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Immune regulation of the metastatic process: implications for therapy

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Abstract

Metastatic disease is the major cause of fatalities in cancer patients, but few therapies are designed to target the metastatic process. Cancer cells must perform a number of steps to successfully establish metastatic foci, including local invasion, intravasation, survival, extravasation, and growth in ectopic tissue. Due to the non-random distribution of metastasis, it has long been recognized that the tissue microenvironment must be an important determinant of colonization. More recently it has been established in animal models that immune cells regulate the metastatic process, including a dominant role for monocytes and macrophages, and emerging roles for neutrophils and various lymphocyte populations. While most research has focused on the early dissemination process, patients usually present clinically with disseminated, if not macroscopic, disease. Identifying pathways by which immune cells regulate growth and therapeutic resistance within metastatic sites is therefore key to the development of pharmacological agents that will significantly extend patient survival.

Keywords

tumor; metastasis; immune; leukocyte; lymphocyte; monocyte; macrophage; neutrophils; chemotherapy; immunotherapy

INTRODUCTION

Although cancer is a disease defined by genetic mutations, genomic instability, and/or chromosomal alterations within malignant cells, solid tumors are comprised of multiple “host” stromal populations, including lymphatic and vascular endothelial cells, fibroblasts and other mesenchymal cells, and a diverse repertoire of immune cells. Cancer cells do not manifest into disease in isolation, but rather draft and corrupt resident and recruited normal cell types that can promote or restrain tumor progression in a highly context dependent manner. The understanding of how these stromal populations influence each step of tumor development, from initiation to growth and progression has become more pronounced as the complexity of tumor modeling has increased (Hanahan and Coussens 2012).

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Cancer related fatalities are usually associated with disseminated metastatic disease, not growth of the primary tumor (Valastyan and Weinberg 2011), and thus understanding the process of metastasis is important for the development of efficacious therapeutic interventions. Metastasis occurs through a sequential cascade of steps: local invasion, intravasation, and survival in circulation, followed by extravasation, survival, and growth at the ectopic site. As with primary tumor development, each of these steps involves interplay between neoplastic cells and their microenvironment. Immune cells in particular play an important role navigating neoplastic cells through the endothelial layer, and interrupting this process diminishes the formation of metastatic lesions in animal models (Joyce and Pollard 2009; Kitamura et al. 2015a).

Unfortunately, the majority of human cancers have already disseminated when the primary tumor is detected, having entered the circulatory system prior to diagnosis (Friberg and Nystrom 2015; Massagé and Obenauf 2016). It is therefore the later steps of the metastatic process that are most clinically relevant, but paradoxically are the least understood. A few pre-clinical studies have shown that whether quiescent cancer cells will establish overt metastatic lesions or remain in a dormant state during a latency period may depend upon immune surveillance (Eyles et al. 2010; Romero et al. 2014). Growth at the metastatic site can also be regulated by myeloid cells, as is well described for bone metastasis (Cook et al. 2014). Finally, the primary tumor microenvironment dictates response to all forms of therapeutic intervention, and thus understanding the role of these cells in metastatic lesions could assist in the development of new treatment strategies (Coussens et al. 2013; Coffelt and de Visser 2015; Klemm and Joyce 2015; Ruffell and Coussens 2015). Here we will discuss the role of immune cells in regulating the metastatic process of solid tumors (Figure 1), with a detailed examination of the establishment of overt metastasis and its implications for therapy. Due to the unique interplay between the immune system and hematological malignancies these will not be discussed (Meads et al. 2009; Nelson and Paulos 2015).

KEY PLAYERS

Monocytes

Monocytes are usually associated with cancer only inasmuch as they are a precursor population to macrophages and dendritic cells (DCs) under inflammatory conditions. However, monocytes are found within the spleen and trafficking through tissue without differentiating into either terminal population (Swirski et al. 2009; Jakubzick et al. 2013). There is evidence that monocytes are functionally important during the early stage of tumor development (Juric et al. 2015), and in solid tumors a monocyte gene expression signature can be a negative prognostic indicator (Gentles et al. 2015). Much of the literature on myeloid-derived suppressor cells may also be attributable to Ly6C⁺ monocytes within the spleens of tumor-bearing mice. Finally, recent studies have described opposing roles for Ly6C⁺ classical/inflammatory and Ly6C⁻ patrolling monocytes in mediating the early stages of lung colonization (Qian et al. 2011; Hanna et al. 2015).

Macrophages

Macrophages are present in all mammalian tissues, represented by unique phenotypic and functional resident populations that are critical for tissue development and homeostasis. Under non-pathological conditions, resident macrophages in many tissues originate from embryonic progenitors and are conserved through local proliferation (Wynn et al. 2013). Within tumors, macrophages originate from circulating monocytes and are one of the most prominent populations of immune cells. Although macrophages were originally thought to be part of an anti-tumor response, animal studies and clinical correlations indicate that macrophages promote tumorigenesis under most conditions by promoting invasion, angiogenesis, and survival, while also directly and indirectly suppressing a cytotoxic T cell response (Ruffell et al. 2012; Noy and Pollard 2014). Exceptions to this are found in colorectal cancer and under select therapeutic conditions where the macrophage phenotype is more closely associated with classical activation (Zhang et al. 2012; Klug et al. 2013). Otherwise, targeting macrophages for depletion or repolarization can enhance response to multiple therapeutic modalities (Ruffell and Coussens 2015). In the context of metastasis, macrophages can promote each of the steps of the metastatic cascade (Kitamura et al. 2015a), and a specific association between macrophages and invasion/metastasis has been noted in melanoma, breast, ovarian, colorectal, pancreatic neuroendocrine, and bladder cancer (Ruffell et al. 2012).

Neutrophils

Neutrophils rapidly infiltrate tissues when they are inflamed, and thus may control some of the earliest interactions of the host immune system with the emerging tumor (Blaisdell et al. 2015). Similar to macrophages, neutrophils were originally associated with an anti-tumor immune response consistent with their role during infection and potential for tissue destruction, but more recent studies demonstrating a role in the healing process highlight their ability to promote tumor growth (Kolaczowska and Kubes 2013). This has been shown in experimental tumor models where neutrophils can promote growth through angiogenesis and suppression of T cell immunity (Granot and Fridlender 2015; Liang and Ferrara 2016). In support of these pre-clinical findings, the presence of neutrophils is negatively associated with survival in breast cancer, lung adenocarcinoma, and melanoma (Gentles et al. 2015). This pro-tumor role for neutrophils appears strongly influenced by transforming growth factor- β , which regulates the maturation state of the cell (Fridlender et al. 2009). Thus tumor-associated immature neutrophils can be immunosuppressive and pro-angiogenic, and at least in murine models represent an overlapping population with granulocytic myeloid-derived suppressor cells. More important than the primary tumor may be the role of neutrophils in the metastatic process, as there is evidence that neutrophils can promote local invasion, retention and extravasation at ectopic sites, as well as survival and growth within the metastatic niche (Liang and Ferrara 2016).

T Lymphocytes

T cells have diverse functions within tumors, reflective of the array of subtypes present (Ruffell et al. 2010). In general CD8⁺, T_H1-polarized CD4⁺, and $\gamma\delta$ T cells suppress tumor growth, promote response to therapy, and are associated with improved outcome through

production of interferon- γ (IFN- γ) and direct cytotoxic activity. However, there are exceptions to these statements in murine models and in clinical correlates (Ciampricotti et al. 2012; Gentles et al. 2015). The roles of T_H2, T_H17 and regulatory (T_{reg}) CD4⁺ T cells are even more complex, and the presence of these cells is associated with both a positive and negative prognosis (Fridman et al. 2012). This reflects both tissue and situational specificity. For example, T_{regs} promote the growth of mammary tumors, but suppress the development of colorectal cancer (Chaudhry et al. 2009; Bos et al. 2013). At the same time, T_{regs} can drive colon carcinogenesis in a model of inflammatory bowel disease (Geis et al. 2015). The function of T lymphocytes in the metastatic process is only beginning to be elucidated. As in the primary tumor, most studies find that CD8⁺ T cells are important mediators of immune surveillance (Massagé and Obenauf 2016). Studies have also found that interleukin (IL)-4 producing CD4⁺ T cells and IL-17-producing $\gamma\delta$ T cells promote metastasis by regulating the function of macrophages and neutrophils, respectively (DeNardo et al. 2009; Coffelt et al. 2015).

Natural killer cells

Natural killer (NK) cells were identified based on their ability to spontaneously lyse tumor cells in the absence of B and T lymphocytes, and share similarities with CD8⁺ T cells in terms of their cytotoxic potential (Sabry and Lowdell 2013). However, in primary tumors NK cells appear to play an important, but secondary role to the adaptive immune response. This may reflect limitations in cell numbers, a susceptibility to immune suppression, or a lack of activating signals (Waldhauer and Steinle 2008; Sabry and Lowdell 2013). That said, NK cells could be critical mediators of anti-metastatic immune surveillance (Massagé and Obenauf 2016), and have recently been shown to restrain metastatic tumor cells in a dormant state (Romero et al. 2014).

INVASION AND INTRAVASATION

Macrophages

Macrophages were originally implicated in the metastatic process using mice with null recessive mutation in the colony-stimulating factor (*Csf1*) gene, resulting in the absence of mature macrophages in most tissues, including mammary tumors (Lin et al. 2001). Subsequent studies showed that CSF-1 expression by tumor cells – mediated in part by steroid receptor coactivator-1 (Wang et al. 2009) – was functionally involved in recruiting macrophages and inducing production and shedding of epidermal growth factor (EGF) (Ishihara et al. 2013), resulting in a paracrine loop that induced tumor cell migration towards the vasculature (Wyckoff et al. 2004; Wyckoff et al. 2007). This paracrine loop can be further augmented by exogenous CXCL12 or heregulin B1 (Hernandez et al. 2009), although the mechanism and importance of these at endogenous levels is unclear. These murine studies may be biased by high CSF-1 expression in the models examined, and a second paracrine loop has been described between granulocyte-macrophage CSF (GM-CSF) and CCL18 using human breast cancer cell lines and humanized mice (Su et al. 2014).

Macrophages are also involved regulating the deposition and structure of the extracellular matrix (ECM) tracks used by tumor cells to migrate towards blood vessels (Condeelis and

Segall 2003). This has been demonstrated for SPARC (secreted protein, acidic rich in cysteine), also known as osteonectin: macrophage-derived SPARC doubles the migratory potential of 4T1 cells on fibronectin in vitro, and SPARC-deficient mice implanted with 4T1 mammary tumors display greatly reduced collagen and fibronectin deposition, along with reduced pulmonary metastatic burden (Sangaletti et al. 2008). Macrophage proteases have long been theoretically connected to an invasive tumor phenotype based upon their potential to degrade the ECM (Kessenbrock et al. 2010). While macrophage-derived matrix metalloproteinases (MMPs) have not been demonstrated to enhance cancer cell invasion in vivo, the absence of stromal urokinase/plasminogen activator (uPA) or cathepsin B, S, or Z all restrict local invasion and/or metastasis (Almholt et al. 2005; Vasiljeva et al. 2006; Gocheva et al. 2010b; Akkari et al. 2014). At least for the cathepsins this involves a reduction in the ability of macrophages to promote invasion in vitro (Gocheva et al. 2010b; Akkari et al. 2014). Cathepsins have also been shown to promote angiogenesis, but it is not clear whether this is linked to their role in metastasis (Gocheva et al. 2006; Gocheva et al. 2010a; Ruffell et al. 2013).

Finally, macrophages are important mediators of angiogenesis, as the absence or depletion of macrophages reduces tumor vascularization (Qian and Pollard 2010; Ruffell and Coussens 2015). More important than vascular density for the metastatic process may be the influence of macrophages on vascular structure. Specific deletion of vascular endothelial growth factor-A (*Vegfa*) in macrophages recapitulates the reduction in vessel density observed in MMTV-PyMT bearing the *Csf1* null mutation (Lin et al. 2006; Stockmann et al. 2008); however, deletion of *Vegfa* in macrophages actually reduces tissue hypoxia and increases tumor growth, a phenotype linked to vessel normalization and increased tissue perfusion. Poor pericyte coverage and hypoxia are associated with metastasis in this same model system (Cooke et al. 2012), and it is foreseeable that macrophages may enhance metastasis via this mechanism, rather than through an increase in the number of vessels. However, this has yet to be examined experimentally.

VEGF-A expression by a subset of vascular-associated macrophages expressing Tie2 has also been shown to promote cancer cell intravasation by increasing the permeability of the endothelium (Harney et al. 2015). This appears to be true in patients as well, as the colocalization of macrophages, tumor cells and endothelial cells is associated with breast cancer patients that develop systemic, but not lymph node metastasis (Robinson et al. 2009). This Tie2⁺ macrophage population may be responsible for many of the angiogenic phenotypes associated with the general macrophage population. Critically, neutralizing angiopoietin 2 – the ligand for Tie2 – reduces the association of macrophages with the vasculature and diminishes pulmonary metastasis (Mazzieri et al. 2011). This includes inhibiting the later steps of the metastatic process, although this has not been directly linked to macrophage function.

Neutrophils

As with macrophages, tumor-associated neutrophils have the potential to promote invasion, angiogenesis, and ECM degradation based upon their expression of the requisite molecules (Liang and Ferrara 2016). That said, little is known about the role of neutrophils in the early

steps of the metastatic process, and there is a relative paucity of in vivo data demonstrating functionality. G-CSF expression results in a systemic expansion of the neutrophil population (Casbon et al. 2015) and multiple CXCR1/2 ligands have been implicated in neutrophil recruitment to tumors and increased metastasis, including CXCL1, CXCL2, CXCL5, and CXCL8/IL-8 (Bekes et al. 2011; Toh et al. 2011; Acharyya et al. 2012; Zhou et al. 2014). In some cases this is associated with an increase in MMP expression, and MMP9-deficient mice display reduced metastasis (Yang et al. 2008; Yan et al. 2010); however, to date the importance of MMPs in local invasion has only been demonstrated in xenotransplantation assays (Bekes et al. 2011). While a few studies have shown neutrophil depletion reduces metastasis, this is usually associated with an immunosuppressive phenotype, and it is not clear which stage of the metastatic process is affected (Simpson et al. 2012; Coffelt et al. 2015). For example, neutrophil depletion was reported to have no influence the number of circulating 4T1 cells (Granot et al. 2011), and in another orthotopic model of breast cancer neutrophil depletion reduces both lung and lymph node metastasis without affecting primary tumor growth (Coffelt et al. 2015). Neutrophils have been shown to promote migration of melanoma cells towards the endothelium during ultraviolet-induced inflammation (Bald et al. 2014), but the preponderance of evidence currently favors neutrophils being more involved in the later steps of the metastatic process (Granot et al. 2011; Acharyya et al. 2012; Cools-Lartigue et al. 2013; Coffelt et al. 2015).

T Lymphocytes

CD4⁺ T cells were first described to promote the early steps of metastasis in the MMTV-PyMT model of breast cancer, with an approximate 5-fold reduction in circulating tumor cells and pulmonary metastasis observed in CD4-deficient animals (DeNardo et al. 2009). This was mediated by T_H2-polarized CD4⁺ T cells expressing IL-4 enhancing the pro-tumor phenotype of macrophages, including augmentation of the CSF-1/EGF paracrine loop. IL-4 also promotes cathepsin protease activity in macrophages within pancreatic neuroendocrine tumors, leading to enhanced local invasion (Gocheva et al. 2010b). T_H2-polarization is specific to tumor CD4⁺ T cells in the MMTV-PyMT model (DeNardo et al. 2009), and this appears to be driven by high levels of CCL5 chemokine expression within late stage tumors (Zhang et al. 2015). It is likely that IL-13 will have similar effects on macrophage polarization within tumors given that neutralization of either IL-4 or IL-13 produces an equivalent increase in the efficacy of paclitaxel chemotherapy (Shiao et al. 2015). CD4⁺ T_H2 cells can thus promote invasion indirectly by modulating the phenotype of macrophages.

SURVIVAL AND EXTRAVASATION

Monocytes

Classical Ly6C⁺ inflammatory and non-classical Ly6C⁻ patrolling monocytes appear to have opposing functions in murine models of lung metastasis. True to their name, Ly6C⁻CCR2^{lo}CX₃CR1^{hi} monocytes continually patrol the lumen of the endothelium under steady-state conditions, pausing in response to inflammatory signals that upregulate expression of CX₃CL1 on endothelial cells (Auffray et al. 2007; Carlin et al. 2013). During experimental metastasis Ly6C⁻ monocyte patrolling is reduced within hours of intravenous injection of tumor cells, and the absence of Ly6C⁻ monocytes increases the number of both

experimental and spontaneous pulmonary foci (Hanna et al. 2015). This function may be induced by tumor cell death as Ly6C⁻ monocytes were found to take up tumor cell debris, and another study has shown this occurs within the first 15 minutes following injection (Headley et al. 2016). Ly6C⁻ monocytes also promoted NK cell recruitment to the lungs, which was necessary for their ability to suppress metastasis of B16F10 melanoma cells (Hanna et al. 2015). Interestingly, Ly6C⁻ monocytes have been shown to recruit neutrophils to the vessel lumen during sterile inflammation induced with a toll-like receptor 7 (TLR7) agonist (Carlin et al. 2013), hinting that these cells may incorporate local signals to differentially regulate inflammatory responses in the vasculature.

In contrast to the role of Ly6C⁻ monocytes, studies have demonstrated a pro-metastatic role for Ly6C⁺CCR2^{hi}CX3CR1^{lo} monocytes and their derivative macrophage population in the lung and liver (Kitamura et al. 2015a). Targeting CCR2/CCL2 prevents spontaneous liver metastasis in a pancreatic ductal adenocarcinoma model (Sanford et al. 2013) and diminishes the ability of mammary carcinoma cells to extravasate into the lung parenchyma and form overt metastatic lesions (Qian et al. 2011). In the lung this requires VEGFA expression by Ly6C⁺ monocytes, which increases endothelial permeability and enhances tumor cell transmigration (Qian et al. 2011), similar to the role of VEGFA-expressing Tie2⁺ macrophages in primary tumors (Harney et al. 2015). While it has been suggested that monocytes can promote tumor cell survival in circulation, this is most likely due to enhanced extravasation based upon the absence of a phenotype at 30 minutes post injection (Gil-Bernabe et al. 2012; Hoos et al. 2014). It remains unclear the degree to which Ly6C⁺ monocytes, as opposed to macrophages, are responsible for the increase in endothelial permeability. Kinetic data suggests Ly6C⁺ monocytes extravasate and differentiate into macrophages after their initial recruitment, especially monocytes that have phagocytosed tumor debris (Headley et al. 2016). CCL2 neutralization also reduces metastatic burden when initiated 2 days after tumor cell injection, a time point by which extravasation has already occurred. Similarly, there is a diminution in foci size when the presence or function of macrophages is interfered with (Qian et al. 2009; Qian et al. 2011; Kitamura et al. 2015b; Qian et al. 2015), indicative of an important role for macrophages in the growth phase of metastasis. Detailed imaging studies will be required to dissect the role of monocytes versus macrophages in extravasation and survival in ectopic organs.

Neutrophils

Several studies support a role for neutrophils in promoting cancer cell capture and retention on the endothelium. In a model of liver metastasis neutrophils were found to promote tumor cell adhesion to the endothelium via CD11b, a process that is enhanced by the release of neutrophil extracellular traps (NETs) following LPS exposure (Spicer et al. 2012; Cools-Lartigue et al. 2013). While the use of the anti-Gr-1 antibody for depletion in these studies would also target Ly6C⁺ monocytes, the re-introduction of purified neutrophils was able to reverse the effect of depletion, demonstrating the specificity of these findings (Spicer et al. 2012). Simultaneous intravenous injection of human neutrophils also supports lung metastasis by melanoma cells, with IL-8 (CXCL8) expression by cancer cells directing the interaction between the two cell types (Huh et al. 2010). Finally, G-CSF-driven expansion of

neutrophils in the lung promotes tumor cell extravasation via Bv8/prokineticin-2, a homologue of VEGF-A (Kowanetz et al. 2010).

In contrast to the aforementioned studies, neutrophils have also been shown to suppress pulmonary metastasis in the 4T1 and MMTV-PyMT/cMyc models of mammary carcinoma (Granot et al. 2011). This was mediated by a classical property of neutrophils, the ability to directly kill cells via release of H_2O_2 . Adoptive transfer of neutrophils also suppressed experimental metastasis, and as neutrophils do not appear to be recruited into the lung during this process (Headley et al. 2016), it suggests targeted killing of arrested tumor cells in the vasculature. Indeed, neutrophil depletion reduces the frequency but not the size of metastatic foci. It is notable that the cytotoxic properties of human and mouse neutrophils were not observed under steady-state conditions, and were induced by CCL2, but not G-CSF (Granot et al. 2011). Chemokine expression patterns by tumor cells and other experimental variations may thus explain differences between studies showing a pro- or anti-metastatic role for neutrophils.

T Lymphocytes

In addition to the MMTV-PyMT transgenic model, $CD4^+$ T cells are important in promoting lung metastasis in the MMTV-ErbB2 model of $Her2^+$ breast cancer (Tan et al. 2011). However, rather than T_H2 -polarized cells, only reconstitution with $CD4^+CD25^+$ cells (presumably $FoxP3^+$ T_{regs}) could restore the metastatic phenotype in immunodeficient mice. This was due to preferential expression of receptor activator of nuclear factor- κB ligand (RANKL) by the T_{reg} population. The authors found their model was insensitive to RANKL-induced migration, and that instead a pro-survival phenotype in non-adherent cells in vitro and circulating tumor cells in vivo was sufficient to explain increased metastasis (Tan et al. 2011). It should be noted that intratumoral RANKL injection also promoted metastasis in this model, suggesting that the T_{regs} mediate their effect within the tumor, and not in circulation. However, these findings may be model and/or organ dependent, and RANKL can directly promote mouse and human epithelial cell migration and bone metastasis (Jones et al. 2006).

Natural Killer Cells

As mentioned, $Ly6C^-$ monocytes suppress metastasis by recruiting NK cells to the lung, although whether tumor cell killing occurs in the vasculature or parenchyma is unclear (Hanna et al. 2015). More direct evidence of a role for NK cells in the vasculature can be found in the enhancement of metastasis by platelets and clotting factors in the lung. NK cell depletion ablates the influence of clotting on metastasis, and enhances tumor cell survival at a 24-hour time point even under normal conditions (Palumbo et al. 2005). Although direct killing of tumor cells has not been shown by intravital microscopy, these studies suggest that NK cells provide a level of immune surveillance against tumor cells adhering to the lumen of the endothelium.

ECTOPIC GROWTH

Macrophages

The formation of the pre-metastatic niche is dependent upon the recruitment and activation of CD11b⁺ myeloid cells by endogenous TLR ligands or collagen crosslinking (Hiratsuka et al. 2008; Erler et al. 2009; Kim et al. 2009). CD11b⁺VEGFR1⁺ cells were originally described as the important population of this myeloid niche (Kaplan et al. 2005), and a subsequent study showed that VEGFR1 expression specifically by macrophages is critical for metastasis outgrowth through its ability to drive CSF-1 expression (Qian et al. 2015). The dominant myeloid population supporting survival and growth of metastatic cells thus appears to be recruited macrophage populations that have differentiated from Ly6C⁺ monocytes. Significant differences between tumor cell survival in the presence or absence of recruited macrophages is observed between 12–24 hours, indicating a pro-survival function for macrophages in multiple studies (Qian and Pollard 2010; Gil-Bernabe et al. 2012; Hoos et al. 2014). Mechanistically this has been shown to result from pro-survival signals induced by vascular cell adhesion molecule-1 (VCAM-1) binding to $\alpha 4$ integrins on macrophages (Chen et al. 2011). It seems likely that additional pathways that enhance tumor cell survival at the single cell level have yet to be identified.

There is also evidence that macrophages can promote the growth of metastatic cells, based upon the reduced rate of growth seen 48 hours after experimental metastasis, and the ability of macrophage depletion to reduce foci size even when initiated 4 days after lung seeding (Qian et al. 2009). However, the mechanism underlying this observation remains unidentified, and there may be substantial differences between experimental and spontaneous metastasis. Also unclear is whether metastasis-associated macrophages possess the direct or indirect immunosuppressive phenotype of macrophages seen in primary tumors (Curiel et al. 2004; Kryczek et al. 2006; DeNardo et al. 2011; Ruffell et al. 2014). Finally, resident macrophage populations have not been extensively evaluated in the metastatic process, with the exception of the unique role of osteoclasts in bone reabsorption (Cook et al. 2014). All of these factors will be important to assess experimentally as macrophage-targeted agents are currently being tested clinically in patients with metastatic disease.

Neutrophils

As with macrophages, neutrophils are present in higher numbers in metastatic and pre-metastatic lungs and livers, driven by a G-CSF-dependent systemic expansion of the population (Kowanetz et al. 2010; Yan et al. 2010; Granot et al. 2011; Casbon et al. 2015; Coffelt et al. 2015; Wculek and Malanchi 2015). Neutrophils have now been implicated in promoting the survival and outgrowth of extravasated tumor cells in the lung through two distinct mechanisms: immune suppression and proliferative potential. It is also possible that neutrophils may regulate angiogenesis and vascular structure through Bv8 and/or MMP9 (Kowanetz et al. 2010; Yan et al. 2010), but clear supportive data for this is lacking.

Neutrophils from metastatic lungs suppress proliferation and IFN- γ production by T cells, and at least in vitro this is dependent upon inducible nitric oxide synthase (iNOS) (Yan et al. 2010; Coffelt et al. 2015). Importantly, while neutrophil depletion via an anti-Ly6G antibody

reduces metastasis, this was completely reversed by CD8⁺ T cell-depletion, demonstrating the importance of the immunosuppressive phenotype (Coffelt et al. 2015). However, it should be noted that neutrophil accumulation is observed in most organs, including the lymph node and spleen, and immune suppression within these secondary lymphoid organs, rather than within the metastatic environment may be important. Indeed, peripheral blood and splenic neutrophils from tumor bearing mice are immunosuppressive (Casbon et al. 2015; Coffelt et al. 2015). This is consistent with the literature on Gr-1⁺ myeloid-derived suppressor cells and their ability to suppress systemic anti-tumor immune responses, leading to enhanced tumor growth and metastasis in many tumor models (Gabrilovich and Nagaraj 2009).

In contrast to the above studies, one study has shown that neutrophil depletion can suppress spontaneous metastasis in immunodeficient mice (Wculek and Malanchi 2015). A caveat to this is immunodeficient mice will lack many of the polarization signals that drive the phenotype of neutrophils in cancer. For example, IL-17 expression by either CD4⁺ T_H17 cells or $\gamma\delta$ T cells enhances G-CSF expression and phenotypic changes in neutrophils (Chung et al. 2013; Coffelt et al. 2015). Regardless, neutrophils and neutrophil culture medium were able to enhance the number of CD24⁺CD90⁺ metastatic initiating cells present in the lung using multiple models of experimental metastasis in a leukotriene-dependent manner (Wculek and Malanchi 2015). G-CSF-driven expansion of neutrophils can therefore promote multiple aspects of cancer cell growth within ectopic tissues.

T Lymphocytes

Immune surveillance and editing are well-established concepts in carcinogen-induced tumors, and mice are more susceptible to cancer in the absence of innate and/or adaptive components of the immune system that mediate cytotoxicity (Vesely et al. 2011). Increased tumor growth in immune deficient mice can lead to increased incidence of metastasis; however, less is known about the importance of immune surveillance in specifically suppressing the outgrowth of disseminated cancer cells. This may have been a limitation of the animal models usually employed, and there have now been studies demonstrating the importance of CD8⁺ T cells in maintaining metastatic dormancy using more unique systems. In a spontaneous model of melanoma wherein the cells disseminate but fail to develop into overt metastasis in most animals, CD8⁺ T cell-depletion increased tumor cell proliferation and resulted in observable metastasis in the majority of mice (Eyles et al. 2010). Similar results were seen in a resectable fibrosarcoma model, with CD8⁺ T cell depletion increasing the incidence of metastasis from 0% to 100% (Romero et al. 2014). Interestingly, CD4⁺ T cell-depletion in this model resulted in a 23% rate of incidence, and CD4⁺ T cells have been shown to provide protection against development of hepatocellular carcinoma (Kang et al. 2011). Similar to primary tumors, T cells may provide an important but context specific role in immune surveillance in metastatic disease. Induction of G-CSF expression by IL-17-producing $\gamma\delta$ T cells has already been discussed (Coffelt et al. 2015), but other cytokines and leukocytes are also likely to be involved in directing the immune response. For example, transportation of tumor antigen by resident CD103⁺ DCs in the lung is protective against experimental metastasis (Headley et al. 2016). Given the potential importance of T cells in

maintaining and/or eliminating dormant metastatic cells, understanding the kinetics and mechanism of this process is critical.

Natural Killer Cells

Numerous studies have demonstrated that NK cells protect against experimental and spontaneous metastasis in immunodeficient and immunocompetent murine models. This includes spontaneous metastasis to the lung, liver, and lymph node in models with no apparent role for NK cells in primary tumor growth (Smyth et al. 1999; Smyth et al. 2001; Takeda et al. 2001; Olkhanud et al. 2009; Milsom et al. 2013; Paolino et al. 2014). This protective effect is mediated by the direct cytotoxic activity of NK cells through expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and perforin (Smyth et al. 1999; Takeda et al. 2001; Grosse-Wilde et al. 2008). As a comparison to the importance of cytotoxic T cells, NK cell depletion in the aforementioned resectable fibrosarcoma model resulted a metastatic incidence of 87% (Romero et al. 2014). Despite the number of studies demonstrating the importance of NK cells in suppressing metastasis, NK cell-based immunotherapies have not progressed to the same degree as those targeting T lymphocytes. This may be due to an overreliance on xenografts and other immunodeficient pre-clinical models, as well as the use of cell lines that display susceptibility to NK-mediated lysis due to low expression of MHCI or sensitivity to TRAIL. Investigations employing transgenic models with spontaneous metastasis may help clarify the importance of NK cells, and determine whether these cells represent a viable therapeutic target.

THERAPEUTIC IMPLICATIONS

Targeting key immune pathways to block the dissemination of tumor cells to ectopic organs has shown success in pre-clinical modeling. However, these pathways may be very context specific, as for example, liver metastasis by MC38 colon carcinoma cells is dependent upon CCL2, while metastasis of B16F10 melanoma cells is not (Zhao et al. 2013). Furthermore, while targeting CSF-1 or CCL2 can reduce metastasis in certain models, compensatory mechanisms mitigate the potential of these therapeutic approaches in others (Bonapace et al. 2014; Swierczak et al. 2014). More importantly, therapeutics targeting CSF-1/CSF-1R and CCL2/CCR2 are being used in patients with established metastatic disease, and it will be necessary to determine whether these and other pathways promote the growth of metastatic lesions or mediate therapeutic resistance, as opposed to cancer cell dissemination. As such, it is promising that CSF-1 is an important driver of metastatic outgrowth (Qian et al. 2015), and the size of metastatic foci is reduced by targeting the CSF-1/CSF-1R pathway in combination with paclitaxel chemotherapy (DeNardo et al. 2011; Ruffell et al. 2014). As a general concept it will be important to test immune-targeted agents in combination with other modalities; although early intervention approaches are often effective as single agents, late stage approaches rarely are.

Given the important role of CD8⁺ T cells and NK cells in protecting against metastasis, augmenting the cytotoxic potential of these cells would appear to be the most promising route for therapeutic intervention. Immune checkpoint blockade therapy is already approved for the treatment of patients with advanced metastatic disease, and the goal is now is to

search for approaches that can improve upon the relatively low response rates being observed (Topalian et al. 2015). This includes targeting additional checkpoint molecules beyond programmed death-1 (PD-1) and cytotoxic T lymphocyte antigen-4 (CTLA-4), but also relieving immune suppression within metastatic tumors. Notably, the reduced lung metastatic burden seen following neutrophil depletion or CSF-1R inhibition is CD8⁺ T cell-dependent (DeNardo et al. 2011; Coffelt et al. 2015), and CSF-1R inhibition enhances the response of primary tumors to checkpoint blockade and adoptive cell transfer (Mok et al. 2014; Zhu et al. 2014). Whether these or other combinations will prove efficacious in a metastatic setting remains to be determined.

As agents that target the microenvironment move into the clinic, it is important to keep in mind that treatment approaches will need to be tailored to the specific organ/tissue. It is unclear the degree to which cancer cells reestablish their microenvironment in metastatic organs, but there is probably a complex interplay between the innate suppressive capacity of the cancer cells and the metastatic site. Even mathematical modeling that accounts simply for organ blood flow and T cell trafficking results in a complex interaction between the primary tumor and metastatic lesions during therapy (Poleszczuk et al. 2016). Lung, liver, brain, and bone metastasis are likely to require therapeutic modalities that reflect both the requirements for survival in these organs, as well as the unique roles that resident immune cells have in these tissues. Advanced preclinical modeling of metastatic disease that permit therapies to be evaluated in an adjuvant setting will greatly assist in therapeutic development. As the number of approved immunotherapies expands, this will also be a critical step in selecting rationale combinations that may increase the success rate of subsequent clinical trials.

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Figure 1. Immune regulation of the metastatic cascade

(A) Invasion of tumor cells towards the vasculature is driven by macrophage expression of the chemoattractants EGF and CCL18 (in humans). Macrophage recruitment to the primary tumor and its ability to secrete EGF is dependent upon CSF-1 expression by the tumor cells, resulting in a paracrine loop. This loop is further augmented by the cytokine IL-4, expressed by T_H2-polarized CD4⁺ T cells. IL-4 also drives cathepsin expression by macrophages, which promotes local invasion through an unidentified mechanism. Stromal-derived MMPs are also thought to enhance the invasion process by regulating the structure of the ECM and increasing the bioavailability of growth factors and chemokines. A subset of Tie2⁺

macrophages associated with the endothelium is responsible for increasing vascular permeability and allowing tumors cells to intravasate into the circulation. **(B)** Tumor cells need to survive in circulation and arrest within the lumen of the vasculature in order to invade ectopic organs. Attachment is enhanced by CD11b⁺ neutrophils recruited by IL-8 (CXCL8) or clotting factors/platelets. Neutrophils can then recruit additional cells by binding to ICAM1 on tumor cells, or releasing NETs under inflammatory conditions. Adherent tumor cells also recruit Ly6C⁺ monocytes via CCL2, leading to macrophage differentiation and secretion of VEGF. This increases vascular permeability and promotes tumor cell extravasation. Tumor cells can be directly killed by cytotoxic NK cells recruited to the endothelium by Ly6C⁻ patrolling monocytes responding to CX₃CL1, or by neutrophils stimulated with CCL2 to induce production of H₂O₂. **(C)** Macrophages are important in promoting the outgrowth of metastatic foci, and this is mediated at least in part by survival signals provided by VCAM-1 on tumor cells interacting with α 4 integrins on macrophages. Additional roles for macrophages in angiogenesis and immunosuppression are likely based upon the phenotype in primary tumors, but have not been shown. Neutrophils support the early proliferative potential of metastatic cells through production of leukotrienes, as well as angiogenesis at later stages of growth through expression of Bv8 and possibly MMP9. Neutrophils in metastatic organs have the potential to suppress CD8⁺ T cell responses through iNOS, otherwise CD8⁺ T cells and NK cells can suppress metastasis through direct cytotoxicity. DCs within metastatic organs can suppress metastasis, likely by trafficking to draining lymph nodes and promoting a T cell response.