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Distant Relations: Macrophage Functions in the Metastatic Niche

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Abstract

Tumor associated macrophages are known contributors of tumor progression in the primary tumor via multiple mechanisms. However, recent studies have demonstrated the ability of macrophages to promote secondary tumor development by inhibiting tumoricidal immune response, initiating angiogenesis, remodeling the local matrix, and directly communicating with cancer cells. In this review, we discuss macrophage functions in establishing distant metastases including formation of the pre-metastatic niche, extravasation of circulating cancer cells, and colonization of secondary metastases. A more thorough understanding of metastasis associated macrophages and their associated mechanisms of metastatic progression may lead to novel therapeutic intervention to prevent further metastatic development and tumor re-seeding.

Keywords

macrophage; metastasis; cancer; extravasation; metastatic niche

1.0 Macrophages are key contributors to metastatic progression

There is now a wealth of research demonstrating the importance of macrophage populations within the primary tumor to promote metastatic progression. These tumor associated macrophages (TAMs) act via multiple mechanisms to promote tumor growth, inhibit tumoricidal immune response, initiate angiogenesis, and activate matrix remodeling [1–8]. A less studied but growing area of research is to understand how macrophages at the metastatic site contribute to the later stages of metastasis including secondary site arrest, extravasation, and early colonization. Recent work suggests that metastasis associated macrophages (MAMs), which include both tissue resident macrophages and bone marrow derived macrophages (BMDMs), promote metastasis in the secondary site. Depletion of macrophages at any stage of metastatic progression decreases the metastatic potential of disseminated cancer cells, indicating that interactions with MAMs influence processes such

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as cancer cell adhesion to the vasculature, transmigration of the endothelium, and colonization of secondary tumors [9–12]. In this review, we will discuss the various functions of macrophages in establishing distant metastases including formation of the pre-metastatic niche, extravasation of circulating cancer cells, and colonization of secondary metastases. A more thorough understanding of these MAMs and their associated mechanisms of metastatic progression may lead to novel therapeutic intervention to prevent further metastatic development and tumor re-seeding

1.1 Macrophage phenotypes and activation influence cancer progression

The mechanisms underlying macrophage recruitment and activation during the immune response provide insights into the potential contributions of macrophages in the tumor microenvironment. Macrophages represent a diverse population of immune cells that are generally classified as tissue resident macrophages or BMDMs (Table 1). Resident macrophages are derived from yolk sac or fetal liver progenitors to aid in embryonic development and ultimately differentiate into several subtypes to perform tissue-specific functions, phagocytose wastes and foreign bodies, and initiate the immune response [13]. BMDMs are typically recruited to tissues as myeloid-derived cells, which fully differentiate within the tissue and can be polarized in response to signals sensed within the tissue. Traditional macrophage polarization is classified as classical “M1” activation or alternative “M2” activation. Typically, M1 polarization is thought to promote pro-inflammatory, tissue destructive functions and M2 promotes anti-inflammatory, tissue repair functions. Although these definitions imply that M1 and M2 macrophages are distinct entities that perform specialized functions, this classification system is now considered an oversimplification since specific phenotypes are distributed along a continuum to allow for a smooth transition between pro-inflammatory, anti-inflammatory/recovery, and homeostatic states [12,14–16].

However, in many diseased states the balance of polarization is skewed towards chronic inflammation (M1-like) or tissue repair (M2-like). In tumors, cancer cells generally promote the maintenance of a more M2 macrophage polarized state to aid in evading the immune response, promoting angiogenesis, releasing pro-tumoral growth factors, and remodeling the local tissue; however, several M1-associated cytokines such as tumor necrosis factor (TNF)- α and interleukin (IL)-6 may be pro- or anti-tumorigenic depending on the tumor type and the environment [17,18]. In fact, many macrophage subpopulations may contribute to tumor progression and metastasis. While tumor associated macrophages are thought to primarily consist of BMDMs, it is often difficult to distinguish resident macrophages from tumor associated macrophages [7]. A recent mass cytometry-based study of macrophage populations isolated from renal cell carcinomas identified up to 17 distinct macrophage populations [19], further highlighting the need to better understand how phenotypic diversity in macrophages influences cancer progression. Since it is likely that a diverse array of macrophage populations at the metastatic site perform a range of functions to facilitate metastatic colonization, it is important to clearly characterize and not over-generalize macrophage phenotypes and functions to understand how they influence metastasis [16].

2.0 Primary tumor-derived factors influence seeding and regulation of macrophages in the metastatic site

Recent studies of metastatic homing to specific organs highlight the diverse roles of resident macrophages and the recruitment of BMDMs in aiding the formation of distinct pre-metastatic niches (Figure 1, Key Figure). Once localized to the metastatic site, BMDMs create an inflammatory environment where tumor cells colonize, recruit additional BMDMs, and exploit BMDM pro-tumoral functions while the tumor persists (Figure 2). Some metastatic models have revealed that systemic soluble and exosomal signaling from the primary tumor induces resident cells in an organotropic manner to recruit BMDMs before the arrival of cancer cells (Table 2). Understanding how BMDMs are initially recruited to the pre-metastatic niche may help prevent the initiation of this malignant cycle.

2.1 Primary tumor secretome impacts bone marrow derived cell recruitment and function in the secondary site

The ability of cancer cells to localize to specific organs is potentially dependent on the ability of the primary tumor to establish bone marrow derived cell clusters—containing progenitors of BMDMs along with other bone marrow-derived cell types—in the pre-metastatic niche [20–22]. For example, systemic signaling produced by intradermally injected Lewis lung carcinoma (LLC) tumors or LLC conditioned media promotes fibronectin expression in the lungs and liver, which are where intradermally injected LLC tumors generally metastasize. Bone marrow derived cells expressing the fibronectin receptor very late antigen (VLA)-4 localize to these fibronectin-rich regions where they promote neovascularization and metastasis in a vascular endothelial growth factor (VEGF)-1 dependent manner. However, in the absence of primary tumor signals bone marrow-derived cell clusters failed to form, resulting in significantly fewer metastases. Similarly, primary tumor signals from highly metastatic B16 melanomas in the same model initiate pre-metastatic bone marrow derived cluster localization to the lungs, liver, testes, spleen, and kidneys, which are consistent with metastatic burden of B16 tumors. Interestingly, LLC cells injected through the tail vein of the B16-educated mouse model seeded in all traditional B16 affected organs rather than just the lungs and liver, which are the normal sites for LLC seeding [20]. These data highlight the importance of soluble factors derived from the primary tumor in establishing the pre-metastatic niche and contributing to organotropic metastases.

Recent studies have elucidated specific factors within the primary tumor secretome that contribute to initiation of the lung pre-metastatic niche [21,22]. In LLC and B16 tumor secretomes, VEGF-A, TNF- α , and transforming growth factor (TGF)- β induce lung endothelial cell secretion of monocyte chemoattractants S100A8 and S100A9 [21]. In addition to directly recruiting bone marrow derived cells, S100A9 may also increase their retention in fibronectin-rich regions by activating β 2 integrins—including CD11b—which increases leukocyte affinity to fibronectin *in vitro* [23,24]. Activation of the endothelium via VEGF-a, TNF- α , TGF- β , S100A8, and S100A9 is associated with increased endothelial permeability, which enhances extravasation of recruited monocytes and cancer cells *in vivo* [25–29]. In an orthotopically injected MDA-MB-231 mammary tumor model, primary

tumor cell-derived lysyl oxidase (LOX) localized to fibronectin-rich tissues in the lung where it cross-linked collagen IV, resulting in increased affinity to CD11b+/VEGFR1+ monocytes. This binding promoted monocyte expression of the collagen cleaving protein, matrix metalloproteinase (MMP)-2, which aided cancer cell extravasation by reducing invasion impedance and recruiting BMDMs via production of chemotactic collagen fragments [22]. While these data highlight some mechanisms of early bone marrow derived cell recruitment and function correlated with fibronectin expression in the lungs, further investigation is required to better understand how primary tumor secretomes impact organotropic pre-metastatic niche formation.

2.2 Exosomes influence macrophage localization and function

In addition to soluble growth factors, primary tumor-derived exosomes have also been shown to impact the pre-metastatic niche. Exosomes are vesicular carriers secreted by cells for intercellular transport, and exosome integrin expression promotes localization to specific organs [30–33]. As highly phagocytic cells, macrophages engulf exosomes and process the cargo allowing distant, primary cancer cells to alter secondary macrophage phenotypes. Tail vein injection of exosomes from organotropic breast (MDA-MB-231—lung, MDA-MB468—lung and liver) and pancreatic cells (BxPC-3 and HPAF-II—liver) into mice revealed that exosomes with $\alpha 6\beta 4$ and $\alpha 6\beta 1$ integrins predominantly localize to the lungs while exosomes with $\alpha V\beta 5$ were associated with liver localization. In both cases, these exosomes were found colocalized with macrophages in fibronectin-rich environments [33]. Exposure of macrophages to exosomes has been shown to directly promote their ability to further enhance pro-tumoral immune cell recruitment. Exosomes collected from lung cancer cells lines contain microRNA-21 or microRNA-29a which bound as ligands to toll-like receptor (TLR)-7 in mice (TLR-8 in humans) and induced expression of IL-6 and TNF- α in an NF- κ B-dependent manner [34]. Because both TNF- α and IL-6 are cytokines associated with leukocyte recruitment during the immune response and tumor promotion, these studies warrant further investigation into the effects of primary tumor-derived exosomes on MAM function in the metastatic site.

2.3 Primary tumor regulation of resident macrophages in the metastatic site

In addition to enhancing the recruitment of macrophage precursors into the metastatic site, primary tumor-derived factors can also impact tissue resident macrophages within that site. For example, initiation of the bone pre-metastatic niche is often driven by activation of bone resident macrophages—osteoclasts. In normal physiology, osteoclasts resorb bone tissue and act in cooperation with bone-forming osteoblasts to promote bone development [35]. However, signals derived from the primary tumor can activate osteoclasts to promote the formation of osteolytic lesions, which serve as hospitable environments for future cancer cells [36–38]. For example, secreted LOX from hypoxic, estrogen receptor negative (ER-) mammary tumor cells induced the activity of the NFATc1 transcription factor, a key regulator of osteoclastogenesis. By skewing homeostasis towards bone resorption, LOX-induced osteoclastogenesis lead to the formation of pre-metastatic lesions, which serve as hospitable environments for metastatic cancer cells, in both orthotopically transplanted tumor models and in tumor-free mice injected with tumor conditioned media [37]. Similarly, non-small cell lung cancer-derived TGF- β has been shown to induce osteoclastogenesis,

leading to increased metastatic burden in murine models [38,39]. TGF- β -induced osteoclastogenesis stimulates the release of additional soluble factors including TGF- β , creating a feed-forward cycle that continues unless osteoblasts can balance the system. However, continuous stimulation by the primary tumor or the arrival of tumor cells can maintain this osteolytic microenvironment by secreting signals that promote further osteoclastogenesis, suppress osteoblast activity, and directly degrade the bone, generating a “vicious cycle” of tumor progression [36].

The primary tumor secretome can also promote accumulation of lung resident alveolar macrophages that generate a pro-tumorigenic pre-metastatic niche for cancer cells. In the MET-1 mammary tumor model, accumulation of alveolar macrophages was found in the lung, which correlated with increased levels of the leukocyte chemoattractant, C5a. This accumulation of alveolar macrophages occurred before the arrival of cancer cells, indicating that primary tumor signals initiate lung secretion of C5a. Furthermore, depletion of alveolar macrophages significantly reduced lung metastasis of MET-1 cells, which correlated with a shift in the immune profiles from tumor-supportive Th-2 cells to tumoricidal Th-1 cells [40]. Further investigation into how the primary tumor initiates C5a production in the lungs may illuminate mechanisms to prevent this pre-metastatic niche initiation.

In addition to soluble factors, primary tumor-derived exosomes can also impact resident macrophages in distant sites. Isolation of exosomes from liver-tropic pancreatic ductal carcinoma (PAN02) cells shows that PAN02 exosomes containing migration inhibitory factor localize to the liver. Upon arrival, Kupffer cells (liver-resident macrophages) engulf the PAN02 exosomes and activate local fibroblasts to secrete fibronectin, contributing to the formation of pre-metastatic nodules where BMDMs localize [32]. These studies collectively suggest that primary tumor-derived factors can impact the function of resident macrophage in distant sites. Additional studies are needed to delineate the mechanisms of resident macrophage regulation and function in these and other distant tissue sites.

3.0 Macrophages Influence Adhesion, Extravasation, and Early Colonization of Cancer Cells

In the secondary site, MAM interactions with arriving cancer cells have been shown to promote metastatic behavior (Table 3). In many cases, the same mechanisms that macrophages use to recruit leukocytes during inflammation, such as enhancing adhesion and transmigration, are used to recruit cancer cells into the metastatic site. MAMs can further aid secondary tumor formation using the same mechanisms they utilize during tissue repair, such as inhibiting tissue-destructive immune response, promoting cellular growth, initiating angiogenesis, and remodeling the matrix. Furthermore, these MAMs can continually recruit BMDMs to maintain the pro-tumoral immune environment. Since MAMs appear to play major roles throughout metastatic progression, understanding the mechanisms through which MAMs drive tumor cell extravasation and early colonization may reveal therapeutic targets.

3.1 The presence of MAMs during extravasation promotes metastatic colonization

Several studies have provided strong evidence that the presence of MAMs during the metastatic extravasation process is critical to successful formation of a metastatic lesion [9–12]. Qian *et al.* injected breast cancer cells into the tail veins of mice following macrophage depletion with L-clodronate and found that the number of cancer cells arrested in the lung of macrophage-depleted mice dropped significantly over a 36-hour period post injection, suggesting that macrophages aid retention of disseminated cancer cells in the lung. Additionally, doubling times of tumor growth were twice as high in mice with macrophages than those without, highlighting the importance of macrophages in sustaining a pro-tumoral microenvironment. The authors also found that extravascular cancer cells in these models were in direct contact with CD11b+ BMDMs, indicating that adhesion of cancer cells to MAMs aids transmigration of the endothelium [9].

In addition to interacting with disseminated tumor cells, MAMs also prevent tumoricidal cell infiltrate. Headley *et al.* observed shedding of 0.5–25 μ m portions of cytoplasm from cancer cells arrested in the vasculature [12]. These mitochondria-containing “cytoplasts” remained within the lung microvasculature and preceded three waves of monocytic cells: neutrophils (~15 minutes), conventional monocytes (15min–6hrs), and non-alveolar macrophages, patrolling monocytes, and dendritic cells (6–12hrs). Of these phagocytic cells recruited to the metastatic site, non-alveolar macrophages and conventional monocytes were mostly responsible for engulfing the cytoplasts and extravasating into the pre-metastatic niche. Engulfment of cytoplasts increased macrophage expression of adhesion and chemotactic receptors important in cancer progression including vascular cell adhesion molecule (VCAM)-1, exosomal surface protein CD63, and natural killer cell adhesion mediator CD155 [12,41–44]. However, in CCR2 knockout models, immunostimulatory CD130+ dendritic cells and CD8+ T cells dominated the cytoplast-engulfing lung infiltrate, suggesting that CCR2+ BMDMs are important in promoting an immunosuppressive environment [12]. Furthermore, inhibiting recruitment of MAMs via the CCR2-CCL2 axis by genetic or chemical means reduced metastatic burden of tail vein injected MET-1, MDA-MB-231, and melanoma cells in the lungs [9–12], demonstrating the importance of this axis in metastatic progression. Taken together, these studies suggest that the presence of MAMs at the time of extravasation aids formation of metastatic lesions through direct interactions with tumor cells and indirect regulation of immune responses.

3.2 Mechanisms by which macrophages enable metastatic extravasation and colonization

Recent studies have explored potential mechanisms through which macrophages contribute to metastatic progression. MAMs expressing CCR2 are directly implicated in metastatic progression [9–12], and CCR2-mediated regulation of CCL3 has specifically been highlighted as a potential therapeutic target [11]. Stimulation of CCL2/CCR2 induces CCL3 in MAMs, which contributes to MAM retention in the metastatic site in a CCR1-dependent manner. Furthermore, the CCL3/CCR1 axis was found to be important for direct binding between macrophages and tumor cells, in part through an α 4-integrin dependent mechanism [9,11]. α 4 integrins bind to VCAM-1, which is expressed by leukocytes and endothelial cells [45], but can also be overexpressed in cancer cells [46]. Chen *et al.* showed that mammary fat pad injections of the lung tropic MDA231-LM2-4175 breast cancer cell line in

which VCAM-1 levels were knocked down grew at the same rate, but created significantly fewer lung metastases due to decreased binding affinity to macrophages. When modeling extravasation via tail vein injection of MDA231-LM2-4175 cells, reduced VCAM-1 expression did not affect accumulation of cancer cells in the lungs during the week after injection, but significantly decreased formation of metastases. Binding of macrophage $\alpha 4$ integrins to VCAM-1 in breast cancer cells activates downstream PI3K-AKT pro-migratory, pro-survival pathway in the cancer cells, which reduces cancer cell apoptosis and promotes secondary tumor formation [41,42].

Additional macrophage-derived factors, such as VEGF and endothelin-1 (ET-1), have also been implicated in promoting metastatic colonization. In mammary tumor models, monocyte/macrophage VEGF has been shown to contribute to retention of MET-1 mammary tumor cells in the lung. Furthermore, in vitro studies have shown that VEGF promotes endothelial permeability and transendothelial migration of MET-1 cells. Macrophage-derived VEGF has also been linked to cancer cell retention in the lung [10]. Studies using LLC lung cancer and B16 melanoma models suggest that VEGF also acts on VEGFR1-expressing macrophages and endothelial cells to induce production of MMP9 and monocyte chemoattractant, CXCL12, [47]. MMP-9 contributes to tumor progression by increasing vascular permeability [47], degrading the ECM, cleaving growth factors that ultimately aid in tumor proliferation [48], and angiogenesis to nourish the highly metabolic tumor [5]. Furthermore, MAM-induced VEGF-VEGFR1 signaling maintains gradients of the CXCL12 chemokine, which is a strong chemoattractant for monocytes and CXCR4+ tumor cells, contributing to the cycle of BMDM recruitment. [10,20,49]. Macrophage-derived ET-1, a soluble vasoconstrictor, has been shown to contribute to metastatic colonization. Tail-vein injection of muscle-invasive bladder cancer cells that secrete soluble vasoconstrictor, endothelin-1 (ET-1), leads to improved recruitment of BMDMs and ultimately increased metastatic burden compared to ET-1 knock-down controls. Inhibition of macrophage ET-1 receptor ET_aR significantly reduced metastatic burden in mice by preventing downstream production of pro-tumoral cytokines such as IL-6, CCL2, COX-2, and MMPs, which are all associated with BMDM recruitment, activating angiogenesis, and secondary tumor formation [50,51].

Macrophages recruited to secondary locations not only influence extravasation through direct interactions with cancer cells, but can also interact with other cells within the pre-metastatic niche to promote metastasis. As alluded to in previous sections, macrophage-endothelial cell interactions can enhance metastatic potential of cancer cells by increasing endothelial affinity and permeability which aids circulating cancer cell adhesion and transmigration into the tissue [10, 25–29, 47, 52]. Additionally, cancer associated fibroblasts (CAFs) polarize TAMs to perform pro-tumoral functions in the primary site [53]. In the secondary site, CAFs and MAMs collaborate to increase metastatic potential of arriving tumor cells. In both lung and liver metastases, the primary tumor promotes fibroblast secretion of fibronectin, which recruits BMDMs to the secondary site [20,22,32,33]. BMDMs recruited to the liver promote pancreatic ductal carcinoma (PDAC) metastasis by secreting granulins, generating a fibrotic environment by activating hepatic stellate cells into myofibroblasts. These myofibroblasts ultimately increase growth of secondary tumors by secreting periostin that activates Wnt and $\alpha V\beta 3$ -Akt/PKB signaling pathways in PDAC

cells. Depletion of granulins prevented MAM-dependent development of the fibrotic environment and subsequent PDAC metastatic growth while inhibition of periostin significantly reduced metastatic outgrowth of PDAC cells by blocking the CAF/PDAC paracrine loop [54]. Understanding how macrophages promote pro-tumoral functions of other cell types within the pre-metastatic niche—including endothelial cells and CAFs—may illuminate several therapeutic targets that can serve as combination therapy to alleviate metastatic progression.

While these studies highlight the importance of MAM-derived factors in tumor cell extravasation and growth in the metastatic site, given the diversity of mechanisms through which TAMs in the primary tumor site contribute to tumor growth and progression, it is likely that there are additional MAM-related factors that contribute to metastasis. For example, it is widely accepted that TAMs promote intravasation via the CSF-1/EGF paracrine loop [1–8,52]. Specifically, secretion of CSF-1 by cancer cells recruit BMDMs to the primary tumor where they secrete EGF, which contributes to vascular permeability and cancer cell transmigration [52]. Additionally, cancer stem cells have been identified in circulation and may be better suited to infiltrate and colonize the secondary site than other tumor cell populations since they can quickly adapt to new environments [55]. Macrophage crosstalk with cancer stem cells in the primary tumor promotes development, maintenance, self-renewal, and the epithelial to mesenchymal transition of cancer stem cells via several mechanisms which could also promote early tumor development in the secondary site [55,56]. Whether these mechanisms or other known pathways through which primary TAMs function are replicated in the metastatic site remains to be investigated. Understanding the similarities and differences in TAM and MAM function is critical for developing the most effective macrophage-targeted therapies that will target macrophage function in both sites.

3.4 Macrophage-tumor cell hybrids from the primary tumor promote pre-metastatic niche formation

Another potential mechanism by which macrophages aid tumor cell extravasation and colonization is through fusion between TAMs and cancer cells. It has been shown that when macrophages engulf apoptotic cancer cells they incorporate cancer cell DNA into their nucleus. These “hybrids” promote invasion and immune evasion via intrinsic macrophage functions but tumor traits promote increased proliferation [57–59]. Recently, macrophage-tumor cell hybrids were identified in the blood of patients with epithelial cancers such as melanoma (SK-Mel-24, 28, 31) [59], breast (MCF-7, MDA-MB-231), colorectal (HepG2), and ovarian (SKOV-3) cancer [58]. The hybrid cells expressed both M2-like macrophage markers (CD163, CD206, CD204) and epithelial markers (EpCAM, KRT) and were significantly more invasive than either cell type cultured alone [58–59]. Macrophage-melanoma hybrids subcutaneously injected into the hind limbs of nude mice disseminated and produced metastatic lesions in the pancreas, indicating that these hybrids can establish distant metastases [59]. It is hypothesized that these hybrids also contribute to pre-metastatic niche formation, but additional research is needed to understand the roles of macrophage-tumor cell hybrids in pre-metastatic niche formation, extravasation, and colonization and how these mechanisms may be future therapeutic targets.

4.0 Macrophages are a potential therapeutic target in the secondary tumor microenvironment

Numerous strategies are being pursued to target macrophages in the primary tumor, including ablation of TAMs residing in the tumor, impeding further recruitment of BMDMs, and polarizing TAMs toward a tumoricidal phenotype [60,61]. Several of these treatment strategies targeting the CSF1-CSF1R [62], CCL2-CCR2 [63], and VEGF-VEGFR1 [20,52] axis effectively reduce primary tumor burden and metastasis in murine models [11,20]. However, clinical trials targeting TAMs have thus far been less successful [61]. Inhibition of the CSF1/CSF1R pathway decreases metastatic burden in murine models and is generally well tolerated in patients, but is ineffective in reducing recruitment of BMDMs to the primary tumor in clinical trials [61,62]. Recruitment of BMDMs to the primary tumor may be maintained when the CSF1 pathway is inhibited via stromal cell secretion of other BMDM recruitment cytokines. Kumar et al. demonstrated that CSF1 downregulates CAF secretion of CXCL1, which is a granulocyte/monocyte recruiting cytokine. Inhibition of CSF1 alone promotes upregulation of CXCL1 and, therefore, increases recruitment of granulocytes and BMDMs. However, inhibition of CSF1R and CXCL1 receptor, CXCR2, significantly reduces BMDM recruitment and primary tumor growth in mice [64]. These data show the importance of understanding the various mechanisms that contribute to BMDM recruitment and finding the optimal combination of targets to prevent BMDM recruitment to the pre-metastatic niche.

Similarly to CSF1R inhibition, blocking BMDM recruitment via CCL2 has shown promise in pre-clinical studies. In murine models, ablation of CCR2+ MAMs reduces metastatic burden, possibly due to tipping the immune cell infiltrate from pro-tumoral CCR2+ MAMs towards anti-tumoral CD130+ dendritic cells and CD8+ T cells [11,12]. However, because ablating CCR2+ macrophages directly is not currently a clinical option due to necessary homeostatic functions of macrophages, clinical trials have focused on reducing CCL2 levels with anti-CCL2 antibodies. Stage 1 trials with anti-CCL2 monoclonal antibody carlumab demonstrated that it is well tolerated by patients, but carlumab on its own does not consistently suppress free CCL2 levels and ultimately increased CCL2 levels over time [65]. The complications of carlumab treatments are likely due to short-term affinity of carlumab, overcompensation of cells expressing CCL2, and increased sensitivity of macrophages to CCL2 through upregulation of CCR2 [65–67]. For example, some murine models of mammary cancer associate cessation of anti-CCL2 agents with accelerating metastatic progression by increasing influx of monocytes to the primary and secondary tumors. In these models, monocytes/macrophages in the lungs increased metastatic growth of cancer cells in an IL-6/STAT3 dependent manner via VEGF-A driven neovascularization. When both CCL2 and IL-6 are inhibited, there is reduced metastatic burden [67]. While there is much to be understood about how these therapeutics influence macrophage recruitment and function in the secondary site, these data emphasize the importance of not only inhibiting BMDM recruitment, but concurrently targeting MAM functions to effectively target metastasis.

One important consideration for developing approaches that inhibit pro-metastatic macrophage functions is the method of delivery to macrophages in the pre-metastatic tissues. Directly targeting the intrinsic immune-altering functions of macrophages has been

investigated for several inflammatory diseases and should be further examined for cancer metastasis [68–70]. As “big eaters,” macrophages have evolved to rapidly phagocytose and process vesicular transport molecules and foreign bodies. This intrinsic behavior corresponds to observations of macrophages phagocytosing a larger percentage of tumor-derived exosomes [32], cytoplasts [12], and man-made drug delivery vehicles [69] than other cell types. By understanding the surface properties of exosomes and cytoplasts that promote organotropic deposition, more precise targeting of macrophages in tumor-specific pre-metastatic niches may be achieved. Loading these targeted delivery vehicles with agents designed to prevent formation of premetastatic lesions, for example, by blocking macrophage activation of fibroblast-secreted fibronectin, inhibiting the CCR2-CCL2 pathway, or preventing macrophage IL-6 secretion, may prevent the influx and activation of MAMs that initiate the cycle of secondary tumor development. Additionally, better targeted drug delivery to potential metastatic organs may also reduce off-target effects of these drugs, since the pathways exploited for cancer progression are necessary for maintenance of homeostasis.

5.0 Concluding Remarks

Therapeutically targeting macrophages in cancer is complex due to the various roles of macrophages in physiology; however, macrophages influence metastatic progression and may be an integral component in combination therapy. Further research is required to fully understand the mechanistic contributions of macrophages to organotropic metastases, early cancer cell-macrophage interactions in the secondary site, influence of cancer cell and macrophage heterogeneity on metastatic potential, and how these interactions influence the immune response (see “Outstanding Questions”). Understanding these key interactions that drive tumor cell-macrophage interactions throughout the metastatic cascade will provide insights that we can leverage with better drugs and delivery vehicles to skew MAMs towards anti-tumoral phenotypes and break the deadly cycle of metastatic progression.

Outstanding Questions

- Do extracellular matrix molecules within the metastatic niche, such as fibronectin, contribute to MAM recruitment and/or pro-metastatic MAM phenotype?
- What mechanisms determine localization of tumor-derived exosomes within distinct secondary sites? For example, what causes α_6 integrin-expressing exosomes to localize to the lung and α_V -expressing exosome to localize to the liver?
- What is the impact of tumor-derived exosome engulfment on macrophage phenotype and function, and can these responses be modified by altering exosome composition?
- Does the CSF-1/EGF paracrine loop between cancer cells and macrophages, which is critical for intravasation in the primary site, functionally contribute to extravasation and/or colonization in the metastatic site?

- What are the signals and cell types that drive increased CCL2 expression in the metastatic site and promote MAM recruitment?
- How do CCR2-expressing MAMs communicate with the metastatic microenvironment to reduce influx of tumoricidal immune infiltrate?
- What roles might macrophages play in formation, dissemination, and survival of circulating tumor clusters?
- Why do current macrophage-targeted therapies fail in clinical trials and will elucidating mechanisms of interaction between macrophages and cells within the metastatic site result in the development of rational combination therapies that may be more effective?

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Highlights

- Soluble signals and exosomes from the primary tumor promote pre-metastatic niche formation in specific organs by influencing osteoclast (bone), Kupffer cell (liver), or alveolar (lung) macrophage behavior
- Recruitment of BMDMs to the secondary site is correlated with increased metastatic potential of cancer cells, which can be initiated by primary tumor signals such as VEGF-A, TNF- α , TGF- β , and LOX
- Metastasis associated macrophages aid extravasation of cancer cells by increasing endothelial permeability through secretion of IL-6, CCL2, COX-2, and MMPs, binding directly to VCAM-1 on cancer cells, and inhibiting tumoricidal immune cell infiltrate upon arrest
- Macrophages aid secondary tumor growth by secreting MMPs that remodel the matrix and cleave growth factors, promoting VEGF-A driven neovascularization, and recruiting pro-tumoral immune cells via cytokines such as IL-6 and TNF- α
- BMDMs recruited via CCL2 help tumor cells evade early clearance from the secondary site by inhibiting tumoricidal immune cell infiltrate

Key Figure. Macrophage Roles in Organotropic Extravasation

In bone, primary, hypoxic ER- breast tumors initiate metastasis to the bone microenvironment through secretion of LOX which promotes osteoclastogenesis and cross-linking of collagen 4 which aids BMDM adhesion and extravasation [37]. TGF- β secretion by primary tumors also promotes metastasis to the bone by activating osteoclastogenesis and secretion of MMP9 which helps form pre-metastatic lesions [38]. In the liver, Kupffer cells engulf TGF-exosomes from the primary tumor which activates fibroblast-secretion of fibronectin that aids adhesion and extravasation of BMDMs [32,47]. Once in the liver, BMDMs secrete granulin which promotes fibroblast secretion of periostin. Periostin secretion generates a fibrotic environment that supports tumor cell colonization in the liver [54]. LOX secreted by hypoxic ER- mammary cancer cells also localizes to fibronectin-rich regions in the lung, cross-links collagen IV, and promotes BMDM recruitment [22]. BMDMs recruited by CCL2 aid extravasation of cancer cells [9,10,12]. Primary tumor secreted VEGF- α , TGF- β , and TNF- α activate S100A8&9 in endothelial cells which recruits BMDMs that predetermine lung metastases [20,21]. Extravasated BMDMs engulf exosomes containing miR-21 or miR-29a and secrete IL-6 or TNF- α to promote endothelial expression of surface receptors VCAM-1 and E-selectin, which increases BMDM and CTC affinity to the endothelium [34].

Figure 2. Cycle of BMDM Recruitment Maintains Secondary Tumor Microenvironment

There is a “vicious cycle” of osteolytic tumor development in the bone microenvironment [36], but evidence suggests there is also a cycle of BMDM recruitment in the lung and liver microenvironments that aids metastatic colonization. The primary tumor can promote formation of the pre-metastatic niche (PMN) by activating resident macrophages via soluble and exosomal signaling. Bone marrow derived macrophages (BMDMs) are recruited to these regions of increased endothelial activation and increased fibronectin expression [20–22, 32–34, 47]. At the secondary site, BMDMs promote cancer cell retention in the secondary site through direct contact, signaling, and interactions with the endothelium. Tumor cell-MAM interactions further promote tumor growth through secretion of growth factors, promotion of

angiogenesis, and maintenance of the immunosuppressive environment [9–12, 25, 41, 42, 50–52]. As the tumor grows, BMDMs and cancer cells are likely continually recruited to this inflammatory, tumor microenvironment (TME) through increased endothelial permeability, affinity to the endothelium, and maintenance of chemokine gradients.

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Table 1**Macrophage Subsets, Ontogeny, and Function**

Macrophage	Ontogeny	Function	Identifying Markers	References
BMDM	Monocyte-derived	Patrol vasculature for pathogens Recruited as part of the immune response Phagocytose pathogens and clear debris Promote tissue reconstruction	Patrolling: CX ₃ CR1-high CCR2-negative LY6C-low Inflammatory: CD11b-high F4/80 CCR2 LY6C-high	14
Alveolar Macrophages	Yolk sac and fetal liver progenitors	Patrol and eliminate inhaled particles Maintain lung homeostasis	F4/80-low CD11b-low CD11c-high CD68 Siglec F MARCO CD206 Dectin-1 Galectin-3	13
Kupffer Cells	Yolk sac progenitors	Clear pathogens and toxins	F4/80 CD11b-low CD169 CD68 Galecin-3 CD80-low	13
Osteoclasts	Monocyte-derived	Bone resorption Create pre-metastatic niches for disseminated tumor cells	Calcitonin receptor	13
TAMs	Monocyte-derived Resident macrophages?	Promote tumor growth Inhibit tumoricidal immune response Initiate angiogenesis Activate matrix remodeling Aid invasion and intravasation	Murine: LY6C MHC II molecules CX3 CR1 CCR2 L-selectin TIE2 Human: CD14 CD312 CD115 CD16	7
MAMs	Monocyte-derived Resident macrophages?	Aid metastatic functions including pre-metastatic niche formation, extravasation, angiogenesis, and immune evasion	Monocyte derived: F4/80 CD11c-low CD11b VEGFR1-high CCR2-high	9–12

Table 2**Macrophage Interaction in Promoting Pre-Metastatic Niche Formation**

Primary Tumor	Secondary Site	Primary Tumor Signal	Secondary site response/ Macrophage interaction	Reference(s)
Lewis lung carcinoma (LLC) B16 melanoma	Lungs, liver Lungs, liver, spleen, testes, and kidney	Conditioned media from LLC/B16 cells or intradermal injection of LLC/B16 cells	Fibronectin expression pre- determines VLA4+ bone-marrow derived cell cluster localization to organs where each cell type generally metastasizes	20
Lewis Lung Carcinoma (intradermal injection)	Lungs	TNF- α , TGF β , VEGF-a	Stimulates endothelial secretion of S100A8/9 which recruits BMDMs	20,21
Hypoxic ER-breast cancer	Lungs	LOX	Localizes to fibronectin rich areas, cross-links collagen IV, promotes BMDM recruitment	22
Pancreatic (PAN02)	Liver	TGF- β exosomes	Activate Kupffer cells which initiates fibronectin secretion in liver	32,47
Lung-tropic breast cancer (MDA- MB231, MDA-MB-468), osteosarcoma, Rhabdomyosarcoma, Wilms' tumor, and melanoma exosomes	Lung	Apha6 β 4 α 6 β 1	Cancer exosomes expressing α 6 integrins localize in lung with macrophages before cancer cells arrive	33
Liver-tropic breast cancer 9MDA-MB468), uveal melanoma, colorectal cancer, pancreatic cancer(BxPC-3, HPAF-II), and gastric cancer exosomes	Liver	α V β 5	Cancer exosomes expressing β 5 integrins localize in the liver with macrophages before cancer cells arrive	33
A-549 and SK-MES lung cancer lines	RAW 264.7 murine macrophages Lungs of WT and TLR7-/- mouse models	miR-21 and miR29a in exosomes	miR-21 and miR-29a containing exoosomes are phagocytosed by macrophages and activate TLR-7 in macrophage endosomes which induces a pro-metastatic immune response by activating NF- κ B transcription of TNF- α and IL-6	34
Hypoxic ER-breast cancer	Bone	LOX	Activates transcription factors that upregulate osteoclastogenesis	37
Breast Renal NSCLC	Bone	TGF- β	Activate osteoclastogenesis and MMP secretion	38

Table 3

Macrophage Influence on Cancer Cell Arrest, Extravasation, and Colonization

Primary Tumor	Secondary Site	Macrophage population	Macrophage function	Cancer Interaction	Reference(s)
PyMT breast cancer cells	Lungs	CD11b/Ly6C/VEGFR1/CC R2/CSFR1+ macrophages recruited to secondary site via CCL2 axis	Secretion of VEGFa increases permeability of endothelium	This subset of macrophages is in direct contact with extravascular breast cancer cells. Increased vascular permeability increases metastasis	9,10
Polyoma-middle-T MET-1 cancer cells	Lungs	Recruited monocytes	VEGF secretion by recruited monocytes	VEGF expressing monocytes significantly increase retention of MET-1 cells in the lung	10
MDA-MB-231:4175	Lungs	CD11b+ MAMs	Recruited via CCL2 axis which induces MAM CCL3 secretion and MAM retention via CCL3/CCR1 axis	The CCL3/CCR1 axis leads to direct binding of tumor cells to MAMs via $\alpha 4$ integrin binding to VCAM-1 in breast cancer cells	11
B16 Melanoma	Lungs	CCR2+ BMDM	Engulf cytoplasts and upregulate adhesion receptors VCAM-1, CD-38, CD-63, CD-88, CMKLR-1, and CD-155	Melanoma cells secrete shear-induced "cytoplasts" that initiate pro-tumoral immune cell recruitment. Lack of macrophages results in recruitment of anti-tumoral immune cells	12
MMTV-PyMT induced mammary tumor cells	-- Lungs	TAMs	BMDMs are recruited via CSF-1 gradients and secrete EGF to aid tumor cell invasion. MAMs are recruited to the lung in a CSF1 dependent manner	Secrete CSF-1 to recruit macrophages. Migrate along EGF gradients produced by macrophages. Intravasate due to this mechanism	9,25,52
Lung-tropic breast cancer cells (MDA231-LM2-175)	Lungs	CD11b+/VLA4+ BMDMs	Macrophages bind to VCAM-1 with $\alpha 4$ integrins	Binding of breast cancer VCAM-1 to $\alpha 4$ activates pro migratory and survival PI3K-AKT	41,42
--	--	Macrophage activation	Promote endothelial expression of VCAM-1	Aids TAM localization and upregulates $\alpha 4$ integrins in macrophages which tethers breast cancer cells	42
MB49 murine bladder cancer	Lungs	BMDM	Arrive to extravasation site before cancer cells and produce CCL2, COX-2, MMPs and IL-6. Depletion of ET-1 from cancer cells decreased macrophage influx to the lung	Secondary tumor growth, not primary tumor, is affected by ET-1 expression. ET-1 recruits macrophages to secondary site to aid immune response and angiogenesis	50,51
Pancreatic Ductal Carcinoma (PDAC)	Liver	BMDM	Recruited to liver and secrete granulins which promotes myofibroblast differentiation	Myofibroblasts secrete periostin which promotes PDAC metastatic growth via $\alpha V\beta 3$ -AKT/PKB signaling	54