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Immune cell promotion of metastasis

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Abstract
Metastatic disease is the major cause of death from cancer, and immunotherapy and chemotherapy have had limited success in reversing its progression. Data from mouse models suggest that the recruitment of immune suppressive cells to tumors protects metastatic cancer cells from surveillance by killer cells, which nullifies the effects of immunotherapy and thus establishes metastasis. Furthermore, in most cases, tumor-infiltrating immune cells differentiate into cells that promote each step of the metastatic cascade, and thus are novel targets for therapy. In this Review, we describe how tumor-infiltrating immune cells contribute to the metastatic cascade and discuss potential therapeutic strategies to target these cells.

Introduction
Cancer progression ends in metastatic disease, which is the major cause of cancer death. For metastasis to occur from solid malignancies, tumor cells need to undergo a process referred to as the ‘Metastatic Cascade’ [FIG. 1]. At the primary site, tumor cells escape from the anti-tumor immune response and remotely prepare the environment (pre-metastatic niche) of the future metastatic site (pre-metastatic niche). The primary tumor cells also invade into the surrounding parenchyma and intravasate into blood and/or lymphatic vessels, which allows them to circulate and spread. At the metastatic site, the location of which is defined by tumor type and particular tissue environment, these circulating tumor cells extravasate, become established and proliferate to form the deadly metastatic tumor.

During each step of the Metastatic Cascade, mutant and thus potentially immunogenic tumor cells are being exposed to the immune system that can recognize them and restrict their growth1,2. For example, recent reports demonstrate that CD8+ T cells restrict the metastatic outgrowth of cancer cells disseminated from the primary tumor, and that natural killer (NK) cells have a potential to reject metastatic tumor cells through inhibition of TAM family receptors that suppress NK cell activation3,4. Consequently depletion of CD8+ T and NK cells increases breast cancer metastasis without affecting primary tumor growth5. Nevertheless, successful cancers and their metastatic derivatives have developed strategies to overcome these immuno-surveillance
immune mechanisms in part through the recruitment of immune suppressive cells\(^6\).

In addition to the local immune cell recruitment, primary tumors affect the systemic environment, particularly the bone marrow, and alter hematopoiesis, which can influence the growth of other less aggressive primary tumors\(^7\). The tumor-driven systemic processes also prepare distant sites to become pre-metastatic niches, thereby enhancing metastatic efficiency\(^7\). These systemic enhancements of metastasis involve, at least in part, myeloid cells that facilitate the escape of circulating metastatic cells.

Tumor-infiltrating immune cells, particularly myeloid cells such as macrophages, also actively participate in metastatic processes. Macrophages are very plastic cells and exert distinct functions in response to environmental signals. For example, interferon gamma and TLR ligands activate macrophages to eliminate pathogens and in some contexts tumor cells. In contrast, macrophages in response to IL-4 and IL-13 are involved in tissue remodeling and tumor progression\(^8\). Accumulating data suggest that tumor microenvironment polarizes recruited macrophages from potentially tumor reactive to a tumor promoting state. Indeed, these tumor educated macrophages influence every step of the metastatic cascade by promoting tumor cell invasion, intravasation and survival in the circulation, as well as their arrest, extravasation and persistent growth at metastatic sites.

Substantial clinical data has indicated that tumor infiltration of certain immune cell types correlate with poor prognosis of cancer patients\(^9\)-\(^11\), although these studies do not address their roles in tumor metastasis. In this Review, we highlight the role of immune cells in each step of metastatic cascade and describe mechanisms underlying their pro-metastatic functions identified by mouse models. Since immune cells do not contain mutator mechanisms and are required for malignancy, they are attractive targets as part of a combination therapy directed to kill tumor cells and to remove their support systems during regeneration. Here we also discuss how these cells are recruited or differentiate to promote the metastatic process, and how these insights lead to therapeutic strategies to block the pro-metastatic immune cells.

Immune Escape
Tumors develop numerous methods to avoid detection and eradication by the immune system through the modulation of tumor-infiltrating leukocytes, such as immunoregulatory myeloid cells, regulatory T (T\(_{\text{reg}}\)) cells, T helper type 17 (T\(_{\text{H17}}\)) cells, and regulatory B (B\(_{\text{reg}}\)) cells [FIG. 2a]. The impaired immune rejection may increase the number of malignant tumor cells that will egress from the primary tumor. It will also enhance the survival of tumor cells disseminating to the metastatic site (see “Tumor cell survival”).

Myeloid cells.
Most solid tumors recruit macrophages through the production of various cytokines and chemokines, such as colony stimulating factor-1 (CSF-1), vascular endothelial growth factor (VEGF), semaphorin 3A (Sema3A), CC\(^-\)chemokine ligand 2 (CCL2) and CXC-chemokine ligand 12 (CXCL12)\(^12\)-\(^14\). These tumor-associated macrophages (TAMs) are reported to suppress CD8\(^+\) T
cell infiltration and anti-tumor immunity in primary tumors. Mechanistically, TAMs are known to suppress cytotoxic activity of CD8+ T cells directly through their expression of inhibitor ligands such as PD-L1 and B7-H4, or indirectly via CCL22-mediated recruitment of Treg cells (see below). TAMs can also mediate their immunoregulatory functions through other molecules (reviewed in REF.19), although the precise mechanisms underlying TAM-mediated immune suppression in vivo are still unknown. Like TAMs, tumor-associated neutrophils (TANs) can reduce CD8+ T cell activity and increase primary tumor growth, although further studies are required to reveal whether their immunoregulatory functions are required for the tumor metastasis. A type of immature myeloid cells that express CD11b and Gr-1 (Ly-6G/Ly-6C) are also found in the tumor microenvironment. These cells can suppress proliferation and cytokine production of T cells in vitro, and are thus referred to myeloid derived suppressor cells (MDSCs) [Box1]. In a pancreatic tumor mouse model, tumor-derived granulocyte-macrophage colony stimulating factor (GM-CSF) was shown to recruit MDSCs to the primary tumors, where they suppressed CD8+ T cell cytotoxicity. In tumors developed following subcutaneous injection of 3LL lung cancer cells, reduction in MDSC numbers through the administration of neutralizing antibodies specific for Gr-1 or Ly-6G increased the frequency and function of NK and CD8+ T cells and number of apoptotic tumor cells. The treatments also reduced primary tumor growth and metastasis from skin to the lung, suggesting that increased survival of primary tumor cells via immune suppression enhances frequency of successful egress from the primary site. However, the contribution of MDSCs to immune evasion by primary tumors needs to be more precisely defined as these neutralizing antibodies also deplete other myeloid cells, such as monocytes and neutrophils. Neutralization of Gr-1 might also affect the number of tumor-infiltrating plasmacytoid dendritic cells (pDCs) that are reported to increase the numbers of MDSCs and Treg cells in the transplanted breast tumor and in turn increase tumor bone metastasis by reduced CD8+ T cell cytotoxicity.

**Treg Cells.**

Thymus-derived Treg cells are immunosuppressive cells that express interleukin-2 (IL-2) receptor α-chain CD25 and transcription factor forkhead box P3 (FoxP3). In a spontaneous metastasis model of breast cancer, the suppression of lung metastasis was accompanied by reduced numbers of CD4+CD25+ cells in the primary tumors, suggesting that Treg cell recruitment to the primary tumor is necessary for tumor metastasis. This Treg cell recruitment has been shown to depend on tumor-derived CCL22 or CCL5, depending on the tumor model used. Increased secretion of prostaglandin E2 from breast cancer cells also recruits Treg cells to the primary tumor, which increases CD8+ T cell apoptosis and cancer cell bone metastasis. Cytokines also contribute to Treg accumulation in tumors and increased metastasis. Furthermore, immune cell-derived tumor necrosis factor (TNF) was shown to enhance lung metastasis of melanoma cells through expansion of Treg cell numbers. Tumor-derived galectin-1 can promote systemic immunosuppression by regulating expansion and function of Treg cells probably through increasing expression of T-cell regulatory molecule LAT, which enhances breast cancer metastasis.
Although the immune-suppressive mechanisms of T$_{reg}$ cells in the tumor settings are not clear\textsuperscript{31}, it has been suggested that T$_{reg}$ cells can induce apoptosis of NK cells by secretion of $\beta$-galactoside-binding protein (\textbeta GGBP), which increases lung metastasis of breast cancer cells\textsuperscript{32}. It is also reported that T$_{reg}$ cells promote experimental lung metastasis through suppression of NK cell cytotoxicity by direct cell to cell contact and through transforming growth factor $\beta$ (TGF-$\beta$) secretion\textsuperscript{33}. These studies suggest that NK cells can significantly eliminate tumor cells such that their dissemination is restricted but that tumor-induced T$_{reg}$ cell recruitment nullifies this suppression.

**Other adaptive immune cells.**

Recent studies indicate that adaptive immune cells can regulate the immune suppressive phenotype of MDSCs and T$_{reg}$ cells. In a genetically engineered mouse (GEM) model of breast cancer caused by the mammary epithelial expression of Polyoma Middle T (PyMT), loss of TGF-$\beta$ signaling in tumor cells recruits MDSCs to the tumor through CXCL1 and CXCL5 secretion\textsuperscript{34}. The MDSCs secrete IL-6, TGF-$\beta$, and IL-23 that accelerate the accumulation of Tr17 cells. IL-17 from Tr17 cells in turn promotes further recruitment of and immunosuppressive gene expression in the MDSCs, which increases lung metastasis\textsuperscript{34}. In the subcutaneous EL4 lymphoma model, IL-17 from Tr17 cells was shown to recruit MDSCs to the tumor site by prompting tumor-associated fibroblasts to secrete granulocyte-colony stimulating factor (G-CSF), which promotes the immunosuppressive function of the immature myeloid cells\textsuperscript{35}. Thus, Tr17 cell recruitment to tumors appears to indirectly regulate suppress anti-tumor immunity in the primary sites, and to enhance metastasis. On the other hand, Tr17 cells can promote anti-tumor immune responses through recruitments of dendritic cells and cytotoxic cells in some mouse models\textsuperscript{36}. Since Tr17 cells exert their biological activities by interacting with other immune cell types, their roles in tumor metastasis might be affected by the tumor microenvironment and thus depend on tumor types. Indeed, Il17 deficiency increases metastasis in a lung cancer mouse model\textsuperscript{37} but suppresses it in a melanoma or colon cancer model\textsuperscript{38}. B$_{reg}$ cells, which are a specific B cell population of B cells expressing CD25 and B220, may also promote tumor metastasis through immune suppression. Intravenous injection of 4T1 breast cancer cells increases the number of circulating CD25$^+$ B cells, and their depletion by anti-B220 antibody reduces lung metastasis. The role of B$_{reg}$ cells in lung metastases may be to induce T$_{reg}$ cell conversion, as B$_{reg}$ cells have been shown to suppress CD4$^+$ and CD8$^+$ T cell proliferation in vitro by inducing the conversion of CD4$^+$ T cells to FOXP3$^+$ T$_{reg}$ cells via TGF-$\beta$ secretion\textsuperscript{38}.

The above experimental data suggest that tumor cells are eliminated by cytotoxic immune cells before proceeding through the metastatic cascade. Thus recruitment of immune regulatory cells to the primary site appears to be an essential preparatory step for tumor metastasis. In addition to this environmental change at the primary site, some tumors also modulate distant tissues to become tumor-supporting environments before metastasis.

**Pre-metastatic niche formation**
In some cases, primary tumors produce systemic factors to establish metastasis-promoting environments at the sites of future metastasis. Although the cells responsible for the pre-metastatic niche formation are poorly characterized, most results suggest a significant contribution of bone marrow-derived immature myeloid cells [FIG. 2b].

In mice with subcutaneous tumors developed by LLC lung cancer or B16 melanoma cells, bone marrow-derived cells including CD11b+ myeloid cells accumulate in the lungs before metastatic tumor cells are detected, which promotes future metastasis of the cancer cells to the lung[39]. These bone marrow-derived cells express VLA4 (integrin α4β1) and are recruited by its ligand fibronectin deposited at pre-metastatic sites in response to primary tumor-derived factors[39]. In the same models, recruitment of CD11b+ cells to the pre-metastatic lung is also facilitated by expression of myeloid chemoattractants S100A8 and A9 that are induced by VEGFA, TNFα, and TGFβ from distant primary tumors[40], and by serum amyloid A3 induced by the S100A8/A9[41] at the pre-metastatic lung. B16 melanoma derived factors also activate sphingosine-1-phosphate receptor-1 (S1PR1) and its downstream molecule STAT3 in the myeloid cells at the pre-metastatic lung. Since treatment of mice with siRNA against Stat3 or S1pr1 eliminates the myeloid cell clusters, constitutive activation of S1PR1-STAT3 signaling is required for myeloid cells to maintain the pre-metastatic niche[42]. Pre-metastatic niche formation is also reported in other tumor types other than the LLC and B16 tumors. For example, xenografts of human breast cancer cell line MDA-MB-231 induce CD11b+ cell accumulation in the pre-metastatic lung through secretion of lysyl oxidase (LOX) that crosslinks collagen IV and thereby increase adherence of CD11b+ cells[43]. Accumulating reports have revealed that various soluble factors and exosomes from primary tumors can mobilize bone marrow cells to the circulation[7,44]. Once released to the circulation, these immature cells are led to the future metastatic sites by the mechanisms described above.

The above studies also indicate that immature myeloid cells are a major component of pre-metastatic niche because subsets of the recruited CD11b+ cells also express a stem/progenitor marker CD117, and reduced accumulation of these cells prevents later tumor cell metastasis[39,43]. On the other hand, recent studies have revealed contributions of more differentiated myeloid cells to the establishment of pre-metastatic niche. For example, the recruitment of CD11b+Ly6C+ monocytes to the pre-metastatic lung by CCL2 also enhances pulmonary metastasis of B16 cells[45], and CD11b+CD68+F4/80+ macrophages recruited to the lung by primary tumor-induced fibrin clots establish the pre-metastatic niche that enhances breast cancer metastasis[46].

The facts that pre-metastatic myeloid cell accumulation fosters subsequent tumor metastasis raise a question how these cells support disseminating cancer cells. It has been reported that bone marrow derived cells recruited to the pre-metastatic lung form clusters and promote the adherence and growth of subsequently disseminating tumor cells[39]. These cells also secrete metalloproteinase 9 (MMP9) that may promote tumor cell invasion[47]. Interestingly, a recent study indicates that systemic factors from hypoxic breast cancer cells increase CD11b+ cell accumulation and reduce cytotoxic functions of NK cells at the pre-metastatic lung[48]. As discussed above,
myeloid cells especially MDSCs are known to suppress immune reactions. It is therefore likely that the recruited myeloid cells establish a pre-metastatic immunosuppressive environment in the lung to promote tumor metastasis.

Another question is whether non-myeloid cells can also play a role in the establishment of pre-metastatic niches. Tumor-specific CD4+ T cells are reported to induce pre-metastatic niches in the bone by secreting RANKL and thus increasing osteoclastogenesis, which promotes bone metastasis of breast cancer cells\(^{49}\). Interestingly, conditioned medium from 4T1 breast cancer cells increases the amount of Treg cells in the pre-metastatic lung by inducing CCL22 expression in the lung stroma\(^ {32}\). This is an emerging area in this field and further studies are required to evaluate the importance of non-myeloid cells as well as their interactions with myeloid cells in pre-metastatic niche formation.

In conclusion there is substantial evidence that myeloid progenitors are the central cell population that establish the pre-metastatic niche. Since macrophages in the metastatic sites originate from bone marrow-derived monocytes\(^ {50}\), it is possible that primary tumor-derived factors recruit these myeloid progenitors at various differentiation stages to pre-metastatic sites where they can rapidly differentiate into metastasis associated macrophages (MAMs) following tumor cell arrival thereby enhancing tumor cell extravasation and survival including that from immune cell attack (see below). This pre-loading of cells capable of enhancing extravasation and tumor cell survival may provide the mechanism behind the metastasis promoting activity of these pre-metastatic niches.

**Tumor cell egress**

To establish metastasis foci, tumor cells need to egress from the primary sites by migration through the stroma and intravasation into blood vessels whose numbers are increased by de novo development in the tumor microenvironment. Accumulating evidence has indicated that myeloid cells such as TAMs and TANs contribute to these early steps of metastasis [FIG. 3]. Recent studies have revealed key signaling pathways that promote tumor intravasation and the contribution of environmental factors that prompt myeloid cells to help in this process, Direct involvement of lymphocytes in tumor cell egress has not reported although CD4+ T cells do contribute to this process through changing macrophage phenotypes.

**Contribution of macrophages.** Ablation of macrophages by genetic loss of the major lineage regulator of macrophages, CSF-1, impairs the onset of angiogenesis\(^ {51}\), and markedly suppresses lung metastases\(^ {52}\) in the PyMT model of breast cancer. This TAM-induced angiogenesis is promoted in part through their VEGF expression\(^ {53}\). Interestingly, macrophage-selective deletion of Ets2, a direct effector of the CSF-1 signaling pathway, increases expression of anti-angiogenic factor such as thrombospondin (THBS) -1 and THBS-2 in TAMs, thereby suppressing PyMT breast cancer lung metastases by inhibiting angiogenesis in the primary tumors\(^ {54}\). Macrophage-selective deletion of Wnt7b also suppresses the angiogenic switch in the PyMT primary tumors and reduces lung metastasis\(^ {55}\). Furthermore, macrophages were
shown to associate with new blood vessels induced by endothelial cell-derived angiopoietin 2 (ANG2), thereby enhancing angiogenesis and metastatic dissemination of the cancer cells\(^{36}\). These data suggest that TAMs help tumor cell egress by increasing the density of leaky blood vessels that in turn may also provide pro-tumoral factors such as CXCL8 (also known as IL-8) and CXCL2 that increase invasiveness of cancer cells\(^{37}\).

TAMs also directly help local invasion and intravasation of tumor cells. Intravital imaging reveals that cancer cells in PyMT tumors invade surrounding tissues together with TAMs\(^{38}\) and that tumor cell intravasation occurs in association with perivascular TAMs\(^{39}\). In these processes, cancer cells secrete CSF-1 to promote macrophage mobility and their secretion of epidermal growth factor (EGF). The TAM-derived EGF in turn activates EGF receptor in cancer cells, which enhances invasion capability and motility through increasing invadopodium formation and matrix degradation and thereby accelerates invasion and intravasation of cancer cells\(^{40}\).

Mechanistically, activation of Wiskott-Aldrich syndrome protein (WASP) by the CSF-1 receptor (CSF-1R) promotes TAMs to migrate toward CSF-1-producing cancer cells and to release EGF from their cell surface\(^{61}\). Genetic deletion of steroid receptor coactivator-1 (SRC-1) also reduces tumor cell CSF-1 production and thereby macrophage numbers and their paracrine action on tumor cell invasion in the primary tumor, which suppresses lung metastasis of PyMT cancer cells without affecting primary tumor growth\(^{62}\). Macrophage-dependent tumor invasion is also triggered by heregulin \(\beta 1\) (HRG\(\beta 1\)) and CXCL12 according to the tumor type through this EGF-dependent mechanism\(^{63}\). These data that has been extended to human breast cancers suggest that EGF/CSF-1 loop, activated by multiple signals from the microenvironment, plays a central role in tumor intravasation. TAMs also promote tumor egress and metastasis through the secretion of CCL18 and osteonectin (also known as SPARC), which modulates the extracellular matrix adhesive properties of cancer cells\(^{64,65}\). In a pancreatic tumor model, TAMs were shown to produce cathepsin B and cathepsin S, which promote cancer cell egress and angiogenesis\(^{66}\). These results indicate that TAMs help tumor cells to enter the blood vessels by cytokine secretion and extracellular matrix remodeling.

Although mechanisms by which macrophages acquire these pro-metastatic properties have not been fully addressed, recent studies shed light on the role of environmental factors in the primary tumors. For example, CD4\(^+\) T cell-derived IL-4 prompts macrophages to express EGF\(^{67}\) and tumor-derived IL-4 increases cathepsin B and cathepsin S production in TAMs\(^{66}\) depending on tumor model. In the PyMT mice, loss of neuropilin-1, a Sema3A receptor, in macrophages prevents TAM infiltration into the hypoxic tumor area, which suppresses their pro-angiogenic functions and thus delays tumor progression and lung metastasis\(^{68}\). Administration of COX-2 inhibitor also suppresses expression of pro-metastatic molecules such as VEGF-A and MMP9 in TAMs and inhibits lung metastasis of breast cancer cells\(^{69}\). A recent study shows that activation of IL-1 receptor is required for monocytes from renal cell carcinoma (RCC) patients and for TAMs from human RCC xenografts to promote tumor angiogenesis and invasion\(^{70}\). GM-CSF from human breast cancer cells also skew macrophages to TAM-like phenotype in vitro, and is required for tumor metastasis in a xenograft model\(^{71}\). In contrast,
overexpression of histidine-rich glycoprotein (HRG) in cancer cells skews TAM polarization from a pro-tumorigenic to a tumor-inhibiting phenotype by down-regulation of placental growth factor (PIGF) expression, which normalize the leaky blood vessels and inhibits metastasis\textsuperscript{72}. Therefore the data suggest that the tumor microenvironment polarizes recruited macrophages from a tumor reactive to tumor promoting state. These preclinical studies give rise to an idea that pro-metastatic function of TAMs can be suppressed by targeting signaling pathways that regulate TAM polarization\textsuperscript{73}. The roles of macrophages in normal developmental process might be useful to find such targets, because gene expression profiles of TAMs \textit{are} close to that of trophic macrophages in developmental tissues\textsuperscript{74}.

In support of these pre-clinical findings, direct contact between perivascular TAMs, endothelial cells and cancer cells is found in human breast cancer specimens, and high number of this histological landmark called as “tumor microenvironment for metastasis (TMEM)” is associated with increased risk of metastasis\textsuperscript{75}. Regardless of clinical subtypes or tumor grade, TMEM score in breast cancer specimens correlates with tumor cell expression of Mena\textsuperscript{76} that strongly potentiates EGF signaling in cancer cells\textsuperscript{76} and is required for macrophage-induced \textit{in vitro} intravasation of human breast cancer cells\textsuperscript{77}. These data indicate that interaction between perivascular TAMs and cancer cells accelerates tumor intravasation and thereby increases the risk of systemic tumor cell dissemination. As we discuss later, TAMs also play pivotal roles in supporting the disseminating cancer cells.

\textbf{Role of neutrophils and MDSCs.} Highly metastatic human fibrosarcoma and prostate cancer cells recruit neutrophils to primary tumors, which increases angiogenesis and intravasation of cancer cells through secretion of MMP9\textsuperscript{78}. In a xenograft model of intrahepatic cholangiocarcinoma, tumor-derived CXCL15 recruits neutrophils that promote lung metastasis of the cancer cells\textsuperscript{79}. Similarly UV exposure of melanoma in a GEM model increases neutrophil recruitment to the primary tumor by high mobility group box 1 (HMGB1) derived from the UV-damaged keratinocytes. These neutrophils enhances angiogenesis, cell migration towards endothelial cells, and hence lung metastasis\textsuperscript{80}. However, the involvement of neutrophils in tumor metastasis is controversial, as depletion of neutrophils increases the number of lung metastasis foci in a spontaneous metastasis model of breast cancer\textsuperscript{81}. Furthermore, neutrophils isolated from tumor-bearing mice can kill the tumor cells \textit{in vitro} by generating H\textsubscript{2}O\textsubscript{2}, and thereby suppress lung metastasis \textit{in vivo} when adoptively transferred to the animals that has received intravenous injection of the cancer cells\textsuperscript{81}. Interestingly, TGF-\(\beta\) has been shown to induce a switch from a tumoricidal to a tumor promoting phenotype in neutrophils within subcutaneously developed mesothelioma\textsuperscript{20}. It is therefore possible that the pro-metastatic functions of neutrophils are regulated by specific environmental factors in a similar fashion to TAMs.

In the PyMT tumor bearing mice, loss of TGF-\(\beta\) signaling in tumor cells increases CXCL5 secretion and recruits CD11b\textsuperscript{+}Gr-1\textsuperscript{+} MDSCs to the invasion front of the tumors. The CD11b\textsuperscript{+}Gr-1\textsuperscript{+} cells promote tumor cell invasion \textit{in vitro} through the expression of MMPs\textsuperscript{82}. In the 4T1 mammary tumor model, macrophage migration inhibitory factor (MIF) produced by cancer cells
increases the number of CD11b^+Ly6C^hi cells within the primary tumors, and depletion of the CD11b^+Ly6C^hi cells by anti-Gr1 antibody treatment reduces lung metastasis of cancer cells. In a GEM model of melanoma, CXCL15 recruits CD11b^+Gr1^hi cells to the primary tumor, and depletion of the cells by anti-Ly6G antibody treatment reduces dissemination of melanoma cells. Although these results suggest that MDSCs promote local invasion and therefore metastasis, it is difficult to evaluate the exact contribution of MDSCs to the process because CD11b and Gr-1 are not specific markers for MDSCs as discussed above. Since CD11b^Gr-1^ cells can differentiate into CD11b^F4/80^ macrophages at the tumor sites when transferred to tumor-bearing mice, depletion of CD11b^Gr-1^ cells might also affect TAM-dependent processes.

Data supporting the role of neutrophils in cancer invasion and extravasation therefore is conflicting with results suggesting both tumor promoting and inhibiting effects. Part of this problem may be definition since MDSCs and neutrophils are often defined by the same marker (Gr-1) and antibody ablation therefore might eliminate or change the balance of both populations. Furthermore, some models such as the 4T1 breast cancer one produces excessive amounts of the neutrophil growth factor G-CSF that can cause a dramatic shift in circulating and tumor associated neutrophils not usually found in tumors. Thus caution needs to be exercised especially when the tumor models involve xenografted ones that consist of homogenous highly selected cells without the complex cellular ecology and tumor cell heterogeneity found in spontaneously evolving cancers.

**Tumor cell survival**

Circulating tumor cells arrest in microvessels in the distant tissues. These cancer cells need to survive in the vessel as well as at the disseminating site to develop metastasis foci. Platelets, macrophages, and Treg cells are reported to protect the disseminating cancer cells from the stress of immune attack and the stress of a non-hostile environment [FIG. 4a].

It has been suggested that platelets have a role in metastatic processes after intravasation. Indeed, intravenously injected melanoma cells develop fewer metastatic foci in the lung of platelet-depleted mice than in normal mice. Moreover, genetic loss of Gαq (a G protein essential for platelet activation), prothrombin (an essential protease precursor for fibrin formation), or fibrinogen (a fibrin precursor important for clot formation) reduces the number of residual cancer cells in the lung 24 hours after intravenous injection. The precipitous loss of embolus cancer cells in these gene-targeted mice is rescued by NK cell ablation. These results indicate that platelet activation and the resultant fibrin clot formation promotes the early survival of cancer cells that are lodged at the metastatic sites by shielding them from NK cells.

Clot formation also enhances tumor cell survival by recruiting macrophages. Genetic or pharmacological inhibition of platelet coagulation reduces cancer cell survival in the lung, as well as the recruitment of CD11b^CD68^F4/80^ macrophages to the tumor-challenged lung. Ablation of these CD11b^ macrophages suppresses tumor cell survival without affecting
clot formation indicating they are sequentially in a linear pathway\textsuperscript{46}. Mechanistically, tumor-initiated clot formation activates endothelial cells to express vascular cell adhesion molecule-1 (VCAM-1) and vascular adhesion protein-1 (VAP-1), which recruits macrophages\textsuperscript{50}. The macrophages expressing integrin $\alpha 4$ (CD49d) bind to cancer cells and transmit survival signals via VCAM-1 to cancer cells, which increases lung metastasis\textsuperscript{91}. A recent report also indicates that efficient tumor cell survival at the metastatic site requires the selectin ligand-mediated recruitment of Ly6C$^+$ monocytes\textsuperscript{92}, progenitor cells of macrophages in the metastatic sites\textsuperscript{50}.

Although the roles of T\textsubscript{reg} cells at metastatic sites are not clear, they may also involve in tumor cell survival. CD4$^+$CD25$^+$FoxP3$^+$ cells isolated from metastatic lymph nodes of melanoma patients inhibit proliferation and cytokine production of CD4$^+$ and CD8$^+$ T cells in vitro\textsuperscript{93}. Likewise, CD4$^+$CD25$^+$FoxP3$^+$ cells from malignant ascites of ovarian cancer patients inhibit T cell proliferation, cytokine production, and cytotoxicity\textsuperscript{18}. It is therefore likely that T\textsubscript{reg} cells at the metastatic sites protect disseminated cancer cells from immune responses. It is noteworthy that T\textsubscript{reg} cells in a primary mammary tumor secrete RANKL, which activates its receptor RANK on cancer cells and promotes lung metastasis without affecting primary tumor growth. Although the precise mechanism(s) is still unknown, activation of RANK increases survival of circulating metastasis-initiating cancer cells\textsuperscript{26}.

As discussed above, pre-clinical studies suggest that immune cells help tumor cells at the metastatic sites by evoking survival signals and by protecting them from immune attack. However, the precise mechanisms underlying such supportive functions are still unclear. A recent study shows that circulating tumor cells from breast cancer patients contain a malignant population that can initiate metastatic foci in mice. These cells express high level of CD47 and hepatocyte growth factor receptor MET\textsuperscript{94} that can inhibit phagocytosis by innate immune cells and suppress apoptosis of cancer cells respectively. This clinical study might provide clues to investigate the contributions of these molecules to disseminating cancer cell survival and possible involvement of immune cells.

**Extravasation and persistent metastatic growth**

To establish metastatic foci the arrested tumor cells must extravasate and grow at distant sites. It has reported that only a very few of the circulating tumor cells establish metastatic foci in cancer patients despite thousands being released into the circulation every day\textsuperscript{95}. Thus the metastatic steps after tumor egress (i.e., survival, extravasation and metastatic growth) are rate-limiting processes for metastasis. Macrophages, neutrophils, and platelets have been reported to promote these inefficient steps of metastasis [FIG. 4b], whereas an active contribution of lymphocytes has not been reported.

**Contribution of metastasis-associated macrophages.** In the lung of the PyMT mice with spontaneous metastatic lung foci, there are at least two macrophage populations: “resident” CD11c$^+$ alveolar macrophages, which also exist in the normal lung and have a role in the host defense\textsuperscript{85} and “recruited” MAMs. MAMs are characterized by the expression of CD11b,
VEGFR1, CXCR3 and CCR2, and the absence of Gr-1, TIE-2 and CD11c. Since depletion of CD11b+ macrophages but not CD11c+ macrophages reduces the number and size of lung foci developed by intravenously injected breast cancer cells, the recruitment of the MAMs is essential for the dissemination and growth of tumor cells at the metastasis site.

*Ex vivo* imaging of the metastatic lung has revealed that macrophages directly contact with extravasating cancer cells, and loss of these macrophages dramatically reduces the number of cancer cells that have emigrated from the blood vessels. Macrophage-selective deletion of *Vegfa* reduces lung metastasis *in vivo*, and suppresses extravasation of cancer cells and permeability of endothelial monolayers *in vitro*, a process required for efficient metastasis. These data indicate that MAMs promote extravasation of cancer cells at least in part through secretion of VEGF-A. In addition to extravasation, MAMs also promote persistent growth of metastatic foci because pharmacological or genetic depletion of macrophages following tumor cell seeding suppresses the lung metastatic loads, and enhance survival of mice. It is reported that enhanced angiogenesis by macrophages via Tie-2-mediated signaling promote outgrowth of micro-metastatic foci in some models. MAMs also increase survival of the disseminated MDA-MB-231 human breast cancer cells through engagement of VCAM-1 on tumor cells, which results in signaling through AKT for cell survival.

Macrophages are diverse cells and consist of several subtypes that play distinct roles in the tumor microenvironment and thus MAMs are another distinct subpopulation that are differentiated in the metastatic environment and acquire pro-metastatic functions. Adoptively transferred inflammatory monocytes preferentially migrate to the metastatic lung rather than the primary tumors and differentiate into MAMs. Neutralization of CCL2, one of the CCR2 ligands, suppresses this recruitment of inflammatory monocytes and MAM accumulation in the foci, thereby reducing tumor extravasation. Of note, a similar population of macrophages in the liver that are recruited by CCL2 and are required for liver metastasis from colorectal cancers has recently been described.

A recent clinical study shows that high number of circulating inflammatory monocytes correlates with shortened survival of the patients with pancreatic cancer. The above-mentioned pre-clinical studies indicate that the inflammatory monocytes are recruited by the CCL2-CCR2 axis to the site of metastasis where they differentiate into MAMs and promote extravasation and survival of tumor cells. These roles of MAMs at metastatic sites have recently been defined and thus further studies are required to understand the mechanisms of action of the metastatic promoting activities of MAMs as this population is an attractive therapeutic target.

**Contribution of other myeloid cell types.** Recent studies suggest that neutrophils enhance entrapment and retention of circulating tumor cells at the metastatic sites. Adoptive transfer of neutrophils into mice an hour after tumor cell injection increased cancer cell retention in the lungs by enhancing extravasation. In this model, entrapped cancer cells secrete CXCL8 to promote neutrophil–cancer cell interaction by increasing neutrophil expression of Mac-1 (integrin αM), which binds to ICAM-1 on cancer cells. The
attachment to the neutrophils via Mac-1/ICAM-1 interaction is also required for lung cancer cells to adhere to liver sinusoid and form metastatic foci\textsuperscript{102}. Neutrophils also produce a unique structure called the neutrophil extracellular trap (NET) that is composed of extruded DNA and antimicrobial proteins. In a mouse model of post-operative infection, injected cancer cells are trapped in the NET that had formed in liver and lung capillaries, which promotes development of micro-metastases\textsuperscript{103}.

Genetic loss of Munc13-4, which is a protein essential for the secretion of granules containing ATP by platelets, markedly reduces the numbers of extravasating melanoma cells and also vascular permeability\textsuperscript{104}. Moreover, genetic ablation of P2Y\textsubscript{2}, a purinergic receptor activated by ATP, prevents vascular leakage and suppresses tumor extravasation. Since reduced expression of P2Y\textsubscript{2} in endothelial cells suppresses platelet-dependent tumor extravasation \textit{in vitro}, these results indicate that platelets loosen the endothelial barrier by releasing ATP-containing granules, which enhances extravasation of cancer cells\textsuperscript{104}. Moreover, platelets promote extravasation of colon and breast cancer cells by inducing epithelial–mesenchymal-like transition through secretion of TGF-β and through a direct contact with cancer cells that increases their survival\textsuperscript{105}.

Each of these studies described above has been performed in isolation and in different models. However, the effects of different immune cell types in promoting extravasation and survival of circulating tumor cells are similar. Since many of these immune cells in other contexts work in concert, it is likely that they do during metastatic seeding. Noteworthy are recent studies that indicate platelets arrest tumor cells in capillaries and provide a survival signal, which in turn allows a chemotactic gradient of CCL2 to be established to recruit IMs that differentiate to MAMs that promote the extravasation step and survival through cell-to-cell contact\textsuperscript{46,50,97}. Multicellular imaging \textit{in vivo} will be important to determine the spatial and temporal relationships of these immune cell types and whether particular combination enhance metastatic frequency.

\textbf{Therapeutic targeting pro-metastatic immune cells}

Eradication of metastatic tumors is the holy grail of tumor immunology but so far until recently there has been little success. Recently however, these recent significant advances in antitumor therapy have been attained using augmentation of tumoricidal T cell responses. This strategy is mostly based on the fact that T cell responses are negatively regulated in tumors by inhibitory receptors such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD1). Thus neutralization of their signals will enhance T-cell responses to eliminate tumor cells. Indeed, antibodies against CTLA4 or PD1 have been clinically tested and have resulted in prolonged overall survival of cancer patients [Box2]. Similarly, a recent preclinical study suggests that inhibition of T\textsubscript{reg} cells and MDSCs by inactivation of p110δ isoform of phosphoinositide 3-kinase (PI(3)Kδ) induces immune-mediated rejection of various types of tumors\textsuperscript{106}. Encouragingly, a clinical trial shows that idelalisib, a selective PI(3)Kδ inhibitor, is tolerated and effective in chronic lymphocytic leukaemia\textsuperscript{107} although its therapeutic effect on metastatic disease has not been tested yet.
Furthermore, a recent study demonstrates that a minor CD103+ dendritic cell subset in the PyMT tumor can efficiently activates CD8+ T cells and is expanded by FLT3L, suggesting that expansion of the CD103+ dendritic cells can be another strategy to improve T cell therapy efficacy. Moreover, clever engineering of hematopoietic stem cells with vectors allowed tumor-infiltrating monocytes and macrophages to express Interferon-α inhibited MDA breast cancer and PyMT tumor growth and mouse experimental metastases associated with an increased numbers of T cells infiltrating into the metastatic lesions. The successes of these clinical and pre-clinical trials imply that it is possible to enhance antitumor immunity by enhancing T cell activation or blockade of immunosuppressive cells and that these strategies alone or in combination might have clinical effects in the treatment of metastases.

Another possible therapeutic strategy is withdrawal of microenvironmental support for metastatic cancer cells by targeting pro-metastatic immune cells, in particular macrophages. Therefore, the signaling molecules that mediate macrophage recruitment and/or activation described above are all potentially important targets for the treatment of metastatic disease. A recent clinical trial shows that neutralizing antibody against CSF-1R reduces TAM numbers and gives marked clinical benefit to patients with diffuse-type giant cell tumor, a type of sarcoma disease caused by aberrant over-expression of CSF-1. A phase I trial using an oral CSF-1R inhibitor on advanced and metastatic cancer was recently completed (ClinicalTrials.gov; ID: NCT01316822). Moreover, several phase I clinical trials of CSF-1 and CSF-1R inhibitors are in the stage of recruiting patients for advanced solid tumors (ClinicalTrials.gov; ID: NCT01316822 ARRY-382, NCT01346358 IMC-CS4, NCT01444404, and NCT01004861). In addition, the dramatic response to a CSF-1R inhibitor found in a GEM mouse model of glioma with evidence that macrophages are re-polarized by GM-CSF to become anti-tumoral is resulting in these inhibitors entering into a clinical trial in glioma. All together these trials may provide evidence for the benefits of targeting CSF-1R pathways in macrophages for the treatment of metastatic disease.

The CCL2-CCR2 pathway is also an attractive target since it is one of the major chemokine signaling pathways that mediates TAM recruitment in pre-clinical models. Although humanized monoclonal CCL2 neutralizing antibody (CNT0888) is ineffective in suppressing serum CCL2 level or tumor progression, a recent clinical trial shows that blockade of CCR2 has a potential to suppress metastatic growth of cancer. In this trial, anti-CCR2 antibody (MLN1202) reduced the levels of urinary N-telopeptide of type I collagen (uNTx), a marker of bone resorption induced by metastatic cancer cells, in 14 out of 43 patients with bone metastases (ClinicalTrials.gov ID: NCT01015 560). Interestingly, trabectedin, an approved drug used in the clinic to treat human ovarian cancer and soft tissue sarcoma, modulates TAM recruitment by inhibiting CCL2 expression, suggesting that the therapeutic effects of this drug might be exerted through inhibiting TAM recruitment. However, application of CCL2 signal inhibitors should be considered carefully because cessation of anti-CCL2 treatment causes influx of monocytes into the metastatic sites that enhances local angiogenesis and metastatic outgrowth and thereby increases mortality.
Although clinical trials targeting pro-metastatic immune cells are still limited, remarkable suppressive effects observed in the above mentioned studies strongly suggest that macrophage-targeting therapy may be an effective strategy. Thus, a better understanding of mechanisms underlying macrophage recruitment and activation in pre-clinical models of specific cancer type may guide more rational design for future trials. Of note, numerous lines of evidence in pre-clinical models indicate that macrophage targeting such as neutralization of CSF-1 enhances the efficacy of conventional therapeutic modalities used in treating metastatic disease, including anti-angiogenesis therapy and cytotoxic chemotherapy. Interestingly, a recent study shows that TAMs in human breast cancer express IL-10 and blockade of IL-10 is equivalent to CSF-1 neutralization in enhancing efficacy of chemotherapy. These particular immunosuppressive roles of infiltrating macrophages also suggests that targeting specific components of the system might result in less toxicity due to auto-immune responses than blocking inhibitory receptors such as CTLA4 on all expressing cells. Thus it is likely that combining these conventional therapeutic modalities with immunotherapy and macrophage-modulating therapy may eventually lead to effective therapeutic strategies to control or eradicate metastatic cancer. However, most of these preclinical studies have not been tested specifically in metastatic tumors or with metastasis as an end-point.

Conclusions and perspectives

Many animal studies indicate that the recruitment of immunoregulatory cells to the primary tumor site protects cancer cells from cytotoxic cells and that this increases the probability of tumor cell survival and dissemination. Detailed knowledge about the mechanisms underlying immunoregulatory cell recruitment and their suppressive functions in the primary tumor and metastatic sites will lead to more effective immunotherapies. Current results indicate that individual suppressor cell types are recruited to the primary or metastatic sites by distinct chemoattractant: for example, CCL2 recruits monocytes and macrophages, CXCL1 and CXCL5 recruit MDSCs, and CCL5 and CCL22 recruit T_{reg} cells. It is therefore possible that certain types of tumor predominantly recruit specific type of suppressor cells to evade anti-metastatic immune responses. Thus, therapeutic approaches will need to assess the immune cell profile in individual cancers so that drug targeting can be precisely tailored to maximize response.

The data discussed here also indicate direct roles for myeloid cells, particularly macrophages, in promoting every step of the metastatic cascade. Thus macrophages are particularly attractive targets for therapeutic intervention, as their diploid nature and consequent low mutation rates, suggest that they will not easily evolve drug resistance. Current therapies, however, are likely to be limited as they either target all macrophages (for example, CSF-1R inhibition) or target essential processes such as monocyte egress from the bone marrow (for example, CCR2 inhibition). Thus a more detailed understanding of the effector mechanisms and essential cellular interactions between the various cell types in the tumor microenvironment is needed to allow for more precise therapeutic intervention.
Cancer cells exhibit high mutation rates that generate very heterogeneous tumors containing innumerable mutants. These mutant cells provide a reservoir of drug resistant cells able to prosper after the selective pressure of chemotherapy. The immune system however, can evolve equally rapidly and is capable of eliminating very large cell masses as seen for example following fetal death when tolerance to the allogeneic fetus is broken. Thus given the high mutation rate of cancer cells immunotherapy maybe the only effective strategy for complete elimination of already drug resistant metastatic cells. Immunotherapy will by necessity require targeting immunosuppressive mechanism provided by the recruited immune (and perhaps other resident) cells. Of course upon targeting tumor cells, owing to their high mutation rates, will evolve to replace their environmental support, perhaps by recruiting other immune cell types such as monocytes and/or neutrophils, as has been suggested in the 4T1 transplantable model of breast cancer following CSF-1R inhibition. Consequently, modeling the evolution and ecology of the tumor microenvironment with a particular emphasis on tumor heterogeneity holds promise for effectively targeting different aspects of the supporting tumor microenvironment, sequentially or synergistically. Such approaches can remove the microenvironmental support for metastatic tumor cells and at the same time enable immune targeting of the now vulnerable tumor cells. Such strategies together with those such as radiation or chemotherapy to reduce tumor burden and expose tumor antigens or vaccination to tumor antigens hold the promise of success in eradicating metastatic disease.
They are here reported to inhibit immune responses. Since the immature myeloid cells possess the potential to inhibit immune responses, they are also referred to myeloid-derived suppressor cells (MDSCs)\(^{120}\).

MDSCs are a heterogeneous population of cells and are subdivided into two major subsets in mice based on their expression of lymphocyte antigens Ly6C and Ly6G; CD11b\(^+\)Ly6C\(^{-}\)Ly6G\(^{hi}\) and CD11b\(^+\)Ly6C\(^+\)Ly6G\(^{lo}\) cells. Based on the morphology of these subsets, they are also termed monocytic MDSCs (CD11b\(^+\)Ly6C\(^+\)Ly6G\(^{lo}\) cells) and granulocytic MDSCs (CD11b\(^+\)Ly6C\(^+\)Ly6G\(^{hi}\) cells). A recent report shows that monocytic MDSCs can differentiate into granulocytic MDSCs\(^{121}\). In humans MDSCs are identified as CD11b\(^+\)CD33\(^+\)HLA-DR\(^{–}\) and are subdivided into CD14\(^{+}\)CD15\(^{–}\) monocytes, CD14\(^{–}\)CD15\(^{+}\) granulocytic MDSCs, and CD14\(^{–}\)CD15\(^{–}\) monocytic MDSCs.

Both MDSC subsets in mice express inducible nitric oxide synthase (iNOS) and arginase 1 (ARG1) at different levels and suppress immune effector cell functions by producing reactive oxygen species. The immunoregulatory functions of MDSCs have been recently reviewed elsewhere\(^{122}\). However, direct evidence showing a pro-metastatic role for MDSCs through immunosuppression is lacking, even though CD11b\(^+\)Gr1\(^+\) cells have been reported to promote metastatic processes. It is also noteworthy that the MDSCs cultured in vitro differentiate into neutrophils, dendritic cells and macrophages\(^{122}\). Moreover, adoptively transferred MDSCs migrate to the tumor site where they lose their Gr1 expression and express the macrophage marker F4/80 within 48 hours\(^{165}\). These results strongly suggest that MDSCs can be progenitor cells for tumor-associated macrophages and that the monocytic MDSCs are probably Ly6C\(^{hi}\) (recognized by anti-GR1 antibody) monocytes that are known to be immunosuppressive characteristics.

When effector CD8\(^{+}\) T cells and helper CD4\(^+\) T cells receive co-stimulatory signals from antigen-presenting cells, they mediate antitumor immunity and kill cancer cells. At the same time, however, the stimulated T cells upregulate expression of CTLA4, which dampens their activities to protect against hyperimmune activation. Moreover, many cancer cells and myeloid cells express PD1 ligand 1 (PD-L1) and activate PD1-mediated signaling in T cells, which downregulates T cell activities\(^{123}\). These inhibitory signals, (known as immune checkpoints), prevent effector T cells from eliminating cancer cells, and thus they are important targets to boost antitumor immune responses. Two humanized antibodies against CTLA4, ipilimumab and tremelimumab, have undergone Phase III clinical trials in patients with advanced melanoma and show overall survival benefits. In 2011, ipilimumab was approved by US Food and Drug Administration (FDA) because of its benefit to long-term survival of metastatic melanoma patients. Several antibodies against PD1 or PD-L1 have also been developed and clinically tested in Phase I trials for advanced solid tumors, including melanoma, non-small cell lung carcinoma, renal cell carcinoma, prostate cancer and colon cancer. Although the therapeutic effects
depend on the compounds and types of tumors, targeting the PD1–PD-L1 signaling pathway results in the regression of tumors. Some of these compounds are in Phase II trials\cite{124}. However, it is still unknown which cell types induce CTLA4- or PD1-mediated immune suppression in tumors because numerous cells in the tumor microenvironment, including macrophages, express CTLA4 ligands and PD-L1\cite{119}.

**Figure legends**

**Figure 1| A long journey to develop metastatic tumor.** Most malignant solid tumors metastasize from the primary organ to another such as lung, liver, bone and brain. To establish the metastatic tumor, cancer cells undertake several steps known as metastatic cascade. First, cancer cells escape from tumoricidal immune reaction by killer cells and produce systemic factors that establish a tumor-supportive environment (pre-metastatic niche) in future metastatic site (dashed arrow). The tumor cells also change the microenvironment of the primary site to increase the density of blood vessels (angiogenesis), which enhances tumor cell egress from the primary site by invasion through the surrounding stroma and intrusion into blood vessels (intravasation). The circulating tumor cells are then arrested in microvessels in the metastatic site where they need to survive. At the metastatic site, the arrested tumor cells escape from the vessel (extravasation), survive at the metastatic niche, and proliferate to form the deadly metastatic tumor.

**Figure 2| Preparation for a metastatic journey.** a| In the primary tumors, cancer cells secrete chemokines and cytokines to recruit tumor-associated macrophages (TAMs), neutrophils (TANs), myeloid-derived suppressor cells (MDSCs), and regulatory T cells (T\textsubscript{reg}). These cells directly suppress cytotoxic functions of NK and CD8\textsuperscript{+} T cells, which increase tumor growth, invasion and egress from the primary sites. Accumulation of T\textsubscript{reg} is also promoted by TAMs and regulatory B cells (B\textsubscript{reg}). MDSCs create paracrine network with T\textsubscript{H}17 cells and cancer-associated fibroblasts (CAFs), and are recruited via plasmacytoid dendritic cells (pDCs)-mediated mechanism. b| The primary tumor also produces systemic factors that induce chemotactic protein expression and extracellular matrix (ECM) remodeling in the metastatic sites before tumor cell arrival. These environmental changes recruit immature myeloid cells (iMCs) that promote subsequent outgrowth of metastasizing cancer cell. TAMs and T\textsubscript{reg} cells are also recruited to the pre-metastatic niche by primary tumor-derived fibrin clot and CCL22, respectively, and promote future metastasis.

**Figure 3| Promotion of the first step of metastasis.** a| Tumor-infiltrating macrophages become pro-angiogenic through the response to colony-stimulating factor 1 (CSF-1) and angiopoietin 2 (ANG2), and promote vessel network formation that is necessary for hematogenous dissemination. TAM-mediated angiogenesis is also induced by VEGF and WNT7b secreted from TAMs. b| Near blood vessel, cancer cells secrete CSF-1 to prompt TAMs to produce epidermal growth factor (EGF), which in turn activate EGF receptor on cancer cells and increase their invasiveness. This EGF/CSF-1 loop is triggered by fibroblast-derived factors. Several environmental factors including interleukin-4 (IL-4) promote the differentiation of macrophages to tumor-
promoting TAMs that produce proteinase cathepsin (CTS), the ECM regulator SPARC, and chemokine CCL18 to accelerate intravasation of cancer cells. TANs and MDSCs also help cancer cells to enter the vessels.

**Figure 4| Helping to overcome rate-limiting steps of metastasis.** a] After leaving the primary sites, tumor cells need to survive in the circulation and at the metastatic sites where tumor cells are arrested. This process is helped by fibrin clot formation by platelets and survival signals from TAMs and Treg. b] At the metastatic sites, cancer cells trapped in emboli secrete chemokine CCL2 to recruit inflammatory monocytes (IMs) toward metastatic sites where the IMs differentiate to metastasis-associated macrophages (MAMs). The MAMs secrete vascular endothelial growth factor (VEGF) and increase vascular permeability, which promotes extravasation of cancer cells. The MAMs also involve in persistent growth of emigrated cancer cells. Cancer extravasation and retention at the metastatic sites are also supported by direct interaction with tumor-associated neutrophils (TANs) recruited by CXCL8. TANs also enhance entrapment of circulating cancer cells by producing neutrophil extracellular trap (NET). Platelets also increase vascular permeability and following tumor extravasation by releasing ATP containing vesicles.

**Notes**

**Metastasis**
The spread of malignant tumor cells from the primary tumor site to the distant organs through the lymphatic or blood vessels and grow there expansively to develop deadly secondary tumor. Individual tumors express different tissue tropisms for metastasis that may be in part due to systemic education of different tissues to form pre-metastatic niches.

**Tumor microenvironment**
The tumor-surrounding environment consists of stromal cells such as endothelial cells, immune cells, fibroblasts and extracellular matrix. It also contains chemokines, cytokines and growth factors derived from tumor cells and tumor-educated stromal cells.

**Regulatory T (T_{reg}) cells**
A distinct population of immunosuppressive CD4^+ T cells characterized as CD25^+FoxP3^+.

**T helper type 17 (T_{H17}) cells**
A subset of CD4^+ T helper (T_{H}) cells that produce interleukin-17 (IL-17). T_{H17} cells are differentiated from naïve T cells and play critical roles in the adaptive immune responses against pathogens.

**Regulatory B (B_{reg}) cells**
A B cell subpopulation that possesses immune suppressive functions and may protect the host from autoimmune diseases. In mouse mammary tumors, CD19^+B220^+CD25^+ cells are isolated as immunoregulatory cells and termed tumor-evoked B_{reg} cells.

**Tumor-associated macrophages (TAMs)**
A distinct population of macrophages in the tumor microenvironment that promotes tumor development and progression. **TAMs** can be identified by the following marker profile (a marker that is unique to mouse or human are shown as ‘m’ or ‘h’); CD11b⁺, CD14⁺, CD23⁺ (h), CD31⁻, CD34⁻, CD45⁺, CD68⁺, CD117⁺, CD133⁻, CD146⁻, CD163⁺ (h), CD204⁺, CD206⁺, CCR2⁺, CSF1R⁺, CXCR4⁺ (h), F4/80⁺ (m), GR1⁻ (m), MHC II⁺, VEGFR1⁺, VEGFR2⁻. This TAM is a generic term that covers several identifiable sub-populations with different functions, including metastasis-associated macrophages Tie-2 expressing pro-angiogenic macrophages in the primary tumor (see REF. 95). Thus each population will have a set of canonical macrophage markers such as the CSF1R and GR1 negativity but differentially express other markers as VEGFR1⁻.

**Myeloid-derived suppressor cells (MDSCs)**
A heterogeneous population of myeloid cells that are increased in most of patients and animal models with cancer. These cells are subdivided to monocytic and granulocytic MDSCs that are characterized by CD11b⁺Ly6C⁺Ly6G⁻ and CD11b⁺Ly6C⁻Ly6G⁺ in mice respectively (see Box1 and REF. 21).

**Plasmacytoid dendritic cells (pDCs)**
A minor population of dendritic cells that link innate and adaptive immune responses. Although pDCs possess pro-inflammatory properties such as cytokine secretion, they also have immunosuppressive activities. In mice, pDCs are characterized by expression of B220, CD11c, Singlec-H, PDCA-1, and Gr-1, and absence of CD11b.

**Metastasis-associated macrophages (MAMs)**
A distinct subset of TAMs that are recruited to the metastatic sites and promote tumor cell dissemination and outgrowth. MAMs originate from inflammatory monocytes and characterized by as CD11b⁺Ly6C⁻ the following marker profile in mice; CD11b⁺, CD31⁻, CD45⁺, CCR2⁺, CXCR4⁺, F4/80⁻, Ly6C⁻, Ly6G⁻, Tie2⁻, VEGFR1⁻, VEGFR2⁻ that are recruited to the metastatic sites and promote tumor cell dissemination and outgrowth.

**Inflammatory monocytes (IMs)**
A subset of monocytes that is recruited to inflammatory sites. They are characterized as CD11b⁺Ly6C⁺ in mice and CD14⁺CD16⁻ in humans. Inflammatory monocytes also express high level of CC-chemokine receptor 2 (CCR2) and colony stimulating factor 1 receptor (CSF1R; also known as CD115), but not a neutrophil marker Ly6G. In mouse tumor models, IMs are recruited to the primary or metastasis sites by CCL2 and differentiate to the TAMs or MAMs respectively (see REFs. 50 and 125). These cells are derived from bone marrow or to a lesser extent from spleen.

**Neutrophil extracellular trap (NET)**
An extracellular fiber structures consists of extruded DNA and antimicrobial proteins released from neutrophils in response to inflammatory stimuli. A major function of NET is to trap and kill pathogens.
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