

Macrophages: Key orchestrators of a tumor microenvironment defined by therapeutic resistance

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ABSTRACT

Macrophages have emerged as promising therapeutic targets in cancer. Within tumor tissue, macrophages foster tumor development, invasion, and metastasis. As the phenotype of macrophages is inherently pliable and dependent on cues received from the surrounding microenvironment, macrophages co-evolve with malignant and other non-malignant cells during cancer progression. In doing so, they establish a microenvironment that is therapeutically resistant and thwarts the productivity of T cell immunosurveillance. Strategies designed to delete, inhibit, or redirect macrophages with anti-tumor activity are being explored to reverse the pro-tumor properties of macrophages that are commonly observed in cancer. In this review, we discuss our current understanding of the mechanisms that regulate macrophage recruitment to tumors, their impact on the tumor microenvironment, and their promise as therapeutic targets for improving the efficacy of cytotoxic- and immune-based therapies.

1. Introduction

Macrophages are differentiated cells arising from the mononuclear myeloid lineage and can be found within many tissues where they mediate important biological functions including host defense against pathogens, removal of cellular debris, wound repair, tissue remodeling, and general maintenance of tissue homeostasis (Gordon and Martinez, 2010). Tissue-resident macrophages largely arise from the yolk-sac and seed tissues (e.g. liver, brain, skin, heart, kidneys, and pancreas) during the earliest stages of embryonic development. These macrophages persist into adulthood (Davies et al., 2013; Epelman et al., 2014). In cancer, embryonic-derived tissue macrophages have been found to support tumor development and progression (Zhu et al., 2017). However, tissue macrophages can also arise from tissue-infiltrating peripheral blood monocytes which develop in the bone marrow and are attracted to tissues by chemokines (Fig. 1). Under normal homeostatic conditions, inflammatory monocytes arising from the peripheral blood traverse healthy tissue, but may then re-circulate back into the

peripheral blood (Jakubzick et al., 2014). However, in an inflamed setting, monocytes infiltrate tissues and differentiate into macrophages. In cancer, tumor-infiltrating monocytes contribute to therapeutic resistance and immune suppression (Kalbasi et al., 2016; Mitchem et al., 2013; Nywening et al., 2016).

Within tissues, the phenotype and function (i.e. polarization status) of macrophages is directed by signals received from their surrounding microenvironment (Epelman et al., 2014). For example, macrophage phagocytosis of cellular material within tissues can imprint macrophages with a unique transcriptional signature and in doing so, contribute to the heterogeneity of tissue macrophages (A-Gonzalez et al., 2017). This inherent plasticity in macrophage biology has major implications and can be a critical determinant of the natural evolution of a disease, such as cancer.

Monocyte frequency in the peripheral blood has been suggested as a prognostic factor in patients with cancer (Liu et al., 2017). Monocytes are precursors for tumor-associated macrophages and their increased presence within tumors is most often associated with a poor prognosis

Abbreviations: CCL, C C chemokine ligand; CCR, C C chemokine receptor; CSF, colony stimulating factor; CTL, cytotoxic T lymphocyte; CTLA-4, cytotoxic T lymphocyte antigen 4; CXCL, C-X-C motif chemokine ligand; CXCR, C-X-C motif chemokine receptor; FAK, focal adhesion kinase; GM-CSF, granulocyte-macrophage colony stimulating factor; HDAC, histone deacetylase; HIF, Hypoxia inducible factor; IDO, indoleamine 2,3 dioxygenase; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; JAK, janus kinase; LIF, leukemia inhibitory factor; LPS, lipopolysaccharide; mAb, monoclonal antibody; MHC, major histocompatibility complex; MMP, matrix metalloproteinase; PD-1, programmed cell death-1; STAT, signal transducer and activator of transcription; TGF, transforming growth factor; TIMP, tissue inhibitor of matrix metalloproteinase; TLR, toll-like receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor

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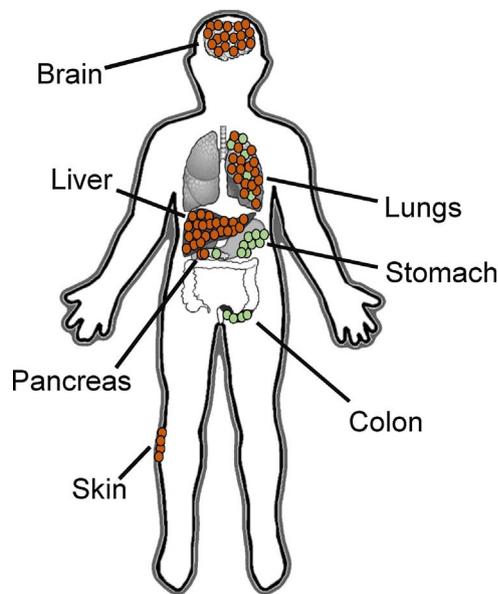


Fig. 1. Origins of tissue resident macrophages. In the steady state, tissue macrophages are either (i) yolk sac- or fetal liver-derived (shown in brown) or (ii) monocyte-derived from the bone marrow (shown in green). Macrophages residing in the brain, liver, lungs, and red pulp of the spleen are predominately derived from the yolk sac with Langerhans cells of the skin derived from fetal liver. In contrast, resident macrophages in the gastrointestinal tract are mainly monocyte-derived. In the setting of inflammation, monocyte-derived macrophages infiltrate tissues and co-exist with tissue resident macrophages. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Qian and Pollard, 2010). Consistent with this notion, in patients with pancreatic cancer, low peripheral blood monocyte levels correlate with improved overall survival (Sanford et al., 2013). Similarly, resected cancers displaying a heavy infiltrate of macrophages show a greater likelihood for disease recurrence (Balermipas et al., 2014; Mahmoud et al., 2012; Shabo et al., 2009; Shabo et al., 2008; Wei et al., 2014). Macrophage infiltration in solid tumors is also associated with lower overall survival rates (DeNardo et al., 2011). The prognostic significance defined by the presence of macrophages in tumors is due to the capacity of macrophages to support many of the hallmarks of cancer (Hanahan and Weinberg, 2011) and mediate protective and pathogenic functions that can contribute to the development of an immunosuppressive microenvironment that surrounds malignant cells (Murray and Wynn, 2011). This microenvironment can thwart the development and productivity of anti-tumor immunity and inhibit the efficacy of cytotoxic therapies. Thus, macrophages are pivotal determinants of cancer biology.

During the earliest stages of tumor development, macrophages may, in some settings, acquire a tumor-suppressing phenotype that is associated with the production of pro-inflammatory cytokines such as interleukin (IL)-12, which can support anti-tumor cytotoxic T lymphocyte (CTL) recruitment and activation (Zaynagetdinov et al., 2011). Tumor-suppressive macrophages can produce inducible nitric oxide synthase (iNOS) and demonstrate tumoricidal activity (Eue et al., 1998). In lung cancer, the phenotype of tumor-infiltrating macrophages has been suggested as a prognostic indicator of overall survival (Yuan et al., 2015). Thus, tumor biology is most significantly impacted by the phenotype of tumor-infiltrating macrophages, rather than the mere presence of macrophages.

Given the nearly universal involvement of macrophages in cancer development and progression, macrophages have emerged as a promising therapeutic target for cancer. In this review, we discuss our current and emerging understanding of factors that regulate macrophage biology in tumors and strategies to manipulate macrophages for therapeutic benefit.

2. Macrophage plasticity

Within tissues, the specific phenotype (i.e. polarization status) of activated macrophages is determined by signals received from the surrounding microenvironment. The extracellular milieu that encompasses cells can produce a broad range of cellular signals and as such, this milieu is critical for instructing macrophages with vastly distinct functional properties. Phenotypical profiles of macrophages are manifested by differential expression of cell surface markers, production of cytokines and enzymes, and biological functions (e.g. metabolism, phagocytosis, and antigen presentation). However, given the expansive array of potential macrophage phenotypes, classification and characterization of macrophage subtypes has been challenging (Murray et al., 2014). One common classification strategy relies on *in vitro* findings determined using distinct cytokine stimuli to activate macrophages. Using this cytokine-differentiation approach, macrophages have been broadly classified as either M1 (i.e. classically activated), or M2 (i.e. alternatively activated) macrophages (Mantovani et al., 2002; Martinez and Gordon, 2014).

Traditionally, M1 macrophages are polarized by a combination of the inflammatory cytokine interferon- γ (IFN- γ) and the component of the outer membrane of gram negative bacteria, lipopolysaccharide (LPS). Alternatively, stimulation via granulocyte-macrophage colony stimulating factor (GM-CSF) can direct macrophages with an M1 phenotype (Joshi et al., 2014; Martinez and Gordon, 2014; Mills et al., 2000). M1 macrophages are pro-inflammatory and characterized by glycolytic metabolism (Galvan-Pena and O'Neill, 2014), the expression of CD86, MHC class II, and iNOS, as well as the production of specific cytokines, including IL-1, IL-6, IL-12, and TNF- α , as well as the production of reactive oxygen and nitric oxygen species. M1 macrophages are crucial for effective control of bacterial infections, but can also mediate anti-tumor activity (Qian and Pollard, 2010).

Conversely, M2 macrophages are traditionally polarized by anti-inflammatory cytokines, such as IL-4 and IL-13, or alternatively, by colony stimulating factor 1 (CSF1) (Joshi et al., 2014; Martinez and Gordon, 2014; Mills et al., 2000). These macrophages are characterized by increased fatty acid oxidation (Galvan-Pena and O'Neill, 2014), express high levels of macrophage scavenger receptor 1 (CD204), the mannose receptor (CD206), arginase 1, and low levels of MHC class II. M2 macrophages produce high levels of the cytokine IL-10, as well as vascular endothelial growth factor (VEGF) and matrix metalloproteinase 9 (MMP9). In addition to their involvement in anti-inflammatory processes, M2 macrophages have been associated with pro-tumorigenic functions, including angiogenesis and promoting tumor growth, survival and metastasis (Qian and Pollard, 2010), and have been shown to be important in wound healing responses (Epelman et al., 2014). M2 macrophages have also been further classified into subsets: M2a, M2b, M2c, and M2d (Murray et al., 2014). This further classification illustrates the complexity of phenotypes that can be manifested by macrophages and has led to the terminology of 'M1-like' and 'M2-like' to describe this range of biological phenotypes that can be observed *in vivo*. In general, tumor-associated macrophages have been described as displaying an M2-like phenotype, based on high expression of the mannose receptor and production of IL-10 and VEGF, with a relatively low production of cytokines attributed to M1-like macrophages, such as IL-1, IL-6, IL-12, and TNF- α .

The use of the M1/M2 classification for macrophages provides an easy, albeit oversimplified, strategy for defining macrophage biology. However, a major shortcoming of this strategy is the inability to account for variations in macrophage phenotype based on spatial localization within tissues. For example, TIE2+ macrophages, which reside adjacent to endothelial cells, show specific functional properties. Specifically, they are involved in regulation of angiogenesis in cancer (Chen et al., 2016a; Mazziere et al., 2011). Thus, while the M1/M2 classification is a mainstay for many investigators as they seek to describe the biology of macrophages in cancer, it is critical to be mindful

of particular factors that can influence macrophage phenotype and function including macrophage origin, spatial localization within tissues, and pathological state of the tissue. Each of these elements must be considered to accurately classify macrophages in cancer.

In this review, we classify macrophages broadly into *tumor-promoting* or *tumor-suppressing* macrophages. These extreme subsets are based on the capacity of macrophages to mediate a spectrum of activities that define tissue inflammation, tumorigenesis, wound healing, angiogenesis, and fibrosis (Davies et al., 2013; Epelman et al., 2014; Long and Beatty, 2013). Here, we also discuss ways to shift the polarity of tumor-infiltrating macrophages from *tumor-promoting* to *tumor-suppressing*.

2.1. Mechanisms that recruit macrophages to tumors

Monocytes are recruited to solid tumors via various mechanisms, including chemokine-driven chemotaxis and extracellular matrix components acting as chemoattractants. Because the majority of monocytes infiltrating tumors are Ly6C^{hi} inflammatory monocytes that express high levels of CCR (C-C chemokine receptor) 2, trafficking and recruitment of monocytes to tumor tissue is significantly controlled by CCL (C-C chemokine ligand) 2 (Epelman et al., 2014; Franklin et al., 2014; Mitchem et al., 2013; Qian et al., 2011). CCL2 production is commonly upregulated in solid tumors (Lim et al., 2016) and has a significant role in fostering monocyte recruitment to tumors. In addition, CCL2 can enhance monocyte mobilization from the bone marrow to the peripheral blood (Sanford et al., 2013). However, other chemokines can also interact with CCR2 and in doing so, promote monocyte infiltration into tissues (Lim et al., 2016). For example, CCL7 can bind CCR2 and support the recruitment of CCR2 expressing monocytes (Shi and Pamer, 2011). In addition, other chemokines such as CCL5 that are produced by solid tumors can act as chemoattractants for monocytes (Balkwill, 2004; Murdoch et al., 2004). Blockade of CCR5 has been found to induce tumor regressions in both mice and humans with colorectal carcinoma that is dependent on a shift in the polarity of tumor-infiltrating macrophages from tumor promoting to tumor suppressing (Halama et al., 2016). Thus, chemokines are important regulators of monocyte recruitment to tumors.

In addition to chemokines, extracellular matrix components present within the stroma that surrounds malignant cells can act to recruit and retain monocytes as macrophages within tissues. For example, type I collagen is significantly overproduced in many solid malignancies and can act as a chemoattractant for monocytes. Consistent with this, monocytes and macrophages are commonly lured to type I collagen rich regions (Parks et al., 2004). Moreover, the extent of macrophage migration in tissues occurs in a type I collagen concentration dependent manner (Postlethwaite and Kang, 1976). Notably, this collagen-dependent recruitment does not require intact collagen fibers, as collagen fragments can also attract monocytes and macrophages (Mundy et al., 1981). The ability of collagen fragments to recruit monocytes and macrophages has particular relevance in cancer as collagen within the tumor microenvironment is in a constant state of production and breakdown.

Like type I collagen, hyaluronic acid (HA) has also been shown to contribute to monocyte mobilization and trafficking into tissues (Kobayashi et al., 2010). HA is deposited in the stroma of many cancers and is involved in enhancing the stiffness of the matrix. Disrupting HA production by conditional gene targeting of the HA synthase 2 gene in stromal fibroblasts has been found to impair the recruitment of macrophages to tumors (Kobayashi et al., 2010). Thus, the extracellular matrix that surrounds malignant cells is critical to the recruitment of monocytes and their retention in cancer tissues.

2.2. Soluble factors produced by malignant and non-malignant cells regulate macrophage biology

Macrophages are a key component of cancer-associated inflammation which is a major contributor to many of the hallmarks of cancer (Hanahan and Weinberg, 2011). During the earliest stages of tumor development, macrophages may initially display a tumor-suppressing phenotype due to the presence of pro-inflammatory cytokines present within an inflamed tissue. These early tumor-suppressing macrophages can act to promote anti-tumor immune responses that may seek to help eliminate tumor cells (Zaynagetdinov et al., 2011). However, this early inflammatory response can also stimulate the expression of adhesion molecules, such as ICAM-1, that can subsequently, serve as a chemoattractant for further recruitment of macrophages (Liou et al., 2015). With tumor progression, macrophages within tumors almost invariably assume a *tumor-promoting* phenotype (Qian and Pollard, 2010).

The interaction between macrophages and tumor cells has commonly been studied under *in vitro* conditions using conditioned media produced during tumor cell culture. In this setting, macrophages up-regulate expression of markers associated with an M2-like phenotype, including IL-10, VEGF, and MMP9 (Sica and Mantovani, 2012). This process occurs independent of direct cell-to-cell interaction and thus, illustrates the importance of tumor-derived factors in regulating macrophage biology.

Within the tumor microenvironment, factors such as hypoxia may also influence the phenotype and function of macrophages. For instance, hypoxia-induced factors, including Semaphorin 3a, are known to attract macrophages to and retain them within tumor regions that demonstrate low oxygen tension (Casazza et al., 2013). To this end, hypoxia is well-recognized to stimulate a transcriptional response pathway that is mediated in large part by hypoxia-inducible factor (HIF) proteins, HIF1 and HIF2. HIF1 induces the release of the chemoattractant SDF1 α , also known as CXCL (C-X-C ligand) 12, and in doing so, supports the recruitment of CXCR (C-X-C receptor) 4+ macrophage populations and the subsequent release of soluble VEGF through increased macrophage-derived MMP-9 activity (Du et al., 2008). This hypoxia-regulated response promotes macrophage entrapment within hypoxic regions of tumors and supports angiogenesis and immunosuppression (Casazza et al., 2013; Du et al., 2008). However, hypoxia can also influence the phenotypic fate of macrophages by promoting anaerobic glycolysis within tissues and the subsequent release of lactic acid. Colegio et al. showed that lactic acid can serve as a metabolic substrate for tumor-associated macrophages and as a result, stimulate a HIF1 α -dependent and IL-4-independent increase in macrophage expression of tumor-promoting factors including arginase 1 and VEGF (Colegio et al., 2014). HIF1 α has also been shown to support macrophage glycolysis and ATP generation important for chronic inflammatory states (Cramer et al., 2003). In addition, the shift in the metabolic state of macrophages induced in the setting of hypoxia may be critical to defining their response to pro-inflammatory stimuli (Mills et al., 2016). Together, these findings highlight the complexity of molecules within the tumor microenvironment that can contribute to macrophage behavior.

Cytokines produced by non-malignant cells present within the tumor microenvironment are also important determinants of macrophage phenotype. In this regard, IL-4 and IL-13, leukemia inhibitory factor, IL-6, and lactic acid have each demonstrated roles in regulating macrophage polarization *in vivo* (Colegio et al., 2014; Duluc et al., 2007; Hagemann et al., 2006; Roca et al., 2009; Stein et al., 1992). Similarly, transforming growth factor-beta (TGF- β) can impact macrophage function within tumors by inhibiting tumor-infiltrating macrophages from acquiring an M1-like phenotype (Haak-Frendscho et al., 1990). Thus, macrophage biology is intricately controlled within tumors by both malignant and non-malignant cells.

3. Macrophage biology within the tumor microenvironment

During cancer development, malignant cells often become encased in dense fibrosis, referred to as desmoplasia. This fibrotic reaction is observed in many types of solid tumors and is driven by an overproduction and accumulation of excess extracellular matrix proteins, including fibrillar collagen type I and type III proteins and fibronectin (Cox and Erler, 2014; Wynn, 2008). Tumor fibrosis is dependent on fibroblasts which are the major producers of extracellular matrix proteins (Wynn and Ramalingam, 2012). Like macrophages, fibroblasts can be induced with either a pro- or anti-fibrotic phenotype. Fibroblast biology is also pliable and is dependent on factors present within their surrounding microenvironment (Cox and Erler, 2014; Wynn and Ramalingam, 2012). As such, macrophages and fibroblasts may co-evolve within tumors with the resulting tumor fibrosis contributing to tumor growth, survival, and metastasis.

3.1. Tumor fibrosis regulates macrophage phenotype

The recruitment, differentiation status and activation of macrophages within tissues is strongly influenced by extracellular matrix proteins (Egeblad et al., 2010; Kobayashi et al., 2010; Mundy et al., 1981; Postlethwaite and Kang, 1976). In particular, the rigidity of the extracellular matrix can alter the polarization state and subsequently, the function of macrophages (McWhorter et al., 2015). For example, increased extracellular matrix rigidity can facilitate the polarization of macrophages toward an M2-like phenotype as seen by an increased expression of CD204 and arginase 1 (McWhorter et al., 2013; Stahl et al., 2013). Similarly, culturing macrophages *in vitro* on a type I collagen matrix has been found to decrease the tumoricidal activity of macrophages (Kaplan, 1983). Glycosaminoglycans (e.g. hyaluronic acid), which are key extracellular matrix components of tumor fibrosis, can also influence macrophage phenotype (Rayahin et al., 2015) by inducing an M2-like polarization of tumor-associated macrophages (Kuang et al., 2007). Thus, the matrix that surrounds malignant cells can shape the phenotype of tumor-infiltrating macrophages.

Aside from the physical aspect that fibrosis contributes to the tumor microenvironment, it can also promote hypoxia which in turn, can paralyze the migration of macrophages within tumor tissues as discussed above and skew their differentiation toward a tumor-promoting phenotype (Henze and Mazzone, 2016). Under hypoxic conditions, tumor cells may respond by releasing exosomes containing microRNAs, which can induce macrophages with tumor-promoting properties (Chen et al., 2017). Activated cancer-associated fibroblasts have also been shown to contribute to the differentiation of monocytes and the polarity of tumor-associated macrophages by releasing soluble factors, such as IL-6. Here, IL-6 produced by fibroblasts has been shown to stimulate the differentiation of monocytes into macrophages (Chomarat et al., 2000). Moreover, when cultured in fibroblast conditioned media, monocytes have been found to differentiate into macrophages with tumor promoting properties (Mathew et al., 2016). This potential cross-talk between fibroblasts and macrophages provides further support for their co-evolution within tumors as malignant cells seek to orchestrate a microenvironment that is conducive to tumor development and progression.

3.2. Macrophage regulate fibroblast phenotype and activation

While fibroblasts can influence macrophage phenotype, macrophages can also impact the biology of fibroblasts. For example, during earliest stages of tumor development, IL-1 β produced by macrophages has been found to activate cancer associated fibroblasts, which in turn can stimulate recruitment and polarization of a new wave of tumor infiltrating macrophages (Erez et al., 2010). This reciprocal interaction between macrophages and fibroblasts is appreciated in the context of wound repair and tissue fibrosis where macrophages produce TGF- β

and platelet-derived growth factor which together activate fibroblasts and promote their proliferation (Pakyari et al., 2013). Similarly, in the setting of cancer, activated fibroblasts are potent contributors to fibrosis and can be activated by tissue macrophages via TGF- β (Pickup et al., 2013). This cross-talk between macrophages and fibroblasts has also recently been shown to be a mechanism for altering the liver microenvironment with increased susceptibility to cancer cell metastasis (Costa-Silva et al., 2015). Thus, reciprocal interactions between fibroblasts and macrophages are fundamental to tumor development and cancer cell spread to distant organs.

3.3. Macrophage orchestrate extracellular matrix production and remodeling

The accumulation of fibrosis in tumor tissue is regulated by the balance of extracellular matrix protein production and degradation. Macrophages are major orchestrators of this balance. For example, Afik and colleagues showed in a mouse model of colorectal cancer that tumor-associated macrophages can contribute directly to extracellular matrix production as well as the cross-linking of collagen fibers (Afik et al., 2016). Macrophages are also major producers of matrix metalloproteinases (MMPs) as well as tissue inhibitor of matrix metalloproteinases (TIMPs). These proteins act to support and inhibit, respectively, the breakdown of extracellular matrix. For instance, MMP9 is a gelatinase produced by macrophages that can selectively degrade type IV collagen associated with the basement membrane and in doing so, support tumor invasion and subsequent metastasis (Huang et al., 2002). The ability of macrophages to both support the deposition and breakdown of fibrosis is a remarkable property dependent on their phenotype. This has been clearly demonstrated in models of liver fibrosis where macrophages are required for both deposition of fibrosis in the setting of chemical injury as well as subsequent resolution of fibrosis upon removal of the chemical insult (Duffield et al., 2005). In this model of chemical-induced liver fibrosis, collagen degradation and tissue repair was shown to be dependent on macrophage production of MMP13 (Fallowfield et al., 2007). Similarly, in a model of pancreatic cancer, it has been found that macrophages can stimulate the production of MMP13 in tumor tissue, and in doing so, shift tumors from chemotherapy resistant to chemotherapy sensitive (Long et al., 2016). In the steady-state, this cross-talk between macrophages and fibrosis is dependent, at least in part, on elevated activity of focal adhesion kinase (FAK) which has been shown to be an important determinant of the fibrotic and immunosuppressive microenvironment observed in pancreatic cancer (Jiang et al., 2016). Disruption of FAK activity alters the expression of myeloid chemoattractants within tumors and with sustained inhibition is associated with a decrease in myeloid recruitment and fibrosis (Jiang et al., 2016). Thus, macrophages hold a strong governance over tissue matrix composition which can influence the natural history of cancer progression and therapeutic outcomes.

4. Macrophages as targets for cancer therapy

Macrophages are major contributors of therapeutic resistance in solid tumors. For example, macrophages can support malignant cell survival in the setting of cytotoxic stress by regulating angiogenesis and by inducing an immunosuppressive gene signature in tumors (Chen et al., 2016a; Kaneda et al., 2016; Mazzieri et al., 2011). In addition, macrophages can contribute to the physical architecture (i.e. collagen and HA) of the tumor microenvironment which can limit effective delivery of cytotoxic drugs (Olive et al., 2009; Provenzano et al., 2012). For these reasons, macrophages have emerged as a promising therapeutic target in cancer.

Strategies to target macrophages in cancer have focused on approaches that are designed to (i) *deplete*, (ii) *inhibit* or (iii) *redirect* macrophages (Liu et al., 2017) (Fig. 2). Macrophages can be *depleted* from the tumor microenvironment by blocking their recruitment using

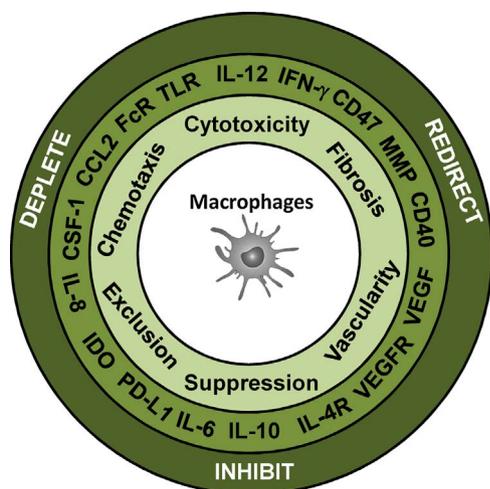


Fig. 2. Targeting macrophage biology in cancer. Macrophages can mediate an array of biological activities in cancer (e.g. chemotaxis, direct cytotoxicity against tumor cells, regulation of fibrosis and vascularity as well as suppression and exclusion of T cells. Multiple molecules are associated with each of these activities and serve as targets for therapeutic intervention involving strategies that inhibit, deplete or redirect macrophages in cancer.

inhibitors of chemokine signaling pathways (e.g. CCR2), by blocking growth factors (e.g. CSF1/CSF-1R) important for macrophage survival, or by directly targeting macrophage subsets for cell death (e.g. trabectedin) (Germano et al., 2013; Ries et al., 2014; Sanford et al., 2013). The functions of tumor-infiltrating macrophages can also be inhibited by targeting signaling pathways (e.g. JAK/STAT), cytokines secreted by macrophages (e.g. IL-6) and immunosuppressive molecules produced by macrophages (e.g. IDO, arginase I) (Chang et al., 2001; Lesina et al., 2011; Long et al., 2017; Munn and Mellor, 2007). Finally, the inherent pliability of tumor-associated macrophages offers an opportunity to redirect macrophages with anti-tumor properties. For example, macrophages can be harnessed for anti-tumor activity by disengaging molecules that inhibit macrophage biology (e.g. CD47, HDAC IIa) or by directly activating macrophages with anti-tumor properties (e.g. IFN- γ , CD40, TLR agonists) (Beatty et al., 2017; Chao et al., 2012b; Guerriero et al., 2017). Here, we discuss each of these strategies for targeting macrophages for cancer therapy.

4.1. Depleting macrophages from tumors

4.1.1. Targeting the CCL2/CCR2 pathway

Peripheral blood monocytes are actively recruited to tumors by chemokines produced within the tumor microenvironment. Thus, strategies to disrupt chemokine signaling are being studied as an approach to restrict myeloid infiltration into tumors and their subsequent pro-tumor functions. CCR2 is expressed at elevated levels on a subset of monocytes which infiltrate tumors in response to the chemokine CCL2. In melanoma, overexpression of CCL2 by tumor cells is associated with an enhanced presence of tumor-associated macrophages and rapid tumor progression (Gazzaniga et al., 2007). In CCR2 deficient mice, macrophage recruitment to orthotopically implanted pancreatic cancer is also markedly reduced and is associated with slowed tumor growth (Sanford et al., 2013).

The importance of CCL2 in regulating macrophage recruitment to tumors has been studied using neutralizing antibodies to CCL2. For example, neutralization of CCL2 with a monoclonal antibody (mAb) decreases overall tumor burden in models of pancreas, prostate, breast and lung cancer (Loberg et al., 2007; Mitchem et al., 2013; Qian et al., 2011; Zhao et al., 2013). Similarly, neutralizing CCL2 mAbs have been shown to decrease macrophage accumulation in a model of glioma and in doing so, improve overall survival (Zhu et al., 2011).

CCL2/CCR2 blockade can also improve the efficacy of cytotoxic therapies. For example, CCL2 neutralization enhances the efficacy of radiotherapy in a model of pancreas cancer by blocking the infiltration of pro-tumor macrophages which are recruited in response to radiation treatment to support tumor cell survival (Kalbasi et al., 2016). CCR2 inhibitors have also been shown in some settings to stimulate T cell infiltration raising the possibility that this therapeutic target may combine with inhibitors of immune checkpoint molecules (e.g. PD-1 and CTLA-4) that act to enhance the activity of endogenous anti-tumor T cell immunity (Mitchem et al., 2013). In this study, blockade of CCR2 signaling with a small molecule inhibitor was found to enhance the efficacy of chemotherapy in mice. Similarly, an early phase clinical study has demonstrated promise for combining CCR2 inhibitors with chemotherapy for treatment of patients with locally advanced and borderline resectable pancreatic carcinoma (Nywening et al., 2016). Thus, derailing monocyte recruitment to tumors by inhibiting chemokine signaling via CCR2 has emerged as an important therapeutic approach in cancer.

4.1.2. Targeting CSF-1/CSF-1R

The survival of macrophages within tumors is dependent on growth factors such as colony-stimulating factor 1 (CSF-1). Strategies to disrupt CSF-1 signaling via the CSF-1 receptor (CSF-1R) produces macrophage depletion in some tumors. In a model of pancreatic cancer, blockade of CSF1R signaling depleted macrophages within tumors, and when combined with chemotherapy, significantly slowed tumor growth (Mitchem et al., 2013). Genetic deletion of CSF-1 using CSF-1 $^{-/-}$ mice is also associated with decreased accumulation of macrophages within tumors and reduced tumor outgrowth (Pyonteck et al., 2012).

The importance of CSF-1 for macrophage survival in tumors has been seen across several malignancies. However, in some settings, inhibition of CSF-1 signaling is associated with a shift in macrophage phenotype rather than a decrease in macrophage survival. For example, in a model of glioma, CSF-1R inhibition using a small molecule inhibitor was found to redirect tumor-infiltrating macrophages with anti-tumor activity (Pyonteck et al., 2013). In this case, macrophage survival in the absence of CSF-1R signaling was supported by additional signals, including IFN- γ and GM-CSF, present within the tumor microenvironment. In a model of liver cancer, CSF1R blockade was also observed to shift the phenotype of macrophages from tumor-promoting to tumor-suppressing, resulting in decreased tumor growth (Ao et al., 2017).

Depletion of tumor-infiltrating macrophages using CSF-1R blockade has been found to induce T cell dependent anti-tumor activity (DeNardo et al., 2011; Zhu et al., 2014). Based on this finding, several groups have recently demonstrated the potential to combine CSF-1R blockade to deplete macrophages with immunotherapy, including dendritic cell vaccination and antagonistic antibodies targeting PD-1 and CTLA-4 (Dammeijer et al., 2017; Holmgaard et al., 2016; Mao et al., 2016). Based on this work, ongoing clinical studies are evaluating the use of both antibodies and small molecule inhibitors to target the CSF1/CSF1R pathway as a strategy for altering macrophage biology in tumors and improving the activity of both cytotoxic chemotherapy and immunotherapy.

4.2. Inhibiting pro-tumor activity by tumor macrophages

Tumor-infiltrating macrophages commonly show activation through multiple signaling pathways including signal transducer and activator of transcription-3 (STAT3) which is associated with pro-carcinogenic inflammation (Corcoran et al., 2011; Lesina et al., 2011; Yu et al., 2009). Elevated phosphorylated STAT3 levels in tumors is correlated with decreased overall survival in patients with advanced solid malignancies (Nagathihalli et al., 2015). While multiple stimuli can activate STAT3 signaling including VEGF, LIF, IL-17, IL-11 and IL-6 among others, IL-6 is a potent and major regulator of STAT3 activation. However, blockade of IL-6 signaling using IL-6 receptor blocking

antibodies or genetic deletion of IL-6 only partially inhibits STAT3 activation in tumors with little impact on STAT3 activation in myeloid cells (Long et al., 2017). This finding supports the notion that multiple signaling pathways may converge to induce STAT3 activation in macrophages *in vivo*.

The skewing of macrophages toward an M2-like phenotype via IL-4 and IL-13 signaling is directed through the STAT6 pathway. Like STAT3, STAT6 expression is upregulated in macrophages *in vivo* and has been shown to enhance tumor growth in a mouse model of pancreas cancer (Yan et al., 2016). In breast cancer, STAT6 activation inhibits T cell-dependent anti-tumor immunity (Sasaki et al., 2008). Tumor-derived soluble factors can also stimulate STAT6 activation in macrophages leading to an increase in CD206 expression associated with an M2-like phenotype (Chen et al., 2016b). In addition, STAT3 and STAT6 signaling pathways have been shown to synergize to upregulate cathepsin secretion by macrophages which can support tumor cell invasion and metastasis (Yan et al., 2016). Thus, strategies to selectively inhibit STAT activation in macrophages may yield therapeutic benefit.

Inhibition of STAT signaling has been shown to improve the efficacy of cytotoxic chemotherapy (Long et al., 2017; Nagathihalli et al., 2015) and to inhibit the progression of premalignant lesions to advanced carcinoma (Corcoran et al., 2011; Lesina et al., 2011). As a result, ongoing clinical trials are evaluating the use of selective JAK1 inhibitors as well as non-selective JAK1/2 inhibitors as strategies to reverse STAT signaling in tumors (Liu et al., 2017). More selective strategies to reverse STAT activation mediated by distinct molecules, such as VEGF and IL-6, are also being evaluated. For example, IL-6 receptor blockade has emerged as a therapeutic approach to inhibit macrophage activation associated with cytokine release syndrome seen in the setting of adoptive cell therapy with chimeric antigen receptor-modified T cells (Maude et al., 2014). Blockade of IL-6 receptor can also enhance the activity of chemotherapy in a spontaneous model of pancreatic cancer [Long et al. MCT 2017]. Thus, the STAT pathways and the stimuli that mediate their activation are important targets for modulating macrophage and tumor biology.

4.3. Redirecting tumor-infiltrating macrophages with anti-tumor activity

The inherent plasticity of macrophage phenotype suggests the possibility that tumor-infiltrating macrophages may be redirected with anti-tumor activity. Strategies to shift the phenotype of macrophages in cancer have addressed approaches to (i) deliver activation signals (e.g. CD40, IFN- γ , TLR agonists) and (ii) relieve inhibitory signals (e.g. CSF1R, CD47, HDAC IIa) that control the phenotypical fate of macrophages (Beatty et al., 2017; Chao et al., 2012b; Guerriero et al., 2017; Pyonteck et al., 2013).

Under *in vitro* conditions, macrophages can be induced with anti-tumor activity upon exposure to IFN- γ and LPS. In this setting, macrophages mediate anti-tumor activity by releasing tumoricidal soluble factors, including reactive oxygen and nitrogen species as well as cytokines, such as TNF- α (Long and Beatty, 2013). Macrophages stimulated with IFN- γ and LPS can also directly mediate tumor cell lysis as well as phagocytose tumor cells (Feng et al., 2015).

IFN- γ is well-recognized as a key activator of macrophage anti-tumor activity. However, toxicity associated with systemic delivery of IFN- γ in patients has precluded its development as a therapeutic approach (Zaidi and Merlino, 2011). Nonetheless, strategies capable of stimulating the production of IFN- γ *in vivo* can also redirect tumor-infiltrating macrophages with anti-tumor activity. For example, treatment with an agonistic CD40 monoclonal antibody stimulates a cytokine release syndrome marked by increased levels of IFN- γ . In this setting, tumor infiltrating monocytes are redirected by systemic IFN- γ to mediate anti-tumor activity and enhance the sensitivity of tumors to cytotoxic chemotherapy (Beatty et al., 2011; Long et al., 2016).

Toll-like receptor (TLR) agonists have also been studied for their capacity to activate macrophages with tumor suppressive activity.

Activation of macrophages with TLR agonists can stimulate the release of soluble factors capable of inducing tumor cell death (Buhtoiarov et al., 2006). In addition, TLR agonists have been found to stimulate macrophages to phagocytose tumor cells (Feng et al., 2015). One example of a TLR agonist that has been studied for its ability to activate macrophages with anti-tumor activity is CpG (Krieg, 2008). CpG is a short synthetic single-stranded DNA molecule containing unmethylated cytosine (C) and guanine (G) deoxynucleotides that activates macrophages via TLR9. Repeated administration of CpG *in vivo* produces a macrophage activation syndrome (Behrens et al., 2011). As monotherapy, CpG has shown activity in some cancer models. However, in combination with additional macrophage-directed therapies, CpG has produced potent anti-tumor responses. For example, in combination with anti-IL-10 receptor blockade, CpG treatment has been shown to induce a rapid macrophage-dependent tumor debulking within one day of treatment in multiple models of cancer (Guiducci et al., 2005). Similarly, in combination with anti-CD40, CpG has demonstrated macrophage-dependent anti-tumor activity with macrophages mediating direct killing of tumor cells in a model of melanoma (Buhtoiarov et al., 2006). The capacity of CpG to activate macrophages with tumor-suppressive properties has led to its translation for cancer therapy. When delivered intratumorally or via intradermal/subcutaneous routes of delivery, TLR9 agonists have been generally well-tolerated with the most common adverse events associated with local injection-site reactions and a systemic flu-like reaction that occurs within 24 h and generally resolves within 48 h of treatment (Krieg, 2007). Treatment with a TLR9 agonist in patients with metastatic solid tumors has produced stable disease as the best overall response indicating that additional therapeutic maneuvers will be required to realize the potential immunomodulatory activity of TLR9 agonists (Weihrach et al., 2015). Nonetheless, TLR9 and other TLR agonists represent a promising category of macrophage-targeted therapies for redirecting tumor-infiltrating myeloid cells with tumor suppressive activity.

As macrophage phenotype is regulated by a balance of activating and inhibitory signals, strategies that block inhibitory cues (e.g. HDAC IIa and CD47) have also been studied for their capacity to redirect macrophages with anti-tumor activity. For example, inhibition of class IIa histone deacetylases (HDAC IIa) can induce macrophages with phagocytic and immunostimulatory properties capable of reducing tumor burden and suppressing metastasis in a spontaneous model of breast carcinoma (Guerriero et al., 2017). By altering macrophage phenotype, HDAC IIa inhibition was also found to enhance the efficacy of cytotoxic chemotherapy and immune checkpoint blockade, suggesting a role for macrophage phenotype in defining therapeutic efficacy in cancer.

Upon entry into the tumor microenvironment, myeloid cells encounter a variety of factors that can influence their ultimate fate. Some of these factors (e.g. CSF1), as discussed above, may shape the activity of macrophages. However, other signals act to directly inhibit the activity of macrophages. For example, CD47 is a membrane bound protein that interacts with Sirp-alpha expressed on macrophages to inhibit phagocytosis. CD47 expression is increased on many solid tumors (Willingham et al., 2012) and has been studied as an immune checkpoint molecule capable of regulating macrophage anti-tumor activity (Chao et al., 2012a). In xenograft studies, blockade of CD47, using CD47 blocking antibodies or engineered high affinity Sirp-alpha monomers, has shown capacity to stimulate macrophages with robust anti-tumor activity capable of inhibiting tumor growth (Gholamin et al., 2017; Weiskopf et al., 2013). CD47 blockade is associated with enhanced macrophage phagocytosis of tumor cells and has been suggested to increase tumor immunogenicity through improved cross-presentation of tumor antigens to tumor-specific T cells (Liu et al., 2015). Multiple clinical trials are ongoing to study the safety and preliminary efficacy of this strategy for cancer.

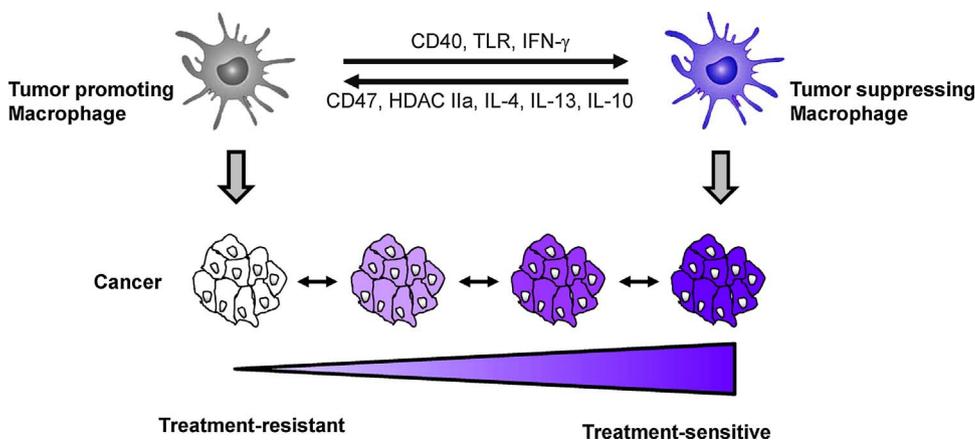


Fig. 3. Macrophages regulate therapeutic resistance in cancer. Macrophage phenotype is associated with cancer sensitivity to therapeutic intervention. Tumor promoting macrophages orchestrate a microenvironment that is supportive of tumor resistance. However, the inherent pliability of macrophage phenotype presents an opportunity to shift the polarity of macrophages from tumor-promoting to tumor-suppressing by targeting molecules (e.g. CD40, TLRs, IFN- γ , CD47, HDAC IIa, IL-4, IL-13, and IL-10) involved in regulating macrophage phenotype. Tumor suppressing macrophages can prime malignant cells for increased sensitivity to cytotoxic and immune based therapies as well as modulate the stromal microenvironment for enhanced therapeutic efficacy.

4.4. Translating macrophage-directed therapies in cancer

As major orchestrators of the tumor microenvironment, macrophages shape the sensitivity of cancer to cytotoxic- and immune-based therapies (Fig. 3). For example, the fibrotic reaction that surrounds malignant cells can limit the effectiveness of drug delivery. Macrophages, though, can be induced to modulate this fibrotic reaction. Specifically, systemic activation of the CD40 pathway stimulates macrophages to facilitate fibrosis degradation in an MMP-dependent manner. In doing so, macrophages condition the tumor microenvironment with enhanced sensitivity to cytotoxic chemotherapy (Beatty et al., 2011; Beatty et al., 2013; Long et al., 2016). Similarly, HDAC IIa inhibition modulates macrophage phenotype leading to normalization of tumor vasculature and enhanced sensitivity to chemotherapy (Guerriero et al., 2017). CCR2 inhibition by blocking macrophage recruitment in response to cytotoxic stress has also been shown to enhance the efficacy of radiation therapy and chemotherapy (Kalbasi et al., 2016; Mitchem et al., 2013). Thus, macrophages are promising targets for reversing therapeutic resistance and improving the tumor debulking potential of cytotoxic therapies.

Macrophages are also being studied for their capacity to regulate the efficacy of T cell immunotherapy. For example, tumor-associated macrophages can inhibit productive T cell immunosurveillance in cancer by producing multiple immunoregulatory enzymes, such as arginase I and indoleamine 2,3 dioxygenase which deplete key amino acids (arginine and tryptophan, respectively) required for effector T cell activity (Chang et al., 2001; Munn and Mellor, 2007). Macrophages can also regulate T cell entry into tumors and the efficacy of vaccine therapies (Beatty et al., 2015). In addition, macrophages can directly suppress effector T cells via the expression of immune checkpoint molecules (e.g. PD-L1) (Kuang et al., 2009). Thus, strategies that deplete, inhibit or redirect macrophages are being combined with inhibitors of immune checkpoint molecules (e.g. PD-1/PD-L1 and CTLA-4 blocking antibodies) in an effort to harness the treatment response durability that can be achieved with T cell immunotherapy (Beatty and Gladney, 2015; Liu et al., 2017).

5. Conclusions

Macrophages are key components of the innate immune system and are necessary for normal tissue function. Their capacity to acquire tumor-promoting or tumor-suppressing activity is important for shaping cancer cell biology. During tumor development and progression, macrophages almost invariably assume tumor-promoting activity that supports malignant cell survival. Preclinical modeling of macrophages in cancer has produced an in-depth understanding of the inherent plasticity of macrophage phenotype, mechanisms of macrophage-dependent pro-tumor activity, and the discreet signals that regulate

macrophage recruitment to tissues and their subsequent survival. This knowledge has revealed novel opportunities for intervening on macrophage biology to improve the efficacy of cancer therapies, including cytotoxic chemotherapy and immunotherapy. Nonetheless, many knowledge gaps still exist particularly in better identifying macrophage subsets *in vivo* that have therapeutic importance as well as defining how a tumor and its surrounding microenvironment adapts in the setting of macrophage-directed therapies. Investigation into this biology will be a critical next step for successfully translating macrophages as targets for cancer therapy.

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