal tissues by eliminating infected epithelial cells that may be drivers of neoplastic transformation. It will thus be important to determine how tissue-resident MAIT cells acquire both their specific functional profiles and their tissue-homing specificity (are these hardwired during thymic selection, or is there plasticity that depends on cues from the local environment?), as well as to understand the role of tissue-specific microbiomes for MAIT cell recruitment and retention. Equally important is understanding how MAIT cells home to tissues that may not contain a resident microbiome, such as ovaries and prostate [27], as well as fetal tissues in utero [26].

Similarly, future studies will need to further dissect the factors that contribute to IL-17 versus IFN-γ production by MAIT cells. Despite the current paradigm that Th1 and Th17 responses comprise distinct immunological pathways, a significant fraction of primary human MAIT cells from mucosal tissues show production of both IFN-γ and IL-17A after mitogen stimulation [25, 28]. Consistent with this, we observed that a breast duct-derived human MAIT cell clone made both IL-17A and IFN-γ in response to mitogen, but only made IL-17A in response to *E. coli*-exposed breast carcinoma cells [28]. A similar, but less dramatic, finding was reported using MAIT cells from the female genital tract [25]. Thus, the nature of certain activation signals may facilitate an IL-17-biased response by mucosal MAIT cells, and understanding these determinants may shed light on the role of IL-17 producing MAIT cells that infiltrate tumors [57].

Also of importance are the observations that tumor-infiltrated MAIT cells appeared functionally exhausted in HCC, with reduced expression of both Th1 and Th17 cytokines, as well as cytotoxins, and upregulated expression of inhibitory markers such as CTLA-4, PD-1, and TIM-3 [62]. Since this does not seem to be a consistent observation for MAIT cells in other cancers (e.g., CRC), it will be of great interest to determine why different epithelial-derived tumors may have different effects on tumor-infiltrating MAIT cells. A further question is the role of the balance between riboflavin and folic acid metabolites in MAIT cell functioning during tumorigenesis. In addition to how the microbial makeup affects the relative abundance of activating versus inhibitory antigens, it may be important to consider the con-

Table 5.1 Summary of findings from published studies relating to MAIT cells in tumors of epithelial origin

Tumor	*Finding*	*Reference*
Colon	**MAIT Cell Presence**	
	Circulating MAIT cells	
	No difference in MAIT cell frequency compared to controls.	55
	Decreased MAIT cell frequency compared to controls.	57, 59
	MAIT TILs	
	MAIT cells increased at tumor compared to controls.	55, 56, 57, 58
	Disease Correlations	
	Inverse correlation with circulating MAIT cells and N staging.	59
	MAIT cell infiltrate is pronounced in early and advanced CRC.	57
	MAIT cell accumulation at tumor leads to worse patient outcome.	58
	MAIT Cell Phenotype and Function	
	Circulating MAIT cells	
	MAIT cells express high levels of tissue homing chemokine receptors CCR6 and CXCR6	57, 59
	MAIT cells have decreased IFN-γ and TNF-α.	57
	MAIT cells have increased IL-17A.	57
	MAIT cells show no changes in IFN-γ, IL-17A and TNF-α.[a]	59
	MAIT TILs	
	MAIT cells express high levels of tissue homing chemokine receptor CCR6.	57
	MAIT cells have decreased IFN-γ.	55
	MAIT cells are uncompromised (no significant differences) in IL-17A, IL-2, granzyme B, perforin, TNF-α, and CD107a detection.	55, 56
	Positive correlation between MAIT TCR transcripts and IL-17A and IFN-γ, but not TNF-α.	57
	MAIT cells are dysfunctional in IFN-γ production from colorectal liver metastases (CRLM) compared to healthy hepatic tissue.	18 (review), 60
Liver	**MAIT Cell Presence**	
	Circulating MAIT cells	
	Decreased MAIT cell frequency compared to controls.	62
	No difference in MAIT cell frequency compared to controls.	59
	MAIT TILs	
	Decreased MAIT cell frequency compared to peritumor/normal tissue.	61, 62
	Disease Correlations	
	MAIT cell tumor infiltration correlated with unfavorable clinical outcomes in HCC.	62
	MAIT Cell Phenotype and Function	
	Circulating MAIT cells	
	MAIT cells express decreased CCR6, CXCR6, and CCR9 compared to healthy controls.	62
	MAIT TILs	
	MAIT cells express decreased CCR6, CXCR6, and CCR9 compared to healthy controls.	62
	MAIT cells have decreased IFN-γ, IL-17A, granzyme B, and perforin.	62
	MAIT cells produced IL-8.	62
	MAIT cells expressed exhaustion and inhibition markers.	62

[a]Author's show 10 aggregated mucosal-associated cancer samples (3 colon, 3 lung, 4 gastric) together without distinguishing type.

tribution that dietary riboflavin and folic acid compounds can play in MAIT cell activation. This is particularly intriguing regarding folic acid, as Ebbing and colleagues demonstrated that large doses of folic acid (and vitamin B_{12}) may increase cancer risk [68]. With many questions remaining about MAIT cells and their role in tumor initiation and progression, this field is ripe for exploration and has the potential to provide highly novel approaches for clinical or therapeutic intervention.

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Fibrocytes in the Tumor Microenvironment

6

David Roife, Jason B. Fleming, and Richard H. Gomer

Abstract

Tumors have long been compared to chronic wounds that do not heal, since they share many of the same molecular and cellular processes. In normal wounds, healing processes lead to restoration of cellular architecture, while in malignant tumors, these healing processes become dysregulated and contribute to growth and invasion of neoplastic cells into the surrounding tissues. Fibrocytes are fibroblast-like cells that differentiate from bone marrow-derived $CD14^+$ circulating monocytes and aid wound healing. Although most monocytes will differentiate into macrophages after extravasating into a tissue, signals present in a wound environment can cause some monocytes to differentiate into fibrocytes. The fibrocytes secrete matrix proteins and inflammatory cytokines, activate local fibroblasts to proliferate and increase extracellular matrix production, and promote angiogenesis, and because fibrocytes are contractile, they also help wound contraction. There is now emerging evidence that fibrocytes are present in the tumor microenvironment, attracted by the chronic tissue damage and cytokines from both cancer cells and other immune cells. Fibrocytes may aid in the survival and spread of neoplastic cells, so these wound-healing cells may be a promising target for anticancer research in future studies.

Keywords

Tumor · Microenvironment · Stroma · Cancer · Fibrocyte · Fibroblast · Myofibroblast · Monocyte · Scar · Wound · Fibrosis · Benign · Malignant · Metastasis

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6.1 Introduction

A major advance in the understanding of tumor biology came in 1986 when Dvorak et al. published an essay detailing the similarities between tumors and chronic wounds that do not heal [1]. In the wound environment, repair of tissue damage was originally thought to be dependent upon fibroblasts which were quiescent cells resident in the local tissues that only became activated after

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tissue damage occurred. These local cells would then migrate to the site of injury, proliferate, and engage in tissue repair. Another school of thought held the possibility that the wound environment is populated in part by cells that originated from distant sites. Sir James Paget in his *Lectures on Surgical Pathology* first published the observation of circular mononuclear cells entering a wound site from the blood and transforming themselves into long spindle-shaped cells in the wound bed [2]. These cells were characterized in 1994 when Bucala et al. discovered the presence of large numbers of fibroblast-like cells coinciding with the entry of circulating inflammatory cells in wound chamber experiments. These cells, termed fibrocytes, were found to enter sites of tissue injury and contribute to connective tissue and scar formation [3].

Over the past 25 years, there has been a significant amount of progress on research of the differentiation and roles of fibrocytes in physiologic and pathologic processes. Fibrocytes differentiate from a $CD14^+$ peripheral blood monocyte precursor population [4]. Fibrocytes express both hematopoietic (CD45, MHC II, CD34) and connective tissue markers (collagens I and III and fibronectin) [3, 4]. Mature fibrocytes secrete inflammatory cytokines and extracellular matrix proteins to promote angiogenesis and wound contraction [5, 6]. Fibrocytes can be specifically identified in culture by their unique co-expression of CD45RO, 25F9, and S100A8/A9, but not PM-2K. Fibrocytes also change expression of some markers with longer time in culture after differentiation from monocytes, including a loss of CD34, increased expression of Mac-2/galectin 3, and expression of CD49c [7]. Fibrocytes can further become activated by local inflammatory signals such as TGF-β and begin expressing α-smooth muscle actin, transitioning into myofibroblasts, as has been shown in bronchial asthma [8]. Fibrocytes are antigen-presenting cells, participating in parts of the innate response to tissue damage and invasion [9]. Since their identification, fibrocytes have been found in a variety of disease processes, including aberrant wound repair such as hypertrophic scarring and keloid formation, fibrosing diseases such as idiopathic pulmonary fibrosis and myelofibrosis, and autoimmunity [10–15]. More recently, studies have also identified fibrocytes in neoplastic processes. In this chapter, we briefly review our understanding of fibrocytes in the tumor microenvironment.

6.2 Fibrocytes in Benign Tumors

One of the first studies that described CD34-positive spindle-shaped cells in benign and malignant tumors was the pathological study of skin lesions by Kirchmann et al. in 1994, the same year as the discovery of fibrocytes by Bucala and colleagues [16]. CD34-positive spindle-shaped cells, which were likely fibrocytes, were found around trichoepithelioma, a benign skin tumor with follicular differentiation. Basal cell cancers were also surrounded by similar spindle cells; however, the spindle-shaped cells did not appear to express CD34, leading the authors to conclude that a loss of CD34 expression was an indicator of malignancy [16]. This was a finding that was reproduced in many studies to come. Fibrocytes identified by spindle-shaped morphology and CD34 expression were observed surrounding breast ducts containing intraepithelial hyperplasia, ductal carcinoma in situ (DCIS), fibroadenomas, and phyllodes tumors [17]. Of note, there was an observed loss of CD34 positivity as DCIS specimens were higher in grade and a variable expression of αSMA in the different tumors [17]. Given these pathologic observations, one hypothesis is that local factors from the tumor influence the further differentiation of a fibrocyte into a myofibroblast, including loss of CD34 and gain of αSMA. As we will discuss more in the next section, the mechanisms and prognostic effects of this transition are not known at this time, but this staining pattern may be a method of differentiating benign from malignant processes. CD34-positive spindle-shaped cells have been observed to be interspersed in benign tumors of adipose tissue such as lipomas, angiolipomas, angiomyolipomas, and intramuscular lipomas [18]. Although

this study did not specify that these cells were fibrocytes, the presence of CD34 staining on a spindle-shaped cell strongly suggests that it is a fibrocyte.

6.3 Fibrocytes in Malignant Tumors

In 1997, Suster et al. reported one of the earliest descriptions of what were likely fibrocytes in malignant tumors [18]. They described $CD34^+$ spindle-shaped cells that were present not only on benign fatty tumors but also on locally aggressive and malignant fatty tumors such as atypical lipomatous tumors, well-differentiated liposarcomas, myxoid liposarcomas, and the sarcomatous component of dedifferentiated liposarcomas [18]. Since then, multiple studies that we will describe below found $CD34^+$ fibrocytes in malignancies. These studies also often observed that the spindle-shaped cells show less CD34 expression in proximity to the tumor and more expression of αSMA [17, 19–24]. This may in fact reflect the transition of a fibrocyte to a myofibroblast in reaction to cytokines expressed by the tumor. This pattern has been found in pancreatic cancer, ductal carcinoma in situ and invasive ductal carcinoma of the breast, invasive lobular carcinoma of the breast, high-grade cervical intraepithelial neoplasia and squamous cell carcinoma of the cervix, squamous cell carcinomas of the oropharynx and larynx, and urothelial carcinoma of the bladder [17, 19–23, 25, 26]. Figure 6.1 shows immunofluorescent staining of fibrocytes in pancreatic adenocarcinoma and invasive ductal carcinoma of the breast. Instead of staining for CD34, we identified fibrocytes by colocalization of collagen I and CD45RO. We have found this to be a more accurate method to identify fibrocytes, as we and other groups have found CD34 expression to be lost as fibrocytes become more mature and activated.

To understand the possible contribution of fibrocytes to tumor growth, we will discuss the stroma of pancreatic adenocarcinoma in more detail, as it is known to have a strong desmoplastic component, in that the majority of the tumor volume is fibrotic stroma rather than neoplastic cells [27]. The tumor cells of solid tumors such as pancreatic cancer attract fibrocytes through paracrine signaling. These solid tumors produce Th2 cytokines such as interleukin-4 and interleukin-13, and high expression of these cytokines has been linked to cancer cell survival, invasion, and metastasis and poor prognosis [27–33].

We previously found that Th2 cytokines such as IL-4 and IL-13 promote fibrocyte differentiation from monocytes [34]. This desmoplastic reaction also promotes angiogenesis, resulting in numerous disorganized, small, leaky blood vessels and capillaries that provide the tumor cells

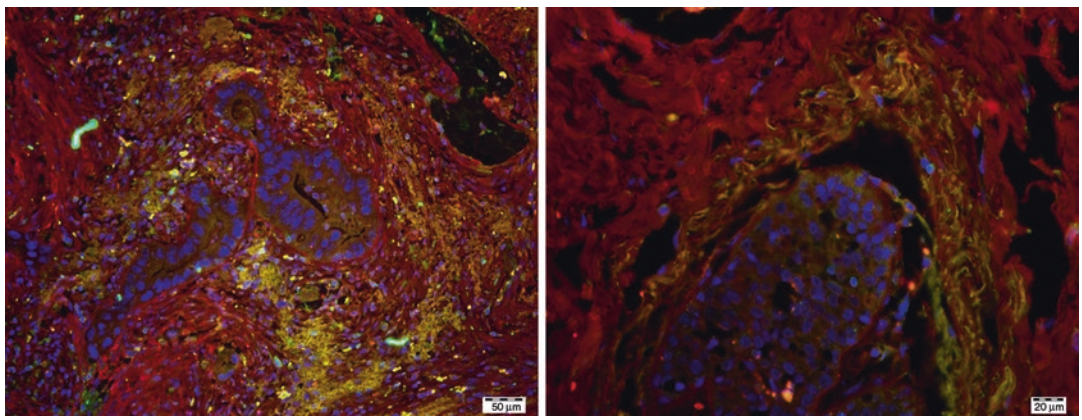

Fig. 6.1 Fibrocytes surrounding neoplastic ducts in pancreatic adenocarcinoma (left) and invasive ductal carcinoma of the breast (right). The fibrocytes are identified by co-expression (yellow) of Collagen-I (red) and CD45RO (green). DAPI staining of nuclei is shown in blue. Bar is 50 µm on left and 20 µm on right

with oxygen [35]. This environment created by the tumor cells and the tumor stroma drives forward its own progression in a positive feedback cycle by production of factors such as transforming growth factor β (TGF-β), matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) [36]. In pancreatic ductal adenocarcinoma, α-SMA$^+$ myofibroblasts form thick sheets surrounding the infiltrating tumor cells. In the pancreatic tissue surrounding the tumor, there is a local concentration of CD34+ fibrocytes around the infiltrating border of the carcinoma. The gradient from tumor-free tissue to invasive carcinoma reveals an abrupt loss of the CD34 positivity [19]. One explanation for this could be that after CD34$^+$ fibrocytes are recruited to the edge of the expanding tumor, they begin secreting ECM and become trapped by abundant ECM and new fibrocytes, myofibroblasts, and stellate cells being recruited around them. Once they are trapped and come under the influence of paracrine signals such as TGF-β, they lose CD34 expression and increase α-SMA expression, thereby becoming a contractile myofibroblast and secreting even greater amounts of ECM in a futile attempt to seal this ever-growing wound environment, unknowingly also contributing to tumor survival by secreting growth factors and promoting angiogenesis. Fibrocytes secrete many of the growth factors found in the tumor microenvironment, such as PDGF, VEGF, TGF-β, MMP9, and FGF, and these could enhance neoplastic cell proliferation by a paracrine effect [36, 37]. ECM proteins such as collagen and fibronectin that are produced by fibrocytes have been shown to contribute to proliferation, survival, metastasis, and drug resistance of cancer cells [38–45]. Bone marrow-derived fibrocytes were also found to be the source of cancer-associated fibroblasts (CAFs) in a mouse model of gastric cancer and caused significantly larger tumors when inoculated along with the cancer cell line [46].

Fibrocytes also appear to contribute to resistance of cancer to therapeutic drugs. In mouse models of malignant pleural mesothelioma and lung cancer, fibrocytes were found to mediate resistance to bevacizumab, an FDA-approved VEGF inhibitor used as a treatment for several cancers, by production of fibroblast growth factor 2 (FGF2) [47].

6.4 Fibrocytes in Metastasis

Following in his aforementioned father's footsteps, the surgeon Stephen Paget contributed a profound theory to cancer research in his "seed and soil" hypothesis. He suggested that the metastasis of cancer is not coincidental and just a matter of random travel through blood vessels of lymphatics but that a receptive microenvironment must be present for malignant cells to establish a metastatic tumor [48]. Recent studies have shown that fibrocytes are one of the cell types that are necessary for tumor metastases. In a mouse model of melanoma, fibrocytes were found to prepare lungs for pulmonary metastases by recruiting Ly-6G monocytes, and the process was dependent on MMP9, CCL2, CCR2, and CCR5 [49, 50]. Fibrocytes have also been observed in liver metastases in a mouse model of colon cancer, and in this study, fibrocyte recruitment was dependent both on CCR1$^+$ neutrophils and subsequent expression of MMP2 and MMP9 [51]. Another study found that myeloid-derived suppressor cells (MDSCs) may ultimately be the source of fibrocytes in cancer metastases and that by inhibiting monocyte differentiation from these MDSCs by knocking out Krüppel-like factor 4 (KLF4) in a mouse model, there were significantly less fibrocytes and myofibroblasts in the lungs and significantly fewer pulmonary metastases of melanoma and breast cancer cell lines [52].

In addition to being present in the stroma surrounding solid tumors, one study has identified higher numbers of fibrocytes in the circulating blood of metastatic cancer patients. These fibrocytes were identified as a novel subset of circulating MDSCs which were expanded in metastatic pediatric sarcoma patients but absent from healthy controls [53]. These cells expressed previously described fibrocyte markers including CD34, CD45, HLA-DR, αSMA (for activated fibrocytes), collagen I/V, MMP9, S100A8/A9,

fibronectin, and LSP-1 and had the capability to produce ECM and promote angiogenesis; however, these cells functioned as immune suppressors rather than antigen-presenting cells as in healthy controls. In the same study, fibrocytes from cancer patients inhibited T-cell proliferation in vitro through production of indoleamine oxidase [53]. Taken together, this work suggests that fibrocytes may have a role in global immune suppression in addition to their local effects in the tumor microenvironment and as a result help metastatic tumor cells initiate new tumors.

6.5 Conclusion and Future Directions

Fibrocytes appear to play an important role in the tumor microenvironment, likely participating in most if not all of the hallmarks of cancer as described by Hanahan and Weinberg, which include activating invasion and metastasis, inducing angiogenesis, resisting cell death, sustaining proliferative signaling, and evading growth suppressors [54]. As demonstrated by the recent successes with immunotherapy, the next frontier of cancer treatment is to continue learning how to harness the normal cells of the body in order to prevent and fight cancer growth. Further research in controlling fibrocytes and their signals for differentiation may become an attractive target for new methods of anticancer therapeutics.

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Models for Monocytic Cells in the Tumor Microenvironment

7

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Abstract

Monocytes (Mos) are immune cells that critically regulate cancer, enabling tumor growth and modulating metastasis. Mos can give rise to tumor-associated macrophages (TAMs) and Mo-derived dendritic cells (moDCs), all of which shape the tumor microenvironment (TME). Thus, understanding their roles in the TME is key for improved immunotherapy. Concurrently, various biological and mechanical factors including changes in local cytokines, extracellular matrix production, and metabolic changes in the TME affect the roles of monocytic cells. As such, relevant TME models are critical to achieve meaningful insight on the precise functions, mechanisms, and effects of monocytic cells. Notably, murine models have yielded significant insight into human Mo biology. However, many of these results have yet to be confirmed in humans, reinforcing the need for improved in vitro human TME models for the development of cancer interventions. Thus, this chapter (1) summarizes current insight on the tumor biology of Mos, TAMs, and moDCs, (2) highlights key therapeutic applications relevant to these cells, and (3) discusses various TME models to study their TME-related activity. We conclude with a perspective on the future research trajectory of this topic.

Keywords

Monocytes · Macrophages · Monocyte-derived dendritic cells · Ontogeny · Differentiation and commitment · Heterogeneity · Cancer · 2D versus 3D ·

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7.1 Introduction

Monocytes (Mos) traffic through vasculature to tissues during steady state and at increased rates during inflammation from cancer [1]. Upon entering cancer-associated tissue, Mos can give rise to tumor-associated macrophages (TAMs) and Mo-derived dendritic cells (moDCs) [1]. Subpopulations of all these cells shape the tumor microenvironment (TME) [1]. Thus, understanding Mo ontogeny and heterogeneity enables improved insight into their roles in the TME and the proper creation and interpretation of human models. Of note, we discuss Mo ontogeny and heterogeneity based on findings derived from human and murine models while recognizing that most murine-derived findings are yet to be validated in humans.

7.1.1 Monocyte Ontogeny

In children and adults, Mos derive from hematopoietic stem cells (HSCs) in the bone marrow [2]. Monopoiesis, a series of differentiation and commitment steps, drives their development [3, 4] and involves intermediary lineage-committed cells including common Mo progenitors (cMoPs), granulocyte-macrophage progenitors (GMPs), and macrophage-dendritic cell progenitors (MDPs) [2]. The sequential transcription of PU.1 and then IRF8 and KLF4 governs monopoiesis [5–7]. GMPs comprise multiple progenitors that differentiate along a spectrum of macrophage (MΦ) or dendritic cell (DC) lineage phenotypes [8]. However, GMPs phenotypically overlap with cMoPs and MDPs [9], suggesting that current definitions oversimplify ontogeny complexities.

Recently, advanced techniques in RNA sequencing (RNA-seq), epigenetic profiling, and fate mapping strategies have facilitated more in-depth understanding of the development hierarchy of Mo ontogeny in normal [10, 11] and cancer [12] settings. Also, the fate of TME-associated Mos is heavily influenced by TME-related cues such as cytokines (M-CSF, GM-CSF, and IL-13) [13–15] and extracellular matrix (ECM) [16], which may differ across cancer types [17, 18]. Thus, TME models must account for these parameters and complexities while remaining adaptable to new discoveries regarding the effect of cancer cells on the differentiation and commitment of cells of monocytic origin.

7.1.2 Monocyte Heterogeneity

Early studies established classical (Cla) ($CD14^+CD16^-$), non-classical (NC) ($CD14^{lo}CD16^+$), and intermediate (Int) ($CD14^+CD16^+$) [19] Mo subsets within the peripheral blood of humans. A developmental relationship, triggered by M-CSF [20], has been observed from the Cla, through Int, to NC subsets [1, 21, 22]. Although gradual transitions across subsets blur their distinctions, the CD14/CD16 nomenclature has proven useful in many studies. Subsets based on differential expression of CX3CR1 [23], CCR2 [24, 25], or 6-sulfo $LacNAc^+$ and $Fc\varepsilon RI^+$ [26–30] were later identified, pointing toward a growing appreciation of diverse Mo subpopulations in humans.

Subset definitions inevitably shift, particularly for Int Mos [31]. Indeed, advanced techniques such as high-dimensional mass cytometry (CyTOF) that allows multiplexed analysis of >40 protein markers in single cells revealed that CCR2, CD11c, CD36, and HLADR can improve the gating purity of Int Mos [32]. Single-cell transcriptional (scRNA-seq) profiling also identified Int Mo sub-clusters, of which 70% are Cla (Mono1) and NC (Mono2) clusters and 30% are Mono3 and Mono4 that, respectively, regulate cell cycle/trafficking and expression of NK/T cell activation genes [33]. In fact, colorectal cancer patients have increased Int Mo percentages, with these being higher in patients with localized disease versus (vs.) those with advanced metastasis [34]. Future studies must thus validate the existence and functions of Mo subpopulations both in healthy and cancer conditions.

Mos also differ by their tissue localization, including their retention within the vasculature of multiple organs [23, 35]. In steady-state conditions, Cla Mos are recruited to tissues where they can differentiate into MΦs or moDCs [11, 36]. On the other hand, NC Mos mainly patrol vasculature [37] through LFA-1 and CX3CR1 [38], scavenging cellular debris and flagging damaged endothelial cells (ECs) for disposal by neutrophils [39]. In inflamed conditions, both Mo subsets increase their trafficking to tissues [40–42]. Here, NC Mos traffic more slowly than Cla Mos [42] and can also give rise to MΦs that secrete inflammatory cytokines [38, 43, 44]. Specifically in cancer, patrolling/non-patrolling Mos can differently modulate primary tumor growth, cancer cell extravasation, and metastatic seeding, with these subsets commonly associated with having pro-tumor or anti-tumor effects, respectively [45, 46].

For the purposes of this review, we adopt the Cla/NC subset nomenclature and further identify Mos by patrolling/non-patrolling classes. The functional term "proinflammatory"/"inflammatory" is avoided as it disregards anti-inflammatory properties of an alleged "(pro)inflammatory" cell [47] and prematurely ascribes cells with ex vivo characterized functions, while they often remain to be validated in vivo. Mos are distinguished from MΦs/DCs as far as evidence is clear. However, where classifications are unclear, we refer to cells of monocytic origin to avoid confusion [42]. Finally, although there is evidence that some human Mo subsets are corollary to murine subsets, there is growing evidence of the heterogeneity between human and murine Mo subpopulations, particularly with regard to cell function [48]. Importantly, human-relevant models are required to fully clarify if murine-derived findings necessarily translate to humans.

7.2 Monocyte Functions in Cancer

Mos have an extensive role repertoire where environmental cues such as cytokines activate distinct transcriptional programs to direct their specific activities in the TME [49]. Here, we discuss current evidence of these various roles (Fig. 7.1) and present outstanding areas that remain to be clarified.

7.2.1 Recruitment to Tumors

Mos are recruited throughout the tumor lifespan, from the early stage of primary tumor growth [50, 51] to late-onset metastases [45, 52, 53]. CCL2/CCR2 signals chiefly recruit Mo to tumors [50, 53], with CCL2 expression correlating with the presence or amount of neoplasia [54]. Many studies in Mo recruitment also implicate modulation by CXCL8/IL-8, CCL5/RANTES, and vascular endothelial growth factor (VEGF) signaling [55, 56], as well as tumor microvasculature upregulation of angiopoietin-2 (Ang-2), CX3CL1, ICAM-1, selectins, and VCAM-1 [57, 58]. Moreover, Cla Mo recruitment could be evolutionarily conserved across tumors as adoptively transferred human Mos traffic to murine tumors [53]. Mos deploy to primary tumors primarily from the bone marrow [59], but the precise mechanism of their trafficking to tissues could differ for different anatomical locations and cancer types [42]. Such features and processes should be considered when modeling the human TME.

7.2.2 Tumoricidal Activity

Mos elicit antitumor activity using multiple pathways. For example, Mos expressing SIRPα can directly phagocytose tumor cells expressing low levels of CD47, which normally provides cells with a protective "don't eat me" signal against phagocytotic cells [60, 61]. Notably, Cla Mos are viewed as the most phagocytic subclass, whereas NC Mos chiefly patrol the vasculature and scavenge cell debris [45, 62]. Growing evidence suggests that monocytic cells can also contribute to cancer cell death by cell contact-mediated antibody (Ab)-dependent cellular cytotoxicity (ADCC) and apoptosis [63–67]. For example, granzyme B expression is induced in human Mos that are treated with TLR8 agonists, leading to

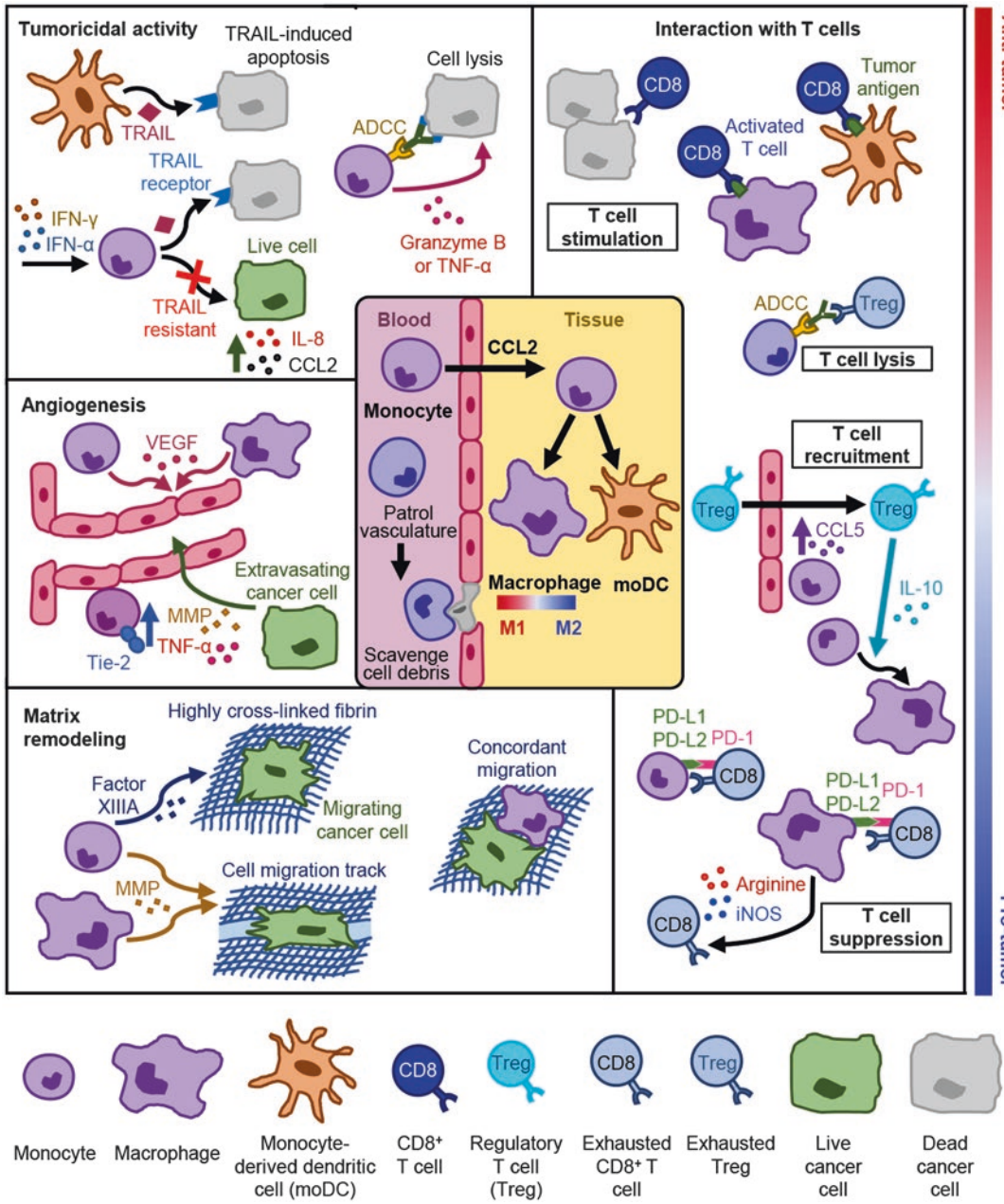

Fig. 7.1 Role of monocytic cells in the tumor microenvironment. Monocytes circulate in vasculature or egress into tissue and differentiate into macrophages/monocyte-derived dendritic cells. These cells display phenotypes along an anti-tumor-to-pro-tumor spectrum. Their roles include the lysis of cancer cells or immunosuppressive regulatory T cells (Tregs), T cell stimulation through Ag presentation, T cell recruitment and immunosuppression, matrix remodeling, and angiogenesis support. (*ADCC* antibody-dependent cellular cytotoxicity, *iNOS* inducible nitric oxide synthase, *MMP* matrix metallopeptidases, *PD-(L)1/2* programmed death-ligand 1/2, *TRAIL* TNF-related apoptosis-inducing ligand)

Mo-mediated ADCC of Ab-coated breast cancer cells [66]. CD16$^+$ Mos engage with Abs bound to cancer cells, inducing Mo secretion of TNF-α and subsequent TNF-α-mediated tumor cell lysis [64]. Mos exposed to IFN-γ and IFN-α can also produce TNF-related apoptosis-inducing ligand (TRAIL) that results in TRAIL-induced cancer cell apoptosis in vitro [67].

Tumor cells alter multiple pathways to evade the tumoricidal activity of Mo-derived cells. To combat the phagocytic functions of SIRPα-expressing Mo-derived cells, solid [60, 61] and hematologic cancer cells [68, 69] upregulate CD47 expression to increase SIRPα inhibitory signaling. Cancers can also be TRAIL-resistant, where a study observed that TRAIL stimulation induces cancer cell lines to secrete cytokines such as IL-8 and CCL2, contributing toward a tumor-supportive TME characterized by heightened accumulation of Mos and increased polarization of myeloid cells toward pro-tumor myeloid-derived suppressor cells (MDSCs) and M2-like MΦs [70]. Additionally, Mos phagocytose tumor-derived microparticles and exosomes, and this suppresses their inflammatory activities [71, 72] and gives rise to immunosuppressive MDSCs [73]. Indeed, many studies in established tumors concur that Mos display only weak or transient tumoricidal activity and, instead, predominantly display (as below-described) pro-tumor functions [74, 75].

7.2.3 Differentiation into TAMs and moDCs

Mos differentiate into TAMs or moDCs depending on the environmental cues of the TME [1, 11, 76]. For example, in the primary tumor, this differentiation process is driven by the exposure of Mos to IL-10 from $CD4^+$ T cells, tumor-synthesized factors including CSF1 and TGFβ, as well as hypoxia due to the poor supply of blood by leaky tumor vessels [76, 77]. scRNA-seq of Mo-derived cells within the TME shows transcriptional profiles or clusters that suggest a transition from blood to intratumoral Mos and then moDCs and TAMs [78]. Notably, although Mos differ from TAMs/moDCs transcriptionally, their phenotypes significantly overlap, and this has led to confusion. For instance, some researchers define $CD11c^+$ Mo-derived cells in the intestines as DCs [79, 80], while others classify these cells to be MΦs [81]. Such findings reinforce that heterogenous populations exist and further study is required to firmly establish unique phenotypes and functions for Mo-derived populations [11].

TAMs are highly abundant within the TME [82] and are viewed to arise from recruited Mos (mostly from the Cla subset and less from NC Mos) [50–53] or from tissue-resident MΦs [11]. However, as seen from the large spectrum of monocytic populations in breast cancer patients [78], there is a need for further studies to better understand the origin of TAMs in the TME. Some studies suggest that TAMs can proliferate [50] and both $CCR2^+$ Mos and resident MΦs contribute to TAM numbers [83]. More recent evidence shows that in some tumor models, $CCR2^-$ mice do not have fewer TAMs [50], suggesting that while CCR2 is fundamental for recruiting Mos to TMEs [50, 53], it is not crucial for amassing TAMs. Such findings also support the notion that TAMs primarily derive from tissue-resident MΦs that are believed to be seeded during waves of embryonic hematopoiesis and to self-renew independently of bone marrow-derived cells during adulthood [11]. Additionally, Mo-TAM differentiation is not fully understood. Cla Mos can differentiate into two populations in the TME that either upregulate DC markers (CD11c and MHCII) or upregulate VCAM-1 plus the murine MΦ marker F4/80 [50, 84]. The differentiation process may further depend on spatiotemporal factors as Mos first localize in deeper regions of the TME but are later found in perivascular sites using sequential CCR2 and CXCR4 signaling pathways in Mo-derived TAMs, respectively [85, 86]. We can speculate that stromal cells secrete factors that drive early recruitment and differentiation of Mos, but cues from the vasculature provide signals that retain monocytic cells within the TME.

TAMs are described with some anti-tumor roles [50, 53] but are generally believed to predominantly play an immunosuppressive role within the TME [87–91]. Higher TAM density at the tumor front correlates with better patient survival [92], suggesting that TAM position in the TME shapes their functions [76, 82, 93]. TAMs are customarily believed to be M2 polarized based on the simplified M1 (IL-1β, IL-6, TNF-α, and CCL3)/M2 (CD163 and CD206) anti-/pro-tumor axis [94, 95], but the M1/M2 dichotomy overlooks how many factors define a MΦ's state [91, 96]. Studies have shown that

M1/M2-like MΦs can co-exist [97, 98] and display mixed M1/M2 functions [99–101], suggesting that the TME supports the emergence of both populations and reinforcing that the M1/M2 concept is likely to be an inaccurate description of Mo-derived cells in the TME. Importantly, this simplistic M1/M2 concept, originally proposed by Mills et al. [102], may stem from how poorly we currently understand polarization cues in the TME because many early studies relied on in vitro protocols that used simple cytokine cocktails or tumor-conditioned media in 2D culture. Also, multiple unique MΦ transcription profiles suggest that specific Mo/MΦ subtypes exist [103] and that TAMs should be grouped or clustered based on high-dimensional analysis (such as scRNA-seq and CyTOF) to account for the complexity in phenotypes, with future studies focused on the function of these subtypes within specific cancers [104, 105].

moDCs form a small fraction of the TME infiltrate [106–108] and often display a phenotype intermediary of Mos and the DC family [109]. In addition to moDCs, other DC subpopulations in the TME include conventional DCs (cDCs) (further categorized by CD103/CD11b expression) and plasmacytoid DCs (pDCs), all of which are efficient cross-presenters of cell-associated antigens (Ags) that can either support or inhibit T cell anti-tumor cytotoxicity in the TME [110]. Specifically for moDCs, these cells show their Mo origins via CD64 and FcγR1 [111, 112] and co-express DC markers (MHCII, CD11b/c) but are viewed as DCs if they have higher MHCII/CD11c expression or dendrite morphology [11]. One study on human breast cancer found that total DCs from the TME ranged from 0% up to 28% of $CD45^+$ leukocytes and clustered closely with Mo/MΦ subsets, supporting the notion that TAMs and DCs are distinct but closely related myeloid subsets in the TME [113].

Polarization within the TME is thought to elicit anti-tumoral effector function through type I IFNs which rapidly mature Mos into tumoricidal moDCs that either produce increased levels of IL-15 to support anti-tumor T helper cell type I responses [114] or express TRAIL to mediate tumor cell apoptosis [115]. Loss of moDCs in tumor-bearing mice can lead to poor chemotherapeutic response [116], and adoptive transfer of Mos [107]/cMoPs [116] can delay tumor growth rates through Ag presentation and drive anti-tumor cytotoxic T cell responses. moDCs are also akin to DCs producing M1-like effector proteins TNF-α and inducible nitric oxide synthase (iNOS) [117] or "inflammatory DCs" [118, 119]. Finally, current understanding of human moDCs is mostly based on studies of in vitro differentiated bone marrow-derived Mos [118]. However, studies in mice show that the specific differentiation cocktail used can result in strikingly different DC subtypes; GM-CSF with IL-4, compared to Flt3L-only differentiation, gives rise to a subtype that more closely resembles in vivo moDCs, whereas the latter gives rise to a phenotype that is typical of cDCs [120]. Thus, consideration should be given to the specific protocols used both in literature and future studies for an improved understanding of moDCs in the TME.

7.2.4 Interaction with Tumor Microenvironment (TME) Matrix

The highly disorganized TME matrix promotes metastasis [121]. The composition of the ECM provides specific biophysical and biochemical cues that influence Mo polarization and activation state [121, 122]. One study showed that THP-1 cells (monocytic cell line) can display spontaneous polarization toward a pro-tumor M2-like phenotype when they are cultured within a 3D in vitro ECM that is rich in hyaluronic acid (HA) [123], an ECM component that is abundant within the TME [124]. Cla Mos can remodel the TME matrix via release of factor XIIIA which cross-links fibrin and provides a scaffold for tumor cells to migrate [125]. In lung cancer patients, densely cross-linked fibrin correlates with $CD14^+$ cells and poor prognosis [125]. $CCR2^+$ Mos that differentiate into MΦs remodel ECM [126] through matrix metallopeptidases (MMP) which degrade collagen and create tracks for cell migration [127]. Moreover, MΦs migrate

concordantly with tumor cells [128, 129], and this contributes to metastasis [128–132]. Interestingly, matrix remodeling is more extensively explored for MΦs [133], due to the short lifespan of Mos in tissue where they promptly differentiate into MΦs [104]. Tumors may also hijack the wound-healing functions of M2-type MΦs that encourage connective tissue cells to reform the ECM to thus shape a pro-tumorigenic TME [134, 135]. Other studies report that Mos give rise to matrix-remodeling programs associated with synthesizing and assembling collagen type I/VI/XIV, which mainly constitute TME ECM [136]. Finally, it would be interesting to understand if undifferentiated Mos can autonomously influence their differentiation into MΦs or M1/M2 polarization by MMP-dependent digestion of the TME ECM, since activated Mos highly produce MMP [49, 137, 138].

7.2.5 Pro-angiogenic Effects

Angiogenesis allows tumors to meet their metabolic needs [139], recruit pro-tumorigenic cell types such as Mos [140], and, in metastatic disease, allows tumor cells to intravasate into tumor vessels to then disseminate from the primary TME [141]. Mos support angiogenesis via VEGF family members, such as VEGF-A, coercing tissue-resident ECs and VEGFR2/CD34$^+$ circulating endothelial progenitor cells to form angiogenic sprouts [142–145]. In vitro, Mos from renal cell carcinoma (RCC) patients are observed to produce more VEGF and better support angiogenesis compared to normal Mos [146]. Studies have also identified a cluster of Mos around tumor blood vessels that express higher levels of Tie-2 than Mos residing elsewhere in the TME or that remain in circulation [147]. Tie-2$^+$ Mos are often studied for their pro-angiogenic role in human cancers as their frequency correlates with tumor vessel density, tumor grade, lymph node status, and frequency of metastasis (TNM stage) [148–150]. Ang-2, overexpressed by tumor vasculature [151], is believed to recruit Tie-2$^+$ CD16$^+$ Mos and augment their production of pro-angiogenic enzymes such as cathepsin B [140, 152]. Tie-2$^+$ Mos secrete other pro-angiogenic factors (MMP and TNF-α) [140, 147, 153] and mediate tumor release of VEGF to recruit other pro-angiogenic Mo-derived cells [154]. MΦs expressing Tie-2 also associate with increased vessel maturation [85], where their depletion by clodronate is linked to the anti-angiogenic effects that was observed in mice [155]. However, future studies should clarify if Tie-2$^+$ MΦs represent polarized tissue-resident MΦs or differentiated Tie-2$^+$ Mos, so that anti-angiogenic therapies can target specific monocytic cell types that mainly drive angiogenesis in the TME. One study provides evidence that Tie-2$^+$CD14$^+$CD45$^+$ MΦ-like cells are specifically found in the blood circulation of cancer patients, and not healthy individuals, suggesting that Tie-2$^+$ cells are bone marrow-derived [156].

7.2.6 Establishing the Pre-metastatic Niche

Beyond their roles in the primary TME, monocytic cells have an important role in establishing the pre-metastatic niche (Pre-MN), distant sites from the primary tumor within the body which enhance the homing of circulating tumor cells (CTCs) in the process of metastasis [157, 158]. Studies of lung metastasis show that CTCs arrest in target tissue vessels [159], enabling tumor-secreted CCL2 to generate a chemoattractive gradient that recruits CCR2$^+$ Mos [53, 160]. These Mos enhance CTC extravasation in part by VEGF secretion, which elevates vascular permeability [161]. This study also found that the genetic or chemical inhibition of CCR2$^+$ VEGFR1$^+$ MΦ (derived from recruited Mos) inhibits metastatic seeding [53, 161]. Other studies have identified a population of metastasis-associated MΦs (MAMs) which promote the extravasation and survival of metastasizing cancer cells by suppressing CD8$^+$ T cell cytotoxicity through superoxide production, thus supporting the establishment of Pre-MNs [52]. Growing evidence further suggests that the primary TME influences the formation of a unique population of MDSCs from particular Mo subsets within the bone

marrow through the systemic release of GM-CSF, G-CSF, IL-6, or tumor-derived extracellular vesicles [162]. These same factors drive systemic monocytosis in cancer and also increase Mo-derived MDSCs which have been shown to suppress anti-tumor T cell responses by anti-inflammatory cytokines and reactive oxygen species (ROS) production [162]. Furthermore, MDSCs can act on distant stromal cells to generate the Pre-MN via cytokine release, pro-angiogenic signaling, and metabolic reprogramming [163, 164].

7.2.7 Interaction with T Cells

Monocytic cells profoundly interact with T cells which directly kill malignant cells [51, 97, 165]. In melanoma, Cla Mos give rise to immunosuppressive Mo-derived cells which produce immunosuppressive iNOS and arginase (Arg), inhibiting the infiltration of effector T cells into the TME [166]. In mice which lacked $CD8^+$ T cells [167], inhibiting CCR2 did not change tumor growth, supporting that effector T cells are downstream targets of Cla Mos which can either suppress or activate T cell functions. Mos and TAMs also express immune checkpoints (proteins that place a "break" on the immune system to keep host immunity in check) such as programmed death-ligand 1/2 (PD-L1/2) that bind to PD-1 on $CD8^+$ T cells to impair T cell proliferation and anti-tumor cytotoxicity [87, 88, 168, 169].

Monocytic cells also present Ags in the context of surface MHCI/II [37], in conjunction with their secretion of T cell-activating cytokines (TNF-α, IL-2, IL-15) or metabolites (iNOS, Arg-1), for homeostasis and response to infection [40, 170]. However, their individual presentations of tumor-associated Ags (TAAs) are less studied. One murine study suggests that $F4/80^{hi}$ Mos can present TAAs to $CD8^+$ T cells as efficiently as MΦ/moDCs [107, 171]. Moreover, TAMs, and not pDCs, which are activated to phagocytose tumor cells by addition of CD47-blocking Abs, can activate $CD8^+$ T cells to induce tumor cell lysis [172]. Also, MHCII-restricted interaction of MΦs (pulsed with OVA-specific peptides) and peptide-specific $CD4^+$ T helper cells can instruct M2-M1 polarization of MΦs, thereby facilitating anti-tumor immune attack [173].

TAA presentation mainly occurs at the primary tumor or lymph nodes, but a recent study shows that $MHCII^+$ Mos also present TAAs to $CD4^+$ T cells within inflamed vasculature of renal glomeruli [174], but future studies are needed to confirm the implications of these findings in other cancer models. Additionally, growing evidence suggests that tissue Mos can retain their monocytic profile without becoming MΦs or moDCs and can patrol for Ags presented across tumor vasculature to transport to draining lymph nodes [37, 40]. Such results support the possible notion that circulating Mos can patrol the vasculature for TAAs and present these to effector T cells to prime them for TAA-specific anti-tumor responses. Thus, future studies should better understand the TAA-presenting capabilities of monocytic cells and where such processes occur with respect to the TME.

Mo-derived cells can also regulate recruitment of effector T cells to the TME. Tumor recruitment of Mos correlates inversely with $CD8^+$ T cell numbers, suggesting that the predominant role of Mos in murine tumor models is in restricting T cell entry into the TME. In murine tumors, CCR2-/CSF1R-based reductions of monocytic cells can increase infiltrating $CD8^+$ T cells and reduce tumorigenesis [167, 175]. Pancreatic cancer patients with lower $CCL2^+$ and higher $CD8^+$ cells display improved survival [176]. Monocytic cells can also secrete CCL5 that recruits regulatory T cells (Tregs) [177] which produce cytokines such as IL-10, differentiating Cla Mos into immunosuppressive TAMs [77, 178]. Interestingly, melanoma patients who responded to immune checkpoint therapy (ICT) that blocked cytotoxic T lymphocyte-associated protein 4 (CTLA-4) have more NC Mos in blood and less intratumoral Tregs [179]. Here, NC Mos induced FcRγ-dependent Treg lysis in vitro [179], suggesting that NC Mos possibly compete against pro-tumor Cla Mos.

7.3 Therapeutic Applications Related to Cells of Monocytic Origin

7.3.1 Biomarkers for Prognosis

Monocytic cells have emerged as biomarkers for early cancer diagnosis. Absolute Mo frequency in blood is associated with improved survival in locally advanced cervical cancer [180]. Improved diagnostic power may be gained from discerning between Mo subsets given their distinct roles as pro- or anti-tumorigenic cells. For instance, lower blood frequencies of Cla Mos and their increase in bone marrow are correlated with improved pancreatic cancer patient survival [176]. Higher levels of myeloid marker CCR2 (primarily recruits Mos to tumors) in metastatic tissues compared to primary prostate tumors is linked to TNM pathologic stage [181]. Finally, PD-L1$^+$ Mo-derived cells are a prognostic factor for patient responders to anti-PD-1/PD-L1 therapy in melanoma [182, 183], head and neck squamous cell carcinoma [184], RCC [185], and colorectal [185] and non-small cell lung cancer [184, 186]. These findings support the capability of monocytic cells to determine disease progression and survival at the clinical level and provide the rationale for future studies aimed at better defining subtype phenotypes and numbers to develop improved therapies and outcomes.

In addition, higher lymphocyte-to-Mo ratio (LMoR) positively correlates with improved prognosis in colorectal [187], lung [188], and ovarian cancer [189]. However, a recent study observed that there is a significant variability in the ratio of T cell to MΦ infiltration across different TMEs and that human tumors are vastly heterogenous [190]. The study clearly shows that patient prognosis must consider multiple factors, such as the extent of neoantigen load and the expression of immunomodulatory genes, both across and within immune cell subtypes [191]. Such findings also emphasize the complexity and intricacies of the human TME that must be modeled precisely to represent the tumor immune milieu in specific cancer contexts.

7.3.2 Combinational Therapeutic Strategies

7.3.2.1 Monocyte-Associated Strategies

Many studies in mice show the potential therapeutic advantage of combining strategies to exploit Mo functions. For example, the combined use of anti-CCL2 Abs and cancer vaccines can lead to reduced Mo accumulation in the TME, enhanced T cell effector functions, and reduced tumor volumes [192]. Vascular density can also be effectively reduced by combining anti-VEGF anti-angiogenesis therapy with the inhibition of Mo activity in the TME via anti-Gr1 Abs, as shown in mice [193]. Also, co-administration of Mos and immunostimulatory IFN-α2a/IFN-γ into xenograft murine models gives rise to reduced tumor growth and prolonged survival [194]. Linehan et al.'s work is one of few human-based studies that demonstrates that co-treatment with a CCR2 agonist (inhibits Mo recruitment) and chemotherapeutic drugs can improve overall survival of pancreatic cancer patients [195]. Notably, the majority of murine-derived findings must be clarified more extensively in humans, reinforcing the need for improved in vitro human TME models.

7.3.2.2 TAM- or moDC-Associated Strategies

TAM-based anti-cancer strategies [76, 196] are broadly classified by limiting their recruitment and localization in the TME [197–201], directly depleting TAMs [202, 203], or reprogramming TAM activities [204–212]. For example, targeting CSF1 can reduce CSF1R$^+$CD163$^+$ MΦs in tumor tissues, translating into positive clinical objective responses in diffuse-type giant cell tumor patients [198]. The chemotherapeutic agent trabectedin can deplete TAMs via apoptosis to give rise to reduced tumor vessel density in patient tumor biopsies [203]. Finally, low-dose gamma irradiation can program the differentiation of iNOS$^+$ MΦs, fostering enhanced infiltration and anti-tumor T cell cytotoxicity [206]. TAMs can also be targeted to achieve anti-tumor

effects alongside other cancer or immune cells such as T cells. For instance, CSF1R blockade can enhance MΦ Ag presentation, but potent tumor regression is only elicited when CTLA-4/PD-1 on $CD8^+$ T cells is also blocked [213]. In vitro or ex vivo tumor Ag-loaded DCs are widely used as cancer vaccines, where they stimulate $CD8^+$ T cells and $CD4^+$ T helper cells to elicit anti-tumor immunity [214]. Further, in vivo tumoricidal activity can be achieved by combining DC vaccination with chemotherapy [215, 216] or phototherapy (therapy using light of specific wavelengths to kill tumor cells) [217]. Other combinational strategies are well reviewed elsewhere [218]. These findings suggest that combinational therapies may be required to block multiple immune evasion strategies that tumors utilize to survive within the TME.

7.3.3 Autologous Monocytic Cell Therapy

Autologous cell therapy (ACT) involves harvesting cells from patients, cell manipulation ex vivo, and re-infusion into patients. This approach provides patients with an adequate supply of highly activated Mos [219], tumoricidal effector MΦs [220], and efficient Ag-presenting moDCs [221]. Overall, clinical studies show that ACT is well tolerated in patients without significant toxicity and decreases cancer relapse frequencies for Mos [222, 223], MΦs [224–226], and moDCs [227, 228]. For example, in melanoma patients, moDC ACT induces cell-mediated anticancer immunity [227, 229] and is also proven safe and potentially effective when combined with chemotherapy [230].

7.3.4 Nano-immunotherapy

Nanoparticles (NPs), particles in the size range of 1–1000 nm, can be engineered to regulate Mo and TAM functions [231, 232]. Lipid and cationic NPs encapsulating siRNA against CCR2 have been developed to interrupt the CCL2-CCR2 axis, disabling Mo recruitment to tumor tissues [201, 233]. Glycocalyx-mimicking NPs (GNPs) can bind to lectin receptors on TAMs, increasing TAM secretion of immunostimulatory IL-12 and decreasing secretion of immunosuppressive IL-10/Arg-1/CCL22. Also, the co-administration of GNPs and anti-PD-L1 Abs can synergistically reduce tumor burden in mice [234]. Lipidoid NPs (LNPs) containing PD-L1 siRNA (siLNPs) can silence PD-L1 in liver-resident MΦs and enhance $CD8^+$ T cell cytotoxicity [235], with similar results being observed for moDCs [236]. Mo/MΦs can also serve as cellular "Trojan horses" that deliver therapeutic cargo due to their tumor-homing capabilities. One study shows that Mos which phagocytized gold nanoshells (Au-NS) can accumulate in breast tumors to result in tumor cell death through photoablation of Au-NS-loaded Mo/MΦs [237, 238]. Also, Mos attached with NP "backpacks" of therapeutic cargo can accumulate more in inflamed organs compared to "free backpacks" [239, 240]. Alternatively, Mos can be loaded with a NP complex of cytotoxic mertansine conjugated to a protease-sensitive peptide [241] and, upon entering lung metastases, differentiate into MΦs that upregulate protease and initiate the on-demand release of mertansine into the TME. Figure 7.2 summarizes the above therapeutic applications.

7.4 Experimental Cancer Models for Studying Monocytes

TME models that study Mos may consider different steps of their activity, from trafficking through vasculature, differentiation, and polarization, to effector functions (including phagocytosis, cytokine secretion, and Ag presentation) and interactions with ECM or TME-specific cells (including cancer-associated fibroblasts (CAFs), tumor, and T cells). These models can mimic the primary tumor or Pre-MN and help to better study mechanisms of human cancer and identify unique human markers. Such models should allow for infusion of whole blood or media containing elements from immune subpopulations while mimicking the physiological shear forces experienced

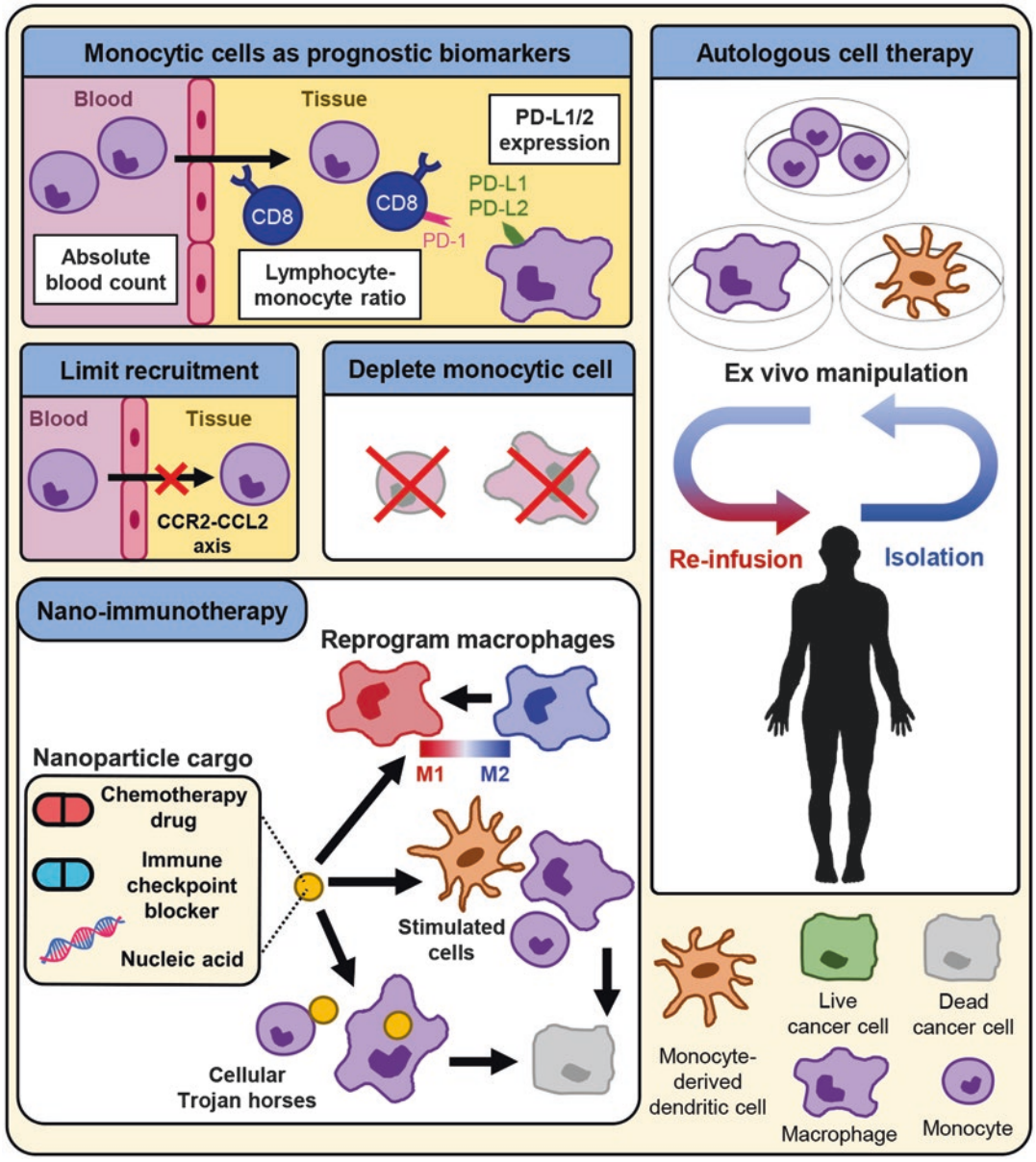

Fig. 7.2 Therapeutic applications and combinational immunotherapy targeting multiple roles of monocytic cells. Monocytes and PD-L1/2^{+} macrophages may be used as prognostic biomarkers for early diagnosis and positive clinical objective response, respectively. Anticancer therapy may be achieved by combinational strategies that limit monocyte recruitment, deplete or reprogram monocytic cells, or employ nanoparticles (encapsulating therapeutic cargo) that exploit the functions of monocytic cells. Monocytic cells can also be used to deliver drug-encapsulating nanoparticles to target sites. Autologous monocytic cells can be manipulated ex vivo and re-infused into patients for anticancer cell therapy

by circulating Mos. Importantly, models should enable the development of human-relevant interventions while complementing preclinical animal models. Here, we describe the progress toward developing such models, existing limitations, and potential solutions.

7.4.1 Conventional 2D In Vitro Cancer Models

Suitable TME models are needed to study the roles and therapeutic applications linked with monocytic cells. Traditionally, these models utilize two-dimensional (2D) cultures of cells in contact with neighboring cells, the culture vessel (made of rigid plastic), and chemically defined medium. These are advantageous in terms of their simple setup and low cost. They also lay important foundations of cancer immunology and TAA discoveries [242]. However, 2D models do not mimic natural tissue structures and fail to recapitulate 3D in vivo cell events [243, 244] which are responsible for cell processes such as differentiation, gene/protein expressions, and others [245–248]. For example, cells in monolayers have relatively free access to signaling molecules and nutrients, which contrasts in vivo environments where barriers to transport, including variations in blood supply, vascular permeability, interstitial fluid flow, and complex matrix interactions that limit diffusion and dynamic cellular consumption rates, generate chemical gradients and unique signaling outcomes that are better recapitulated using 3D culture systems [245, 248]. Alternatively, there are transwell models where cells can be cultured in 2D or 3D settings and which allow for simple cell migration measurements across a filter membrane between upper and lower chambers [249, 250]. However, 2D models often present endpoint readouts that can be confounded by in vitro artifacts such as the non-physiologic constraints of structural materials (such as polycarbonate, polystyrene, or polyester), the lack of mechanical stimuli such as fluid shear stress or mechanical forces, and the absence of cellular, tissue, or ECM heterogeneity seen in patient TMEs. Thus, 3D in vivo and emerging in vitro models in hydrogels or scaffolds can better represent the physical, architectural, and biochemical cues of the in vivo TME.

7.4.2 Conventional 3D Cancer Models

Murine in vivo models are the gold standard of 3D cancer models and, due to their complex nature and feasibility of genetic manipulation, are responsible for many of our recent advances in understanding the TME, particularly in tumor immunology [251]. Such models also facilitate in vivo evaluation of drug pharmacokinetics and enable studies of drug uptake and biodistribution in specific organs [251, 252]. However, murine models raise ethical issues and are costly and time-consuming, and the relevance of results from murine models has been questioned due in part to low conservation between murine vs human tumors and immune systems [253, 254]. Also, despite successful preclinical testing in mice, more than 80% of drug trials in patients fail in early phases, and only 50% of those that pass phase III are approved clinically [255]. Therefore, 3D in vitro models may be improved representations of human cancer and include suspension cultures in non-adherent plates and cultures in scaffold or in gel-like matrix within well plates [256]. Multicellular aggregates/spheroids are a common feature of these models by virtue of their ability to mimic metabolic/chemical gradients, hypoxic conditions, and cell-cell/cell-matrix interactions [247, 257–259]. Aggregates also enable functional studies of monocytic cells in terms of their infiltration of a 3D tumor mass or support of cancer invasion into the 3D TME ECM [260–263].

7.4.3 Comparative Studies of 2D Versus 3D In Vitro Cancer Models

Clearly, 2D and 3D models offer distinct experimental advantages, with the former being more easily adapted for higher-throughput studies and the latter being generally more representative of

in vivo TMEs. However, 2D vs. 3D comparisons reveal disparities in their evaluation of cell immunotherapies [88] and drug sensitivity for both single drugs [264–266] and drug combinations [267]. Other 2D-3D differences include the reduced expression of TAAs and HLA type I by cells grown as a 3D spheroid compared to a 2D monolayer [268]. Cells also differentially express protein(s) when they migrate through a 3D matrix or 2D substrate [244, 250, 269, 270]. For instance, FAK is crucial in 3D, but in 2D, FAK-null cells compensate for migration defects by overexpressing other migration machineries [270]. Morphological [271] and quantitative [269] differences arise between 2D and 3D migration. Loss of diverse phenotypes [272] also results from 2D culturing, and this is especially relevant for monocytic cells which in vivo have a broad spectrum of functional responses to environment cues of the TME. Thus, a 3D environment will more accurately predict in vivo drug responses for different pathways. In the example of the aforementioned FAK pathway that is under-represented in a 2D culture of tumor cells, drugs targeting these pathways may be falsely deemed to be negative in 2D studies. Conversely, 2D studies might yield drugs that are ineffective in clinical studies because compensatory pathways can also emerge under more physiologic 3D settings. Finally, although more studies are needed to confirm that 3D cultures better indicate clinical outcome [252], a 3D model should be strongly considered over simplistic 2D cell monolayers so that experimental conclusions have improved physiological relevance.

7.4.4 Microfluidic Cancer Models

Microfluidic models of the TME could represent an advantageous intermediate step that links the findings of 2D in vitro cell assays, preclinical animal studies, and clinical patient trials. Unlike conventional 3D models, microfluidic technologies capture immune cell processes through spatial compartmentalization [273] and the capability to mimic precise chemokine gradients [274], endothelial barrier function [275, 276], and flow conditions [277, 278]. These models can be built using gels of specific composition (e.g., collagen, fibrin, or various proteoglycans) that more closely mimic the ECM of cancer-specific TMEs. Moreover, because the culture of monocytic cells in a 3D matrix supports their de novo production of ECM [279], such systems yield more physiological 3D environments from an initial setup based on a simple gel. Their small dimensions also allow for experiments that require less reagents and cells [273, 280], making them ideal for testing precious patient specimens. Such systems can be incorporated with vasculature to mimic the transport of circulating immune and tumor cells and their intravasation into vasculature or extravasation into the surrounding matrix [275, 281, 282]. They also enable high-resolution imaging and real-time tracking of cell migration [274, 275, 282, 283], a procedure that may be feasible (e.g., by intravital two-photon imaging) but is technically demanding in animal models [273, 280].

7.4.4.1 Microfluidic Cancer Models to Study Monocytes

Studies have increasingly used microfluidic platforms to gain improved insight on the role of Mos [88, 275, 284–286], TAMs [99, 131, 243, 277, 287, 288], and DCs [289–291] (Table 7.1). Lee et al. revealed the differential capability of PD-L1$^+$ Mos to suppress the anti-tumor efficacy of retrovirally transduced vs. mRNA-electroporated T cells, results that were not shown through 2D cytotoxicity assays [88]. Otano et al. showed the therapeutic boost of anti-sense oligonucleotides against PD-1 to CD8$^+$ T cells that allow them to overcome PD-L1$^+$ Mo suppression [284]. Finally, a vascularized model revealed that Mos reduce cancer cell extravasation independently from their contact with cancer cells and Mos have little effect on cancer cell extravasation once they transmigrate across the microvasculature [275]. Importantly, microfluidic models of the human TME provide a system of improved physiological relevance to validate the above-discussed effects of Mos in 3D which to date have mostly been specific to murine systems, including their effect on the growth of tumor aggregates, ADCC-based tumoricidal activity, and their support toward developing the Pre-MN.

Table 7.1 Microfluidic cancer models for studying monocytes/macrophages/monocyte-derived dendritic cells

Research topic	Cells in co-culture	Description of model setup	Gel matrix	Key findings	Ref.
MΦ impairment of endothelial barrier and effect on cancer cell intravasation	1. RAW 264.7 cell line 2. HT1080 cell line 3. HUVECs (endothelial cells)	One central gel channel (MΦs and HT1080), flanked by two media channels (one has HUVEC monolayer). Cancer cell intravasation was assessed by confocal imaging	2.5 mg/mL rat tail collagen I	MΦs secrete TNF-α, resulting in endothelial barrier impairment, increasing intravasation of HT1080	[276]
MΦ glycolytic activity and support of cancer cell extravasation	1. Human primary Mos differentiated in vitro into MΦs via cancer-/normal cell line-conditioned media 2. Panc1, MiaPaCa2, and HPNE cell lines 3. HUVECs	One central gel channel flanked by two media channels (one has HUVEC monolayer). MΦs and cancer cells were added into the HUVEC-covered channel lumen, and extravasation across the HUVEC layer was tracked by live confocal imaging	2.5 mg/mL rat tail collagen I	Tumor-conditioned MΦs promote cancer cell extravasation compared to control MΦs	[99]
Mo extravasation across vasculature and support of cancer cell extravasation	1. Human primary Mos 2. MDA-MB-231 and MDA-MB-435 cell lines 3. HUVECs 4. NHLFs (fibroblasts)	One central gel channel (vasculature self-assembled from a HUVEC/NHLF co-culture), flanked by media channels. Mos were perfused through vasculature with/without cancer cells, and extravasation across the vasculature was tracked by live confocal imaging	3 mg/mL fibrin	$CCR2^+$ Mos extravasate via actomyosin. Mos reduce cancer cell extravasation in a non-contact-dependent manner, with little impact on extravasation once Mos transmigrate across vasculature	[275]
Non-contact- vs. contact-dependent MΦ support of EMT and dispersion of A549 cell aggregates	1. Human primary Mos differentiated/polarized in vitro into M0, M1, M2a, M2b, and M2c subtypes 2. A549 cell line 3. HUVECs	Two inner gel channels (MΦs and A549 aggregates in one or separate channels), flanked by two media channels (one has HUVEC monolayer). Aggregate dispersion was tracked by live confocal imaging	2.5 mg/mL rat tail collagen I	M2a MΦ subset supports A549 aggregate dispersion via ICAM-1 and β2 integrin contact-dependent mechanisms	[131, 298]
MΦ-derived cytokines that support cancer cell migration	1. THP-1 cell line differentiated into M2-like MΦs using PMA 2. MRC-5 cell line 3. CL1-0 (fibroblasts)	Three multi-layer cell culture chambers (each with 1 cell type). Microvalves connected chambers and were opened/closed by pneumatic conduits embedded in the second layer. Migration was tracked by live differential interference contrast (DIC) imaging	–	MΦs enhance cancer cell migration but restrain the tumor-promoting effect of myofibroblasts through TNF-α	[299]
Mo PD-L1 expression and suppression of engineered T cells	1. Human primary Mos 2. HepG2 cell line 3. Retrovirally transduced (Tdx)/mRNA-electroporated (EP) T cells	One central gel channel (Mos and HepG2 aggregates), flanked by two media channels (T cells added in one media channel and migrated toward HepG2 targets). HepG2 death was tracked by live confocal imaging	2.5 mg/mL rat tail collagen I	$PD\text{-}L1^+$ Mos suppress Tdx but not EP T cells. PD-1 knockdown in Tdx T cells overcomes Mo suppression	[88, 284]

Patient-specific screen of immune checkpoint blockade	1. Patient-derived/murine-derived organotypic tumor spheroids (PDOTS/MDOTS)	One central gel channel (PDOTS/MDOTS), flanked by two media channels. Spheroids were cultured with/without various immune checkpoint blocking Abs for downstream characterization	2.5 mg/mL rat tail collagen I	PD-L1/2$^+$ MΦs are seen in PDOTS/MDOTS. PDOTS exposed to dual PD-1/CTLA-4 blockade show increased M0 MΦs than single blockade	[286, 288]
MΦ migration toward cancer cells	1. RAW 264.7 cell line 2. MDA-MB-231 cell line	Three inner gel channels flanked by two media channels. Migration was tracked by live confocal imaging	4 mg/mL Matrigel (RAW 264.7) and rat tail collagen I (MBA-MB-231)	MΦs invade into neighboring gels containing MDA-MB-231 but not into empty gels	[285]
MΦ M2 polarization and migration toward cancer cells	1. RAW 264.7 cell line 2. T24 cell line 3. HUVECs 4. BJ-5Ta (fibroblasts)	Four innermost culture chambers linked by conduits to an outermost media chamber that could be linked to media perfusion equipment. Migration was assessed by live fluorescence imaging	2.5 mg/mL (approximated) Matrigel	MΦs express high Arg-1 in the quadruple cell culture and migrate toward cancer cells	[287]
MΦ M2 polarization and migration under interstitial flow (IF)	1. BMDM (bone marrow-derived MΦs) 2. RAW 264.7 cell line	One central gel channel (MΦs), flanked by two media channels. To generate IF, a media reservoir was added directly above the inlets of 1 fluidic channel. Migration was tracked by live phase-contrast imaging	2.5 mg/mL rat tail collagen I	IF supports MΦ M2 polarization via integrin/Src and STAT3/6 and enhances MΦ migration activity	[277]
DC contact with T cells under IF	1. Adherent murine DC cell lines (MutuDC) 2. CD4$^+$ and CD8$^+$ T cell hybridomas	One flow channel (DC monolayer) with two inlets/outlets that were connected to syringe pumps (to insert fluids and cells). Migration was tracked by confocal imaging	–	DCs interact more transiently with CD8$^+$ T cells vs. CD4$^+$ T cells and more strongly with specific vs. non-specific T cells	[290]
DC migration toward cancer cells	1. Human primary Mos treated (3 days) with M-CSF and IFN-α2b 2. SW620 cell line	One central gel chamber (DCs) linked by conduits to two chambers (tumor cells) and two outer media channels. DC migration was tracked by fluorescence imaging	3 mg/mL rat tail collagen I	IFN-α2b-treated DCs migrate toward and phagocytose tumor cells that were pre-treated with drugs	[289]
DC migration under precise chemokine gradients	1. Murine DCs matured from LPS-treated BM-derived cells	One central gel channel (DCs), flanked by two media channels (chemokines added to create a gradient across the central channel). Migration was tracked by live fluorescence imaging	1.5 mg/mL rat tail collagen I	DCs respond to small CCL19/CCL21 gradients, but CCL21 directs migration more potently than CCL19	[291]

7.4.4.2 Microfluidic Cancer Models to Study Monocyte-Derived Cells

Complex TME models have successfully captured the in vivo profile of Mo-derived cells in the TME. In one of the more cellularly complex TAM-associated models, MΦs upregulate Arg-1 in their quadruple cell culture with ECs, fibroblasts, and bladder cancer cells, analogous to their activation in vivo [287]. The same model capably screens for chemotherapy regimens. Other models characterize TAM supportive capabilities in cancer cell extravasation [99] or intravasation [276] across EC barriers. The impact of specific Mo-derived MΦ subsets can also be elucidated as shown by Bai et al., where a subset of M2-like MΦs (specifically, the M2a MΦ subset) show the capability to mediate contact-dependent epithelial-to-mesenchymal (EMT) transition of tumor aggregates [131].

Microfluidic models have been developed for the general DC population, but no study that specifically focused on moDCs has been performed. One model reveals that CXCR4 mediates the migration of IFN-α2b-conditioned DCs toward cancer cells that were treated with epigenetic drugs [289]. Other models provide insight on DC chemotaxis under precise CCL19/21 gradients [291], cell volume changes under hyperosmotic stress [292], and contact with T cells under different shear stresses [290]. Other areas such as the effect of DCs on T cell activation (via TAA presentation) or immunosuppression or DC trafficking between the TME and draining lymphatics should also be explored in microfluidic models of the human TME.

7.4.4.3 Patient-Derived Microfluidic Cancer Models

Microfluidic models have the great advantage of allowing the culture of patient-derived explants such as patient-derived organotypic tumor spheroids (PDOTS) that retain the relevant immune cell types found in tumor tissues. Jenkins et al. developed an ex vivo system that retains key features of patient-specific immune TMEs, showing the presence of $CD14^+$ monocytic cells and heterogenous PD-L1 expression which is reminiscent of in vivo Mo-derived cell profiles [286]. Further, Aref et al. demonstrated the capability of such platforms to screen ICT, a form of therapy which includes the use of Abs against complementary checkpoint proteins (e.g., between PD-L1 and PD-1) to inhibit checkpoint protein signaling. Through the platform, authors could observe an expansion of both $CD8^+$ T cells and naïve M0 MΦs within in vitro PDOTS that received dual checkpoint blockade against CTLA-4 and PD-1 [288]. Because such ex vivo models mimic the patient-specific TME, they have the potential to predict patient-specific responses to immunotherapies.

7.5 Future Directions

Despite recent progress, multiple areas remain to be clarified of monocytic cells in the TME, ranging from questions of their ontogeny, heterogeneity, and functions. At the same time, the versatility of these cells presents an opportunity to exploit combinational approaches to achieve superior cancer immunotherapy. For example, NPs are candidate therapies that can simultaneously modulate multiple roles of Mos, which include inhibiting their recruitment to tumors, differentiation into pro-tumor MΦs/moDCs, and potential tumoricidal activity in the TME. Current understanding has mostly derived from studies performed in murine models and remains to be validated in human settings. Therefore, improved physiologically relevant TME models are needed for investigating human-relevant monocytic cell biology and developing human-relevant therapeutic strategies.

While simplistic 2D and traditional 3D (transwell) in vitro cell cultures are scalable and robust, their relevance in vivo is limited by their lack of biological functionality. Conversely, animal models replicate function at both organ and multi-organ levels but are inherently flawed due to human-murine species differences. As such, we support that microfluidic human TME models combine the best features of both models by culturing human cells in tissue-specific conditions that are designed to mimic human-relevant biological and physical cues of the TME. To date, microfluidic human TME models have been developed to study Mos, TAMs, and moDCs

(Table 7.1), all of which are capable of mimicking relevant tumor-immune interactions in a controlled setting that is unique to the specific cancer. Future model developments can draw from the growing understanding of the biochemical and biophysical properties of the TME, such as the impact of tumor interstitial flow and the function of tumor lymphatics. Incorporating these elements in TME models would enhance the physiological accuracy of TME models and enable deeper characterizations of monocytic cells in the TME to design and screen immunotherapies. Moreover, by incorporating patient specimens, scientific understanding can be specific to the patient's pathology and can be applied for developing patient-specific treatments.

The emergence of microfluidic human TME models highly complements ongoing immunophenotypic studies that utilize advanced techniques such as CyTOF, RNA-seq, and single-cell analysis [113, 190, 293]. Gubin et al. observed multiple subpopulations of Mos/MΦs (distinguishable by markers such as CD206, CX3CR1, and CD1d) that evolve over the course of ICT. These findings further suggest that ICT contributes toward broader remodeling of the TME, supporting that circulatory Mos/early MΦs are more important than pre-polarized intratumoral MΦs in tumor progression [113]. Such findings also highlight the intricacies and complexity of the TME that must thus be meaningfully recapitulated through a precise and controlled mimic of environmental cues in human cancer-specific TMEs.

Notably, one can envision future organ-on-a-chip technology, for example, of the human brain [294], to be integrated with tumor spheroids to model primary or metastatic TMEs. Patient tumor samples, as well as patient-derived monocytic cells, can be incorporated into such models to explore patient-specific tumor progression and response to novel immunotherapies [295, 296]. Moreover, immunophenotyping of parallel devices at different time points can be utilized to capture, in detail, human responses to immunotherapy over time, an area of study that is currently not possible due to ethical concerns and practical limitations of repeat patient biopsies.

As such, research groups have increasingly focused on the development of culture reactors to extend the lifetime of in vitro and ex vivo cultures and on the design of high-throughput and automated systems toward the aim of establishing standardized platforms for clinical precision medicine applications [252]. These microfluidic models could complement existing in vivo preclinical studies while reducing the economical and ethical burden of preclinical investigations. Further, by developing several organ-specific TME models and connecting these using appropriate perfusion conduits [297], a comprehensive model of the human system can be built to study the dynamic functions of Mos across different cancer stages and cancer-specific TMEs.

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Myeloid-Derived Suppressor Cells in the Tumor Microenvironment

8

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Abstract

Myeloid-derived suppressor cells (MDSCs) represent a heterogenous population of immature myeloid cells capable of modulating immune responses. In the context of cancer, MDSCs are abnormally produced and recruited to the tumor microenvironment (TME) to aid in the establishment of an immunosuppressive TME that facilitates tumor escape. Additionally, MDSCs contribute to non-immunologic aspects of tumor biology, including tumor angiogenesis and metastasis. The clinical significance of MDSCs has recently been appreciated as numerous studies have suggested a correlation between circulating and intratumoral MDSC frequencies and tumor stage, progression, and treatment resistance. In this chapter, we review MDSC characterization, development, expansion, and mechanisms that facilitate immunosuppression and tumor progression. Furthermore, we highlight studies demonstrating the clinical significance of MDSCs in various disease states in addition to strategies that modulate various aspects of MDSC biology for therapeutic gain.

Keywords

Myeloid-derived suppressor cells (MDSC) · Cancer · Tumor microenvironment (TME) · Immunosuppression · Solid tumor · Myelopoiesis · Angiogenesis · Metastasis · Autoimmunity · Therapy resistance · Therapeutic strategies · STAT3 · S100A9 · Transforming growth factor (TGF)-β · Arginase-1 · Regulatory T cells (Treg)

8.1 Introduction

Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells normally produced and secreted by the bone marrow in response to localized inflammatory states such as infection or trauma to try to restrain hyper-inflammation and protect the host from generation of autoimmunity

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[1, 2]. In the setting of cancer, however, MDSCs are abnormally produced and recruited by tumor-derived factors to tumor sites in order to establish a microenvironment around the tumor that can suppress host immunity (via MDSC expression of suppressive cytokines like TGF-β and surface molecules like PDL1 and PDL2), establish new vasculature (via MDSC expression of VEGF and βFGF isoforms), and remodel tissue with tumor-supportive stromal elements (via MDSC arginase-1, iNOS, and MMP-7/MMP-9/MMP-12 activity) [3]. In fact, MDSCs play a central role in controlling and maintaining the suppressive TME in solid tumors [3]. In models where MDSCs are absent or eliminated, the TME breaks down, allowing access and activation of immune effector cells [4, 5]. Recently, these unique suppressive cells of the tumor microenvironment have gained direct clinical significance as increasing evidence has mounted suggesting a correlation between the frequency of circulating and intratumoral MDSCs and cancer stage, disease progression, and resistance to standard chemo- and radiotherapy [6, 7]. Hence, understanding MDSC biology represents an important step in the quest to enhance anticancer immunity. In this chapter, we will review important aspects of MDSC biology, including their characterization, development and expansion, activation, and the suppressive mechanisms that support cancer growth and progression (highlighted in Fig. 8.1). In addition, we will highlight important studies that have attempted to target or manipulate MDSC biology for therapeutic gain. Finally, we will discuss recent trends and potential future directions concerning targeting and use of MDSCs in both oncologic and non-oncologic diseases.

8.2 Defining Human and Murine MDSCs

8.2.1 Defining MDSCs in the Periphery

In general, *murine* MDSCs are defined by a combination of markers utilized for murine myeloid lineages (Gr-1, Ly6, CD11b, CD49d) in combination with suppressive effector function (e.g., suppression of T-cell proliferation). Early studies in mice led to the identification of a suppressive cell population defined as Gr-1^{+}CD11b^{+}. Further characterization of these Gr-1^{+}CD11b^{+} cells revealed two subsets based on their expression of both Gr-1 [8] and the Ly6 superfamily molecules Ly6G and Ly6C [9, 10], which are preferentially expressed on the surface of granulocytes and monocytes, respectively. However, because Gr-1 mAbs bind both Ly6G and Ly6C, double staining of Ly6G and Ly6C is highly recommended to identify the two distinct populations, specifically the polymorphonuclear (PMN-MDSCs), or sometimes referred to as granulocytic (G-MDSCs), and monocytic (M-MDSCs) subsets. PMN-MDSCs are characterized as CD11b^{+}Ly6G^{+}Ly6C$^{low/int}$, whereas M-MDSCs are CD11b^{+}Ly6G^{-}Ly6C^{high}. In addition, PMN-MDSCs, with their high degree of granularity, are high on the side scatter axis in flow cytometric applications, whereas M-MDSCs present as lower on the side scatter axis. Haile et al. identified CD49d as a novel marker to further aid MDSC subset identification, demonstrating that CD11b^{+}CD49d^{+} phenotypically and functionally resembled M-MDSCs [11]. CD11b^{+}CD49d^{-} cells were more granulocytic compared to their CD11b^{+}CD49d^{+} counterparts, thus representing the PMN-MDSC subset.

In 2016, Bronte et al. proposed the minimal phenotypic characteristics of *human* peripheral blood MDSCs [12]. The mouse equivalent of PMN-MDSC is defined as CD11b^{+}CD14^{-}CD15^{+} or CD11b^{+}CD14^{-}CD66^{+}, whereas M-MDSC is CD11b^{+}CD14^{+}CD15^{-}HLA-DR$^{-/low}$. The myeloid marker CD33 can also be used for differentiation, where PMN-MDSCs stain CD33^{dim} and M-MDSCs present CD33$^{+/hi}$. It is also important to include some form of Lineage (Lin) cocktail (CD3, CD19, and CD56) that can differentiate immature MDSC progenitors from PMN-MDSC and M-MDSC, with cells representing Lin^{-}HLA-DR^{-}CD33^{+} defined as early-stage MDSC.

Unfortunately, phenotypic staining analysis of the abovementioned markers alone for both mouse and human MDSC subsets cannot dis-

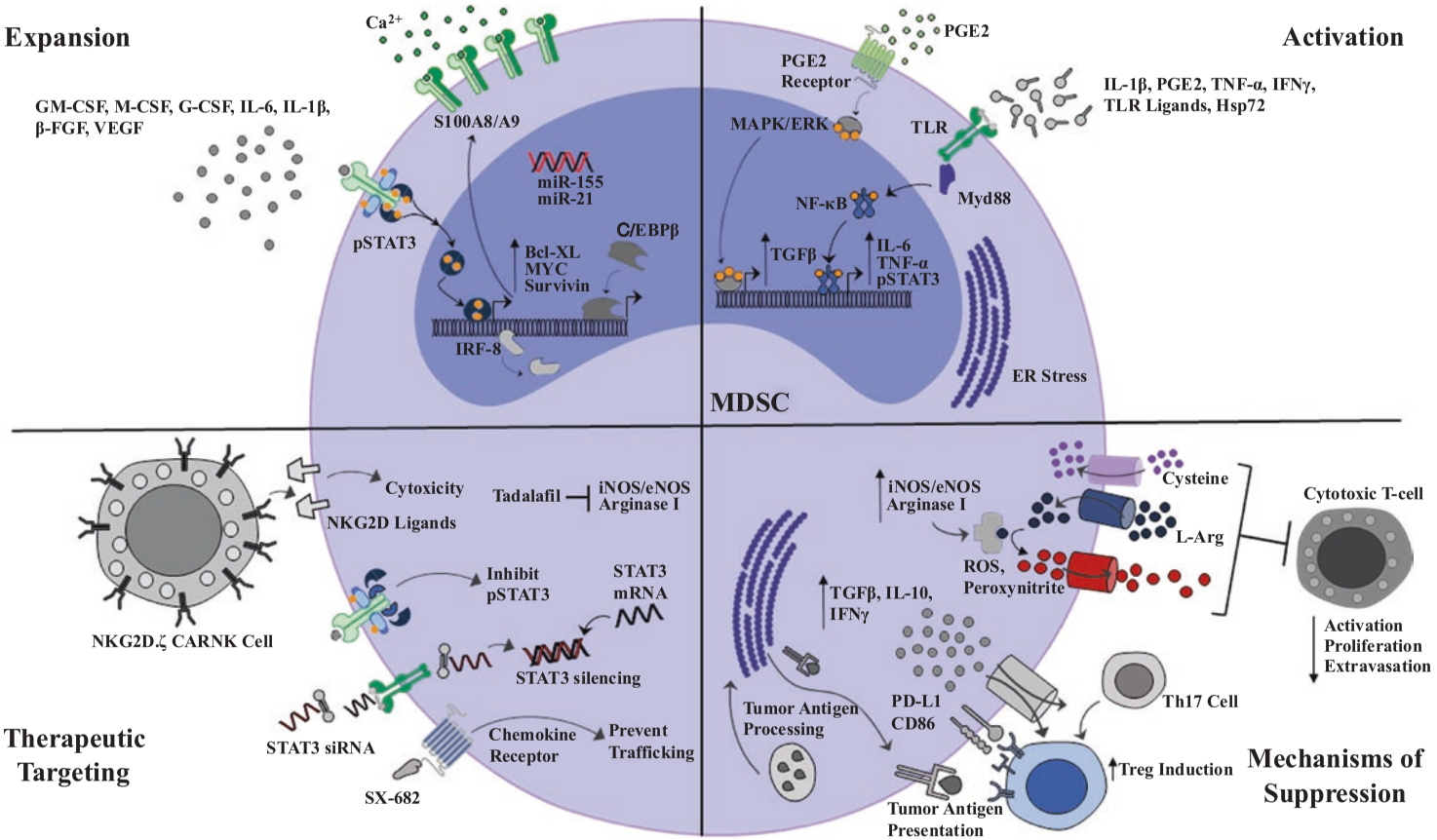

Fig. 8.1 *Expansion*. MDSC expansion is governed by factors secreted by the tumor and tumoral stroma that converge mainly on STAT3 signaling resulting in the upregulation of genes that mediate MDSC survival and proliferation. *Activation*. Once expanded, tumoral stroma secrete factors that converge on MAPK/ERK and NF-κB signaling to upregulate genes that endow MDSCs with their characteristic suppressive capacity. *Mechanisms of suppression*. MDSCs suppress T-cell antitumor responses by sequestering the amino acids cysteine and arginine, in addition to converting arginine to reactive oxygen and nitrogen species that facilitate T-cell suppression. Conversely, MDSCs mediate the induction of Tregs through release of soluble cytokines that further suppress antitumor responses. *Therapeutic targeting*. Efforts to target MDSCs for therapeutic benefit have included inhibiting their expansion and trafficking, inhibiting their suppressive capabilities, and direct elimination of MDSCs from the TME

criminate MDSC subsets from their respective mature cell population. PMN-MDSCs share a common origin with neutrophils and thus present many of the same morphological and phenotypic characteristics. Similarly, M-MDSCs share a common origin with monocytes. In a study that compared $CD11b^{+}Ly6G^{+}Ly6C^{low}$ PMN-MDSCs in tumor-bearing mice to $CD11b^{+}Ly6G^{+}Ly6C^{low}$ neutrophils in tumor-free mice, Youn et al. demonstrated that PMN-MDSCs had significantly higher expression of CD115 and CD244 [13]. In humans, M-MDSC can be differentiated from monocytes by the absence of HLA-DR expression. Recently, the ability to separate PMN-MDSC from neutrophils via a Ficoll gradient separation was described [14]. Despite these proposed differentiating markers, it still is absolutely essential to characterize these MDSC subsets for their immunosuppressive state, both molecularly and in functional suppression assays. MDSCs differ molecularly from mature myelocytes through transcription factors and biochemical signatures associated with immunosuppression [12].

The benchmark suppressive function of MDSCs is their ability to inhibit T-cell proliferation, cytokine production, and cytotoxic functions. MDSCs have demonstrated suppressive effects in vitro on both activated, antigen-specific T cells and naïve, non-specific T cells. Typically, suppression assays examine the ability of purified MDSC populations to suppress either antigen-specific or antigen-non-specific T cells. To induce antigen-independent stimulation, T cells can be stimulated with anti-CD3/CD28 antibodies in the presence of cytokines such as interleukin (IL)-2, IL-7, or IL-15 and assessed for proliferation using ^{3}H-thymidine incorporation, CFSE dilution, or cell trace violet dilution in the presence of MDSCs. Similarly, antigen-specific T cells can be stimulated with cognate antigens or allogeneic leukocytes. MDSC suppressive capacity is also assayed by their ability to inhibit T-cell IFN-γ and IL-2 production. In vivo studies are more technically challenging and include more critical parameters. Marigo et al. described an in vivo mouse protocol where MDSCs are expanded in tumor-bearing mice and tumor control is subsequently assessed to determine MDSC suppressive capacity [15]. In this protocol, the antigen is expressed by the tumor and cross-presented to lymphocytes via dendritic cell vaccination to generate an antigen-specific T-cell population. MDSC suppression was assessed for their ability to inhibit antigen-specific T-cell IFN-γ production and overall tumor control.

8.2.2 Defining MDSCs Within Tumors

Based on the ease of isolation and characterization, most of the early studies defining MDSC subsets focused on circulating MDSCs isolated from blood or spleen. Intratumoral MDSC characterization has been challenging due to the small proportion of MDSCs within a tumor sample as well as the technical challenges of isolating cells from a complex tumor microenvironment (TME) [16]. Comprised of immune infiltrates, stromal cells, connective tissue, and vasculature, the TME facilitates complex, heterotypic interactions that have both acute and chronic impacts on the local components. Further complicating intratumoral MDSC characterization is the presence of both tumor-associated macrophages (TAMs) and neutrophils (TANs) that phenotypically and morphologically resemble M-MDSCs and PMN-MDSCs, respectively. Tissue-resident macrophages are present in all tissues of the body and display high heterotypic and functional diversity [16, 17].

In the mouse, Movahedi et al. demonstrated that $CD11b^{+}Ly6C^{+}$ cells were the exclusive monocytic precursors of TAMs. Further, they demonstrated that tumor-infiltrated myeloid populations could be grouped into at least seven subsets based on their differential expression of MHCII and Ly6C, thus reflecting the high heterogeneity and complexity when characterizing intratumoral suppressive myeloid populations [18]. Similarly, Franklin et al. demonstrated the presence of three intratumoral myeloid populations: tissue-resident macrophages as $CD11b^{+}MHCII^{+}$ and two subsets of TAMs as $CD11b^{low}MHCII^{-}Ly6C^{+}$ or $CD11b^{low}MHCII^{-}Ly6G^{+}$ [19]. Furthermore, TAMs were derived from $CD11b^{+}Ly6C^{+}CCR2^{+}$

circulating monocytes that underwent phenotypic changes characterized by the downregulation of CD11b and Ly6C and upregulation of CD11c, F4/80, and MHCII [19]. MDSCs exhibit plasticity and cross-phenotype skewing with tumor-associated macrophages (TAMs) in the TME in response to tumor-associated hypoxia [20]. Thus, these studies reflect the plasticity of intratumoral myeloid populations and highlight the difficulty in exactly defining and differentiating myeloid subsets within the TME. In the mouse, however, the F4/80 marker has allowed differentiation of murine intratumoral MDSCs, separating PMN-MDSC ($F4/80^-$) from M-MDSC ($F4/80^{low/dim}$) and M-MDSC from TAM ($F4/80^+$) [1].

In *human* intratumoral MDSCs, M-MDSC are defined as $CD11b^+CD33^+CD14^+HLA\text{-}DR^{lo/-}$ and PMN-MDSC as $CD11b^+CD33^+CD15^+CD66^+HLA\text{-}DR^{lo/-}$ [21]. However, analyzing tumor myeloid infiltrates presents the same complexities and challenges as eluded for murine studies: low frequencies of MDSCs within tumor tissue samples and a complex, heterogenous myeloid landscape. Within the TME, human myeloid cells have been classified into four general categories: (1) TAMs, (2) Tie2-expressing monocytes, (3) neutrophils, and (4) MDSCs [21]. PMN-MDSCs share a similar ontogeny with neutrophils and thus complicate clearly characterizing MDSCs within the TME. Indeed, PMN-MDSCs within the TME have been referred to in the literature as pro-tumor, anti-inflammatory neutrophils, and some investigators have proposed that PMN-MDSCs should instead be referred to as "neutrophils with suppressive activity" until further approaches to differentiate these two populations are offered [22]. However, studies have suggested that tumor-associated neutrophils possess both anti-tumor and pro-tumor properties [23]. Thus, differentiating between neutrophils and PMN-MDSCs based simply on suppressive capacity may be misleading. Several differentiating features that can aid in distinguishing intratumoral PMN-MDSCs from neutrophils have been employed. Neutrophils are high density that present with high side scatter axis (SSC) on flow cytometric applications, whereas PMN-MDSCs are lower SSC-density cells [24]. In combination with SSC profiles, CD11b, and CD15, the inclusion of CD16 and CD66b, classic neutrophil markers, has been used to help identify neutrophils [21]. Recently, LOX-1 has also emerged as a marker unique to PMN-MDSC. Condamine et al. demonstrated that $LOX\text{-}1^+$ PMN cells isolated from peripheral blood had a gene expression profile similar to PMN-MDSCs and suppressed T-cell proliferation in vitro [25]. In addition, 15–50% of CD15+ cells isolated from various solid tumors were $LOX\text{-}1^+$.

8.3 MDSC Development and Expansion

In healthy individuals, hematopoietic stem cells (HSCs) in the bone marrow give rise to common myeloid progenitor cells (MPCs) and immature myeloid cells (IMCs) that then differentiate into mature macrophages, dendritic cells, and granulocytes in peripheral organs. During acute inflammatory stimuli, normal activation of these mature cell populations results in marked phagocytosis and release of pro-inflammatory signals, in addition to remodeling of tissues after the inflammatory state is resolved [26]. Thus, normal myelopoiesis and myeloid differentiation is a tightly regulated process that controls and limits inflammatory responses. However, in pathological conditions such as cancer, chronic inflammatory signals secreted by the tumor microenvironment reprogram myelopoiesis and serve to exacerbate tumor progression [27]. Tumor-derived factors in the form of growth factors, cytokines, chemokines, and other inflammatory mediators facilitate the expansion of an immature myeloid population characterized by defective antigen presentation and secretion of several factors that suppress the resultant antitumor response [27]. Thus, myeloid-derived suppressor cells (MDSCs) were so named to encompass both their characteristic immature state and ability to suppress antitumor responses [28].

The conversion of HSCs into MDSCs remains a process that is incompletely understood but is proposed to be mediated by two general signals. The first signal facilitates the expansion and

impaired differentiation of myeloid cells and includes factors that are produced primarily by tumor and bone marrow stromal cells. The second signal activates MDSCs and results in their suppressive capabilities [29, 30].

8.3.1 Signal 1

The same factors that govern normal myelopoiesis are also proposed to induce the mobilization and expansion of MDSCs. These include granulocyte/macrophage colony-stimulating factor (GM-CSF), macrophage CSF (M-CSF), granulocyte CSF (G-CSF), IL-6, IL-1β, beta-fibroblast growth factor (β-FGF), and vascular endothelial growth factor (VEGF) [31–33]. Tumor and stromal cells produce and secrete these factors resulting in autocrine and paracrine loops that further drive tumor progression [33]. The colony-stimulating factors represent a group of cytokines essential in modulating myelopoiesis. GM-CSF functions on a more global level, displaying pleiotropic and widespread effects on hematopoietic cells, whereas G-CSF and M-CSF are relatively more lineage specific [34]. In particular, GM-CSF has repeatedly demonstrated to be a key mediator of MDSC expansion both in vitro and in vivo [35–40]. Interestingly, preclinical and clinical evidence suggests that the effect of GM-CSF is dose-dependent. In vitro, murine bone marrow cells cultured in high GM-CSF concentrations generated MDSCs along with neutrophils in 3–4 days, whereas lower concentrations of GM-CSF required 8–10 days to generate MDSCs [37]. Results of clinical trials studying GM-CSF as an adjuvant in cancer vaccination were reviewed by Parmiani et al. [41]. At low concentrations, GM-CSF potentiated a vaccine-induced antitumor response, whereas at higher doses, an immunosuppressive effect was observed [41]. In addition to dose-specific effects, GM-CSF preferentially expanded highly suppressive M-MDSCs in a mammary tumor mouse model [8]. In the same study, G-CSF preferentially expanded PMN-MDSCs that resulted in a less immunosuppressive environment [8].

Vascular endothelial growth factor (VEGF), a key mediator of angiogenesis in tumors, is also a potent inducer of MDSC expansion. Gabrilovich et al. were the first to demonstrate that VEGF produced by breast and colon cancer cells significantly affected the functional maturation of progenitor stem cells [42]. Subsequent studies revealed that activation of VEGF receptor (VEGFR) in the bone marrow of mice leads to myeloid expansion [43]. In this same study, neutralizing GM-CSF activity via antibodies, or by use of GM-CSF-null hematopoietic cells, inhibited VEGFR-mediated myeloid progenitor activity. More recently, Horikawa et al. demonstrated that patient high-grade serious ovarian cancer IHC samples that presented high levels of VEGF upregulated genes associated with myeloid cell chemoattractants and matrix metalloproteases (MMPs) [44]. Furthermore, a mouse ovarian tumor cell line (ID8) modified to overexpress *Vegf* induced an increase in intratumoral MDSCs and decrease in effector T cells [44].

In addition to key myeloid-specific growth factors, IL-1β and IL-6 are potent inducers of MDSC expansion. Mammary carcinoma cells transfected to overexpress IL-1β in mice exhibited decreased survival times in addition to elevated levels of splenic MDSCs [45]. Similarly, stomach-specific overexpression of human IL-1β in transgenic mice subsequently leads to spontaneous gastric inflammation and cancer that correlated with recruitment of MDSCs [46]. It is proposed that IL-1β skews MDSC expansion to that of PMN-MDSCs [47]. Due to the pleiotropic nature of IL-1β, it is believed that IL-1β acts to stimulate MDSCs both directly [46, 47] and indirectly via stimulation of growth factors and cytokines [48], including IL-6. Mice deficient in IL-1R exhibited reduced MDSC numbers that were rescued by re-expression of IL-6 [49]. In a hormone-resistant prostate cancer mouse model, IL-6 correlated with both aggressive tumor growth and MDSC recruitment that could be diminished via an IL-6-silencing shRNA [50]. Moreover, IL-6 blocking mAbs resulted in significantly less accumulation of MDSCs in a mouse model of squamous cell carcinoma [51].

In response to these growth factors and cytokines, myeloid progenitor cells initiate a complex transcriptional network that enables their expansion and prevents their differentiation. Many of the abovementioned factors converge on the activation of signal transducer and activator of transcription (STAT) 3 [52]. For this reason, STAT3 is generally considered to be a master transcription factor regulating both the expansion and activation of MDSCs [1]. In general, STAT proteins are intracellular transcription factors that mediate a wide variety of cell functions, including proliferation, apoptosis, and differentiation. In response to appropriate stimuli, transmembrane receptors dimerize and induce *trans*-phosphorylation of their associated Janus kinases (JAKs). The phosphorylated JAKs can subsequently activate downstream targets, including STATs. Upon activation by phosphorylation, STAT proteins form homo- or hetero-dimers with other STAT family members and translocate to the nucleus, where they bind DNA and induce the transcription of multiple gene targets. STAT3 upregulates genes essential for myeloid progenitor cell survival and proliferation, including Bcl-XL, MYC, survivin, and cyclin D1 [1, 53]. Furthermore, STAT3 activation drives cell surface expression of the S100A8/S100A9 dimer, a calcium- and zinc-binding complex that regulates a variety of inflammatory immune responses and serves as a phenotypic and functional marker for MDSCs. Cheng et al. demonstrated mice lacking S100A9 elicited potent antitumor immune responses, which could be reversed by adoptive transfer of wild-type MDSCs from tumor-bearing mice into S100A9-deficient mice [54]. In contrast, overexpression of S100A9 in cultured embryonic stem cells and transgenic mice inhibited the differentiation of DCs and macrophages and resulted in accumulation of MDSCs [54]. As a clinical correlate, an interaction between S100A9 and the common myeloid marker CD33 facilitated the expansion of MDSC in myelodysplastic syndrome patients. STAT3 activation was also shown to modulate interferon regulatory factor-8 (IRF-8), an integral transcriptional factor regulating myeloid terminal differentiation [55]. *Irf8*-deficient mice generated phenotypically and functionally similar immature myeloid populations compared to tumor-induced MDSCs. In contrast, IRF-8 overexpression in mice facilitated a reduction in MDSC levels in the spleen, bone marrow, and tumor site, suggesting an important negative regulatory role for IRF-8 in MDSC accumulation and differentiation [55]. CCAAT-enhancer-binding proteins (C/EBPs) are a family of transcription factors implicated downstream of STAT3 in blocking terminal differentiation as well as expansion of MDSCs. C/EBPs comprise a family of six basic-region leucine zipper transcription factors that homo- or hetero-dimerize with other C/EBPs and transcription factors that enable binding to target DNA. Specifically, C/EBPα and C/EBPε mediate differentiation and maturation of myeloid progenitors, whereas C/EBPβ is only important in regulating emergency myelopoiesis [56, 57]. Mackert et al. demonstrated that C/EBPα was significantly reduced in MDSCs from tumor-bearing mice compared to non-tumor-bearing hosts [58]. Similarly, myeloid lineage-specific deletion of C/EBPα resulted in significantly enhanced MDSC proliferation and expansion as well as increased myeloid progenitors and decreased mature cells [58]. Conversely, Marigo et al. demonstrated that in a myeloid lineage-specific mouse model of C/EBPβ deletion, there was a significant loss of an MDSC-like population and reversal of a tolerogenic state exhibited by tumor-specific T cells [15]. These data suggest that chronic signals secreted via the tumor skew the normal balance of C/EBPs to a profile that sustains myeloid proliferation while blocking terminal differentiation.

Lastly, microRNAs (miRNAs) have also been implicated in facilitating the induction and expansion of MDSCs. miRNAs are endogenous, small non-coding RNAs that modulate gene expression. miRNAs have been proposed to function as either oncogenes or tumor suppressors [59]. More specific to MDSCs, miRNAs can affect the development and differentiation of HSCs to lineage-specific cells [60]. Recently, miR-155 and miR-21 were identified as the two miRNAs highly upregulated during the induction of MDSC from the bone marrow cells via GM-CSF and IL-6 [61]. miR-155 and miR-21

miRNAs down-modulated the expression of the negative regulator of myeloid cell proliferation, SHIP-1, and the tumor suppressor gene PTEN. Down-modulation of either SHIP-1 or PTEN leads to an increase in STAT3 activation. Thus, miR-155 and miR-21 may synergistically enhance the induction of MDSCs via down-modulation of negative regulators, in addition to increasing STAT3 activation [61].

8.3.2 Signal 2

After immature myeloid cell expansion and accumulation, it is thought that these cells then receive an "activation signal" that endows them with suppressive functionality. This activation is mediated by tumoral stroma factors that include IL-1β, PGE_2, TNF-α, toll-like receptor (TLR) ligands, and IFN-γ [29]. Similar to the importance of STAT3 in MDSC induction and expansion, the NF-κB pathway is an essential factor mediating MDSC activation [30]. NF-κB proteins encompass a family of dimeric transcription factors that regulate the expression of genes governing a broad range of immunological processes. In general, stimuli activate an IKK complex, which subsequently phosphorylates IκB proteins that exist in complex with NF-κB proteins. Upon phosphorylation, IκB is ubiquitinated and degraded, freeing the NF-κB proteins to enable translocation to the nucleus, where they bind target DNA sequences and facilitate gene transcription. IL-1β activates MDSCs recruited to tumor sites via an NF-κB-dependent pathway, evidenced by an increase in tumor microenvironment TNF-α and IL-6 [46]. Similarly, it was demonstrated in a mammary tumor mouse model that IL-1β-induced inflammation increased MDSC production of IL-6 and TNF-α via the TLR4/CD14 pathway, which signals through the NF-κB pathway [62]. The timing of myeloid cell expansion in relation to these "activation signals" is not understood and is an area of active investigation.

Liu et al. demonstrated that tumor-derived exosomes (TDEs) were also potent inducers of IL-6 and TNF-α in MDSCs [63]. In this study, TDE-mediated activation was dependent on MyD88, an important adapter protein in the TLR signaling pathway. Analogously, another study demonstrated that TDE membrane-associated heat shock protein 72 (Hsp72) activated MDSC suppressive functions through TLR2/MyD88-dependent mechanisms [64]. Both studies confirmed dependence on the MyD88 pathway with an associated increase in phosphorylated STAT3, suggesting synergy between NF-κB and STAT3 signaling. More recently, Achyut et al. demonstrated the importance of NF-κB signaling in MDSC function within a mouse model of glioblastoma [64]. Conditional deletion of p65 in myeloid cells in this model resulted in decreased intratumoral MDSCs with increased dendritic cells and T cells, further suggesting a role for NF-κB in MDSC expansion.

PGE_2 has also been implicated in the activation of MDSCs. Activation of MDSC functions by PGE_2 exposure requires contact or close proximity between monocytes and melanoma cells and was dependent on COX2 [65]. Cancer patient-derived M-MDSCs treated with PGE_2 resulted in the activation of the p38 MAPK/ERK pathway and an increase in TGF-β secretion, leading to potent suppression of T and NK cell function in vitro [66]. Furthermore, silencing of COX2 via shRNA resulted in reduced MDSC numbers in the spleen and an increase in the number of NK cells in an in vivo model. PGE_2 has also been implicated in MDSC expansion. Sinha et al. demonstrated that bone marrow stem cells stimulated with agonists of the prostaglandin EP2 receptor (EP2R) induced differentiation into murine $Gr\text{-}1^+CD11b^+$ MDSCs [67]. Additionally, EP2R knockout mice exhibited reduced MDSC numbers to wild type, suggesting that EP2 partially mediates MDSC induction and expansion. Blocking PGE_2 production by COX2 inhibitors also reduced MDSC numbers in these models.

In order for MDSCs to exert their tumor-promoting suppressive functions at sites of tumor, they must be able to function within tumor microenvironments that present hostile conditions, including hypoxia, low pH, and oxidative stress. In most cells of the body, these conditions disrupt the protein-folding capacity of the endoplasmic

reticulum (ER) and result in activation of the ER stress response pathway, which when overwhelmed leads to cell dysfunction or death [68]. However, persistent ER stress within tumors paradoxically facilitates tumor progression through effects on both malignant cells and infiltrating cells, such as MDSCs [68]. In tumor-bearing mice, Lee et al. demonstrated that repeat administration of the ER stress inducer thapsigargin resulted in increased mRNA levels of the immunosuppressive factors ARG1, iNOS, and NOX2 in tumor-infiltrating MDSCs [69]. Thus, the TME reinforces MDSC-mediated immunosuppression, though it still may eventually influence MDSC in vivo life span [70].

8.4 Mechanisms of MDSC Suppression

Peripheral MDSCs, such as those in circulation or localized to secondary lymphoid organs, differ in their mechanisms of suppression compared to MDSCs localized to the tumor. In the periphery, MDSC immunosuppression is governed by multiple mechanisms, including production of immunosuppressive metabolites. For example, MDSC suppression has been affiliated with the metabolism of L-arginine. Local L-arginine depletion results in the proliferation arrest of activated T lymphocytes, as well as downregulation of the CD3ζ chain [71, 72]. Indeed, using arginase-1 inhibitors, either in vitro or in tumor-bearing mice, restored T-cell function and resulted in immune-mediated antitumor responses [73]. Similar to arginine, cysteine also serves as an essential amino acid for T lymphocytes, which rely on antigen-presenting cells (APCs) to export soluble cysteine into the milieu. MDSCs harbor a cystine transporter but lack a cysteine transporter. Thus, MDSCs can sequester extracellular cystine from APCs without returning cysteine back to the milieu [74], starving T and NK lymphocytes of this essential amino acid. Oxidative stress via the production of reactive oxygen and nitrogen species by NO synthases also contributes to peripheral T-cell inhibition. Raber et al. demonstrated that PMN-MDSCs and M-MDSCs utilize different NO synthases for the suppression of T lymphocytes [75]. PMN-MDSC inhibited T lymphocyte proliferation via peroxynitrites dependent on endothelial nitric oxide synthase (eNOS), whereas M-MDSCs elicited their effects via the release of NO by iNOS. Molecular mechanisms of reactive oxygen and nitrite species-mediated T lymphocyte suppression include loss of TCR ζ-chain expression [76] and inhibiting T-cell activation [77]. In addition, peroxynitrites impede extravasation of lymphocytes from circulation, thus decreasing the pool of antitumor lymphocytes available at tumor sites [78, 79].

Another major mechanism mediating peripheral immunosuppression is the recruitment and induction of other suppressive or regulatory cells, such as thymus-derived natural T regulatory (nTreg) cells and local tumor-induced Treg (iTreg) cells. In healthy individuals, Tregs are a subset of T cells that play critical roles in immune modulation, specifically maintaining peripheral tolerance and preventing autoimmunity. However, in the context of cancer, Tregs contribute to an immunosuppressive periphery and TME that facilitates tumor escape [80]. Because the TME allows close proximity between MDSCs and Tregs, considerable cross-talk exists that serves to modulate both populations. Indeed, Ghiringhelli et al. demonstrated that immature myeloid cells induced by tumor progression selectively promoted the proliferation of Tregs in a TGF-β-dependent manner in vivo [81]. Huang et al. also demonstrated that MDSCs induce the development of Treg cells in vitro and in tumor-bearing mice and that Treg induction was dependent on MDSC-secreted IL-10 and IFN-γ [82]. Further, MDSCs upregulate ligands for several costimulatory molecules (specifically, CD86 and PD-L1) that additionally provided signals for Treg development. In a mouse model of B-cell lymphoma, MDSCs demonstrated the ability to uptake tumor-associated antigens and present them to facilitate the expansion of tumor-specific Tregs [83]. In addition to inducing the development of Tregs, Hoechst et al. also showed that MDSCs were capable of inducing the transdifferentiation of Th17 T cells into Tregs [84]. Conversely, Tregs can also modulate MDSC

expansion and function. In a mouse model of colitis, Tregs potentiated both the expansion of MDSCs and suppressive functions through a TGF-β-dependent mechanism [85]. Thus, factors secreted by both MDSCs and Tregs exist in positive feedback loops to facilitate the expansion of each population and reinforce the suppressive environment.

In addition to the cross-talk between MDSCs and Tregs, MDSCs have also demonstrated effects on macrophages. In a mammary carcinoma mouse model, MDSCs decreased IL-12 production by macrophages and facilitated the polarization of macrophages to a tumor-promoting M2 phenotype [86]. Beury et al. sought to further define the cross-talk that occurs between macrophages and MDSCs in the context of murine tumor cell lines [87]. They found that IL-6, IL-10, IL-12, TNF-α, and NO are modulated within the cross-talk between macrophages, MDSCs, and tumor that create an immunosuppressive environment.

In contrast to the mechanisms employed by MDSCs in the periphery, MDSCs localized to tumor tissues exhibit several key differences. Studies directly comparing MDSCs from spleens (peripheral tissue) and tumors of the same mouse demonstrated that tumor MDSCs acquire a more suppressive phenotype in the TME characterized by high amounts of NO, arginase-1, and immunosuppressive cytokines. In a mouse model of prostate cancer, Haverkamp et al. demonstrated that MDSCs derived from tumor tissue possessed immediate ability to inhibit T-cell function, whereas MDSCs isolated from the spleens and liver were not suppressive without additional in vitro exposure to suppressive cytokines [88]. Maenhout et al. showed that both PMN-MDSC and M-MDSC isolated from the tumor had much stronger suppressive capacity compared to MDSCs isolated from the spleen, associated with higher nitrogen dioxide production and arginase-1 [89]. Another study examining the metabolic characteristics of tumor-infiltrating MDSCs (TI-MDSCs) reported that TI-MDSCs increased fatty acid uptake and activated fatty acid oxidation (FAO) [90]. In this study, MDSCs isolated from the site of the tumor were able to inhibit antigen non-specific T-cell proliferation, whereas splenic MDSCs did not.

The suppressive mechanisms employed by different MDSC subsets likely depend on multitude of factors that includes the preferential expansion of the MDSC subset and local inflammatory milieu. The relative suppressive capacity of the major MDSC subsets, M-MDSCs and PMN-MDSCs, is a matter of debate, with the literature suggesting mixed results. Traditionally, it has been proposed that M-MDSCs are more suppressive due to the increased levels and higher half-life of suppressive mediators [91]. However, in certain tumor subtypes, PMN-MDSCs represented the more suppressive subset. For example, in a mouse model of pancreatic adenocarcinoma, PMN-MDSCs were preferentially expanded and represented the majority of MDSCs in the bone marrow, blood, spleen, and pancreas [92]. Targeted depletion of PMN-MDSCs in this model resulted in restored antitumor immunity and a reduction in tumor size. In a study analyzing the peripheral blood of patients with head and neck and urological cancers, a higher frequency of PMN-MDSCs was associated with a significant decrease in survival [93]. Additionally, PMN-MDSCs were more suppressive in in vitro T-cell suppression assays compared to the M-MDSCs isolated from the same patients [93]. Similarly, in advanced-stage melanoma patients, PMN-MDSCs isolated from peripheral blood suppressed stimulated T cells more strongly than M-MDSCs and negatively correlated with survival rate [94]. When compared to healthy controls, lung cancer patients had elevated levels of circulating M-MDSCs but not PMN-MDSCs [95]. However, a significant increase in both intratumoral M-MDSCs and PMN-MDSCs was evident compared to peripheral levels in tumor patients. In a breast cancer patient cohort, both PMN-MDSC and M-MDSC peripheral levels were elevated to similar levels when compared to each other and healthy controls [96]. Thus, a tumor-specific understanding of the relevant MDSC subsets in the periphery and TME can inform of potential mechanisms of suppression.

8.5 Non-immunologic Functions of MDSCs

In addition to their immunosuppressive effects, MDSCs also support non-immunologic aspects of tumor biology including tumor angiogenesis and metastasis [33]. During tumor growth, especially solid tumors, the tumor microenvironment requires angiogenesis to provide adequate oxygen and nutrients, as well as the removal of waste products, both of which facilitate optimal growth [97]. Yang et al. demonstrated in murine tumor models that Gr-1$^+$CD11b$^+$ cells co-injected with tumor cells increased tumor angiogenesis and vasculature maturation compared to tumor cells alone [98]. Additionally, Gr-1$^+$CD11b$^+$ cells were found directly incorporated into the new vessel endothelium and that they drastically upregulated endothelial markers. In a mouse model of glioblastoma, intratumoral accumulation of CD11b$^+$ myeloid cells promoted angiogenesis [99]. Interestingly, in both studies, MMP-9 activity was necessary to facilitate angiogenesis. In a murine model of multiple myeloma, PMN-MDSCs, but not M-MDSCs, induced a pro-angiogenic effect using the chick chorioallantoic membrane assay [100]. Furthermore, PMN-MDSCs demonstrated an upregulation of pro-angiogenic factors, including VEGF. Parihar et al. also demonstrated that in several xenograft mouse models including neuroblastoma and rhabdomyosarcoma, co-inoculation of M-MDSC with tumor cells facilitated an increase in TME microvasculature and vascular leakiness compared to tumor alone [101]. Bv8 (also known as prokineticin-2), a VEGF homologue [102], is upregulated in MDSCs and promoted tumor angiogenesis in murine xenograft [103] and transgenic models [104].

Clinical correlative data support the notion of MDSC-mediated tumor metastasis in breast cancer [105], non-small cell lung cancer (NSCLC) [106], melanoma [107], and prostate cancer [108]. Indeed, MDSCs have been readily implicated in tumor invasion. Clark et al. demonstrated in a mouse model of pancreatic ductal adenocarcinoma that MDSCs suppressed early antitumor immune responses that facilitated invasive tumor lesions [109]. The establishment of a pre-metastatic niche that accepts and protects circulating tumor cells in secondary organs is widely accepted to be a key determinant of tumor metastasis [110]. MDSCs have been shown to remodel the secondary organ local microenvironment through secretion of pro-angiogenic and growth factors in addition to inflammatory cytokines [111, 112]. Recruitment of tumor metastases to secondary sites is largely facilitated through chemokines and integrins. In an orthotopic murine model of colorectal carcinoma, VEGF secreted by primary tumor cells stimulated TAMs to produce CXCL1 that subsequently is released into circulation [113]. In response to CXCL1, circulating CXCR2$^+$ MDSCs were then recruited to the liver to establish a pre-metastatic niche that expedited liver metastasis. Similarly, in a mouse model of breast cancer, primary breast tumor-derived chemokine CCL2 resulted in the accumulation of PMN-MDSCs in the lungs [114], resulting in establishment of a pre-metastatic niche. Once at the site of the secondary organ, MDSCs contribute to the remodeling of the extracellular matrix (ECM) to make the local microenvironment more permissive for the seeding of circulating tumor cells. MDSC-mediated factors include chemokines, cytokines, growth factors, and extracellular vesicles, including TGF-β [115], VEGFA [116], S100A8/A9 [117], and MMP-9 that aid in pre-metastatic niche formation [110].

8.6 Clinical Significance of MDSCs

With the recent success of immunotherapies (checkpoint blockage, CAR-T cells) in treating a limited number of cancer types, efforts to translate this success to other tumors have intensified. Because the TME helps evade and inhibit antitumor responses, investigators have begun to assess peripheral and intratumoral MDSCs within the context of clinical trials (reviewed in Table 8.1). Elevated levels of circulating and intratumoral MDSC correlate with poor prognosis in various types of cancer and thus are postulated to correlate to immune escape. In

Table 8.1 Examples of completed and recruiting clinical trials where the frequency and/or suppressive capacity of MDSCs were assessed as primary or secondary outcome measures

Clinical trial number	Study title	Condition(s)	Interventions	Status	Primary/secondary outcome measure (of how many measures)
NCT00499122	NOV-002, Doxorubicin, Cyclophosphamide, and Docetaxel in Women with Newly Diagnosed Stage II or IIIC Breast Cancer	Breast cancer	Cyclophosphamide, Docetaxel, Doxorubicin, NOV 002	Completed, with results	Secondary
NCT00843635	Tadalafil in Treating Patients Undergoing Surgery for Cancer of the Oral Cavity or Oropharynx	HNSCC	Tadalafil	Completed, with results	Primary (1 of 3)
NCT02403778	Ipilimumab and All-Trans Retinoic Acid Combination Treatment of Advanced Melanoma	Advanced melanoma	VESANOID, Ipilimumab	Completed, with results	Primary (2 of 3)
NCT01425749	Study to Assess Safety and Immune Response of Stage IIB–IV Resected Melanoma After Treatment with MAGE-A3 ASCI (Mel55)	Melanoma	recMAGE-A3, AS15 ASCI	Completed, with results	Secondary
NCT01876212	Dendritic Cell Vaccines + Dasatinib for Metastatic Melanoma	Metastatic melanoma	DC vaccine, Dasatinib	Completed, with results	Secondary
NCT01581970	Potentiation of Cetuximab by Tregs Depletion With CSA in Advanced Head and Neck Cancer	HNC, HNSCC	Cyclophosphamide, Cetuximab	Completed, results submitted	Secondary
NCT03188276	The Relationship Between MDSCs and NK Cells Activity of CHC Patient Treated by DAAs	Chronic hepatitis C	Ledipasvir-Sofosbuvir, Daclatasvir-Sofosbuvir	Completed, no results posted	Primary (1 of 1), secondary
NCT02332642	Immunomonitoring of Patients With Metastatic Melanoma (AJCC Stadium IV) Under Chemotherapy (ImmunoPAX)	Metastatic melanoma	Immunomonitoring	Completed, no results posted	Primary
NCT02275039	p53MVA Vaccine and Gemcitabine Hydrochloride in Treating Patients With Recurrent Ovarian Epithelial Cancer	Recurrent ovarian epithelial, fallopian tube, and peritoneal carcinoma	Modified vaccinia virus ankara vaccine expressing p53, gemcitabine hydrochloride	Completed, no results posted	Secondary
NCT02718443	VXM01 Phase I Pilot Study in Patients With Operable Recurrence of a Glioblastoma	Glioblastoma	VXM01	Completed, no results posted	Secondary
NCT01653249	A Phase I Clinical Trial of an HPV Therapeutic Vaccine	Human papillomavirus	Vaccine consisting of four HPV-16 E6 peptides in combination with Candin®	Completed, no results posted	Secondary

NCT04022616	Myeloid-Derived Suppressor Cell Function in Breast Cancer Patients	Breast cancer	Specimen collection	Recruiting	Primary (1 of 1), secondary
NCT02827344	PDL-1 Expression on Circulating Tumor Cells in Non-small Cell Lung Cancer (IMMUNO-PREDICT)	Lung cancer	Blood sample collection for circulating tumor cells and MDSC analysis	Recruiting	Secondary
NCT02669173	Capecitabine + Bevacizumab in Patients With Recurrent Glioblastoma	Glioblastoma	Capecitabine, Bevacizumab	Recruiting	Primary (1 of 1), secondary
NCT02916979	Myeloid-Derived Suppressor Cells and Checkpoint Immune Regulators' Expression in Allogeneic SCT Using FluBuATG (FluBuATG)	Various hematological malignancies	Fludarabine, Busulfan, Rabbit ATG, Methotrexate	Recruiting	Secondary
NCT03486119	A Study for Identification of Predictive Immune Biomarker in Peripheral Blood for Nivolumab Therapy in NSCLC Patients	Non-small cell lung cancer	Nivolumab	Recruiting	Primary (1 of 6)

patients with advanced hepatocellular carcinoma (HCC), frequencies of circulating M-MDSCs were significantly higher compared to early-stage HCC patients [118]. Additionally, patients who underwent curative radiotherapy and had higher frequencies of M-MDSCs posttreatment had significantly shortened relapse-free and overall survival [118]. Similarly, frequencies of peripheral MDSCs increased with advanced cancer stage in patients with breast cancer [96]. In a meta-analysis of 16 different studies involving 1864 cancer patients with GI, HCC, NKT lymphoma, and melanoma tumors, Ai et al. found that increased MDSC frequencies were associated with poor prognoses and decreased overall survival [119]. MDSCs have also been proposed to serve as a prognostic marker in informing the best option for treatment. One such example is the immune checkpoint inhibitor ipilimumab approved for the treatment of metastatic melanoma. Ipilimumab has demonstrated efficacy in some cases, but overall clinical response rates remain low, with 3-year survival rates of about 20% [120]. Hence, efforts to understand factors that govern whether a patient responded or not are needed to enhance ipilimumab efficacy. Meyer et al. reported that metastatic melanoma patients who responded to ipilimumab treatment had significantly lower circulating frequencies of MDSCs compared to nonresponders [121]. In a similar study that aimed to identify baseline peripheral prognostic markers in metastatic melanoma that correlated with clinical outcome following ipilimumab treatment, MDSC frequencies were the strongest stand-alone predictor of clinical response to treatment [122]. This finding was also demonstrated in several other melanoma studies [123, 124] in addition to prostate cancer [125]. Thus, the clinical significance of MDSCs has inspired efforts to therapeutically target this population that could enhance the efficacy of antitumor immunity.

8.7 Therapeutic Targeting of MDSCs

Efforts to therapeutically target MDSCs have attempted to do so by (1) inhibiting MDSC expansion and trafficking, (2) differentiating MDSCs into mature and less suppressive myeloid cells, (3) inhibiting MDSC immunosuppressive function, and (4) depleting MDSCs from the TME [126].

8.7.1 Inhibiting MDSC Expansion and Trafficking

As discussed, MDSC generation occurs by way of abnormal myelopoiesis stimulated via tumor-derived factors. Thus, efforts to modulate or correct this abnormal myelopoiesis could prove beneficial in preventing MDSC accumulation. As discussed previously, STAT3 activation has been demonstrated to play a crucial role in the mobilization and expansion of MDSCs. Hence, agents that can block STAT3 activation in MDSCs could prove a viable option in preventing the expansion of MDSCs. Sunitinib, a small-molecule multitargeted receptor tyrosine kinase inhibitor with antitumor and anti-angiogenic effects [127], has proven a useful agent in targeting STAT3 signaling in MDSCs. In patients with renal cell carcinoma (RCC), treatment with sunitinib resulted in reduced numbers of circulating MDSCs [128]. Xin et al. later demonstrated in mouse models of RCC that sunitinib inhibited STAT3 activity and concomitantly resulted in a significant reduction of MDSCs and Tregs at the site of the tumor [129]. Pretreatment with sunitinib in RCC patients resulted in improved tumor-infiltrating lymphocytes that inversely correlated with intratumoral MDSC numbers [130]. Bevacizumab, an anti-VEGF mAb, has also shown to have effects on MDSC expansion. Rather than having a direct effect on MDSCs, bevacizumab inhibits VEGF at the site of the tumor, thus preventing VEGF as an inducer of MDSC expansion. In a mouse model of RCC, bevacizumab resulted in a decrease of peripheral $CD11b^+$ myeloid cells [131]. Similarly, in patients with metastatic colorectal carcinoma, bevacizumab administered with the chemotherapy drugs 5-fluorouracil and oxaliplatin resulted in a decrease in PMN-MDSC in 15 out of 25 evaluable patients [132]. Given that broad inhibition of STAT signaling may have off-target implications that restrain overall antitumor responses, other methods to more specifically target STAT3

have included the use of STAT3 siRNA or antisense oligonucleotides synthetically attached to a toll-like receptor 9 (TLR9) agonist that selectively targets TLR9+ myeloid cells [133]. STAT3 siRNA directed at TLR9+ cells elicited a potent antitumor response in vivo [133].

In addition to STAT3, targeting PGE_2 and COX2 has reduced MDSC numbers in mouse models. Sinha et al. first demonstrated that the COX2 inhibitor SC58236 reduced MDSC accumulation and delayed primary tumor growth in a mouse model of breast cancer [67]. Subsequently, the COX2 inhibitor celecoxib was effective in reducing MDSC numbers in mouse models of mesothelioma [134] and glioma [135]. Currently, a clinical trial (NCT02432378) is recruiting patients with recurrent ovarian cancer to examine the effects of celecoxib in addition with cisplatin. A secondary outcome measure in this study will be the change in the number of MDSCs in the peritoneal fluid of the patients.

Preventing MDSC trafficking and localization to the tumor may prevent accumulation and subsequent pro-tumor effects within the TME. Studies have implicated the chemokine receptor CXCR2 as a key mediator of MDSC trafficking that facilitates both tumorigenesis and metastasis [113, 136–138]. Highfill et al. demonstrated in a murine model of rhabdomyosarcoma that tumor cells expressing CXCL1 and CXCL2 facilitated trafficking of CXCR2+ MDSCs to the tumor [139]. In mice with a CXCR2-deficient bone marrow compartment, the percentage and absolute numbers of MDSC recruited to the tumor were significantly decreased [139]. Similarly, in mouse models of lung carcinoma, the small-molecule CXCR1/2 inhibitor SX-682 abrogated PMN-MDSC recruitment to the tumor that resulted in potentiated T-cell activation and antitumor immunity [140]. A clinical trial (NCT03161431) is currently evaluating the efficacy of SX-682 to block MDSC recruitment in metastatic melanoma. In addition to CXCR2, CXCR5 can also recruit MDSCs to intratumoral sites [141, 142]. Fusion proteins (CCR5-Ig) directed at all three CCR5 ligands demonstrated reduced MDSC infiltrates in mouse models of melanoma [141] and prostate cancer [142].

8.7.2 Differentiating MDSCs into Mature Cells

As MDSCs represent an immature myeloid population, efforts have been directed at understanding the requirements for their differentiation that may then attenuate their suppressive functions. To this end, all-*trans* retinoic acid (ATRA) has demonstrated a potent differentiator of MDSCs. ATRA is a natural metabolite of vitamin A oxidation and is a well-known regulator of cell differentiation, including the terminal differentiation of promyelocytes into mature neutrophils in patients with acute promyelocytic leukemia [143], and thus is a commonly used antineoplastic in chemotherapy regimens for this disease. Almand et al. demonstrated the utilization of ATRA to differentiate MDSCs into dendritic cells [144] and that differentiation eliminated the inhibitory function of MDSCs in vitro [145]. The mechanism of ATRA-dependent differentiation appears to be neutralization of ROS production in MDSCs via the accumulation of glutathione, both in patients and mice [146]. Subsequent clinical trials have demonstrated the potential of ATRA alone [146] or in combination with other therapies to reduce the number of circulating MDSCs [147, 148]. Although the clinical efficacy of ATRA has been demonstrated in a multitude of other trials given its use in common chemotherapy regimens, because MDSCs were not evaluated in these trials, the effect of ATRA could not be attributed to MDSC reduction [149]. Vitamin D3 has also demonstrated the ability to induce the differentiation of immature myelocytes. In patients with head and neck squamous cell carcinoma (HNSCC), CD34+ progenitor cells believed to be responsible for intratumoral immunosuppression were isolated and cultured in the presence of vitamin D3 and various cytokines [150]. CD34+ cells cultured with the combination of vitamin D3 and cytokines resulted in increased numbers of cells phenotypically similar to mature dendritic cells. In addition, these cells present antigen more efficiently to autologous T cells. In a study examining the clinical efficacy of vitamin D3 in reducing immature CD34+ at tumor sites, Kulbersh et al. found that

patients treated with vitamin D3 displayed reduced immature CD34⁺ numbers, whereas levels of intratumoral dendritic cells increased [151]. HNSCC patients treated with vitamin D3 had increased numbers of intratumoral T cells and had a longer relapse-free survival compared with the controls [152]. In patients with chronic lymphocytic leukemia (CLL), significantly higher levels of circulating M-MDSCs were present in patients with low vitamin D levels compared to those of CLL patients with high vitamin D levels [153]. Tumor-conditioned CLL exosomes facilitated the induction of healthy donor-derived monocytes to MDSCs that was reversed with pretreatment of the CLL exosomes with vitamin D3 [153].

8.7.3 Inhibiting MDSC Suppressive Function

Phosphodiesterase (PDE) inhibitors, such as sildenafil and tadalafil, are pharmacologic agents that increase the intracellular concentrations of secondary messenger molecules cAMP and cGMP. PDE inhibitors have been used clinically with widespread use in non-malignant conditions such as erectile dysfunction. Their utilization for inhibition of MDSC suppressive functions was demonstrated by Serafini et al. [154], where in multiple tumor models, the administration of sildenafil downregulated arginase-1 and NOS expression. This resulted in enhanced intratumoral T-cell infiltration and activation with resultant reduced tumor growth [154]. Subsequent studies demonstrated modulation of the suppressive function of MDSCs with improved antitumor immunity after treatment with PDE inhibitors in mouse models of colonic inflammation-induced tumorigenesis [155] and melanoma [156]. In the clinic, tadalafil has demonstrated safety and efficacy in reducing peripheral and intratumoral MDSC numbers. In patients with HNSCC, tadalafil significantly reduced arginase-1 and iNOS activity compared to controls, in addition to enhancing antitumor immunity [157]. A significant reduction in MDSC numbers was observed in patients treated with tadalafil, suggesting that arginase-1 and iNOS inhibition could interfere with autocrine and paracrine feedback loops that facilitate MDSC myelopoiesis [157]. Similarly, in a phase II clinical trial, tadalafil significantly reduced both MDSC and Treg numbers in HNSCC patients with an increase in CD8⁺ T cells [158]. A study in patients with metastatic melanoma demonstrated that tadalafil achieved stable disease as best response in 25% (3/12) evaluable patients. Moreover, in the responders, M-MDSC peripheral numbers decreased, and NO production was reduced in MDSC-infiltrated metastatic lesions [159].

8.7.4 Depleting MDSCs from the TME

Efforts have also focused on directly eliminating MDSCs at the tumor site to overcome their suppressive effects. Observations in mouse models of various tumors found that cytotoxic agents such as cisplatin [160], 5-fluorouracil [161], gemcitabine [161, 162], and oxaliplatin [163] were effective at selectively reducing MDSC numbers. However, due to their non-specific cytotoxicity and adverse side effects, other groups have developed novel approaches to selectively target intratumoral MDSCs. One such approach is the use of liver X receptor (LXR) agonists. LXR agonism significantly suppressed tumor growth and metastasis in vitro and in vivo [164]. Thus, Tavazoie et al. sought to define the antitumor mechanisms of LXR agonism in various cancer models [165]. LXR agonist RGX-104 resulted in significant tumor growth suppression in an array of cancer animal models, including lung, breast, ovarian, and colon cancer [165]. Tumor suppression was due to RGX-104-mediated reduction of MDSCs. In a phase I dose-escalation trial (NCT02922764) evaluating the safety of RGX-104, peripheral PMN-MDSC and M-MDSC numbers were decreased by an average of 85% in the first cohort of evaluable patients [165]. Another novel approach developed by Parihar et al. is the use of natural killer (NK) cells modified to express a chimeric version of the NK cell-activating receptor NKG2D, herein referred

to as NKG2D.ζ, that targets MDSCs specifically within the TME, as intratumoral MDSCs upregulated activating ligands for NKG2D [101]. Tumors escape wild-type NKG2D-mediated NK cell cytotoxicity by down-modulating the wild-type NKG2D cytotoxic adapter molecule DAP10 [166]. Thus, a synthetic NKG2D construct expressed on the surface of NK cells that bypasses the need for DAP10 could overcome the suppressive effects of the TME and rescue NKG2D-mediated cytotoxicity. Indeed, NKG2D.ζ expressing NK cells exhibited cytotoxicity against autologous and allogeneic MDSCs in vitro and in vivo. In a mouse model of neuroblastoma that recapitulated the suppressive TME, co-injection of NKG2D.ζ NK cells and neuroblastoma-directed chimeric antigen receptor (CAR)-T cells increased the antitumor activity of CAR-T cells when compared to CAR-T cells alone. Thus, NKG2D.ζ NK cells as a combination treatment could enhance current immunotherapeutic treatment modalities by altering the TME to one more permissive of antitumor immunity [101].

8.8 MDSCs in Non-oncologic Conditions

Though studies regarding MDSCs have largely focused on their implications in cancer, it is now being appreciated the role MDSCs might play in non-oncologic settings, such as autoimmunity. Because studies investigating the involvement of MDSCs in autoimmunity are relatively new, much is yet to be understood about the potential role that MDSCs have in either promoting or inhibiting autoimmune disease [167]. Similar to cancer studies, MDSCs in autoimmunity demonstrate the same plasticity and heterogeneity that make definitive characterization difficult. Thus, efforts to compare and contrast studies characterizing MDSCs in autoimmunity have been extremely challenging [167]. In a mouse model of experimental autoimmune encephalitis (EAE) that closely resembles the pathology of multiple sclerosis, adoptive transfer of PMN-MDSCs reversed EAE by suppressing the expansion of autoreactive T cells through PD-L1 [168]. In addition, circulating PMN-MDSC frequency was increased in the periphery of human subjects with MS [168], indicating MDSCs might also play a role in facilitating MS. Conversely, Cantoni et al. found that overall MDSC numbers were decreased in the periphery of MS patients compared to healthy controls. In a study examining MDSCs over the course of MS disease progression, M-MDSCs isolated from patients diagnosed with secondary progressive MS were impaired in their ability to suppress autologous $CD3^+$ T-cell proliferation [169]. Thus, rather than working to eliminate MDSCs or inhibit their suppressive function, potentiating the suppressive capacity of MDSCs in autoimmune settings could prove an efficacious option. A single injection of IFN-β at the clinical onset of EAE enhanced the presence of and promoted the immunosuppressive activity of MDSCs, limiting the severity of EAE [170].

This approach can also be applied in organ transplantation, where host immune rejection of the donor graft limits the effectiveness of transplant. Patients with circulating MDSC frequencies >10% who received a kidney transplant demonstrated 1- and 5-year graft survival rates of 93% and 79%, respectively, whereas patients with MDSC frequencies <10% had 1- and 5-year survival rates of 68% and 36% [171]. Of note, the levels of circulating MDSC in these patients also positively correlated with the levels of Tregs, indicating a generalized state of immune tolerance. Similarly, in models of hematopoietic stem cell transplantation (HSCT), MDSCs have proven useful in limiting graft-versus-host disease (GVHD) and inducing host immune tolerance. Highfill et al. demonstrated that in a fully MHC-mismatched model of HSCT, MDSCs transferred into mice suppressed donor T-cell activation, resulting in reduced GVHD lethality [172]. In 62 patients who received a haploidentical HSCT, donor grafts that displayed higher absolute counts of M-MDSCs and PMN-MDSCs resulted in lower incidences of acute and chronic GVHD [173]. However, because adoptive transfer of MDSCs failed to induce allograft tolerance in recipients [174], research has focused on inducing MDSC expansion within the host as

a means of promoting immune tolerance [175]. Yang et al. demonstrated that murine isolated bone marrow cells could be induced in vitro into M-MDSCs via M-CSF and IFN-γ and that the adoptive transfer of either recipient- or donor-induced MDSCs significantly prolonged allo-skin graft survival in mice [176]. Taken together, these studies highlight the potential to exploit the suppressive capacity of MDSCs for therapeutic benefit in non-oncologic conditions.

8.9 Trends and Future Directions

Data presented in this chapter have highlighted that the expansion and activation of MDSCs in tumor-bearing hosts contribute to multiple aspects of tumor progression, through the inhibition of endogenous and adoptive antitumor responses as well as by promoting tumor vascularization and metastasis. Because MDSCs are highly plastic with variations depending on tumor type, stage, and disease phase, research efforts over the last decade have been directed at defining their phenotype and characterizing their tumor-promoting functions in these varying disease states. Studies highlighted in this chapter emphasize the difficulty in defining MDSCs through cell surface markers alone. Conventional fluorophore-based flow cytometric applications have been limited by the low number of phenotypic markers able to be detected. The relatively recent advent of high-throughput methods, e.g., "-omics" approaches, heavy metal- or synthetic fluorophore-based cytometry, and mass spectrometry (CyTOF), should help further define cell surface markers as well as signaling and gene networks that are characteristic of MDSCs. High-throughput methods are already being utilized to investigate MDSCs in various cancers [177, 178]. In addition, consortiums dedicated to defining and characterizing suppressive myeloid cells within tumor microenvironments have been established. A current example is the Mye-EUNITER consortium (http://www.mye-euniter.eu/), made up of researchers with the primary goal of establishing gold standard protocols and guidelines for defining and characterizing myeloid regulatory cells in cancer, infection, and inflammation. Insights gained from these more global approaches and types of studies will hopefully facilitate a clear consensus on MDSC definition and functional characterization that can be applied across disease and tumor types.

Only recently have the therapeutic implications of MDSCs been expanded, as technology to allow MDSC genetic modification and reprogramming is being developed. As discussed above, efforts to therapeutically target MDSCs have been directed to modulate both the expansion and inhibit the suppressive capacities of MDSCs in the context of cancer. MDSCs have also demonstrated to be prognostic markers that correlate with disease stage and progression, in addition to providing clinicians predictive insight on potential microenvironment-directed treatment options such as checkpoint blockade. Oncologists have also utilized metronomic dosing of chemotherapy that is based on frequent, lower doses of traditional chemotherapeutic agents (as opposed to toxic high-dose chemotherapy) that facilitate a reduction in suppressive immune subsets, including MDSCs. Conversely, potentiating the suppressive capacity of MDSCs for therapeutic gain in conditions such as autoimmunity and stem cell transplant has also been explored, and studies highlighted here have demonstrated the potential MDSCs have to alleviate these disease pathologies [179]. Current efforts are being directed at further understanding and exploiting the suppressive potential of MDSCs in diseases such as multiple sclerosis, GVHD, and other autoimmune disorders. Finally, utilizing MDSCs as a cell platform for genetic manipulation and therapy is also now being explored, given the advent of technology that allows successful genetic reprogramming of primary cells of the myeloid lineage [180].

Ultimately, future efforts directed to further understand MDSC biology should focus on a deeper understanding of the mechanisms behind their pathological expansion, activation, pro-angiogenic capabilities, and immune regulatory mechanisms. Insights gained from these studies will likely provide prognostic markers and novel

therapeutic targets to either inhibit or potentiate their suppressive capacity.

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