

## 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 immunosuppressive cells to tumours protects metastatic cancer cells from surveillance by killer cells, which nullifies the effects of immunotherapy and thus establishes metastasis. Furthermore, in most cases, tumour-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 tumour-infiltrating immune cells contribute to the metastatic cascade and we discuss potential therapeutic strategies to target these cells.

### Metastasis

The spread of malignant tumour cells from the primary tumour site to distant organs (through the lymphatic system or the blood), in which they grow expansively to develop deadly secondary tumours. Individual tumours express different tissue tropisms for metastasis that may partly be due to the systemic education of different tissues to form pre-metastatic niches.

Cancer progression ends in metastatic disease, which is the major cause of cancer death. For metastasis to occur from solid malignancies, tumour cells need to undergo a process that is referred to as the metastatic cascade (FIG. 1). At the primary site, tumour cells escape from the antitumour immune response and remotely prepare the environment of the future metastatic site (pre-metastatic niche). The primary tumour cells invade 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 the tumour type and the particular tissue environment — these circulating tumour cells extravasate, become established and proliferate to form the deadly metastatic tumour.

During each step of the metastatic cascade, mutant and thus potentially immunogenic tumour cells are being exposed to the immune system, which can recognize them and restrict their growth<sup>1,2</sup>. For example, recent reports demonstrate that CD8<sup>+</sup> T cells restrict the metastatic outgrowth of cancer cells disseminated from the primary tumour and that natural killer (NK) cells have the potential to reject metastatic tumour cells when the MERTK (also known as TAM; TYRO3, AXL and MER) tyrosine kinase receptors that suppress NK cell activation are inhibited<sup>3,4</sup>. Depletion of CD8<sup>+</sup> T cells and NK cells consequently increases breast cancer metastasis without affecting primary tumour growth<sup>5</sup>. Nevertheless, successful cancers and their metastatic derivatives have developed strategies to overcome these immune mechanisms partly through the recruitment of immunosuppressive cells<sup>6</sup>.

In addition to the local recruitment of immune cells, primary tumours affect the systemic environment, particularly the bone marrow, and alter haematopoiesis,

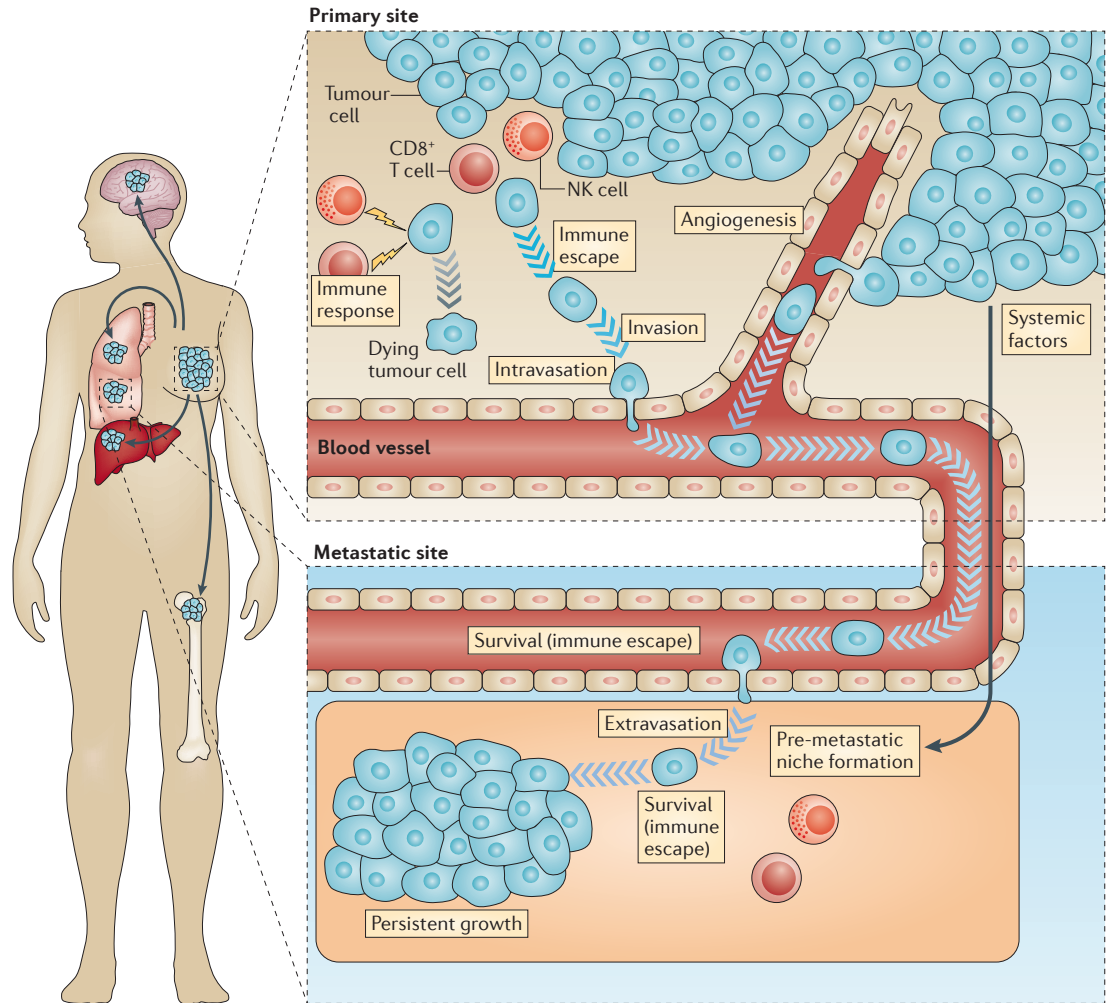
which can influence the growth of other less aggressive primary tumours<sup>7</sup>. The tumour-driven systemic processes also prepare distant sites to become pre-metastatic niches, thereby enhancing metastatic efficiency<sup>7</sup>. These systemic enhancements of metastasis involve, at least partly, myeloid cells that facilitate the escape of circulating metastatic cells from immune detection.

Tumour-infiltrating immune cells, particularly myeloid cells such as macrophages, also actively participate in metastatic processes. Macrophages are very plastic cells and have distinct functions in response to environmental signals. For example, interferon- $\gamma$  (IFN $\gamma$ ) and Toll-like receptor (TLR) ligands activate macrophages to eliminate pathogens and, in some contexts, to eliminate tumour cells. By contrast, macrophages participate in tissue remodelling and tumour progression in response to stimulation with interleukin-4 (IL-4) and IL-13 (REF. 8). Accumulating data suggest that the tumour microenvironment polarizes recruited macrophages from a potentially tumour-reactive state to a tumour-promoting state. Indeed, these 'tumour-educated' macrophages influence every step of the metastatic cascade by promoting tumour cell invasion of the surrounding tissue, intravasation and survival in the circulation, as well as tumour cell arrest, extravasation and persistent growth at metastatic sites.

A substantial amount of clinical data has indicated that tumour infiltration of certain immune cell types correlates with poor prognosis of patients with cancer<sup>9–11</sup>, although these studies do not address the roles of these cells in tumour metastasis. In this Review, we highlight the role of immune cells in each step of the metastatic cascade and describe the mechanisms that underlie their pro-metastatic functions, which have been identified

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**Figure 1 | A long journey to develop metastatic tumours.** Most malignant solid tumours metastasize from the primary organ to another, such as the lungs, liver, bone and brain. To establish the metastatic tumour, cancer cells undertake several steps that are known as the metastatic cascade. First, cancer cells escape from the tumoricidal immune response that is mediated by killer cells, such as CD8<sup>+</sup> T cells and natural killer (NK) cells, and produce systemic factors that establish a tumour-supportive environment (pre-metastatic niche) in the future metastatic site. The tumour cells also change the microenvironment of the primary site to increase the density of blood vessels (angiogenesis), which enhances tumour cell egress from the primary site by invasion through the surrounding stroma and intrusion into blood vessels (intravasation). The circulating tumour cells are then arrested in microvessels in the metastatic site where they need to survive. At the metastatic site, the arrested tumour cells escape from the blood vessel (extravasation), survive at the metastatic niche and proliferate to form the deadly metastatic tumour.

using mouse models. We also discuss how these cells are recruited and/or differentiate to promote the metastatic process, and how these insights are leading to the development of therapeutic strategies that block pro-metastatic immune cells.

**Immune escape**

Tumours develop numerous methods to avoid detection and eradication by the immune system by modulating the recruitment, expansion and function of tumour-infiltrating leukocytes, such as immunoregulatory myeloid cells, regulatory T cells (T<sub>Reg</sub> cells), T helper 17 cells (T<sub>H</sub>17 cells), and regulatory B cells (B<sub>Reg</sub> cells) (FIG. 2a). Thus, impaired immune-mediated rejection of the primary tumour may increase the number of malignant

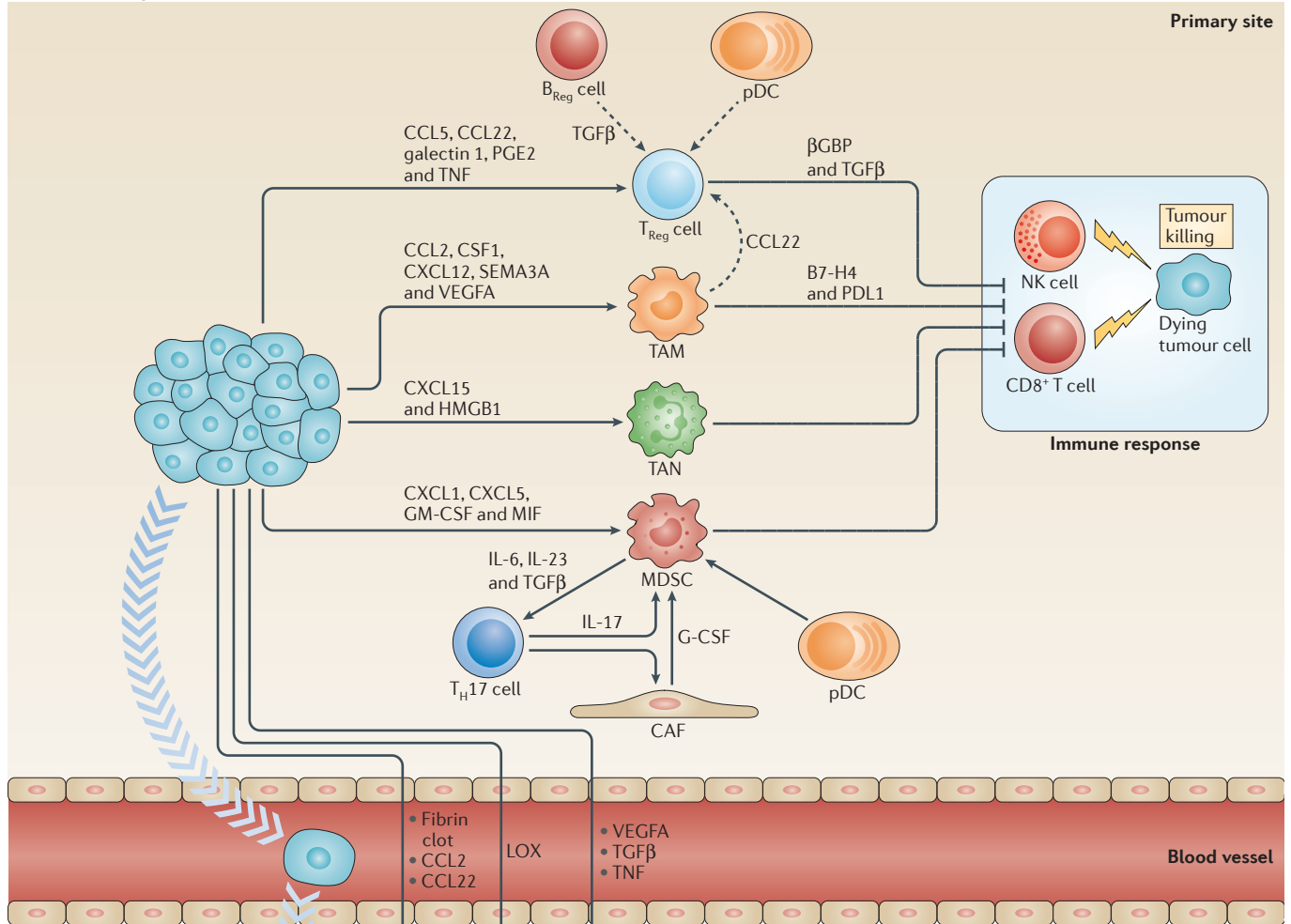
tumour cells that will egress from the primary tumour. It will also enhance the survival of tumour cells disseminating to the metastatic site (see below).

**Myeloid cells.** Most solid tumours recruit macrophages through the production of various cytokines and chemokines, such as colony-stimulating factor 1 (CSF1), vascular endothelial growth factor A (VEGFA), semaphorin 3A (SEMA3A), CC-chemokine ligand 2 (CCL2) and CXC-chemokine ligand 12 (CXCL12)<sup>12–14</sup>. Tumour-associated macrophages (TAMs) are reported to suppress CD8<sup>+</sup> T cell infiltration and antitumour immunity in primary tumours<sup>15</sup>. Mechanistically, TAMs are known to suppress the cytotoxic activity of CD8<sup>+</sup> T cells either directly through their expression of inhibitor

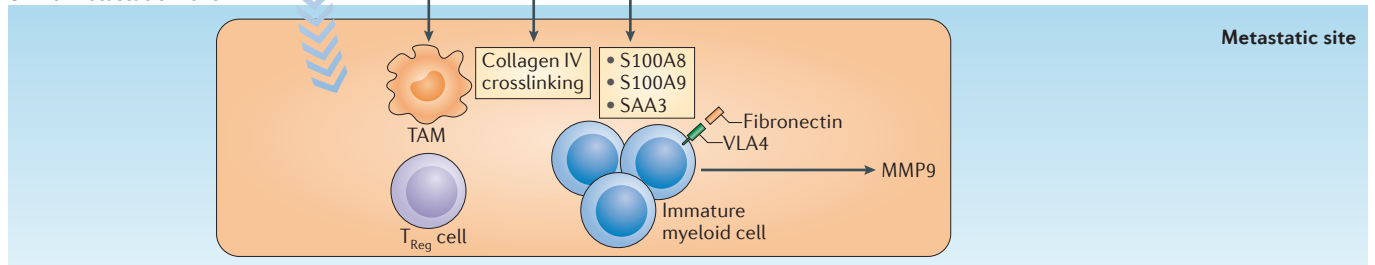
**Tumour microenvironment**

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

**a Immune escape**



**b Pre-metastatic niche**



**Figure 2 | Preparation for a metastatic journey. a** | In the primary tumour, cancer cells secrete chemokines and cytokines to recruit tumour-associated macrophages (TAMs), tumour-associated neutrophils (TANs), myeloid-derived suppressor cells (MDSCs) and regulatory T (T<sub>Reg</sub>) cells. These cells directly suppress the cytotoxic functions of natural killer (NK) cells and CD8<sup>+</sup> T cells through the production and expression of various factors, including β-galactoside-binding protein (βGBP), programmed cell death 1 ligand 1 (PDL1) and B7-H4, which increase tumour growth, invasion and egress from the primary site. Accumulation of T<sub>Reg</sub> cells is also promoted by TAMs through the production of CC-chemokine ligand 22 (CCL22) and by regulatory B (B<sub>Reg</sub>) cells via transforming growth factor-β (TGFβ) secretion. MDSCs secrete interleukin-6 (IL-6), IL-23 and TGFβ that recruits T helper 17 (T<sub>H</sub>17) cells. T<sub>H</sub>17 cells secrete IL-17, which promotes recruitment of MDSCs and the secretion of granulocyte colony-stimulating factor (G-CSF) from cancer-associated fibroblasts (CAFs) that in turn promotes immunosuppressive function of MDSCs. MDSCs are also recruited via a plasmacytoid dendritic cell (pDC)-mediated mechanism.

**b** | The primary tumour also produces systemic factors, such as vascular endothelial growth factor A (VEGFA), TGFβ, tumour necrosis factor (TNF) and lysyl oxidase (LOX), that induce chemotactic protein expression (S100A8, S100A9 and serum amyloid A3 (SAA3)) and extracellular matrix remodelling in the metastatic sites before tumour cell arrival. These environmental changes recruit immature myeloid cells that form clusters and secrete matrix metalloproteinase 9 (MMP9) to promote subsequent outgrowth of metastasizing cancer cell. The immature myeloid cells express very late antigen 4 (VLA4) and are also recruited to the pre-metastatic niche by its ligand fibronectin. TAMs and T<sub>Reg</sub> cells are also recruited to the pre-metastatic niche by primary tumour-derived fibrin clots, and by CCL2 and CCL22, respectively, and these cells promote future metastasis. CSF1, colony-stimulating factor 1; CXCL, CXC-chemokine ligand; GM-CSF, granulocyte-colony-stimulating factor; HMGB1, high mobility group protein B1; MIF, macrophage migration inhibitory factor; PGE2, prostaglandin E2; SEMA3A, semaphorin 3A.

**Regulatory T cells**

( $T_{Reg}$  cells). A distinct population of immunosuppressive  $CD4^+$  T cells that are characterized as  $CD25^+FOXP3^+$ .

**T helper 17 cells**

( $T_H17$  cells). A subset of  $CD4^+$  T helper cells that produce interleukin-17.  $T_H17$  cells have crucial roles in adaptive immune responses to pathogens.

**Regulatory B cells**

( $B_{Reg}$  cells). A subpopulation of B cells that have immunosuppressive functions and that may protect the host from autoimmune diseases. In mouse mammary tumours,  $CD19^+B220^+CD25^+$  cells are isolated as immunoregulatory cells and termed tumour-evoked  $B_{Reg}$  cells.

ligands — such as programmed cell death 1 ligand 1 (PDL1)<sup>16</sup> and B7-H4 (also known as VTCN1)<sup>17</sup> — or indirectly via CCL22-mediated recruitment of  $T_{Reg}$  cells<sup>18</sup> (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. Similarly to TAMs, tumour-associated neutrophils (TANs) can reduce  $CD8^+$  T cell activity and increase primary tumour growth<sup>20</sup>, although further studies are required to reveal whether their immunoregulatory functions are required for tumour metastasis.

A type of immature myeloid cell that expresses CD11b and GR1 (which is comprised of LY6C and LY6G) is also found in the tumour microenvironment. These cells can suppress the proliferation and the cytokine production of T cells *in vitro* and are thus referred to as myeloid-derived suppressor cells (MDSCs)<sup>21</sup> (BOX 1). In a pancreatic tumour mouse model, tumour-derived granulocyte-macrophage colony-stimulating factor (GM-CSF) was shown to recruit MDSCs to the primary tumours, in which they suppressed  $CD8^+$  T cell cytotoxicity<sup>22</sup>. In tumours that developed following subcutaneous injection of 3LL lung cancer cells, reduction in MDSC numbers through the administration of neutralizing antibodies specific for GR1 (which bind both LY6C and LY6G) or for LY6G increased the frequency and function of NK cells and  $CD8^+$  T cells, and the number of apoptotic tumour cells<sup>23</sup>. The treatments also reduced primary tumour growth and metastasis from the skin to the lungs<sup>23</sup>, which suggests that increased survival of primary tumour cells via immune suppression enhances the frequency of successful egress from the primary site. However, the contribution of MDSCs to immune evasion by primary tumours

needs to be more precisely defined, as these neutralizing antibodies also deplete other myeloid cells, such as monocytes and neutrophils. Neutralization of GR1 might also affect the number of tumour-infiltrating plasmacytoid dendritic cells (pDCs), which are reported to increase the numbers of MDSCs and  $T_{Reg}$  cells in a transplanted breast tumour and, in turn, to increase tumour bone metastasis by reducing  $CD8^+$  T cell cytotoxicity<sup>24</sup>.

**$T_{Reg}$  cells.** Thymus-derived  $T_{Reg}$  cells are immunosuppressive cells that express the IL-2 receptor  $\alpha$ -chain (also known as CD25) and the 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 tumours<sup>25</sup>, which suggests that  $T_{Reg}$  cell recruitment to the primary tumour is necessary for tumour metastasis. This  $T_{Reg}$  cell recruitment has been shown to depend on tumour-derived CCL22 (REFS 18,25) or CCL5 (REFS 26,27), depending on the tumour model used. Increased secretion of prostaglandin E2 from breast cancer cells also recruits  $T_{Reg}$  cells to the primary tumour, which increases  $CD8^+$  T cell apoptosis and cancer cell bone metastasis<sup>28</sup>. Furthermore, immune cell-derived tumour necrosis factor (TNF) was shown to enhance lung metastasis of melanoma cells through expansion of  $T_{Reg}$  cell numbers<sup>29</sup>. Tumour-derived galectin 1 can promote systemic immunosuppression by regulating the clonal expansion and the function of  $T_{Reg}$  cells, probably by increasing the expression of the T cell regulatory molecule LAT (linker for activation of T cells), which enhances breast cancer metastasis<sup>30</sup>.

Although the immune-suppressive mechanisms of  $T_{Reg}$  cells in the tumour setting are not clear<sup>31</sup>, it has been suggested that  $T_{Reg}$  cells can induce apoptosis of NK cells by secretion of  $\beta$ -galactoside-binding protein ( $\beta$ GBP), which increases lung metastasis of breast cancer cells<sup>32</sup>. 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<sup>33</sup>. These studies suggest that NK cells can eliminate tumour cells such that their dissemination is restricted, but that tumour-induced  $T_{Reg}$  cell recruitment nullifies this suppression.

**Other adaptive immune cells.** Recent studies indicate that other adaptive immune cells can regulate the immunosuppressive 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 virus middle T (PyMT) antigen, loss of TGF $\beta$  signalling in tumour cells recruits MDSCs to the tumour through CXCL1 and CXCL5 secretion<sup>34</sup>. The MDSCs secrete IL-6, IL-23 and TGF $\beta$  and, which accelerates the accumulation of  $T_H17$  cells. IL-17 from  $T_H17$  cells in turn promotes further recruitment of MDSCs and immunosuppressive gene expression in these cells, which increases lung metastasis<sup>34</sup>. In the subcutaneous EL4 lymphoma model, IL-17 from  $T_H17$  cells recruits MDSCs to the tumour site by prompting

**Box 1 | Myeloid-derived suppressor cells**

It has been reported that tumour-bearing mice show increased numbers of immature myeloid cells in the spleen and in tumours. These cells are characterized by the expression of CD11b and GR1, and by the absence or reduced expression of mature myeloid cell markers. As these immature myeloid cells have the potential to inhibit immune responses, they are also referred to as myeloid-derived suppressor cells (MDSCs)<sup>120</sup>.

MDSCs are a heterogeneous population of cells and are divided into two major subsets in mice on the basis of their expression of the lymphocyte antigens LY6C and LY6G, and their morphology; these subsets are termed monocytic MDSCs ( $CD11b^+LY6C^hiLY6G^{low}$  cells) and granulocytic MDSCs ( $CD11b^+LY6C^{low}LY6G^+$  cells). A recent report shows that monocytic MDSCs can differentiate into granulocytic MDSCs<sup>121</sup>. In humans, MDSCs are identified as  $CD11b^+CD33^+HLA-DR^-$ , and are subdivided into  $CD14^{hi}CD15^-$  monocytic MDSCs and  $CD14^{low}CD15^+$  granulocytic MDSCs.

Both MDSC subsets in mice express inducible nitric oxide synthase (iNOS) and arginase 1 at different levels, and suppress immune effector cell functions by producing reactive oxygen species. The immunoregulatory functions of MDSCs have been recently reviewed elsewhere<sup>122</sup>. 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<sup>23,82,83</sup>. It is also noteworthy that MDSCs cultured *in vitro* differentiate into neutrophils, dendritic cells and macrophages<sup>122</sup>. Moreover, adoptively transferred MDSCs migrate to the tumour site where they lose their GR1 expression and express the macrophage marker F4/80 within 48 hours<sup>85</sup>. These results strongly suggest that MDSCs can be progenitor cells for tumour-associated macrophages and that the monocytic MDSCs are probably  $LY6C^hi$  (recognized by a GR1-specific antibody) monocytes that have immunosuppressive characteristics.

### Tumour-associated macrophages

(TAMs). A distinct population of macrophages in the tumour microenvironment that promotes tumour development and progression. TAMs can be identified by the following marker profile (a marker that is unique to mouse or human is indicated by (m) or (h), respectively): CD11b<sup>+</sup>, CD14<sup>+</sup>, CD23<sup>+</sup> (h), CD34<sup>-</sup>, CD45<sup>+</sup>, CD68<sup>+</sup>, CD117<sup>-</sup>, CD133<sup>-</sup>, CD146<sup>-</sup>, CD163<sup>+</sup> (h), CD204<sup>+</sup>, CD206<sup>+</sup>, CCR2<sup>+</sup>, CSF1R<sup>+</sup>, CXCR4<sup>+</sup> (h), F4/80<sup>+</sup> (m), GR1<sup>-</sup> (m), MHC class II<sup>+</sup>, VEGFR1<sup>+</sup> and VEGFR2<sup>-</sup>. TAM is a generic term that encompasses several identifiable subpopulations with different functions, including TIE2-expressing pro-angiogenic macrophages in the primary tumour. Thus, each population will have a set of canonical macrophage markers, such as CSF1R<sup>+</sup>GR1<sup>-</sup>, but will differentially express other markers, such as VEGFR1.

### Myeloid-derived suppressor cells

(MDSCs). A heterogeneous population of myeloid cells, the numbers of which are increased in most patients with cancer and in animal models of cancer. These cells are subdivided into monocytic and granulocytic MDSCs.

### Plasmacytoid dendritic cells

(pDCs). A small population of DCs that link innate and adaptive immune responses. Although pDCs have pro-inflammatory properties, such as cytokine secretion, they also have immunosuppressive effects. In mice, pDCs are characterized by expression of B220, CD11c, Siglec-H, PDCA1 (also known as CD317) and GR1, and by the absence of CD11b.

### Osteoclastogenesis

A process whereby haematopoietic stem cells differentiate into multinucleated osteoclasts with bone-resorbing activity.

cancer-associated fibroblasts to secrete granulocyte colony-stimulating factor (G-CSF), which promotes the immunosuppressive function of the immature myeloid cells<sup>35</sup>. Thus, T<sub>H</sub>17 cell recruitment to tumours seems to indirectly suppress antitumour immunity in the primary sites and to enhance metastasis. By contrast, T<sub>H</sub>17 cells can promote antitumour immune responses through the recruitment of dendritic cells and cytotoxic cells in some mouse tumour models<sup>36</sup>. As T<sub>H</sub>17 cells exert their biological activities by interacting with other immune cell types, their roles in tumour metastasis might be affected by the tumour microenvironment and thus might depend on the tumour type. Indeed, *Il17* deficiency increases metastasis in a lung cancer mouse model<sup>37</sup> but suppresses metastasis in a melanoma or colon cancer model<sup>36</sup>.

B<sub>Reg</sub> cells, which are a specific B cell population expressing CD25 and B220, may also promote tumour metastasis through immune suppression. Intravenous injection of 4T1 breast cancer cells increases the number of circulating CD25<sup>+</sup> B cells, and their depletion using a B220-specific antibody reduces lung metastasis<sup>38</sup>. The role of tumour-evoked B<sub>Reg</sub> cells in lung metastases may be to induce T<sub>Reg</sub> cell conversion, as B<sub>Reg</sub> cells have been shown to suppress CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation *in vitro* and to induce the conversion of CD4<sup>+</sup> T cells to FOXP3<sup>+</sup> T<sub>Reg</sub> cells via TGFβ secretion<sup>38</sup>.

The above experimental data suggest that tumour cells are eliminated by cytotoxic immune cells before they can proceed through the metastatic cascade. Thus, the recruitment of immunosuppressive cells to the primary site seems to be an essential preparatory step for tumour metastasis.

### Pre-metastatic niche formation

In some cases, primary tumours 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 studies suggest an important contribution of bone marrow-derived immature myeloid cells (FIG. 2b).

In mice with subcutaneous tumours developed by LLC lung cancer or B16 melanoma cells, bone marrow-derived cells including CD11b<sup>+</sup> myeloid cells accumulate in the lungs before metastatic tumour cells are detected, which promotes future metastasis of the cancer cells to the lungs<sup>39</sup>. These bone marrow-derived cells express very late antigen 4 (VLA4; also known as integrin α4β1) and are recruited by its ligand fibronectin, which is deposited at pre-metastatic sites in response to primary tumour-derived factors<sup>39</sup>. In the same models, recruitment of CD11b<sup>+</sup> cells to the pre-metastatic lung is also facilitated by expression of myeloid chemoattractants S100A8 and S100A9, which are induced by VEGFA, TNF and TGFβ from distant primary tumours<sup>40</sup>, and by serum amyloid A3, which is induced by S100A8 and S100A9 (REF. 41) in the pre-metastatic lung. B16 melanoma-derived factors also activate sphingosine-1-phosphate receptor 1 (S1PR1) and its downstream signalling molecule signal transducer

and activator of transcription 3 (STAT3) in the myeloid cells in the pre-metastatic lung<sup>42</sup>. As treatment of mice with small interfering RNA against *Stat3* or *S1pr1* eliminates the myeloid cell clusters, constitutive activation of S1PR1–STAT3 signalling is required for myeloid cells to maintain the pre-metastatic niche<sup>42</sup>.

Pre-metastatic niche formation is also reported in tumour types other than the LLC and B16 tumours. For example, xenografts of the human breast cancer cell line MDA-MB-231 induce CD11b<sup>+</sup> cell accumulation in the pre-metastatic lung through secretion of lysyl oxidase (LOX), which crosslinks collagen IV and thereby increases adherence of CD11b<sup>+</sup> cells<sup>43</sup>. Accumulating reports have revealed that various soluble factors and exosomes from primary tumours can mobilize bone marrow cells into the circulation<sup>7,44</sup>. Once released into the circulation, these immature cells are recruited to the pre-metastatic sites by the mechanisms described above.

The above studies also indicate that immature myeloid cells are a major component of the pre-metastatic niche because subsets of the recruited CD11b<sup>+</sup> cells also express the stem and progenitor cell marker CD117 and reduced accumulation of these cells prevents later tumour cell metastasis<sup>39,43</sup>. Furthermore, recent studies have revealed contributions of more differentiated myeloid cells to the establishment of the pre-metastatic niche. For example, the recruitment of CD11b<sup>+</sup>LY6C<sup>+</sup> monocytes to the pre-metastatic lung by CCL2 also enhances the pulmonary metastasis of B16 cells<sup>45</sup>, and CD11b<sup>+</sup>CD68<sup>+</sup>F4/80<sup>+</sup> macrophages that are recruited to the lung by primary tumour-induced fibrin clots establish the pre-metastatic niche that enhances breast cancer metastasis<sup>46</sup>.

The fact that pre-metastatic myeloid cell accumulation promotes subsequent tumour metastasis raises the question of 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 tumour cells<sup>39</sup>. These cells also secrete matrix metalloproteinase 9 (MMP9), which may promote tumour cell invasion<sup>47</sup>. Interestingly, a recent study indicates that systemic factors from hypoxic breast cancer cells increase CD11b<sup>+</sup> cell accumulation and reduce the cytotoxic functions of NK cells in the pre-metastatic lung<sup>48</sup>. As discussed above, myeloid cells, especially MDSCs, can suppress immune responses and it is therefore likely that the recruited myeloid cells establish a pre-metastatic immunosuppressive environment in the lung to promote tumour metastasis.

Another question is whether non-myeloid cells can also have a role in the establishment of pre-metastatic niches. Tumour-specific CD4<sup>+</sup> T cells are reported to induce pre-metastatic niches in the bone by secreting receptor activator of nuclear factor-κB ligand (RANKL) and thus increasing osteoclastogenesis, which promotes bone metastasis of breast cancer cells<sup>49</sup>. Interestingly, conditioned medium from 4T1 breast cancer cells increases the number of T<sub>Reg</sub> cells in the pre-metastatic lung by inducing CCL22 expression in the lung stroma<sup>32</sup>. This is an emerging area of research 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 progenitor cells are the central cell population that establishes the pre-metastatic niche. As macrophages in the metastatic sites originate from bone marrow-derived monocytes<sup>50</sup>, it is possible that primary tumour-derived factors recruit myeloid progenitor cells at various stages of differentiation to pre-metastatic sites where they can rapidly differentiate into metastasis-associated macrophages (MAMs) following tumour cell arrival, thereby enhancing tumour cell extravasation and survival, including survival from immune cell attack (see below). This pre-loading of the pre-metastatic niche with cells that are capable of enhancing extravasation and tumour cell survival may provide the mechanism that underlies the metastasis-promoting activity of these pre-metastatic niches.

### Tumour cell egress

To establish metastatic foci, tumour cells need to egress from the primary sites by migration through the stroma and by intravasation into blood vessels, the numbers of which are increased by *de novo* development in the tumour 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 the key signalling pathways that promote tumour intravasation and the contribution of environmental factors that prompt myeloid cells to help in this process. Direct involvement of lymphocytes in tumour cell egress has not been reported, although CD4<sup>+</sup> T cells do contribute to this process through the modulation of macrophage phenotypes.

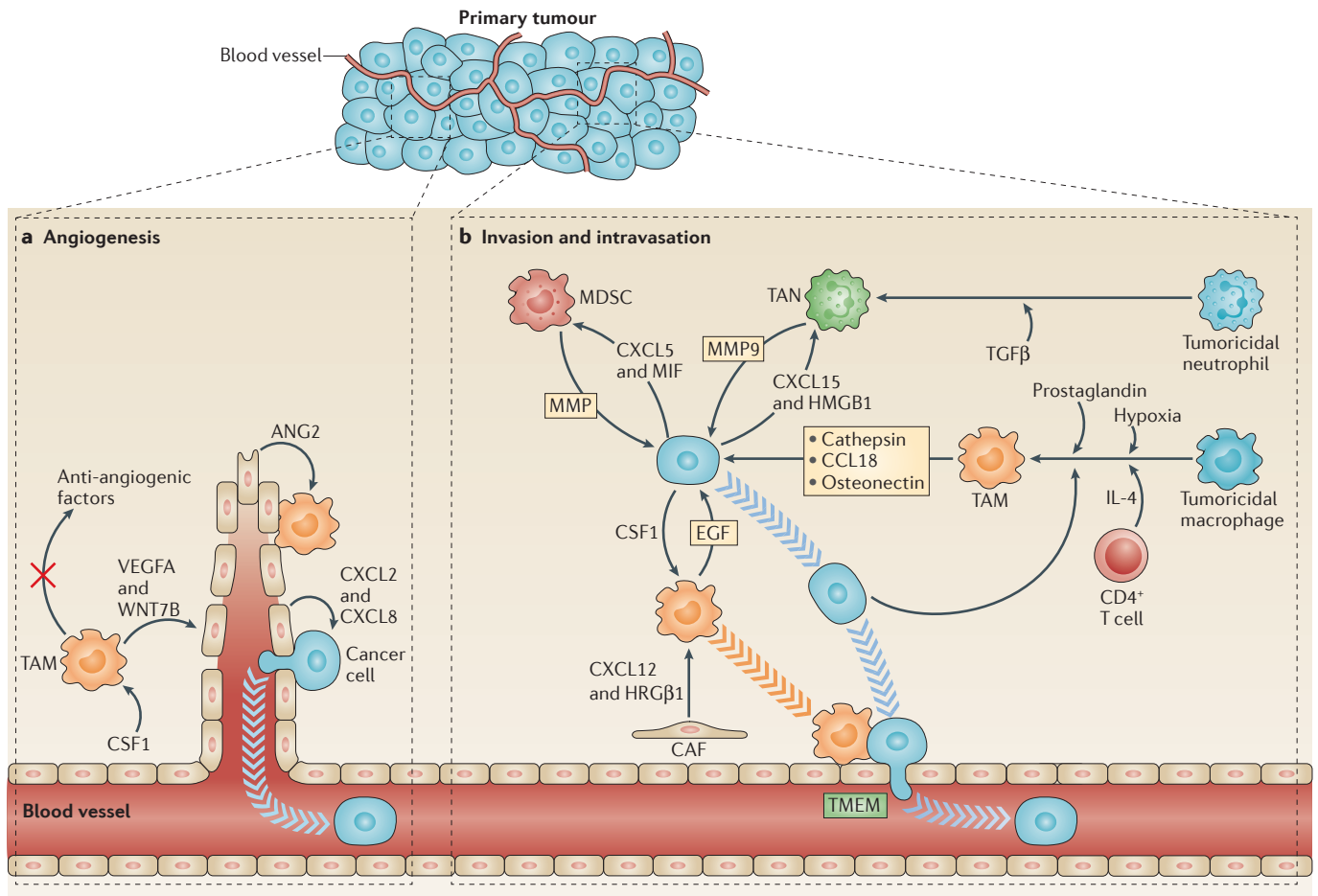
**Contribution of macrophages.** Ablation of macrophages by genetic loss of the major lineage regulator of macrophages, CSF1, impairs the onset of angiogenesis<sup>51</sup> and markedly suppresses lung metastases<sup>52</sup> in the PyMT model of breast cancer. This TAM-induced angiogenesis is partly promoted through VEGFA expression by TAMs<sup>53</sup> (FIG. 2a). Interestingly, macrophage-selective deletion of *Ets2*, which is a direct effector of the CSF1 signalling pathway, increases expression of anti-angiogenic factors such as thrombospondin 1 (THBS1) and THBS2 in TAMs, thereby suppressing PyMT breast cancer lung metastases by inhibiting angiogenesis in the primary tumours<sup>54</sup>. Macrophage-selective deletion of *Wnt7b* also suppresses the angiogenic switch in the PyMT primary tumours and reduces lung metastasis<sup>55</sup>. Furthermore, macrophages were shown to associate with new blood vessels that were induced by endothelial cell-derived angiopoietin 2 (ANG2), thereby enhancing angiogenesis and metastatic dissemination of the cancer cells<sup>56</sup>. These data suggest that TAMs help tumour cell egress by increasing the density of leaky blood vessels that in turn may also provide pro-tumorigenic factors such as CXCL8 (also known as IL-8) and CXCL2, which increases the invasiveness of cancer cells<sup>57</sup>.

TAMs also directly help invasion of the surrounding tissue and intravasation of tumour cells. Intravital imaging reveals that cancer cells in PyMT tumours invade surrounding tissues together with TAMs<sup>58</sup> and that tumour cell intravasation occurs in association with perivascular TAMs<sup>59</sup>. In these processes, cancer cells secrete CSF1 to promote macrophage mobility and their secretion of epidermal growth factor (EGF) (FIG. 2b). TAM-derived EGF in turn activates the EGF receptor in cancer cells, which enhances their invasion capability and motility by increasing invadopodium formation and matrix degradation, thereby accelerating the invasion and the intravasation of cancer cells<sup>60</sup>. Mechanistically, activation of Wiskott–Aldrich syndrome protein (WASP) by the CSF1 receptor (CSF1R) promotes the migration of TAMs towards CSF1-producing cancer cells and stimulates the release EGF from their cell surface<sup>61</sup>. Genetic deletion of steroid receptor co-activator 1 (SRC1; also known as NCOA1) also reduces tumour cell CSF1 production and consequently macrophage numbers and their paracrine action on tumour cell invasion in the primary tumour, which suppresses lung metastasis of PyMT cancer cells without affecting primary tumour growth<sup>62</sup>. Macrophage-dependent tumour invasion is also triggered by heregulin  $\beta$ 1 (HRG $\beta$ 1) and CXCL12, according to the tumour type, through this EGF-dependent mechanism<sup>63</sup>. These data, which have been extended to human breast cancers, suggest that the EGF–CSF1 loop, activated by multiple signals from the microenvironment, has a central role in tumour intravasation.

TAMs also promote tumour egress and metastasis through the secretion of CCL18 and osteonectin (also known as SPARC), which modulates the extracellular matrix adhesive properties of cancer cells<sup>64,65</sup>. In a pancreatic tumour model, TAMs were shown to produce cathepsin B and cathepsin S, which promote cancer cell egress and angiogenesis<sup>66</sup>. These results indicate that TAMs help tumour cells to enter the blood vessels by cytokine secretion and extracellular matrix remodelling.

Although the mechanisms by which macrophages acquire these pro-metastatic properties have not been fully characterized, recent studies shed light on the role of environmental factors in primary tumours. For example, CD4<sup>+</sup> T cell-derived IL-4 prompts macrophages to express EGF<sup>67</sup>, and tumour cell-derived IL-4 increases cathepsin B and cathepsin S production by TAMs<sup>66</sup>, depending on the tumour model. In PyMT mice, loss of neuropilin 1, which is a SEMA3A receptor, in macrophages prevents TAM infiltration into the hypoxic tumour area, which suppresses the pro-angiogenic functions of TAMs and thus delays tumour progression and lung metastasis<sup>68</sup>. Administration of a cyclooxygenase 2 (COX2) inhibitor, which inhibits prostaglandin production, also suppresses expression of pro-metastatic molecules such as VEGFA and MMP9 in TAMs and inhibits lung metastasis of breast cancer cells<sup>69</sup>. A recent study shows that activation of the IL-1 receptor is required for monocytes from patients with renal cell carcinoma (RCC) and for TAMs from human RCC xenografts to promote tumour angiogenesis and invasion<sup>70</sup>. GM-CSF from human breast cancer cells also

**Metastasis-associated macrophages (MAMs).** A distinct subset of tumour-associated macrophages that are recruited to the metastatic sites and promote tumour cell dissemination and outgrowth. MAMs originate from inflammatory monocytes and are characterized by the following marker profile in mice: CD11b<sup>+</sup>, CD31<sup>-</sup>, CD45<sup>+</sup>, CCR2<sup>+</sup>, CXCR4<sup>-</sup>, F4/80<sup>+</sup>, LY6C<sup>-</sup>, LY6G<sup>-</sup>, TIE2<sup>-</sup>, VEGFR1<sup>+</sup> and VEGFR2<sup>-</sup>.



**Figure 3 | Promotion of the first step of metastasis.** **a** | Tumour-associated macrophages (TAMs) become pro-angiogenic through their response to colony-stimulating factor 1 (CSF1), which suppresses anti-angiogenic factor expression, and angiopoietin 2 (ANG2), which enhances their interaction with endothelial cells and promotes the vessel network formation that is necessary for haematogenous dissemination. Tumour angiogenesis is also induced by vascular endothelial growth factor A (VEGFA) and WNT7B secreted by TAMs. TAM-mediated angiogenesis helps the haematogenous dissemination of cancer cells by increasing the density of leaky blood vessels, which in turn provide CXC-chemokine ligand 2 (CXCL2) and CXCL8 that increase invasiveness of cancer cells. **b** | Near blood vessels, cancer cells secrete CSF1 to prompt TAMs to produce epidermal growth factor (EGF), which in turn activates EGF receptor on cancer cells and increases their invasiveness. This EGF–CSF1 loop is triggered by cancer-associated fibroblast (CAF)-derived factors, such as heregulin β1 (HRGβ1) and CXCL12. Cancer cells migrate towards blood vessels with TAMs and interact with endothelial cells, creating a tumour microenvironment for metastasis (TMEM) where the cancer cells intravasate. Several environmental factors, including interleukin-4 (IL-4) from CD4<sup>+</sup> T cells or tumour cells, promote the differentiation of macrophages to tumour-promoting TAMs that engage in the EGF–CSF1 paracrine loop and produce cathepsin proteinases, CC-chemokine ligand 18 (CCL18) and the extracellular matrix (ECM) regulator osteonectin to accelerate migration and intravasation of cancer cells. Tumour-associated neutrophils (TANs) acquire their pro-tumorigenic phenotype via tumour-derived transforming growth factor-β (TGFβ) within the tumour microenvironment. Myeloid-derived suppressor cells (MDSCs) recruited by tumour-derived CXCL5 and macrophage migration inhibitory factor (MIF) also help cancer cells to enter the vessels. HMGB1, high mobility group protein B1; MMP, matrix metalloproteinase.

skews macrophages to a TAM-like phenotype *in vitro* and is required for tumour metastasis in a xenograft model<sup>71</sup>. Furthermore, overexpression of histidine-rich glycoprotein in cancer cells skews TAM polarization from a pro-tumorigenic to a tumour-inhibiting phenotype by downregulating placental growth factor (PGF) expression, which normalizes the leaky blood vessels and inhibits metastasis<sup>72</sup>. Therefore, the data suggest that the tumour microenvironment polarizes recruited

macrophages from a tumour-reactive to a tumour-promoting state. These preclinical studies give rise to an idea that the pro-metastatic function of TAMs can be suppressed by targeting signalling pathways that regulate TAM polarization<sup>73</sup>. Understanding the roles of macrophages in normal developmental processes might be useful in identifying such targets because the gene expression profiles of TAMs are similar to those of trophic macrophages in developmental tissues<sup>74</sup>.

In support of these preclinical findings, direct contact between perivascular TAMs, endothelial cells and tumour cells — which has been termed the ‘tumour microenvironment for metastasis’ (TMEM) — is found in human breast cancer specimens, and a high TMEM score (that is, the number of TAM, endothelial cell and tumour cell interactions) is associated with increased risk of metastasis<sup>75</sup>. Regardless of clinical subtypes or tumour grade, the TMEM score in breast cancer specimens correlates with tumour cell expression of the invasion isoform of MENA (a vasodilator-stimulated phosphoprotein) that strongly potentiates EGF signalling in cancer cells<sup>76</sup> and is required for macrophage-induced *in vitro* intravasation of human breast cancer cells<sup>77</sup>. These data indicate that the interaction between perivascular TAMs and tumour cells accelerates tumour intravasation and thereby increases the risk of systemic tumour cell dissemination. As we discuss below, TAMs also have crucial roles in supporting the disseminating cancer cells.

**Role of neutrophils and MDSCs.** Highly metastatic human fibrosarcoma and prostate cancer cells recruit neutrophils to primary tumours, which increases angiogenesis and intravasation of cancer cells through the secretion of MMP9 (REF. 78). In a xenograft model of intrahepatic cholangiocarcinoma, tumour-derived CXCL15 recruits neutrophils that promote lung metastasis of the cancer cells<sup>79</sup>. Similarly, ultraviolet (UV) exposure of melanoma in a GEM tumour model increases neutrophil recruitment to the primary tumour through expression of high mobility group protein B1 (HMGB1), which is derived from the UV-damaged keratinocytes. These neutrophils enhance angiogenesis, cell migration towards endothelial cells and hence lung metastases<sup>80</sup>.

However, the involvement of neutrophils in tumour metastasis is controversial, as depletion of neutrophils increases the number of lung metastatic foci in a spontaneous metastasis model of breast cancer<sup>81</sup>. Furthermore, neutrophils isolated from tumour-bearing mice can kill the tumour cells *in vitro* by generating H<sub>2</sub>O<sub>2</sub> and can thereby suppress lung metastasis *in vivo* when adoptively transferred into mice that have been intravenously injected with the cancer cells<sup>81</sup>. Interestingly, TGFβ has been shown to induce a switch from a tumoricidal to a tumour-promoting phenotype in neutrophils within subcutaneously developed mesothelioma<sup>20</sup>. It is therefore possible that the pro-metastatic functions of neutrophils are regulated by specific environmental factors in a similar manner to TAMs.

In PyMT-induced tumour-bearing mice, loss of TGFβ signalling in tumour cells increases CXCL5 secretion and recruits CD11b<sup>+</sup>GR1<sup>+</sup> MDSCs to the invasion front of the tumours. The CD11b<sup>+</sup>GR1<sup>+</sup> cells promote tumour cell invasion *in vitro* through the expression of MMPs<sup>82</sup>. In the 4T1 mammary tumour model, macrophage migration inhibitory factor (MIF) produced by cancer cells increases the number of CD11b<sup>+</sup>LY6C<sup>hi</sup> cells within the primary tumours, and depletion of the CD11b<sup>+</sup>LY6C<sup>hi</sup> cells using a GR1-specific antibody

reduces lung metastasis of cancer cells<sup>83</sup>. In a GEM model of melanoma, CXCL5 recruits CD11b<sup>+</sup>GR1<sup>hi</sup> cells to the primary tumour, and depletion of these cells by LY6G-specific antibody treatment reduces dissemination of melanoma cells<sup>84</sup>.

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 GR1 are not specific markers of MDSCs, as discussed above. As CD11b<sup>+</sup>GR1<sup>+</sup> cells can differentiate into CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages at the tumour site when transferred to tumour-bearing mice<sup>85</sup>, depletion of CD11b<sup>+</sup>GR1<sup>+</sup> cells might also affect TAM-dependent processes.

Data supporting the role of neutrophils in cancer invasion and extravasation conflict with results suggesting that neutrophils have tumour-inhibiting effects. Part of this problem may be owing to how the cells are defined, as MDSCs and neutrophils are often defined by expression of the same marker (GR1) and antibody ablation therefore might eliminate or change the balance of both populations of cells. Furthermore, some tumours, such as in the 4T1 breast cancer model, produce excessive amounts of the neutrophil growth factor G-CSF, which can cause a dramatic shift in the number of circulating neutrophils and TANs<sup>86</sup>. Thus, caution needs to be exercised when using tumour models, especially when the tumour models involve xenografted tumours that consist of homogeneous, highly selected cells without the complex cellular ecology and tumour cell heterogeneity that is found in spontaneously evolving cancers.

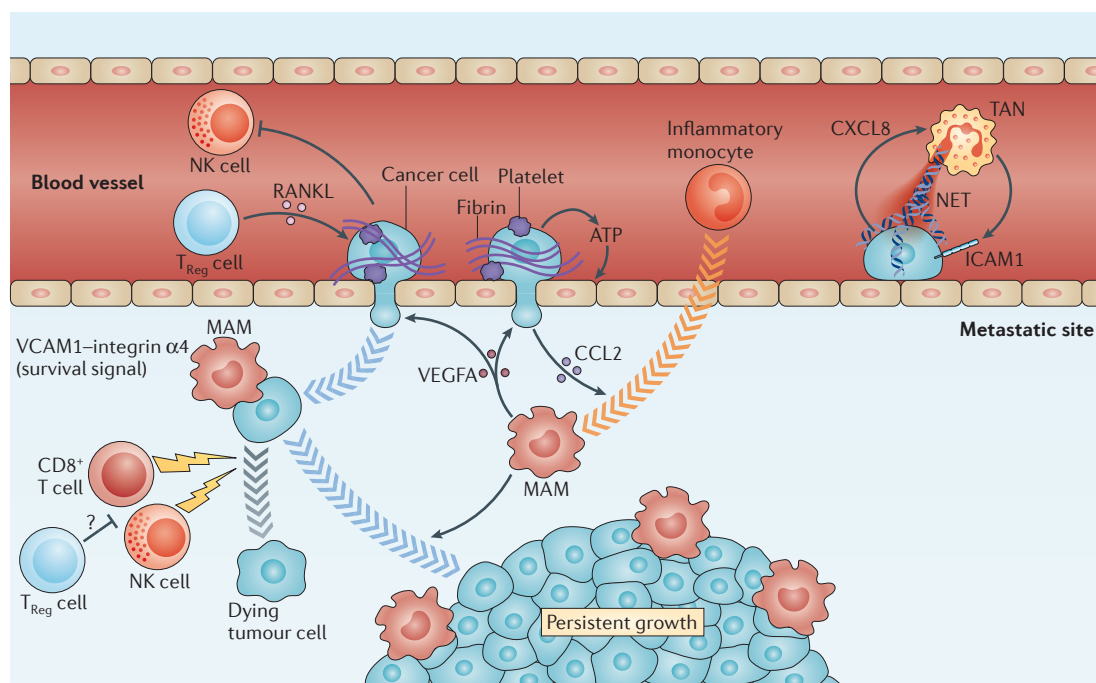
### Tumour cell survival

Circulating tumour cells arrest in microvessels in distant tissues. These cancer cells need to survive in the vessel as well as at the disseminating site to develop metastatic foci. Platelets, macrophages and T<sub>Reg</sub> cells are reported to protect the disseminating cancer cells from immune attack and the stress of a hostile environment (FIG. 4).

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 lungs of platelet-depleted mice than in normal mice<sup>87</sup>. Moreover, genetic loss of Gα<sub>q</sub> (which is a G protein that is essential for platelet activation), prothrombin (which is an essential protease precursor for fibrin formation) or fibrinogen (which is a fibrin precursor that is important for clot formation) reduces the number of residual cancer cells in the lungs 24 hours after their intravenous injection<sup>87</sup>. The precipitous loss of embolus cancer cells in these gene-targeted mice is rescued by NK cell ablation<sup>88,89</sup>. These results indicate that platelet activation and the resultant fibrin clot formation promote the early survival of cancer cells that are lodged at the metastatic sites by shielding them from NK cells.

Clot formation also enhances tumour cell survival by recruiting macrophages. Genetic or pharmacological inhibition of platelet coagulation reduces cancer cell survival in the lungs, as well as the recruitment of CD11b<sup>+</sup>CD68<sup>+</sup>F4/80<sup>+</sup> macrophages to the





**Figure 4 | Helping to overcome the rate-limiting steps of metastasis.** After leaving the primary sites, tumour cells need to survive in the circulation and following arrest at the metastatic sites. This process is helped by fibrin clot formation by platelets and survival signals delivered by them and by tumour-associated macrophages (TAMs) that activate AKT signalling via vascular cell adhesion molecule 1 (VCAM1) on tumour cells, as well as regulatory T ( $T_{Reg}$ ) cells, through the production of receptor activator of nuclear factor- $\kappa$ B ligand (RANKL). At the metastatic sites, cancer cells trapped in emboli secrete CC-chemokine ligand 2 (CCL2) to recruit inflammatory monocytes towards metastatic sites, in which the inflammatory monocytes differentiate into metastasis-associated macrophages (MAMs). MAMs secrete vascular endothelial growth factor A (VEGFA) and increase vascular permeability, which promotes extravasation of cancer cells. MAMs are also involved in the survival and persistent growth of emigrated cancer cells. Cancer cell extravasation and retention at the metastatic sites are also supported by direct interactions through intercellular adhesion molecule 1 (ICAM1) with tumour-associated neutrophils (TANs), which are recruited by CXC-chemokine ligand 8 (CXCL8) that is secreted by the tumour cells. TANs also enhance the entrapment of circulating cancer cells by producing neutrophil extracellular traps (NETs). In addition, platelets increase vascular permeability following tumour extravasation by releasing ATP-containing vesicles. NK cell, natural killer cell.

tumour-challenged lungs. Ablation of these  $CD11b^+$  macrophages suppresses tumour cell survival without affecting clot formation, which indicates that clot formation and macrophage recruitment occur sequentially in a linear pathway<sup>46</sup>. Mechanistically, tumour-initiated clot formation activates endothelial cells to express vascular cell adhesion molecule 1 (VCAM1) and vascular adhesion protein 1 (VAP1; also known as AOC3), which recruit macrophages<sup>90</sup>. The macrophages expressing integrin  $\alpha 4$  (also known as CD49d) bind to cancer cells and transmit survival signals to them via VCAM1, which increases lung metastasis<sup>91</sup>. A recent report also indicates that efficient tumour cell survival at the metastatic site requires the selectin ligand-mediated recruitment of  $LY6C^+$  monocytes<sup>92</sup>, which are progenitor cells of macrophages in the metastatic sites<sup>50</sup>.

Although the role of  $T_{Reg}$  cells at metastatic sites is not clear, these cells may have a role in tumour cell survival.  $CD4^+CD25^+FOXP3^+$  cells isolated from metastatic lymph nodes of patients with melanoma inhibit proliferation and cytokine production of  $CD4^+$  and  $CD8^+$  T cells *in vitro*<sup>93</sup>. Similarly,  $CD4^+CD25^+FOXP3^+$  cells

from malignant ascites of patients with ovarian cancer inhibit T cell proliferation, cytokine production and cytotoxicity<sup>18</sup>. Therefore, it is likely that  $T_{Reg}$  cells at the metastatic sites protect disseminated cancer cells from immune responses. It is noteworthy that  $T_{Reg}$  cells in a primary mammary tumour secrete RANKL, which activates its receptor RANK on cancer cells and promotes lung metastasis without affecting primary tumour growth. Although the precise mechanisms are still unknown, activation of RANK increases the survival of circulating metastasis-initiating cancer cells<sup>26</sup>.

As discussed above, preclinical studies suggest that immune cells help tumour 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 tumour cells from patients with breast cancer contain a malignant population that can initiate metastatic foci in mice. These cells express high levels of CD47 and hepatocyte growth factor receptor (HGFR; also known as MET)<sup>94</sup>, which can inhibit phagocytosis by innate immune cells and

suppress apoptosis of cancer cells, respectively. This clinical study might provide clues about the contributions of these molecules to disseminating cancer cell survival and the possible involvement of immune cells.

### Extravasation and persistent metastatic growth

To establish metastatic foci the arrested tumour cells must extravasate and grow at distant sites. It has been reported that only very few of the circulating tumour cells establish metastatic foci in patients with cancer despite thousands of cells being released into the circulation every day<sup>95</sup>. Thus, the metastatic steps following tumour egress (that is, 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. 4), whereas an active contribution of lymphocytes has not been reported.

**Contribution of MAMs.** In the lungs of PyMT mice with spontaneous metastatic lung foci, there are at least two macrophage populations: 'resident' CD11c<sup>+</sup> alveolar macrophages, which also exist in the normal lungs and have a role in host defence<sup>96</sup>, and 'recruited' MAMs. MAMs are characterized by the expression of CD11b, VEGF receptor 1 (VEGFR1), CXC-chemokine receptor 3 (CXCR3) and CC-chemokine receptor 2 (CCR2), and by the absence of GR1, angiopoietin 1 receptor (TIE2) and CD11c. As depletion of CD11b<sup>+</sup> macrophages but not of CD11c<sup>+</sup> macrophages reduces the number and the size of lung foci that develop following intravenous injection of breast cancer cells, the recruitment of MAMs is essential for the dissemination and the growth of tumour cells at the metastatic site<sup>97</sup>.

*Ex vivo* imaging of the metastatic lung has revealed that macrophages directly contact the extravasating cancer cells, and loss of these macrophages dramatically reduces the number of cancer cells that emigrate from the blood vessels<sup>97</sup>. Macrophage-selective deletion of *Vegfa* reduces lung metastasis *in vivo* and suppresses the extravasation of cancer cells and the permeability of endothelial monolayers *in vitro*<sup>50</sup>, which is a process required for efficient metastasis. These data indicate that MAMs promote extravasation of cancer cells, at least partly, through secretion of VEGFA. In addition to extravasation, MAMs also promote persistent growth of metastatic foci because pharmacological or genetic depletion of macrophages following tumour cell seeding suppresses the lung metastatic load and enhances the survival of mice<sup>97</sup>. It has been reported that enhanced angiogenesis by macrophages, via a TIE2-mediated mechanism, promotes outgrowth of micrometastatic foci in some models<sup>56</sup>. MAMs also increase the survival of disseminated MDA-MB-231 human breast cancer cells<sup>97</sup> through engagement of VCAM1 on tumour cells, which results in signalling for cell survival through AKT<sup>91</sup>.

Macrophages are diverse cells and consist of several subtypes that have distinct roles in the tumour micro-environment<sup>98</sup>; thus, MAMs are another distinct subpopulation that differentiate in the metastatic environment and acquire pro-metastatic functions.

Adoptively transferred inflammatory monocytes preferentially migrate to the metastatic lungs rather than to the primary tumours and differentiate into MAMs<sup>50</sup>. Neutralization of CCL2, which is one of the CCR2 ligands, suppresses this recruitment of inflammatory monocytes and the accumulation of MAMs in the metastatic foci, thereby reducing tumour extravasation<sup>50</sup>. Of note, a similar population of macrophages in the liver, which are recruited by CCL2 and are required for liver metastasis from colorectal cancers, has recently been described<sup>99</sup>. A recent clinical study shows that a high number of circulating inflammatory monocytes correlates with shortened survival of patients with pancreatic cancer<sup>100</sup>.

Taken together, these data indicate that 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 tumour cells. These roles of MAMs at metastatic sites have only recently been defined and thus further studies are required to understand their metastasis-promoting mechanisms of action, as this macrophage population is an attractive therapeutic target.

**Contribution of other myeloid cell types.** Recent studies suggest that neutrophils enhance entrapment and retention of circulating tumour cells at metastatic sites. Adoptive transfer of neutrophils into mice 1 hour after tumour cell injection increased cancer cell retention in the lungs by enhancing extravasation<sup>101</sup>. In this model, entrapped cancer cells secrete CXCL8, promoting neutrophil–cancer cell interaction by increasing neutrophil expression of integrin  $\alpha$ M, which binds to intercellular adhesion molecule 1 (ICAM1) on cancer cells. The attachment of the cancer cells to the neutrophils via integrin  $\alpha$ M–ICAM1 interaction is also required for lung cancer cells to adhere to liver sinusoids and to form metastatic foci<sup>102</sup>. Neutrophils also produce a unique structure called a neutrophil extracellular trap (NET), which is composed of extruded DNA and antimicrobial proteins. In a mouse model of postoperative infection, injected cancer cells have been shown to become trapped in NETs that had formed in liver and lung capillaries, which promotes development of micrometastases<sup>103</sup>.

Genetic loss of MUNC134 (also known as UNC13D), which is a protein that is essential for the secretion by platelets of granules containing ATP, markedly reduces the numbers of extravasating melanoma cells and also vascular permeability<sup>104</sup>. Moreover, genetic ablation of P2Y purinoceptor 2 (P2Y<sub>2</sub>), which is a purinergic receptor activated by ATP, prevents vascular leakage and suppresses tumour extravasation. As reduced expression of P2Y<sub>2</sub> in endothelial cells suppresses platelet-dependent tumour extravasation *in vitro*, these results indicate that platelets loosen the endothelial barrier by releasing ATP-containing granules, which enhances extravasation of cancer cells<sup>104</sup>. Moreover, platelets promote extravasation of colon and breast cancer cells by inducing epithelial–mesenchymal-like transition through secretion of TGF $\beta$  and through direct contact with cancer cells, which increases their survival<sup>105</sup>.

#### Inflammatory monocytes

A subset of monocytes that is recruited to inflammatory sites. They are characterized as CD11b<sup>+</sup>LY6C<sup>+</sup> in mice and CD14<sup>+</sup>CD16<sup>-</sup> in humans. Inflammatory monocytes also express high level of CCR2 and CSF1R but not the neutrophil marker LY6G. In mouse tumour models, inflammatory monocytes are recruited to the primary or metastatic sites by CCL2 and differentiate into tumour-associated macrophages or metastasis-associated macrophages, respectively.

#### Neutrophil extracellular trap

(NET). An extracellular fibre structure that consists of extruded DNA and antimicrobial proteins released from neutrophils in response to inflammatory stimuli. A major function of NETs is to trap and kill pathogens.

**Box 2 | Immune enhancement by immune checkpoint blockade**

When tumour-specific effector CD8<sup>+</sup> T cells and CD4<sup>+</sup> T helper cells receive co-stimulatory signals from antigen-presenting cells, they mediate antitumour immunity and kill cancer cells. However, at the same time, the stimulated T cells upregulate expression of cytotoxic T lymphocyte antigen 4 (CTLA4), which dampens their activities to protect against hyper-immune activation. Moreover, many cancer cells and myeloid cells express programmed cell death 1 ligand 1 (PDL1) and activate programmed cell death protein 1 (PD1)-mediated signalling in T cells, which downregulates T cell activities<sup>123</sup>. These inhibitory signals (known as immune checkpoints) prevent effector T cells from eliminating cancer cells and thus they are important targets to boost antitumour immune responses. Two humanized antibodies against CTLA4, ipilimumab and tremelimumab, have undergone Phase III clinical trials in patients with advanced melanoma and have shown overall survival benefits. In 2011, ipilimumab was approved by US Food and Drug Administration (FDA) because of its benefit to long-term survival of patients with metastatic melanoma. Several antibodies against PD1 or PDL1 have also been developed and clinically tested in Phase I clinical trials for advanced solid tumours, including melanoma, non-small-cell lung carcinoma, renal cell carcinoma, prostate cancer and colon cancer. Although the therapeutic effects depend on the compounds and the type of tumour, targeting the PD1–PDL1 signalling pathway results in the regression of tumours. Some of these compounds are in Phase II clinical trials<sup>124</sup>. However, it is still unknown which cell types induce CTLA4- or PD1-mediated immune suppression in tumours because numerous cells in the tumour microenvironment, including macrophages, express CTLA4 ligands and PDL1 (REF. 19).

Each of the studies described above has been carried out in isolation and in different models. However, the effects of different immune cell types in promoting extravasation and survival of circulating tumour cells are similar. As many of these immune cells work together in other contexts, it is likely that they also work together during metastatic seeding. Recent studies indicate that platelets arrest tumour cells in capillaries and provide a survival signal, which in turn allows a chemotactic gradient of CCL2 to be established to recruit inflammatory monocytes. These monocytes differentiate into MAMs that promote extravasation and cancer cell survival through cell-to-cell contact<sup>46,50,97</sup> (FIG. 4). Multicellular imaging *in vivo* will be important in determining the spatial and temporal relationships of these immune cell types and whether particular combinations of cells enhance metastatic frequency.

**Targeting pro-metastatic immune cells**

Eradication of metastatic tumours is the holy grail of tumour immunology but until recently there has been little success. These recent important advances in antitumour therapy have been attained by augmenting tumoricidal T cell responses. This strategy has mostly made use of the fact that T cell responses are negatively regulated in tumours by inhibitory receptors such as cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed cell death protein 1 (PD1). Thus, neutralization of signals through these receptors enhances T cell responses to eliminate tumour cells. Indeed, antibodies against CTLA4 or PD1 have been clinically tested and have resulted in prolonged overall survival of patients with cancer (BOX 2).

Similarly, a recent preclinical study suggests that inhibition of T<sub>Reg</sub> cells and MDSCs by inactivation of p110δ isoform of phosphoinositide 3-kinase (PI3Kδ) induces immune-mediated rejection of various types

of tumours<sup>106</sup>. Encouragingly, a clinical trial shows that idelalisib (Zydelig; Gilead), which is a selective PI3Kδ inhibitor, is tolerated and effective in patients with chronic lymphocytic leukaemia<sup>107</sup>, although its therapeutic effect on metastatic disease has not been tested yet. Furthermore, a recent study showed that the small population of CD103<sup>+</sup> dendritic cells in the PyMT tumour model, which is expanded by administration of FMS-related tyrosine kinase 3 ligand (FLT3L), can efficiently activate CD8<sup>+</sup> T cells, suggesting that expansion of the CD103<sup>+</sup> dendritic cell population can be another strategy to improve the efficacy of T cell-targeting therapies<sup>108</sup>. Moreover, the engineering of haematopoietic stem cells with vectors that allowed tumour-infiltrating monocytes and macrophages to express IFNα resulted in the inhibition of MDA breast cancer and PyMT tumour growth and experimental metastases. This inhibition was associated with increased numbers of T cells infiltrating into the metastatic lesions<sup>109</sup>. The successes of these clinical and preclinical trials imply that it is possible to enhance antitumour 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 signalling molecules described above that mediate macrophage recruitment and/or activation are all potentially important targets for the treatment of metastatic disease<sup>110</sup>. A recent clinical trial showed that neutralizing antibodies against CSF1R reduced TAM numbers and had a marked clinical benefit in patients with diffuse-type giant cell tumour, which is a type of sarcoma associated with aberrant overexpression of CSF1 (REF. 111). A Phase I clinical trial using an oral CSF1R inhibitor in advanced and metastatic cancer was recently completed (ClinicalTrials.gov identifier: NCT01316822). Moreover, several Phase I clinical trials of CSF1 and CSF1R inhibitors are in the stage of recruiting patients with advanced solid tumours (ClinicalTrials.gov identifiers: NCT01316822 (ARRY-382), NCT01346358 (IMC-CS4), NCT01444404 and NCT01004861). In addition, the dramatic response to a CSF1R inhibitor that was observed in a GEM mouse model of glioma, with evidence that macrophages are repolarized by GM-CSF to become antitumorigenic<sup>112</sup>, has resulted in these inhibitors entering a clinical trial for patients with glioma. Together, these trials may provide evidence for the benefits of targeting the CSF1R pathway in macrophages for the treatment of metastatic disease.

The CCL2–CCR2 pathway is also an attractive target as it is one of the major chemokine signalling pathways that mediates TAM recruitment in preclinical models. Although a humanized monoclonal CCL2-neutralizing antibody (CNT0888) is ineffective in suppressing serum CCL2 levels or tumour progression<sup>113</sup>, a recent clinical trial shows that blockade of CCR2 has the potential to suppress metastatic growth of cancer.

In this clinical trial, a CCR2-specific antibody (MLN1202) reduced the levels of urinary N-telopeptide of type I collagen, which is a marker of bone resorption induced by metastatic cancer cells, in 14 out of 43 patients with bone metastases (ClinicalTrials.gov identifier: NCT01015560). Interestingly, trabectedin (Yondelis; Zeltia and Johnson & Johnson), which is an approved drug used in the clinic to treat human ovarian cancer and soft tissue sarcoma, modulates TAM recruitment by inhibiting CCL2 expression, which suggests that the therapeutic effects of this drug might be exerted by inhibiting TAM recruitment<sup>114,115</sup>, as well as by directly affecting monocyte and macrophage viability. However, the use of inhibitors of CCL2 should be carefully considered because cessation of CCL2-specific treatment has been shown in a mouse model to cause an influx of monocytes into metastatic sites, which enhanced local angiogenesis and metastatic outgrowth and thereby increased mortality<sup>116</sup>.

Although clinical trials targeting pro-metastatic immune cells are still limited, the remarkable suppressive effects observed in the above-mentioned studies strongly suggest that a macrophage-targeting therapy may be an effective strategy. Thus, a better understanding of the mechanisms underlying macrophage recruitment and activation in preclinical models of specific cancer types may guide more rational designs for future trials. Of note, numerous lines of evidence in preclinical models indicate that macrophage targeting, such as via neutralization of CSF1, enhances the efficacy of the conventional therapeutic modalities used in treating metastatic disease, including angiogenesis-targeted therapy and cytotoxic chemotherapy<sup>8</sup>. Importantly, a recent study showed that TAMs in human breast cancer express IL-10 and that blockade of IL-10 was equivalent to CSF1 neutralization in enhancing the efficacy of chemotherapy<sup>117</sup>. The particular immunosuppressive roles of infiltrating macrophages also suggest that targeting specific components of the immune system might result in less toxicity than blocking inhibitory receptors such as CTLA4 on all cells that express this receptor, which could result in increased autoimmune responses. Thus, it is likely that combining 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 specifically tested in metastatic tumours or with metastasis as an end-point.

### Conclusions and perspectives

Many animal studies indicate that the recruitment of immunosuppressive cells to the primary tumour site protects cancer cells from being killed by cytotoxic cells and that this increases the probability of tumour cell survival and dissemination. Detailed knowledge about the mechanisms that underlie immunoregulatory cell recruitment and their suppressive functions in the primary tumour and metastatic sites should lead to more effective immunotherapies. Current results

indicate that individual suppressor cell types are recruited to the primary or metastatic sites by distinct chemoattractants; for example, CCL2 recruits monocytes and macrophages; CXCL1 and CXCL5 recruit MDSCs; and CCL5 and CCL22 recruit T<sub>Reg</sub> cells. It is therefore possible that certain types of tumour predominantly recruit specific types 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 the response.

The data discussed in this Review 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. However, current therapies are likely to be limited as they either target all macrophages (for example, CSF1R inhibition) or they 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 the essential cellular interactions between the various cell types in the tumour microenvironment is needed to allow for more precise therapeutic intervention.

Cancer cells have high mutation rates that generate very heterogeneous tumours containing innumerable mutants. These mutant cells provide a reservoir of drug-resistant cells that are able to prosper after the selective pressure of chemotherapy. However, the immune system can evolve equally rapidly and is capable of eliminating very large cell masses, as seen following fetal death when tolerance to the allogeneic fetus is broken, for example. Thus, given the high mutation rate of cancer cells, immunotherapy may be the only effective strategy for complete elimination of already drug-resistant metastatic cells. Immunotherapy will, by necessity, require targeting the immunosuppressive mechanisms that are provided by the recruited immune (and perhaps other resident) cells. Of course, tumour cells will evolve following targeting 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 CSF1R inhibition<sup>86</sup>. Consequently, modelling the evolution and the ecology of the tumour microenvironment with a particular emphasis on tumour heterogeneity<sup>118</sup> holds promise for effectively targeting different aspects of the supporting tumour microenvironment, sequentially or synergistically. Such approaches can remove the microenvironmental support for metastatic tumour cells and at the same time enable immune targeting of the now vulnerable tumour cells. Such strategies, together with more traditional treatments, such as radiation or chemotherapy, that reduce tumour burden and expose tumour antigens<sup>119</sup> or with vaccination using tumour antigens holds promise for successfully eradicating metastatic disease.

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**Competing interests statement**

The authors declare no competing interests.

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