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One microenvironment does not fit all: heterogeneity beyond cancer cells

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Abstract

Human cancers exhibit formidable molecular heterogeneity, to a large extent accounting for the incomplete and transitory efficacy of current anti-cancer therapies. However, neoplastic cells alone do not manifest the disease, but conscript a battery of non-tumor cells to enable and sustain hallmark capabilities of cancer. Escaping immunosurveillance is one of such capabilities. Tumors evolve immunosuppressive microenvironment to subvert anti-tumor immunity. In this review, we will focus on tumor-associated myeloid cells, which constitute an essential part of the immune microenvironment and reciprocally interact with cancer cells to establish malignancy toward metastasis. The diversity and plasticity of these cells constitute another layer of heterogeneity, beyond the heterogeneity of cancer cells themselves. We envision that immune microenvironment co-evolves with the genetic heterogeneity of tumor. Addressing the question of how genetically distinct tumors shape and are shaped by unique immune microenvironment will provide an attractive rationale to develop novel immunotherapeutic modalities. Here, we discuss the complex nature of tumor microenvironment, with an emphasis on the cellular and functional heterogeneity among tumor-associated myeloid cells as well as immune environment heterogeneity in the context of a full spectrum of human breast cancers.

Keywords

Tumor microenvironment; tumor-associated myeloid cells; macrophages; neutrophils; myeloid-derived suppressor cells; breast cancer; inter-tumor heterogeneity

1. Introduction

Tumors are not just mixture of neoplastic cells but contain multiple stromal cells and extracellular matrix components that together assemble an organ-like structure whereby interacting with the entire organism at a systemic level [1,2]. Bidirectional crosstalk between cancer and stromal cells has been shown to drive tumor growth and metastasis [3–6]. Molecular profiling of various tumor stromal components has yielded prognostic information, highlighting the tumor microenvironment as a crucial determinant of tumor development [7–10]. It has become increasingly evident that such interaction resembles the processes often occurring in inflammation [11–13], wound healing [14,15] and developing organs [1], with tumors hijacking normal tissue homeostasis to support disease progression. Although our discussion in this review applies to all cancer types, we will give a special emphasis on breast cancer, as it is one of the best studied cancer type with regard to tumor

microenvironment. Studies in other cancer types will also be discussed if they demonstrate points that are not yet elucidated in breast cancer.

Breast cancer is the most commonly identified and one of the deadliest neoplasms in women worldwide [16]. Whole-genome sequencing and transcriptomic profiling have revealed the molecular heterogeneity in human breast cancers, classifying the disease into different subtypes with distinct gene expression profiles and clinical outcome [17–22]. Moreover, dramatic heterogeneity also exists within the same breast tumor and among different cells either due to clonal evolution [23] or hierarchy of differentiation statuses [24,25]. What remains poorly understood, however, is the manner in which immune microenvironment evolves in the context of inter- and intra-tumor heterogeneity. Recent genomic analysis of human breast cancer unraveled genetic traits associated with molecular networks of immune response [26]. This raised the possibility that tumor-induced immune aberrations may be intrinsically encoded in and predisposed by the cancer genome.

While the immune system represents a powerful force against tumor formation and progression, tumors acquire the ability to blunt anti-tumor immunity during cancer evolution [11,27–30]. The importance of reversing such immunosuppressive mechanisms is well typified by the recent efficacies exhibited by anti-PD1, -PDL1, -CTLA4 therapy [31–33], although the exact mechanism behind such success remains to be elucidated [34]. Among cancer-promoting inflammatory effectors, tumor-associated myeloid cells (TAMCs) encompass a heterogeneous population of cells that orchestrate tumor-induced immunosuppression as well as many other hallmarks of cancer [35–37]. Targeting TAMCs in tumor microenvironment represents a promising therapeutic strategy not only because of their stable genome unlike cancer cells, but because of their profound plasticity allowing re-education of TAMCs towards anti-tumorigenic phenotype [4].

In this review, we discuss pleiotropic roles played by immune cells as an integral partner for tumor progression. We chose to focus our discussion on specific TAMCs, namely tumor-associated macrophages (TAMs), neutrophils (TANs), and myeloid-derived suppressor cells (MDSCs) as they are commonly associated with breast cancer progression. Mechanisms employed by other myeloid cells including dendritic cells [38–40] and mast cells [41,42] are not covered in this article and readers are referred to excellent reviews covering this topic. Herein, we ascribe the multifaceted tumor-promoting role of TAMCs to its intrinsic versatility and demonstrate evidence of how plasticity could be therapeutically exploited for treating cancer. Lastly, we describe the diversity of tumor immune microenvironment as the potential cause and consequence of inter-tumor heterogeneity, particularly in perspective of diverse breast cancer.

2. Tumor-associated myeloid cells (TAMCs)

2.1 Tumor-associated macrophages (TAMs)

Macrophage is a tissue-resident cell type derived from mononuclear phagocyte system, playing a pivotal role in anti-microbial immunity, tissue homeostasis, and organ development [43–45]. As a crucial mediator of cancer-related inflammation, characterization of tumor stroma revealed tumor-associated macrophage (TAM) as a prominent component of

leukocyte infiltrate in most types of malignancy influencing human patient prognosis [46,47]. In particular, breast cancer is characterized by abundant infiltration of TAMs, and studies with preclinical models have revealed diverse molecular mechanisms by which TAMs utilize to interact with and influence tumor cells to support their progression [48,49] (Table 1). Various hallmarks of cancer have been attributed to pro-tumoral functions of macrophages, with their ability to contribute to each and every step of metastatic cascade including tumor angiogenesis, invasion, migration, colonization at distant organ, immune suppression as well as regulation of anti-cancer therapies [50–52]. While the extreme plasticity of macrophages relate to their multifaceted roles in tumor progression, such adaptive responses also open therapeutic windows to re-educate these myeloid cells toward anti-tumor cells [53].

2.1.1 Impact of macrophage infiltration in human cancer—All of the clinical studies regarding tumor-associated macrophages have consistently associated their density and expression signature with poor clinical outcomes including decreased survival [47,54,55], with the exception of non-small cell lung carcinoma [56–58]. The presence of TAMs and related factors has been linked to particular functional roles in human tumors, with strong association with tumor vasculature [59–65]. In breast cancer, it has been reported that macrophages can constitute as much as 50% of the total cell mass [66]. A meta-analysis performed by Bingle and coworkers showed that an increased macrophage density was associated with poor prognosis in more than 80% of breast cancer cases [47]. The presence of proliferating macrophages as well as macrophage colony-stimulating factor 1 response signature have been specifically associated with high grade, hormone-receptor negative breast cancer and poor clinical outcome [67–69]. Importantly, recent report suggests that $CD68^{high}$, $CD4^{high}$, $CD8^{low}$ immune signature predicts reduced overall survival and recurrence-free survival, independently of conventional histopathologic parameters [70].

2.1.2 The role of macrophages in tumor progression

Tumor vascularization: Vascularization nourishes oxygenation and nutrition which ensures tumor growth above a certain size. It is well characterized that microenvironment supports tumor angiogenesis through an array of mechanisms, among which TAM is an obligate regulator [51,71]. Through genetic depletion of macrophage population as well as their derived growth factors, multiple studies have confirmed the functional importance of macrophage in angiogenesis. One of the well-characterized subset identified in primary tumor stroma is the Tie2+ macrophage which specifically governs the process of angiogenic switch [72]. In a mouse breast tumor model of MMTV-PyMT, Lin and Pollard identified that colony stimulating factor-1 (CSF1)-directed macrophages are recruited before malignant conversion and promote tumor angiogenesis by producing VEGF [73]. The crucial role of VEGF-A in angiogenesis was determined by an experiment in which ectopic expression of VEGF-A restored high-density vessel network and tumor growth in MMTV-PyMT model where macrophages were depleted [74]. Zabuawala et al proposed that transcription factor Ets2 in TAMs directly regulate tumor angiogenesis and metastasis through repression of a transcriptional program implicated with endogenous angiogenesis inhibitors [75]. Consistent with these findings, expression of VEGF by macrophages has been found to be up-regulated

in poorly vascularized areas of human breast cancers [76]. Interestingly, CSF-1 was shown to regulate differentiation of monocytes from Tie2- to Tie2+ phenotype and augmented their angiogenic as well as chemotactic response to angiopoietin-2, a ligand for Tie2 receptor expressed by endothelial cells [77]. On the contrary, Eubank et al demonstrated that granulocyte macrophage colony-stimulating factor (GM-CSF) inhibited VEGF activity and slowed tumor growth and metastasis by producing soluble VEGF receptor-1, indicating a variation of macrophage function under different microenvironmental cues [78].

Invasion and migration: A wide variety of molecules derived from TAM have been identified to promote invasive as well as migratory activity of breast cancer cells. Utilizing MMTV-PyMT model, Wyckoff et al demonstrated that a paracrine loop which consists of tumor-derived CSF-1 and macrophage-derived EGF mechanistically regulates tumor cell migration into surrounding connective tissue [79]. Blockade of either CSF-1- or EGF-stimulated signaling abrogated the migration of both cell types. Subsequently, DeNardo et al showed in the same mouse model in which IL-4 secreting type 2 CD4+ T cell promotes the invasion and migration of malignant mammary epithelial cells via skewing phenotype and effector function of tumor-associated macrophages toward M2 phenotype [80]. These macrophages in turn facilitated tumor metastasis through activation of epidermal growth factor receptor signaling in cancer cells. Similarly, Su et al reported a similar mutual interaction between TAM and human breast cancer cells essential for metastasis [81]. Mesenchymal-like breast cancer cells activate macrophages to a TAM-like phenotype by GM-CSF and macrophages in turn secrete CCL18 to directly induce epithelial-mesenchymal transition (EMT) of tumor cells. It has also been shown that macrophage-derived CCL18 promotes invasion of human breast cancer cells acting via its receptor PITPNM3, which mediates the extracellular matrix adherence and migration [82]. One alternative mechanism by which TAMs support breast cancer invasion is through macrophage-secreted exosomes which deliver invasion-potentiating miRNAs to cancer cells [83]. IL-4-activated macrophages promoted invasiveness of co-cultured human breast cancer cell lines without direct cell-cell interaction. Treatment of macrophages with miR-223 antisense oligonucleotide inhibited Mef2c-β-catenin-dependent cancer cell invasion. Gene expression profiling of invasion-associated macrophages identified enrichment of Wnt signaling molecules which potentiate the motility of PyMT breast tumor cells [84].

Intravasation: Several lines of evidence suggest that macrophage promotes intravasation, a key step in the metastatic spread of tumor cells [85,86]. Utilizing intravital real-time imaging, studies visualized macrophage-assisted vessel permeability and dissemination of tumor cells into blood stream *in vivo*. In agreement, tumor microenvironment of metastasis (TMEM), an anatomical structure comprised of macrophages, endothelial cells and tumor cells is predictive of distant metastasis of human breast cancer patients [87].

Metastasis: Metastasis is a highly inefficient process in that less than 0.1% of circulating tumor cells are predicted to withstand the harsh stresses of infiltrating and colonizing at distant organ [88]. Numerous studies have identified macrophage as a critical enhancer of this process at metastatic site. For instance, Qian et al demonstrated that CCL2-driven recruitment of Gr1+ inflammatory monocytes promotes pulmonary seeding of PyMT breast

cancer cells [89]. More specifically, monocyte-derived VEGF promoted the extravasation of tumor cells [89]. Along the same lines, previous studies determined a distinct subpopulation of macrophage originated from inflammatory monocytes mediated the extravasation and colonization of tumor cells [90]. Furthermore, Kitamura et al showed activation of CCL2-CCR2 signaling promotes secretion of CCL3 from metastasis-associated macrophages (MAM) and this is responsible for the retention of MAM to promote lung metastasis [91]. Ferjan i et al proposed an alternative mechanism by which macrophages are recruited to secondary organ to support metastatic growth [92]. Tumor cell-clot formation induced the activation and expression of vascular cell adhesion molecule-1 (VCAM-1) and vascular adhesion protein-1 (VAP-1) in lung endothelial cells, which were required for the homing myeloid cell. Blockade of endothelial activation with a VCAM-1 blocking antibody or a VAP-1 small molecule inhibitor diminished myeloid cell recruitment and tumor cell survival. Interestingly, VCAM-1 expression by breast cancer cells has been associated with lung metastasis [93]. Chen et al demonstrated that VCAM-1-mediated juxtacrine interaction between pulmonary macrophages and disseminated cancer cells supports tumor survival. Clustering of cell surface VCAM-1 triggered Akt activation and protected cancer cells from pro-apoptotic cytokines such as TRAIL. However, anti-metastatic functions of inflammatory monocytes have also been reported [94]. In a mouse model of spontaneous melanoma expressing human RET oncogene, CD11b+ Ly6C high monocytes inhibited tumor cell proliferation through a reactive oxygen species-dependent mechanism. It was shown that regulatory CD4+ T cell-derived IL-10 favored tumor progression by inhibiting recruitment and/or differentiation of inflammatory monocytes in the skin.

Immune suppression: Although less established in human cancer, increasing evidence suggests that macrophages support tumor growth by means of immune evasion [51]. Specific subset of macrophage with potent immunosuppressive activities has been identified [54] and several mechanisms have emerged to support this process. In a mouse model of breast cancer, macrophages residing in hypoxic region of primary tumor were identified to suppress function of T cells in a HIF1-α dependent manner [95]. It is established that suppression of T cell activity is dependent, at least in part, on metabolic activities of TAM through arginase 1 or iNOS expression [95,96]. In a mouse model of mammary and cervical tumors, restoration of cytotoxic CD8+ T cell function was observed with tumor regression under genetic as well as pharmacological depletion of tumor-associated macrophages and their growth factor CSF-1[97,98]. Furthermore, Bloch et al demonstrated that in patients with glioblastoma, circulating monocytes and tumor-infiltrating macrophages had elevated expression of immunosuppressive ligand B7-H1. [99] Stimulation of monocytes with either glioma-conditioned media or IL-10 alone significantly increased B7-H1 expression which induced apoptosis of cytotoxic T cells. Alternatively, macrophages suppress CD4+ and CD8+ T cells effector function by recruiting natural regulatory cells (nTreg) [100,101] as well as by inducing CD4+ regulatory cells (iTreg) [102] in the tumor microenvironment.

2.1.3 Plasticity and heterogeneity of macrophages

Polarization of macrophage: M1-M2 linear scheme and beyond: Plasticity and heterogeneity are the overarching characteristics of macrophages. Macrophages acquire distinct phenotype and function, with their capacity to integrate various distinct signals

emanating from surrounding microenvironment [103]. Polarization of macrophage results in distinct expression of surface receptors and effector functions through a repertoire of cytokine and chemokine production [104].

In a simplistic point of view, macrophages adopt two opposite polarization states, mirroring and mediating T helper type 1 (Th1) T helper type 2 (Th2) classification. Macrophage exposure to Th1 cytokines (e.g. IFN-γ) alone or in combination with bacterial components (e.g. Toll-like receptor ligands) polarize into M1 (classically activated) macrophages with anti-microbial and tissue destructive properties. These classically activated macrophages efficiently produce cytotoxic molecules (e.g. reactive oxygen and nitrogen intermediates) and pro-inflammatory cytokines (e.g. IL-1β, TNF, IL-6), performing as potent phagocytic and antigen-presenting cells. M1 macrophages are characterized by high secretion of IL-12 and low expression of IL-10 [103]. On the other hand, immunomodulatory signals such as IL-4 and IL-13 inhibit the classical activation and trigger the alternative form of macrophage polarization, namely M2 macrophage [105–108]. In general, M2 macrophages resolve inflammation, mediate parasite clearance, and promote wound healing, angiogenesis and tissue remodeling [109]. In contrast to M1 macrophages, they are characterized by high production of IL-10 and low expression of IL-12 as well as with poor antigen presenting capacity. Mechanistically, polarization into two different phenotypes requires distinct signaling pathways: M1 polarization relies on activation of ERK, NF-κB, and STAT1 signaling, whereas M2 polarization depends on activation of STAT3 and STAT6 pathways [107,110]. NF-κB and STAT1 signaling suppresses the activation of STAT3 and STAT6 and vice versa [110–112]. Two different subsets of macrophage also differ in their ability to engage in communication with innate and adaptive immune cells [104]. While M1 macrophage recruit Th1 lymphocytes by producing chemokines including CXCL9 and CXCL10, M2 macrophages attract regulatory T cells, Th2 lymphocytes, basophil, and eosinophil by secreting CCL17, CCL22, and CCL24 [107,113,114]. It has been shown that macrophages can also be polarized toward an M2-like state which partially shares M2 signature features, further refining the diverse forms of alternative activation. Several classes of molecules orienting “M2-like” properties have been identified; antibody immune complex together with LPS or IL-1β give rise to M2b macrophage and immunosuppressive cytokines (e.g. IL-10, TGF-β) drive M2c form [104,109].

While the description of activation with two opposite states offers a mechanistic framework for understanding macrophage polarization, the concept tends to oversimplify the diversity of macrophage phenotypes. Consequently, the emerging paradigm proposes M1- and M2-polarizations as the extremes of a spectrum of activation states [115,116]. Heterogeneous gene expression signatures in macrophage could be ascribed to simultaneous activation of different signaling pathways induced in complex tissue microenvironments. In line with this scenario, macrophages with intermediate or only partially overlapping phenotypes of IFN-γ (M1) and IL-4 (M2)-induced macrophage have been observed *in vivo* [117–121]. For instance, monocytes infected with human cytomegalovirus infection displayed an atypical M1/M2 reprogramming through activation of both NF-kB and PI3K signaling pathways [122,123]. Similarly, a mixed profile of M1/M2 polarization was observed in CD11c+ adipose tissue macrophages from obese mice [124]. Peritoneal macrophages induced by D1-DMBA-3 mammary tumors display neither M1 nor M2 characteristics [125]. A new

macrophage phenotype which differs distinctly from conventional M1 and M2 state has been identified in response to oxidized phospholipids [126]. Importantly, a recent study done by Xue and coworkers demonstrated a spectrum of human macrophage activation states, expanding the current M1-M2 paradigm [127]. The authors investigated the transcriptional changes triggered in human monocyte-derived macrophages by 28 different stimuli or their combinations. They identified 49 transcriptional modules with similar profiles of transcriptional induction in response to different stimuli. The transcriptional responses to these 28 stimuli could be organized into at least 10 clusters that represented distinct activation states.

Polarization of tumor-associated macrophages: Multifaceted role of TAM is dependent on the diverse functional state that is regulated by environmental factors such as hypoxia, local concentrations of cytokines and chemokines, and immune microenvironment [128]. Although macrophage infiltrates in developing tumor may exert diverse functions, macrophages isolated from metastatic murine and human tumors generally display an M2-polarization state [129,130]. Among various signaling factors expressed within tumor microenvironment, IL-10, prostaglandin E2 (PGE2), TGF-β, CCL2, CSF-1 have been reported to induce alternative activation of macrophages [13,131,132]. TAMs with M2 phenotype largely show IL-12 low and IL-10 high phenotype, impaired antigen presentation and tumoricidal capacity, poor expression of reactive nitrogen intermediates, and high production of angiogenic factors (e.g., VEGF, EGF, and semaphoring 4D), metalloproteases (e.g., MMP9), and cathepsins [125,133–135]. Diverse transgenic mouse model systems revealed the intrinsic signaling networks regulating TAM polarization. For instance, Src homology 2-containing inositol-5′-phosphatase-1 (SHIP1)-deficient [136] and Notch signaling-deficient macrophages displayed M2-biased phenotype [137], whereas p50 Nuclear factor-κB- (NF-κB) deficiency showed defective capacity to mount M2 activation [138].

Polarization of TAM is shaped by different immune and stromal cells in tumor microenvironment as well as oncogenic pathways of cancer cells as they regulate distinct cytokines/chemokines. In a mouse model of mammary carcinoma, MDSCs were shown to promote tumor progression by inducing M2 polarization of TAMs [139]. Erez and colleagues proposed an association between distinct inflammatory gene signature from CAFs and TAM recruitment, angiogenesis, and tumor growth in K14-HPV16 mouse model of squamous carcinogenesis [140]. With the same model, Andreu and coworkers found B cells shaping TAM phenotype and fostering cancer development by activating Fcγ receptors on resident and recruited myeloid cells [141]. Moreover, utilizing MMTV-PyMT model of mammary carcinogenesis, DeNardo et al demonstrated that CD4+ T lymphocytes expressing M2-polarizing cytokines, IL-4 and IL-13, potentiated tumor metastasis by modulating the bioeffector functions of TAMs [80]. Importantly, several studies established the direct link between epithelial-mesenchymal transition (EMT) pathway and M2-macrophage. Utilizing human breast cancer cell lines, Su et al demonstrated that production of GM-CSF by mesenchymal-like cancer cells activate nearby macrophages toward M2 phenotype [81]. Similarly, Hsu et al showed that among the downstream targets of transcriptional repressor

Snail were discrete cytokines specific for macrophage recruitment and polarization including CCL2 and CCL5 [142].

It should be noted that macrophages display a progression of functional changes in response to temporal evolution of microenvironment milieu. A shift in monocyte-macrophage phenotypes during the course of several diseases has been observed including the case of cancer [143–145]. For instance, in early phase of tumorigenesis high production of pro-inflammatory mediators (e.g. TNF, ROS) from M1 macrophages appears to support neoplastic transformation [130], whereas in established cancers the M2 macrophages with immunosuppressive and tissue remodeling activities promote immune escape and tumor progression [130,146–149]. This suggests that functional state of macrophage during tumor progression differs that during tumor initiation.

TAM heterogeneity in tumor microenvironment: Accumulating evidence suggests that each of extrinsic activities including tumor angiogenesis, invasion, metastasis, and immunosuppression are ascribed to a unique subpopulation of macrophage [54]. Given the extreme plasticity of these myeloid cells, phenotype of TAM differs within different areas of the same tumor [53,128,150]. Several studies have confirmed differential migratory capacity of TAM in different compartments of the tumor where perivascular TAMs exhibit stronger migration compared to the ones in avascular regions [151,152]. For example, Wyckoff et al showed perivascular macrophages which are present in large numbers at the margins of mouse mammary tumors reciprocally interact with cancer cells, resulting in coordinated migration of both cell types [79]. Corroboration of the “motile” and “sessile” macrophages has shown different activation status between these two subsets in which the vessel-distal TAMs express higher levels of M2 markers than vessel-proximal TAMs [152,84,153]. Further reports indicate differentially activated TAMs residing in distinctively oxygenated tumor regions within the same tumor [154,155]. In a mammary adenocarcinoma model, Movahedi et al showed MHC-II low TAMs expressing M2-assocaited markers (e.g., IL-4Rα, stabilin-1, CD204, and CD206) are found in most hypoxic tumor regions, whereas MHC-II high TAMs with M1-like phenotype reside in normoxic regions [154]. The authors identified up to seven phenotypically distinct macrophage subpopulations within tumors, further demonstrating the complexity of macrophage population [154]. Indeed, macrophage phenotypes are closely associated with hypoxic environment (e.g. necrotic and poorly vascularized region) [48] in which debris in this area attracts macrophages [156]. Most solid tumors contain regions of hypoxia resulting from an abnormal vascularization as well as a high metabolic activity [157]. Turner et al showed that once recruited to the tumor, macrophages are trapped in necrotic tumor areas [158], possibly due to abortion of chemotactic response following de-phosphorylation of VEGFR and CCR2 by mitogen-activated protein kinase phosphatase (MKP-1) [159,160]. Up-regulation of transcription factor HIF-1α and HIF-2α expression induces hypoxia-responsive gene expression in macrophages, highly implicated with aggressive behavior of tumor cells including pro-angiogenic factors (e.g., VEGF) [76,161].

Recently, Franklin et al delineated the phenotype, function, and ontogeny of two TAM populations in MMTV-PyMT model; namely mammary tissue macrophages (MTMs) and tumor-associated macrophages (TAMs) [98]. MTMs defined as MHC II high CD11b high

Mr1c+ cells populate in untransformed wild-type mammary gland and decrease upon tumor growth, whereas TAMs identified as MHC II high CD11b low Vcam1+ cells increase. Accumulation of these two subsets of macrophage showed differential dependencies on monocytes, TAMs requiring less input from blood CCR2+ inflammatory monocytes and having higher proliferative capacity. Interestingly, TAMs were not alternatively-activated macrophages typically associated with tumor progression. Instead, MTMs more closely resembled M2 macrophages with higher expression of M2 markers including Ym1 and Fizz1. Utilizing $CD11c^{cre}$ $Rbpj^{fl/fl}$ transgenic mice that deletes Notch pathway selectively in TAMs, the authors further showed that TAM differentiation from blood monocytes was dependent on Notch signaling whose abrogation resulted in loss of TAMs, reduced tumor burden and increased tumor immune surveillance by functional restoration of GzmB+ CD8+ T cells.

Repolarizing TAM as a therapeutic strategy: Most therapeutic modalities against cancer are to target various aspects of tumor cells directly. Targeting tumor microenvironment has become an attractive strategy since genetically stable stromal cells are less prone to develop resistance mechanisms. Inherent plasticity of stromal cells enables in situ reprogramming toward an anti-tumoral phenotype, an alternative option to simply depleting and destroying them. Such depletion strategy has been reported with limited benefits including the case of angiogenesis inhibitors [162]. Recent repolarizing approach combined with conventional anti-tumor therapies has been made with clinical success by utilizing high versatile nature of macrophages. These studies collectively suggest that cancer immune surveillance can be strictly governed by innate immunity and does not necessarily require the adaptive arm. For instance, macrophage-mediated cancer cell phagocytosis could be restored upon blockade of CD47, a “don t eat me” signal overexpressed by B cell non-Hodgkin lymphoma cells (NHL) [163]. Treatment of human NHL-engrafted mice with anti-CD47 antibody synergized with rituximab, which resulted in elimination of lymphoma and cure of disease [163]. It was previously shown that up-regulation of CD47 is an important mechanism by which hematopoietic stem cells are protected from phagocytosis during inflammation-mediated mobilization, and cancer cells co-opt this mechanism in order to evade macrophage killing [164]. In the case of pancreatic ductal adenocarcinoma, tumor-bearing mice and humans were effectively treated with CD40 agonist [165]. While previous studies have identified the importance of CD40-mediated licensing of antigen-presenting cells in developing T cell-dependent anti-tumor immunity [166], Beatty et al demonstrated that systemic activation of CD40 with anti-CD40 monoclonal antibody induced macrophage-mediated tumor regression without engagement of T cells [165]. Efficacy of anti-CSF1R was shown against glioblastoma multiforme in which upon administration of a CSF-1 receptor inhibitor, macrophages were polarized to M1 phenotype, rather than depleted to evoke anti-tumor response [167]. However, anti-CSF1R treatment depletes macrophages in pre-clinical mouse models of breast cancer and it has no effect on primary tumor growth unless used in combination with chemotherapies [70]. These observations suggest that with adequate immunological stimulation, macrophages in tumor microenvironment can be “re-educated” to establish robust consequences for mitigating tumor growth.

2.2 Tumor-associated neutrophils (TANs)

Neutrophils are one of the most abundant leukocytes circulating in human peripheral blood. They are well-established for providing the first line of defense against invading pathogens through potent antimicrobial substances [168–170]. Recent studies suggest a pleiotropic role for neutrophils, with their ability to synthesize a variety of effector molecules in response to different environmental signals [171,172]. Along with other leukocytes they play an essential role in directing hematopoiesis, wound healing, and angiogenesis [173,174]. While neutrophils are generally considered a homogeneous population, emerging studies point to the heterogeneity of neutrophils [175–179]. Although frequently present at tumor, neutrophils have largely been neglected in cancer-related inflammation partly due to their short life span and fully differentiated state. Currently, the role of neutrophils in tumor progression is controversial, since both pro- and anti-tumoral functions have been reported [180–182] (Table 2). It has been suggested that different degree of activation may underlie the dual effects of neutrophils on tumor cell behavior [180]. As one of prominent tumor-infiltrating myeloid cells displaying versatility whose function can be polarized toward distinct phenotypes, neutrophils have come to light as a potential therapeutic target in cancer.

2.2.1 Impact of neutrophil infiltration in human cancer—Early studies demonstrated increased infiltration of neutrophils in biopsies of cancer patients compared to surrounding non-cancerous tissue or from that of healthy controls [183–186]. Multiple studies have described that the presence of TAN is associated with poor clinical outcome in various tumor settings. In renal cell carcinoma, intra-tumoral neutrophils defined as CD66b+ cells were an independent predictor of mortality [187]. Similarly, neutrophil infiltration in bronchoalveolar cell carcinoma (BAC) , head and neck squamous cell carcinomas (HNSCC), gastric adenocarcinoma and hepatocellular carcinoma positively correlated with poor patient survival [183,188–190]. Elevated expression of neutrophil elastase in human breast cancer showed a strong relation with poor prognosis [191,192] and low response rate to tamoxifen treatment [193]. Moreover, high neutrophil-to-lymphocyte ratio (NLR) is a strong predictor of mortality in several malignancies, including breast cancer [194–196]. Gentles et al recently suggested that polymorphonuclear (PMN) cell signatures emerged as the most significant adverse cancer-wide prognostic populations among diverse tumor-infiltrating leukocytes [197]. Along the same line, preclinical studies experimenting PMN depletion confirmed the detrimental nature of TANs. Together, these studies indicate that presence of intra-tumoral neutrophils may have a profound effect on clinical outcome as well as on tumor growth.

2.2.2 The role of neutrophils in tumor progression

Proliferation: Several molecules expressed by neutrophil have been identified to promote tumor cell proliferation. In a mouse model of squamous carcinoma elicited by HPV16 oncogenes, Coussens et al reported that transgenic mice devoid of MMP9 showed reduced keratinocyte hyperproliferation and a decreased incidence of invasive tumors [198]. Bone marrow transplantation of MMP-expressing wild type cells rescued tumor growth in MMP9 knockout mice. The authors further determined that MMP9 is predominantly expressed by neutrophils, macrophages, and mast cells, highlighting the essential role of bone marrow-derived cells in regulation of tumor cell proliferation. Wada et al showed that neutrophil

elastase (NE) released from activated neutrophils stimulated proliferation and invasion of esophageal cancer cells by releasing growth factors present on the surface of cancer cell [199]. A specific NE inhibitor, sivelestat, significantly abrogated the NE-induced growth advantage of cancer cells by inhibiting the release of TGF-α, PDGF-AA, PDGF-BB and VEGF. Recently, Houghton et al demonstrated that NE secreted by TAN promotes lung tumor growth in the Lox-Stop-Lox (LSL)-K-ras mouse model of lung adenocarcinoma [200]. Interestingly, NE did not function as a traditional matrix-degrading enzyme which releases bioactive molecules sequestered in extracellular matrix, but directly induced tumor cell proliferation upon entering tumor cell endosomes. NE degraded IRS-1, a homeostatic binding partner of PI3K pathway activity, resulting in hyper-activation of PI3K signaling and tumor cell proliferation.

Tumor Vascularization: Neutrophils have shown to be a potent stimulator of angiogenesis by secreting pro-angiogenic factors in non-tumor model systems [174,201]. In parallel, there is mounting evidence suggesting a significant role of TAN in supporting tumor vascularization. Bergers et al discovered in RIP1-tag2 model of pancreatic islet carcinoma that MMP9 contributes to angiogenic switch through liberating angiogenesis inducer VEGF which was sequestered in normal and hyperplastic pancreatic islets [202]. The enhanced bioavailability of VEGF elicited angiogenesis by ligand-dependent activation of its receptor on endothelial cells. Genetic depletion of MMP9 as well as application of MMP inhibitor markedly inhibited initial angiogenic switching and growth of nascent tumor cells. A further study determined that the major source of MMP9 was the inflammatory cells including neutrophils and macrophages in close apposition to vasculature [203]. Antibody-mediated depletion of MMP9-expressiong neutrophils significantly reduced the frequency of initial angiogenic switching in dysplasias, highlighting a crucial role of TAN at early stages of carcinogenesis. Bv8 (prokinectin-2) is a protein structurally similar to endocrine gland-derived VEGF that are implicated with tumor angiogenesis. Utilizing the RIP1-tag2 model, Shojaei et al identified G-CSF-elicited Bv8 protein as a crucial mediator of neutrophil-dependent angiogenesis [204,205]. Early treatment of mice with anti-Bv8 antibody resulted in a significant decrease in the number of angiogenic islets as well as mobilization of CD11b + Gr1+ cells to neoplastic lesions. However, the treatment did not affect tumor vascularization or burden when applied at later stages of tumor progression, suggesting a similar stage-dependent role of Bv8 in tumor angiogenesis. Moreover, Shojaei et al described that G-CSF-induced Bv8 endow tumor refractoriness to anti-angiogenic therapy [206]. While treatment of refractory tumors with anti-G-CSF or anti-Bv8 augmented the effect to anti-VEGF-A therapy, G-CSF delivery to sensitive tumors reduced responsiveness through induction of Bv8-dependent angiogenesis.

Invasion and migration: Tumor-associated neutrophils can act as a modulator of invasion and migration of cancer cells via a positive-regulatory loop. For instance, when co-cultured with MDA-MB-231 and T47D human breast cancer cell lines, human neutrophils secreted oncostatin M in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by breast cancer cells [207]. Importantly, neutrophil-derived oncostatin M in turn induced vascular endothelial growth factor (VEGF) from breast cancer cells and increased both detachment and invasiveness of cancer cells. Strell et al also showed a reciprocal

interaction between neutrophils and cancer cells that enhance migratory activity of both cell types [208]. The authors showed that secretion of IL-8 and GRO-α by MDA-MB-468 breast cancer cells attracted neutrophils towards tumor cells and enabled direct cell-cell interaction. Neutrophil expressed beta(2)-integrins, the ligands of intercellular adhesion molecule (ICAM)-1 that were expressed by cancer cells. Upon ligation, neutrophil-induced ICAM-1 clustering triggered tumor cell migration through phosphorylation of focal adhesion molecules including focal adhesion kinase via Src kinase as well as activation of p38 MAPK pathway through Rho kinase. Encouraged by the observation of high neutrophil infiltration in human solid tumors and its correlation with metastasis, Wu et al demonstrated that tumor supernatant derived from hepatocellular, cervical, colorectal, and gastric carcinoma cell lines provoked production of inflammatory cytokines and enhanced survival of neutrophils [209]. Tumor-educated neutrophils promoted cancer cell motility via a contact-dependent mechanism. Importantly, the effects of culture supernatants were reversed upon blocking interaction between tumor-derived hyaluronan and neutrophil-expressed TLR4, highlighting the importance of tumor-neutrophil crosstalk during neoplastic progression.

EMT and intravasation: Acquisition of mesenchymal characteristics and access to blood stream has been associated with intratumoral neutrophils. Grosse-Steffen et al observed a connection between PMN-derived NE and epithelial-mesenchymal transition (EMT) in pancreatic cancers *in vitro* [210]. In alignment with the observation that EMT phenotype coincides with the PMN infiltrate in biopsies of human pancreatic ductal adenocarcinoma, co-culture with either neutrophils or neutrophil-derived NE induced dyshesion of cancer cells with the loss of E-cadherin and downregulation of keratin. In parallel, up-regulation of transcription factor TWIST and appearance of β-catenin and ZEB1 in nucleus were observed. Utilizing an *in vivo* model of human fibrosarcoma and prostate carcinoma, Bekes et al demonstrated that high propensity of certain tumor variants to intravasate and metastasize strongly correlated with their pronounced angiogenic potential via recruitment of MMP9-positive neutrophils [211]. Primary tumors formed by highly disseminating variants exhibited enhanced angiogenesis and elevated infiltration by MMP9-expressing neutrophils, compared with tumors composed of low disseminating variants. Inhibition of neutrophil influx by anti-IL-8 antibody resulted in coordinated diminishment of tumor intravasation as well as angiogenesis.

Metastasis: Importantly, studies have indicated that neutrophils can be usurped by tumors to perpetuate metastasis at secondary organ. Direct cell-cell interaction between neutrophils and cancer cells seems to be a crucial event during extravasation. Utilizing an *in vitro* trans-endothelial migration model, Wu et al demonstrated that human neutrophils (PMN) enable human breast tumor cell line MDA-MB-231 to cross endothelial barrie r[212]. PMN treated with tumor-conditioned medium showed functional alteration including low cytocidal function, delayed apoptosis, and upregulated expression of adhesion molecules. Blockade of adhesion molecules mediating the attachment of two cell types, CD11b and CD18 on PMN and ICAM-1 on cancer cells, significantly attenuated PMN-mediated tumor cell migration. Huh et al reported an existence of similar physical interaction between neutrophils and cancer cells *in vivo* [213]. Co-injection of melanoma cells with human neutrophils increased cancer cell retention at lung where entrapped melanoma cells secreted high levels of IL-8,

increasing both recruitment of neutrophils and their beta(2) integrin expression. Interaction of ICAM-1 on melanoma cells and Mac-1 on neutrophils promoted tethering of circulating tumor cells to vascular endothelium, whose disruption by anti-IL8 treatment decreased trans-endothelial migration and lung metastasis. Spicer et al has also showed the importance of Mac-1/ICAM-1 interaction in liver metastasis of lewis lung carcinoma [214].

Several additional mechanisms have been identified whereby neutrophils facilitate metastasis. Utilizing K14cre;Cdh1F/F;Trp53F/F (KEP) mice, a conditional model of invasive lobular breast cancer, Coffelt et al demonstrated that γδ T cell/IL-17/neutrophil axis drives multi-organ breast cancer metastasis [215]. KEP mammary tumors did not exhibit significant increase of G-CSF or GM-CSF. Instead, IL-1β-elicited IL-17 expression from γδ T cells induced G-CSF-dependent expansion and polarization of immunosuppressive neutrophils. Neutralization of IL-17, G-CSF, and γδ T cells reduced cKit+ neutrophil accumulation and reversed pro-metastatic neutrophil phenotype. Moreover, the absence of γδ T cells and neutrophils reduced lymph node as well as pulmonary metastasis without affecting primary tumor growth. Importantly, combined depletion of neutrophils and CD8+ T cells reversed the metastasis phenotype of neutrophil depletion alone, suggesting that neutrophils facilitate metastasis by suppressing cytotoxic T cells. Kowanetz et al proposed that mobilization of Bv8-expressing Ly6G+ Ly6C+ granulocytes generates pre-metastatic niche formation and facilitate colonization by breast cancer cells [216]. The authors claimed that immunosuppression did not play a role in this process since inhibiting mobilization and function of myeloid cells had similar effects between immune-competent and immune-deficient mice. Tumor-derived G-CSF was sufficient to induce pre-metastatic microenvironment by mobilizing granulocytes that express pro-metastatic factors such as S100A8/9 and MMP9. Interestingly, Bv8 promoted lung metastasis through PKR1-mediated stimulation of tumor cell extravasation.

2.2.3 Plasticity and heterogeneity of neutrophils

Repolarizing TAN as a therapeutic strategy: Mirroring plasticity and heterogeneity of macrophages, increasing evidence suggests the phenotypic and functional variation of neutrophils in both tumor and non-tumor settings [169,171]. It has been reported that murine neutrophils exhibit a previously unappreciated immune-regulatory role during acute and chronic microbial infections [217,218]. Cancer serves as a representative example highlighting the versatile nature of neutrophils [169]. In general, tumor-infiltrating neutrophils are skewed toward pro-tumoral phenotype and this can be reversed for treatment of cancer by manipulating the cytokine/chemokine milieu.

For instance, Fridlender and colleagues described the existence of N1- and N2-tumor-associated neutrophils, in analogy to M1- and M2-tumor associated macrophages [219]. Utilizing a syngeneic murine mesothelioma and orthotopic LSL-K-ras tumor model, the authors demonstrated that while treatment with SM16, a TGF-β receptor kinase antagonist, decreased tumor growth with abundant tumor infiltration by CD11b+ myeloid cells, depletion of neutrophils by anti-Ly6G antibody reversed the therapeutic effect of SM16, suggesting that neutrophils participate in the anti-tumor activity of TGF-β blockade. Tumor killing activities of theses neutrophils include the enhanced expression of immunoactivating

cytokines and chemokines (e.g., FAS, TNF-α, CCL3), production of oxygen radicals (e.g., superoxide, H_20_2) and activation of CD8+ CTLs. The neutrophil plasticity was regulated by molecules in tumor microenvironment, where immunosuppressive cytokine TGFβ induced N2 pro-tumoral neutrophils and lack of TGFβ enabled the generation of N1 anti-tumoral neutrophils. Another study from Jablonska et al similarly depicts the differential polarization of neutrophils under distinct tumor microenvironment. The authors demonstrated that constitutively produced endogenous IFN-β regulates neutrophil phenotype as an important mediator of innate tumor surveillance [220]. Utilizing B16F10 melanoma or MCA205 fibrosarcoma cells, it was shown that tumors grew more rapidly with better-developed blood vessels in IFN-β-deficient mice than in control mice. These tumors exhibited elevated infiltration by CD11b+ Gr1+ neutrophils with higher expression of pro-angiogenic factors VEGF and MMP9 and the homing receptor CXCR4. *In vitro* treatment of these TAN with IFN-β restored expression level of proangiogenic factors to control levels. Furthermore, depletion of neutrophils inhibited tumor growth in both control and IFN-β-deficient mice, indicating that variable levels of endogenous IFN-β may imprint neutrophil phenotype to either restrict tumor angiogenesis (N1 neutrophils) or enhance angiogenesis (N2 neutrophils).

Dual functions of TAN in tumor progression: Emerging evidence points to the fact that tumor-associated myeloid cells (TAMCs) are indispensable ingredient of cancer whose function can occur locally in or around the tumor microenvironment as well as systematically at secondary organs. Yet controversy still surrounds the exact identity and function of TAMCs as they have been reported, in extreme cases like tumor-associated neutrophils (TAN), with bi-functional behaviors to both oppose and potentiate tumor progression (Table 2). Emergence of different phenotypes in same cell type strongly illuminates the context-dependency and inherent plasticity of these cells. Pulling together these disparate ideas remains to be unraveled. Hence, an important research goal might be the investigation of cause behind such heterogeneous role and of strategies to develop effective immunotherapeutic modalities that can tune them into becoming anti-tumoral.

Several molecules (e.g., NE, ROS, and MMP9) illustrate the dual functions of TAN. At variance with the finding of Houghton et al in which NE induced tumor cell proliferation [200], cancer cell uptake of NE was shown to potentiate antitumor immunity in breast cancer cell lines [221]. The authors argued that NE uptake resulted in increased expression of low molecular weight cyclin E (CCNE) and susceptibility of breast cancer cells to lysis by CCNE-specific cytotoxic T lymphocytes.

While multiple studies strongly suggest the pro-angiogenic/metastatic role of neutrophil-derived MMP9, anti-tumoral effects of MMP9 have also been reported in the literature [222], which may potentially explain the failure of broad-spectrum MMP inhibitors in clinical trials. Utilizing models of breast cancer, Leifler et al reported a dose-dependent decrease of tumor growth and angiogenesis after intra-tumoral injection of adenoviruses carrying MMP9 gene. Adenoviral gene transfer of MMP9 (AdMMP-9) altered the cytokine profile of stroma *in vivo,* accompanied by massive infiltration of neutrophils and activation of anti-tumoral macrophages. Importantly, neutrophil depletion prior to gene transfer

abolished the therapeutic effects of AdMMP9, indicating that neutrophils exerted an anti-tumoral action.

Lastly, neutrophil-derived reactive oxygen and nitrogen species may possibly play dual roles during tumor progression [223–225]. In mice orthotopically implanted with 4T1 breast cancer cells, Granot et al showed that neutrophil-produced ROS suppressed metastatic process [223]. Depletion of neutrophils that accumulated in pre-metastatic lung increased the metastatic burden. It was proposed that tumor-entrained neutrophils (TEN) prevent tumor cell seeding at metastatic organ through an H_20_2-dependent cytotoxic mechanism. TEN was shown to acquire tumoricidal ability following stimulation by G-CSF and tumor-derived CCL2. On the contrary, studies have shown that genetic mutation frequency of tumor correlates with the degree of neutrophil infiltration and inducible nitric oxide synthase (iNOS) activity [224]. Utilizing a series of Mutatect tumor cell lines, Haqqani et al spotted hypoxanthine phosphoribosyl transferase (*Hprt*) locus as a measure of genotoxicity. It was shown that neutrophils predominantly infiltrating Mutatect tumors stained positively for iNOS, suggesting a potential source of mutagenic factors. Regulation of IL-8 expression of Mutatect tumors was sufficient to alter the level of tumor-infiltrating neutrophil as well as that of genotoxicity.

It has been suggested that unlike macrophages that display alternative activation, N1- and N2- neutrophils are cells with a simply different degree of activation (i.e. fully activated or weakly activated neutrophils, respectively). It is hypothesized that neutrophils elaborate molecules in different concentrations, rather than to use different substances, to dictate tumor behavior. Different effects over a dose range may explain the reported contradictory roles of TAN-derived molecules in tumor progression: with modest concentrations being genotoxic, and with excessive concentrations being cytotoxic to tumor cells [180]. A biphasic response was demonstrated in melanoma cell lines where increasing levels of transduced IL-8 facilitated tumor formation, whereas at very high levels of IL-8 tumor growth was impaired dependent on massive infiltration of neutrophils [226].

Earlier studies have documented the natural tumoricidal ability displayed by neutrophils, opening the possibility of exploiting neutrophils as endogenous agents for treating cancer [181,182]. Cytokine gene transfer strategies in which engineered tumors overexpress cytokines induced massive recruitment of neutrophils to tumors, leading to the rejection of tumor cells and establishment of anti-tumor immunity against wild-type parental tumor [227,228]. Tumor destruction by activated neutrophils is achieved through their release of a variety of factors including ROS, membrane-perforating agents, and cytokines (e.g., TNF-α, IL-1β, and IFNs) which result in activation of cytotoxic immune cells (e.g., NK cells, CD8+ T cells) which evoke immunological memory against tumors or antibody-dependent cellular cytotoxicity (ADCC) [181]. However, it remains to be carefully assessed the consequence of employing cytotoxic neutrophils to kill cancerous cells since it may also be injurious to neighboring host tissues, as detrimental effects by highly activated neutrophils have been observed for human inflammatory diseases.

2.3 Myeloid-derived suppressor cells (MDSCs)

In addition to macrophages and neutrophils, dramatic accumulation of myeloid cells defined as myeloid-derived suppressor cells (MDSCs) accompanies tumor progression has been reported in both murine and human patients. MDSCs are a heterogeneous population of cells composed of immature myeloid and progenitor cells prevented from differentiating into macrophages, dendritic cells, and granulocytes [229–231]. Hallmark of MDSCs is their potent ability to subvert antitumor-immunity by frustrating T cell cytotoxicity, contributing to immunosuppressive microenvironment favorable for tumor progression [229–231]. In addition to cancer, involvement of MDSCs in progression of other pathological conditions such as infection [232], sepsis, trauma [233], autoimmunity [234]and inflammatory bowel disease [235] has been reported. It has become increasingly clear that minimizing MDSC-mediated immunosuppression is critical for developing anti-tumor immune response as well as improving efficacy of current immunotherapies [236–238]. Besides their inherent immunosuppressive function, non-immunological functions of MDSCs have been implicated in supporting tumor growth through directly enhancing angiogenesis, invasion, and establishing pre-metastatic niche [36,229] (Table 3). However, phenotypic characterization of MDSCs still remains contentious due to intrinsic cellular heterogeneity and lack of standardization of phenotypic marker profiles [239,240].

2.3.1 Impact of MDSC accumulation in human cancer—High number of MDSCs has been proposed to inversely correlate with prognosis and overall survival in cancer patients [241]. Initial experiments quantifying different immune cell subsets in peripheral blood of patients with breast cancer and other malignancies showed that compared to healthy volunteers, the level of immature myeloid cells which lack mature markers of hematopoietic cells substantially increased while that of mature dendritic cells decreased [242]. It is reported that increased circulating MDSCs identified as immature Lin HLADR CD11b+ CD33+ cells correlate with clinical cancer stage and metastatic tumor burden in human breast cancer patients [241]. Furthermore, surgical resection of tumors resulted in decreased number of MDSC in peripheral blood and reversal of T cell suppression, suggesting a direct connection between MDSC frequency and the extent of disease [242]. Collectively, these clinical studies strongly suggest that MDSCs hold potential prognostic significance in breast cancer.

2.3.2 Identification and subset characterization of MDSCs—MDSCs are particularly noted for their morphological, phenotypic, and functional heterogeneity [239]. In mice, MDSCs are identified by the co-expression of myeloid lineage differentiation markers CD11b and Gr1; thus they are also called CD11b+ Gr-1+cells. MDSCs are roughly divided into two subpopulations: granulocytic (G-MDSC) and monocytic MDSCs (M-MDSC), as defined by morphology and expression of cell surface markers. G-MDSCs are CD11b+ Ly6G+ $Ly6C^{low/-}$ with granulocyte-like morphology, while M-MDSCs are cells with CD11b+ Ly6G- $Ly6C^{high}$ resembling monocyte morphology [231]. Although G-MDSC and M-MDSC share a common phenotype with neutrophil and inflammatory monocyte, respectively, these populations are functionally different from their normal counterpart in that they are immunosuppressive [35,243]. Definitive identification of MDSCs in human cancer patients has been complicated by the lack of specific markers. Nevertheless, human

MDSCs include granulocytic and monocytic subsets similar to mouse MDSCs. Human MDSCs are generally defined as cells expressing either or both of common myeloid markers CD33 or CD11b, lacking makers of mature myeloid and lymphoid cells (Lin HLA-DR), and endowed with ability to suppress T cell function [240,244,245]. Recently, more rigorous markers have been identified to distinguish MDSC subsets, including M-MDSC expression of CD14 and G-MDSC expression of CD15 [229]. However, different MDSC phenotypes were identified in patients with various types of tumor, suggesting an existence of diverse cellular subsets across different tumor settings [240].

In addition to their phenotypic and morphological differences, two subsets differ in the mechanisms by which they suppress immune response and the content of immunosuppressive molecules [246]. G-MDSCs suppress antigen-specific CD8+ T cell function mainly by releasing reactive oxygen species (ROS), which require prolonged cell-cell contact between MDSC and T cells [230,247]. On the contrary, M-MDSCs-mediated immunosuppression is antigen-independent, primarily involving expression of nitric oxide synthase 2 (NOS2), arginase (ARG1) and a variety of immunosuppressive cytokines [230]. It has been demonstrated that M-MDSCs are more potent immune-suppressors than G-MDSCs per cell basis [248–250]. Although the proportion of G-MDSCs and M-MDSCs are highly variable across different tumor models [239], G-MDSC has been shown to be the group predominantly increased in blood and peripheral lymphoid organs of most murine tumor models [250].

2.3.3 The role of MDSCs in tumor progression

Immune suppression: The tumor-host interaction triggers accumulation, abnormal differentiation, and pro-tumoral polarization of myeloid cells, resulting in construction of a highly immune suppressive microenvironment. MDSCs are central mediators among various host components orchestrating the event contributing to tumor progression.

A wide variety of mechanisms has been suggested that MDSCs employ to inhibit different aspects of anti-tumorigenic T cells [35,251,252]. MDSCs inhibit T cell activation and function by either downregulating or dissociating CD3-associated δ chain from T cell receptor (TcR) through production of reactive oxygen and nitrogen species (ROS and RNS) [253,254]. Alternative mechanisms utilized by MDSCs to suppress T cell activation and proliferation include: disruption of IL-2 receptor signaling [255], prevention of antigen/MHC peptide recognition by nitrating TcR [247] and MHC class I molecules [256], and depletion of amino acids including arginine and I-cysteine present in microenvironment [257,258]. MDSCs have also been shown to induce T cell apoptosis. Moreover, MDSCs disrupt T cell migration to either lymph nodes or tumor site through production of ADAM17 which diminishes the homing receptor CD62L on T cells [259] and peroxynitrite modification of the chemoattractant CCL2 [260], respectively. In addition, MDSCs are shown to inhibit cytotoxicity of natural killer cells and their production of IFN-γ, through a cell-contact-dependent mechanism [261–263].

Besides their inherent T cell suppressive function, MDSCs amplify immunosuppressive network via cross-talk with other immune cells [264]. They are stimulatory to immune regulators such as Treg [265,266] and tumor-associated macrophages [139].

Non-immunological functions: Critical evaluation of the role of MDSCs is hampered by inexplicit identification of such cells. Simultaneous definition of MDSC identity as well as function is missing in many of the literatures: MDSCs are identified as cells merely double positive for CD11b and Gr1 without further discrimination between two different subsets and demonstration of immunosuppressive activities which define the “identity” of these cells [267]. Nonetheless, CD11b+ Gr1+ cells seem to play a pivotal role in promoting tumor progression. In this article, we review the role of MDSCs identified by CD11b+ Gr1+ cells either associated with immature status claimed by the authors or studied with previously known MDSC-expanding tumor models.

Tumor vascularization: Analogous to tumor-associated macrophages and neutrophils, MDSCs significantly contribute to tumor vascularization [268–271]. Utilizing cell lines of MC26 colorectal cancer and 3LL lewis lung carcinoma, Yang et al demonstrated that CD11b + Gr1+ cells promote tumor angiogenesis [268]. Authors stated that MMP9 derived from these myeloid cells increased VEGF bioavailability in primary tumors. Interestingly, CD11b + Gr1+ cells also enhanced angiogenesis by directly incorporating into vessels following differentiation into cells with endothelial properties. As further characterized by H&E staining, CD11b+ Gr1+ cells included the whole spectrum of myeloid cells undergoing several stages of polymorphonuclear development, suggesting a possible involvement of granulocytic MDSCs in this process. Boelte et al by taking advantage of the same mouse tumor models has demonstrated that hypoxia-induced upregulation of Rgs2 signaling in MDSCs mediated their pro-angiogenic function via production of MCP1, a known regulator of angiogenesis [269].

In addition, MDSCs have been specifically implicated with tumor vasculogenesis. Tumor vasculature can arise either by sprouting of nearby blood vessels (angiogenesis) or from colonization by circulating endothelial cells primarily derived from bone marrow (vasculogenesis). In a mouse model where local angiogenesis is abrogated, Ahn et al proposed that CD11b+ myelomonocytic cells but not endothelial progenitor cells were recruited into irradiated tumors or tumors grown in pre-irradiated tumor bed [270]. Tumor growth as well as vasculogenesis was selectively impaired in irradiated tissues when MMP9 or MMP9-expressing CD11b+ myelomonocytic cells were either genetically or chemically ablated, suggesting a critical role of CD11b+ myeloid cells and MMP9 in this process.

Invasion: MMP9-expressing MDSCs have also been found to promote tumor invasion. Utilizing a mouse model of mammary carcinoma, Yang et al showed that inactivation of TGFβ signaling causes recruitment of CD11b+ Gr1+ immature myeloid cells to the invasive front of the tumor, primarily attributed to two chemokine receptor axes, SDF-1/CXCR4 and CXCL5/CXCR2 [272]. CD11b+ Gr1+ cells promoted invasiveness of cancer cells by producing MMP9. Interestingly, recruitment of CCR1+ myeloid cells has been observed in SMAD4-deficient intestinal tumors [273]. In this study, Kitamra et al demonstrated that induction of CCL9 secretion following TGFβ blockade in epithelial cells was responsible for the attraction of immature myeloid cells expressing MMP9 and MMP2.

Metastasis: In order to metastasize to distant organs, cancer cells require acquisition and reversion of motile phenotype at primary and metastatic tumor, respectively. Using a

spontaneous murine model of melanoma, Toh et al argued that a granulocytic subset of MDSCs preferentially infiltrates the primary tumor and promotes cancer cell dissemination by inducing EMT [274]. MDSCs attracted by CXCL5 do so by activating TGFβ, EGF, and HGF signaling pathways. Importantly, reduction of metastasis was only seen if G-MDSCs were depleted before clinical manifestation of the primary tumor, suggesting an important role of MDSCs in early dissemination of tumor cells. Conversely, myeloid progenitor cells were shown to promote mesenchymal-to-epithelial transition (MET). Utilizing mouse models of spontaneous breast cancer, Gao et al demonstrated that versican-expressing CD11b+ Ly6C high monocytic cells stimulated MET of tumor cells by attenuating phosphor-Smad2 levels, which resulted in accelerated tumor cell proliferation at metastatic site [275].

Pre-metastatic niche formation is one of the major mechanisms utilized by primary tumors to make disseminated tumor cells permissive for metastatic spread [276–278]. MDSCs are critical regulators for preparing the foreign microenvironment (“soil”) before tumor cells (“seeds”) arrive and ultimately adapt to distant organs. Yan et al proposed that CD11b+ Gr1+ cells in 4T1 bearing mice changes pre-metastatic lung into an inflammatory and proliferative microenvironment with aberrant vasculature that supports tumor extravasation and subsequent growth of tumor cells [279]. Similarly, in mouse mammary tumor models of E0771 and MMTV-PyMT, Sceneay et al demonstrated that hypoxia in the primary tumor provides secreted cytokines and growth factors capable of creating a pre-metastatic lung by enhancement of CD11b+ Ly6G+ LyC med cell infiltration and reduction in the cytotoxic effector functions of NK cell population [280].

Regulation of anti-tumor therapies: Accumulating evidence extends the role of MDSCs to the regulation of anti-tumor therapies, strengthening the rationale of combining therapy targeting MDSC population with conventional treatment. For instance, anti-VEGF refractoriness is attributed to angiogenic function of CD11b+ Gr1+ myeloid cells [281]. Shojaei et al demonstrated that tumors refractory to anti-VEGF therapy (EL4 and LLC) were associated with increased infiltration of tumor tissue by CD11b+ Gr1+ cells, compared to tumors sensitive to the treatment (TIB6 and B16F1). Recruitment of these myeloid cells was also sufficient to confer refractoriness to the sensitive tumors. Gene expression analysis of CD11b+ Gr1+ cells revealed elevation of genes implicated in differentiation and/or activation of myeloid cells. Bruchard et al demonstrated that while chemotherapeutic drugs such as gemcitabine and 5FU kill both cancer cells and MDSCs, these drugs show ambivalent anti-tumor effect in that they activate MDSC function as well [282]. Chemotherapy-triggered activation of NLRP3 inflammasome in MDSCs led to release of IL-1β, enhancing the capacity of CD4+ T cells to produce IL-17 and potentiating the IL-17-dependent proangiogenic effect. Acharyya et al proposed a network of endothelial-carcinoma-myeloid cell signaling, which was shown to link clinical phenomena between breast cancer chemo-resistance and metastasis [283]. The granulocytic subset of CD11b+ Gr1+ cells promoted survival of metastatic cancer cells in lung through production of S100A8/9. Efficacy of chemotherapeutic agents was augmented upon blockade of granulocytic cell recruitment by cancer cell-secreted CXCL1/2.

Regulation of tumor-initiating potential: For years, self-renewal and differentiation capacity of stem cells have been mainly attributed to the intrinsic properties of stem cells. It is only recently that we have recognized the role of microenvironment in stem cell behavior across our body. Diverse immune cell types have been reported to play a crucial role, as essential components in stem cell niche even in the absence of pathogenic invasion, in ensuring organ development [284–287], tissue homeostasis [288] and regeneration following injury [289,290]. Importantly, myeloid cells have been caught in the act of regulating stem cell function in cancer settings. Our group has recently demonstrated that breast tumors induce the accumulation of myeloid-derived suppressor cells (MDSC) to promote tumor-initiating cell frequency [291]. Utilizing syngeneic mammary tumor models and patient-derived tumor xenografts, we reported that breast tumor-initiating cells (TIC) display elevated level of G-CSF compared to non-TICs, therefore enriching TIC s ability to induce MDSC expansion. Importantly, MDSCs recruited to tumor microenvironment reciprocally enhance TIC features by activating Notch pathway in cancer cells, forming a feedforward loop. This suggests tumor-initiating potential is tightly linked to tumor-induced immunosuppression, and provides scientific basis for deploying MDSC-targeting agents as part of combination therapy approaches for patients with breast cancer. Along with the same line, the cooperation between immune system and cancer stem cell has also been delineated with different immune cell type [292] as well as in different context of cancer [293].

2.3.4 Plasticity and heterogeneity of MDSCs

Differentiation potential of MDSCs: Multiple studies have confirmed the ability of MDSCs to differentiate into distinct tumor stromal cells, strongly suggesting the versatility and their relationship with other myeloid cells in tumor microenvironment. For example, utilizing a model of tumor EL4 ascites, Corzo et al demonstrated the potentials of MDSC differentiation were dependent on the context of microenvironment [294]. Despite phenotypic and morphological similarity, profound differences of MDSC were observed between tumor site and spleen. While spleen MDSCs differentiated into both macrophages and dendritic cells, tumor MDSCs were only capable of differentiating into macrophages. MDSCs from these two different sites also differ in their ability to suppress T cells: the former suppressed antigen-specific CD8+ T cells, while the latter inhibited both antigen-specific and non-specific T cell activity. The authors identified that hypoxia-inducible factor (HIF-1α) was responsible for the observed effects of MDSC differentiation and function. Interestingly, macrophages derived from MDSC in hypoxic conditions did not show preferential polarization to either the M1 or M2 type; although these MDSC expressed high levels of genes associated with both types.

The importance of MDSC differentiation was also underscored at bone metastatic sites [295–297]. Utilizing spontaneous bone metastasis model of mammary carcinoma, Sawant et al showed that tumor-induced MDSCs exacerbate cancer-associated bone destruction by directly differentiating into functional osteoclasts [295]. Nitric oxide signaling was critical for MDSC differentiation. Importantly, differentiation into osteoclast was not recapitulated with MDSCs derived from naive or tumor-bearing mice that lacked bone metastasis, indicating the significance of cross-talk between tumor cells and MDSCs in this process.

The functional role of MDSCs serving as direct osteoclast progenitor was also reported in the study of multiple myeloma [297].

Following studies also effectively exhibit the dynamic plasticity of MDSCs in tumor microenvironment. Previously, it was shown that M-MDSC has the potential to differentiate into G-MDSC, suggesting an alternative mechanism by which G-MDSCs are expanded in tumor-bearing host [298]. Youn et al demonstrated that in cancer the normal pathway of monocyte differentiation toward macrophages and DCs is altered to preferential differentiation toward G-MDSCs both *in vitro* and *in vivo*. This process was mediated by epigenetic silencing of the retinoblastoma gene controlled by histone deacetylase 2 (HDAC-2). Furthermore, utilizing MC26 and 3LL tumor model, Yang et al showed that CD11b+ Gr1+ cells were able to differentiate into endothelial-like cells, thereby directly incorporating into the tumor endothelium and promoting angiogenesis [268]. The expression of endothelial markers, VE-cadherin and VEGFR2, was dramatically increased in tumor-infiltrating CD11b+ Gr1+ cells. Acquisition of endothelial cell properties was recapitulated in pro-angiogenic culture conditions *in vitro*. CD11b+ Gr1+ VEGFR2+ cells as functional progenitor of endothelial cells are regarded as potentially the third subset of MDSCs following granulocytic and monocytic groups [229].

Repolarizing MDSCs as a therapeutic strategy: Therapeutic strategies taking advantage of inherent plasticity of myeloid cells have also been demonstrated in the context of MDSC biology [265]. Ma et al pinpointed an essential role of paired-immunoglobulin receptor signaling in the polarization of M-MDSC. Using a mouse model of lewis lung carcinoma, authors argued that the balance between PIR-A and PIR-B signaling modulates the polarization of MDSCs. PIR-B-deficient M-MDSCs underwent a specific transition towards M1 phenotype with high expression of iNOS and TNF-α, resulting in decreased function of immunosuppression and regulatory T cell activation with retarded primary tumor growth and metastasis.

It is evident that MDSCs are an important component in the regulation of host immune responses. However, the exact nature of these cells is still far from clear. Controversy arises from the heterogeneous and plastic traits of MDSCs which create confusion in the identification as well as the dissection of origin and fate of these cells [240]. Previous studies have shown that MDSCs are capable of differentiating into mature functionally competent macrophages and dendritic cells under culture conditions without tumor-derived factors or when adoptively transferred into tumor-free recipients [230]. These observations highlight the fact that MDSCs include precursors of mononuclear phagocytic cells and, more importantly, MDSCs can restore their ability to differentiate into mature myeloid cells in the absence of tumor-derived factors. Elucidation of molecular mechanisms underlying abnormal differentiation and function of MDSCs and polarization of these myeloid cells toward anti-tumor phenotype will not only advance our understanding of MDSC functionality in pathological conditions and but also open new therapeutic opportunities.

3. Breast cancer

Heterogeneity is an ominous feature of breast cancer. Breast cancer is not a single disease, but a collection of diseases with distinct histological features and genetic variability, resulting in diverse clinical outcomes. With the advent of high-throughput technologies such as gene expression profiling and massively parallel sequencing, molecular background of breast cancer has been comprehensively scrutinized, facilitating the development of personalized treatment approaches and therapeutic agents that target specific molecular aberrations. Nevertheless, tumor heterogeneity imposes critical impediments to successful clinical response [299–303]. As an integral determinant as well as consequence of tumor genetic heterogeneity, several provocative but ill-defined questions may arise regarding immune microenvironment in breast cancers. How does the immune microenvironment vary among breast tumors of diverse genetic and epigenetic background? What is the most critical immune cell type both in frequency and in function in a certain context of breast tumor? And how does tumor evolve dependencies on certain immune cells? Deciphering immune environment heterogeneity in the context of diverse breast cancers will enable successful creation of personalized immunotherapies that target deleterious immune cells as well as reinvigorate anti-tumorigenic T cells.

3.1 Inter-tumor heterogeneity of breast cancer cells

A number of genomic and transcriptomic studies have been performed shedding new light on the previously unknown heterogeneity across the full spectrum of breast cancers. Results suggest that such molecular heterogeneity can occur either between different individuals with same type of tumor (inter-tumor heterogeneity) or within the same individual (intra-tumor heterogeneity) [303,304]. Moreover, intra-tumor heterogeneity itself can exist either between different geographical regions of the tumor (spatial) or as molecular evolution of a tumor over time (temporal) under selective pressure applied by surrounding microenvironment as well as therapeutic interventions [304,300]. For instance, differential expression level of oestrogen receptor (ER), progesterone receptor (PR), and HER2 has been observed among patients with breast cancers, as well as between primary tumors and matched metastatic lesion within the same patient [305].

Gene expression profiles have been determined that stratify breast carcinomas largely into four intrinsic subtypes: luminal A, luminal B, HER2-enriched and basal-like (triple-negative) breast cancers with distinct proliferation index, pathological grade, and patient outcome [19,20]. Specifically within triple-negative breast cancers, six additional subclasses have been classified with distinct sensitivity profiles to anti-cancer drugs [306]. Further refinement of molecular classification and characterization of human breast cancers has been achieved, emphasizing the genetic heterogeneity occurring at various molecular levels [17,18,307,308].

3.2 Inter-tumor heterogeneity of tumor microenvironment

Apart from cancer cells, stromal cells are highly variable and heterogeneous between different tumors and within same tumor. Gene expression analysis revealed that tumor-associated stroma undergoes extensive molecular evolution during cancer progression [10,7].

It is no longer a matter of debate that characteristics of stromal compartment, especially those of immune cells are of high prognostic significance for patients with breast cancer [70,309–312]. The term “immune contexture” has been coined to describe the location, density, and functional orientation of different immune populations residing in tumor microenvironment, as the parameters strongly relate to cancer outcome [309]. However, the extent of immune heterogeneity and its clinical implications in context of diverse breast cancers have not been revealed. Rigorous examination of various immune infiltrates and their function driving tumor progression are warranted.

Several retrospective studies based on histopathological examination and/or bioinformatic analysis of human-tumor interactions reveal specific stromal component as crucial modulator of cancer progression, demonstrating the tumor heterogeneity in relation to microenvironment. Utilizing laser capture microdissection to compare gene expression profiles of primary breast tumor stroma, Finak et al developed a 26-gene predictor associated with disease outcome [310]. Interestingly, breast tumors were tri-partitioned by stromal expression profiles, with the good-outcome cluster overexpressing a distinct set of immune-related genes, including T cell and NK cell markers indicative of a Th1 type immunity. Poor-outcome cluster instead showed markers of an increased hypoxic and angiogenic response, as well as a decrease in chemokines that stimulate migration and/or survival of cytotoxic lymphocytes. Similarly, Bergamaschi et al divided invasive breast cancers into four main classes based upon 278 ECM-derived genes and such classification was representative of distinct clinical outcome [313]. It was shown that ECM classification of breast cancers was partly independent of the intrinsic molecular subtypes. One group of tumors mostly associated with low-grade and ER-positive tumors was defined by the over expression of protease inhibitors belonging to serpin family and had a favorable clinical outcome. However, tumors with ECM1 signature overrepresented by basal-like tumors had a poorer prognosis and were associated with high lymphoid infiltration and high expression of metallopeptidases.

Recently, pan-leukocyte analyses were conducted in a variety of cancer type, identifying frequency and functional orientation of specific immune cells as important determinant of patient clinical outcome. Gentles et al analyzed associations between clinical outcomes and relative abundance of diverse tumor-associated leukocyte subsets by applying CIBERSORT, a computational approach inferring leukocyte representation in bulk tumor transcriptome [190]. Pooling multiple cancer types provided global leukocyte prognostic patterns where increasing levels of T cell populations generally correlated with superior survival, while high infiltration of myeloid populations primarily correlated with poorer survival. Intra-tumoral γδ T cell and polymorphonuclear (PMN) cell signatures yielded as the most significant favorable and adverse cancer-wide prognostic populations, respectively. Specifically in breast and lung cancer, authors identified reciprocal survival associations of PMN cell and plasma cell signatures. Likewise, Bindea et al calculated the composition of 28 different immune infiltrates in colorectal cancer at different tumor stage and at different geographical regions of the tumor using quantitative real-time PCR and tissue microarrays [314]. The authors demonstrated that immune infiltrates vary considerably as a function of time and space. Different immune cell populations had distinct impact on clinical outcome along tumor progression, with B cells associated with prolonged survival, while macrophages with

no significant influence on tumor recurrence despite high density. In the settings of breast cancer, Ruffell et al presented a detailed description of immune cell infiltrates in both breast tumors and nonadjacent normal breast tissues derived from patients who either had or had not received neoadjuvant chemotherapy before surgery [315]. Utilizing flow cytometry combined with confocal immunofluorescence and immunohistochemical analysis, the study revealed that activated T lymphocytes predominated in breast tumor, while myeloid cells were more prevalent in normal tissues. Interestingly, increased percentage of infiltrating myeloid cells and concomitant increase of CD8/CD4 T cell ratio were observed in residual tumors treated with chemotherapy, suggesting a possible reconstruction of immune microenvironment following anti-cancer therapy.

3.3 Oncogenic pathways of cancer: a potential driver of inter-tumor immune heterogeneity

Multiple factors may govern immune environment heterogeneity across breast tumors. Among others, tumor-intrinsic pathways may provide a compelling framework upon which tumors construct inflammatory microenvironment. Many human cancers are known to regulate a complex chemokine and cytokine network influencing tumor properties such as tumor cell growth, survival, and metastasis [316]. Furthermore, related to local production of chemokines by tumor and stromal cells are the extent and phenotype of immune infiltrates that constitute immune microenvironment. Provocatively, numerous studies have supported the notion that inflammatory response operate downstream of oncogenic pathways, indicating these signaling programs unlikely to be confined to cell-autonomous effects [13,317]. Several distinct oncogenic pathways have been suggested to drive the recruitment of TAMCs through production of cytokines/chemokines.

Utilizing multiple genetically engineered mouse models that recapitulate human lung cancer, Xu et al have demonstrated a dichotomy of immune microenvironment in different subtypes of lung cancer [318]. In contrast to murine *Kras* and *Kras;p53* adenocarcinoma models (ADC) that predominantly contain macrophages, lung squamous cell carcinomas (SCC) driven by biallelic inactivation of both *Lkb1* and *Pten* show distinct accumulation of TANs. Similarly, enrichment for TANs specifically in the areas of SCC tumors, but not in the adjacent ADC tumors was also observed in the *Kras;Lkb1* mouse model, which has the admixed features of ADC and SCC histology, substantiating the concept that distinct oncogenic mutations in cancer may directly shape the immune microenvironment in different ways.

Several studies implicate activated *Ras* oncogenic signaling in elaboration of cytokines from tumor cells, thereby generating an inflammatory microenvironment favorable for tumor progression. Sparmann et al showed a transcriptional upregulation of the chemokine interleukin-8 (CXCL-8/IL-8) by oncogenic *H-Ras* signaling [319]. The production of IL-8 mediated specific recruitment of neutrophils to the tumor and subsequent mobilization of endothelial cells for onset of tumor vascularization. Similarly, Ji et al demonstrated that *K-ras* activation in the bronchiolar epithelium is associated with an inflammatory response characterized by an abundant infiltration of macrophages and neutrophils [320]. *K-ras* activated lung cancer cells directly produced chemokines MIP-2, LIX and KC responsible for neutrophil attraction to the site of tumorigenesis. However, chemokines for macrophages

were not produced by cancer cells, suggesting an alternative source of driving macrophage recruitment.

In the settings of breast cancer, aberrant TGFβ signaling has been shown to specifically drive the recruitment of CD11b+ Gr1+ immature myeloid cells to the invasive front of primary tumor, promoting tumor invasion and metastasis [272]. Utilizing a mammary tumor model with type II TGFβ receptor gene (Tgfbr2) deletion, Yang et al observed a significant infiltration of CD11b+ Gr-1+cells in the PyVmT/Tgfbr2MGKO, compared with Tgfbr2flox/flox control tumors. Interestingly, similar involvement of dysregulated TGF-β pathway in recruiting CCR1+ immature myeloid cells has been reported in mouse model of intestinal tumor [273].

Epithelial-mesenchymal-transition pathway (EMT) may be specifically associated with the recruitment and polarization of tumor-associated macrophages. Hsu et al demonstrated that acetylation of Snail transcription factor activates transcription of immune response genes, modulating the cytokinome in the tumor microenvironment [142]. Of note, many of the target genes of Snail as transcription activator were chemo-attractants for tumor-associated macrophages, including *TNFA*, *CCL2*, and *CCL5.* It was shown that ectopic expression of Snail in 4T1 cancer cells exhibit increased TAM infiltration, microvascular density and metastasis. Similarly, Low-Marchelli et al reported that expression of Twist 1, a well-known EMT-inducing transcription factor, in human mammary epithelial cells promoted angiogenesis by recruiting macrophage to tumor through CCL2 secretion [321]. The authors proposed that among transcriptional regulators of EMT pathway, direct induction of CCL2 transcription was specific to Twist1. The relationship between EMT pathway and monocyte/macrophage population was also observed in ovarian tumors [322].

A variety of other oncogenic pathways has been associated with production of chemokines and cytokines that potentially regulate myeloid cell recruitment. *Myc* is a pleiotropic transcription factor overexpressed in many human cancers and is known to regulate many extracellular aspects of the tumor tissue. Utilizing mouse model of pancreatic islet tumor, Soucek et al showed that activation of *Myc* in beta cell triggers rapid induction of multiple chemokines and recruitment of mast cells to the tumor site which are required for angiogenesis and macroscopic tumor expansion [323]. *RET/PTC* oncogenes which are specific to papillary thyroid carcinoma have also been implicated with induction of immune response. Borrello et al showed that *RET/PTC1*oncogene, when exogenously expressed in primary normal human thyrocytes, induced the expression of a large set of genes involved in inflammation and tumor invasion, including those encoding chemokines, cytokines, and matrix-degrading enzymes [324].

What remains largely obscure is whether specific subtypes of breast cancer show preferential evolution of immune environment or conversely whether breast cancers can be “extrinsically” subtyped in respective of immune infiltrates and functions. Our group has recently reported that distinct oncogenic pathway can regulate production of specific cytokines that in turn recruit certain immune cell type to the tumor [291]. Utilizing a variety of syngeneic mammary tumors models and patient-derived xenografts, we provided evidence that only a subset of breast cancers stimulates myeloid-derived suppressor cells

(MDSC) accumulation, challenging the current paradigm that MDSCs are universally significant player in malignant settings. Mechanistic studies identified mTOR signaling in cancer cells dictates breast tumor s ability to induce MDSC accumulation through regulating G-CSF production. This suggests that local and systemic increase of MDSC is a stable trait of each individual tumor, and highlights the tentative personalized immunotherapeutic strategy to treat breast cancer patients based on immune as well as genetic makeup of the tumor. Future studies are warranted to determine immune components playing distinct role in other tumor contexts.

3.4 Host-regulated immunity

While somatic differences in tumor cells may influence host immune response, accumulating evidence indicate that inter-tumor heterogeneity may arise at the level of germ-line polymorphisms at immune system regulatory genes as well as be regulated by the environment in nature. The threshold of immune system activation is deterministically modulated by genetic variation at genomic regulatory region, as demonstrated by human autoimmune disorders [325] and F1 crosses of inbred mouse strains [326]. Furthermore, studies show that variation of human immune system is largely driven by non-heritable traits [327]. These hypotheses argue a combined analysis of tumor genomics, host genetics and epidemiological history upon investigation of immune microenvironment heterogeneity as they might collectively influence the extent of both pro- and anti-tumoral actions of tumor-infiltrating immune cells.

3.4 Co-evolution of cancer cells and immune system

Complex collaborative interaction between cancer cells and stromal cells drive the evolution in both compartments [328]. Tumorigenic and metastatic progression share many features with the evolution of ecosystems. Selective pressures are placed on cancer cells, with cells of the highest survival and proliferative advantage continuously prevailing towards malignancy [329]. Importantly, tumors persistently shape local and systemic microenvironment, thereby constructing an abnormal ecosystem [328]. Indeed, it has been shown that tumor-associated stroma undergoes extensive molecular evolution during cancer progression, similar to the extent that of tumor cells [7,10]. Conversely, tumor microenvironment has been proposed to be a major factor driving tumor molecular heterogeneity [303]. Experimental data point to the evidence that tumor-infiltrating immune cells engage in tumor evolutionary process. For instance, tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) have been shown as an extrinsic stimulator influencing in cancer stem cell population, metastatic occurrence, drug resistance, or patient survival [81,330,293,331]. As such, interplay between cancer cells and stromal cells represents a robust target for cancer therapy.

3.5 Integration of pre-clinical model systems: future directions in breast tumor immunology

Inter-tumor heterogeneity of immune microenvironment still remains poorly understood in human cancer. This is particularly important for breast cancer as it is noted for substantial inter-tumor genetic heterogeneity. Although previous studies provide valuable insights regarding immunological diversity among different cancers, current methodologies forcibly

retain inherent limitations. For instance, retrospective studies utilizing immune-based biomarkers prevent comprehensive evaluation of immune infiltrates as well as their functional state in tumor stroma. Quantification of tumor immune infiltrates by gene expression profiles in predefined cell subsets may not accurately estimate their exact abundance and functions, for immune cell-specific genes could also be expressed by various cell types under vastly heterogeneous and dynamic tumor microenvironment.

Recently emerged experimental systems have empowered searches for therapeutic targets of breast cancer. Despite species differences, pre-clinical murine models have shown useful for studying clinically relevant breast tumor biology *in vivo* under genetically controlled and immune competent conditions [332–334]. Several studies have confirmed that many of the defining characteristics of human breast cancer subtypes are conserved among murine mammary tumor models [335]. The complex forms of this disease as a whole, however, have not yet been fully taken into account in tumor immunology [336]. Of note, the majority of current studies incorporate only one or a few models of breast tumor and/or together with tumors originated from different tissues, preventing context-specific analysis of the immune microenvironment. We believe that an integrated and multi-systems approach will provide a basic framework to better understand the aberrant immune regulation in specific contexts of breast cancer [336]. Combination of humanized models may offer additional benefit to the system [337–339].

4. Concluding remarks

In recent years, there has been a heightened interest in TAMCs and their biological function in tumor pathobiology. TAMCs are a heterogeneous population of cells whose functions go far beyond immune tolerance. Co-opted by tumor, TAMCs take part in every step of metastatic cascade, increasing the chance of cancer cells to colonize at distant organ. Diversity and plasticity are defining characteristics of TAMCs, providing both challenges and opportunities to the clinic. Experimental data clearly suggest that targeting TAMCs either by ablation or in situ reprogramming can be beneficial in disease management. Selective manipulation of pro-tumoral subsets would yield favorable outcomes by avoiding systemic toxicities as well as achieving maximal killing of tumor cells. This is of great clinical importance in breast cancer biology as breast tumors exhibit intensive heterogeneity in both tumor and immune compartment. Further studies are necessary to determine the full picture of immune microenvironment heterogeneity across various breast tumors, and unravel the molecular mechanism of how tumors with distinct genetic and epigenetic background differ in their ability to induce immune dysfunction. Investigation of such context-dependent functionality of TAMCs will shed light on developing personalized immunotherapies to treat each individual patient with higher efficacy.

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Table 1

The role of tumor-associated macrophages (TAM) in breast cancer

Role	Identification Isolation	Tumor model	Experimental system Species	Reference
Angiogenesis	IHC: F4/80+ FC: CD11b+ F4/80+	MMTV-PyMT	Mouse, In vivo	74
	IF: F4/80+ FC: c-fms-YFP+	Met1, MVT-1	Mouse, In vivo	75
Migration	MP: Lys-GFP+, F4/80+	MMTV-PyMT MTLn3	Mouse, In vivo	79
	FC: CD11b+ Gr1- F4/80+	MMTV-PyMT	Mouse, In vivo	80
	FC: CD68+ CD206 high HLA-DR low (monocyte-derived macrophages)	MCF7, BT474, T47D, BT549, MB 436, MDA-MB-231	Human, In vitro Human, In vivo (humanized mice) Human patient samples	81
Invasion	IF/FC: CD68+ (monocyte-derived macrophages) Density gradient centrifugation	MDA-MB-231, MDA-MB-435s, BT-474, SKBR3, MCF-7	Human, In vitro Human patient samples	82
	IF: CD68+ (monocyte-deri ved macrophages)	SKBR3, MDA-MB-231	Human, In vitro	83
	Dextran+ F4/80+	MMTV-PyMT	Mouse, In vivo	84
Intravasation	MP: dextran+/Lys-GFP+/c-fms-GFP+	MMTV-PyMT	Mouse, In vivo	85
Metastasis	FC: CSF1R-eGFP+ CD11b+Gr1-F4/80+	MMTV-PyMT , Met-1, MDA-MB-231	Mouse, In vivo	90
	FC: Resident lung macrophage: F4/80+ CD11b- CD11c+ Ly6C- Metastasis-associated macrophage: CD11b+ F4/80+ CD11c- Ly6C-	MMTV-PyMT, E0771, MDAMB-231	Mouse, In vivo Human, In vivo	91
	IF: CD11b+ (together with Gr1+ or CX3CR1-GFP+ or CSF1R-GFP+)	4T1, Met-1 MDA-MB-231	Mouse, In vivo	92
	FC: CD45+ F4/80+ IF: CD68+	MDA-MB-231, CN34, 4T1	Mouse, In vivo Human, In vivo	93
Immunosuppression	FC: F4/80+ Bone marrow-derived macrophages	MMTV-PyMT	Mouse, In vivo	95

Table 2

The role of tumor-associated neutrophils (TAN) in breast cancer

Role	Identification Isolation	Tumor model	Experimental system Species	Reference
Anti-tumor immunity	N/A (neutrophil elastase)	Cyclin E-overexpressing breast cancer: MCF-7, MDA-MB-231, T47D, and MDA-MB-453	Human, In vitro Human patients	221
	IHC: Ly6G+	MCF-7 MMTV-PyMT	Human, In vivo Mouse, In vivo	222
	IHC, FC: Ly6G+ Density-gradient centrifugation	4T1, MMTV-Wnt1, MMTV-PyMT, 66Cl4, MCF-7, MDA-MB-231	Mouse, In vivo Human, In vivo Human patient samples	223
	IHC: Ly6G+ FC: CD11b+ Ly6G+	MMTV-PyMT	Mouse, In vivo	225
Invasion	Density- gradient centrifugation	MDA-MB-231, T47D	Human, In vitro	207
Migration	Density- gradient centrifugation	MDA-MB-468	Human, In vitro	208
	Density- gradient centrifugation	MDA-MB-231	Human, In vitro	212
Metastasis	FC: CD11b+ Ly6G+ Ly6C+ F4/80-	K14cre;Cdh1F/F;Trp53F/F (KEP)	Mouse, In vivo	215
	FC: CD11b+ Ly6G+ Ly6C+	67NR, 168FARN, 4TO7, 66c14, 4T1, MDA-MB-231	Mouse, In vivo Human, In vivo	216

Table 3

The role of myeloid-derived suppressor cells (MDSC) in breast cancer

Role	Identification Isolation	Tumor model	Experimental system Species	Reference
Angiogenesis	FC: CD11b+ Gr1+	4T1	Mouse, In vivo	271
Invasion	FC: CD11b+ Gr1+	MMTV-PyVmT/Tgfbr2^{MGKO}, 4T1	Mouse, In vivo	272
Metastasis	IF: Gr1+ FC: CD11b+ Gr1+ (CD11b+ Ly6C high)	MMTV-PyMT MDA-MB-231	Mouse, In vivo Human, In vivo Human patient samples	275
	FC: CD11b+ Gr1+	4T1	Mouse, In vivo	279
	FC: CD11b+ Ly6Cmed Ly6G+	E0771, MMTV-PyMT	Mouse, In vivo	280
	FC: CD11b+ Gr1+	4T1	Mouse, In vivo	295
Immunosuppression	FC: CD11b+ Gr1+	4T1	Mouse, In vivo	139
	FC: CD11b+ Gr1+	4T1	Mouse, In vivo	258
	FC: CD11b+ Gr1+	4T1, DA-3, D1-DMBA-3	Mouse, In vivo	259
	FC: CD11b+ Gr1+	4T1	Mouse, In vivo	263
Cancer stem cell-ness	IF: S100A8 FC: CD11b+ Ly6G+ Ly6C low-med	67NR, 4T07, 4T1, P53-null tumor models, MMTV-Wnt1, MMTV-Wnt1- iFGFR, p53 pten −/−	Mouse, In vivo Human, In vitro Human, In vivo (patient-derived xenografts)	291