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Imaging interactions between macrophages and tumour cells that are involved in metastasis *in vivo* and *in vitro*

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Summary

Tumour-associated macrophages participate in several protumour functions including tumour growth and angiogenesis, and facilitate almost every step of the metastatic cascade. Interfering with macrophage functions may therefore provide an important strategy in the clinical management of cancer and metastatic disease. Our understanding of macrophage functions has been greatly expanded by direct observations of macrophage–carcinoma cell interactions using light microscopy. Imaging approaches include intravital microscopy of tumours in mouse models of cancer and visualization of macrophage–carcinoma cell interactions in *in vitro* assays; whether atop 2D substrates, embedded in 3D matrices or in more complex assemblies of multiple cell types that mimic specific topologies of the tumour microenvironment. Such imaging and reconstitution approaches have provided us with a wealth of information on the motile behaviour and physical associations between macrophages and carcinoma cells and the role of the tumour microenvironment in influencing the movement of these cells. Finally, high-resolution imaging techniques have permitted researchers to correlate motility patterns with specific gene signatures and biochemical pathways in cells, pointing to potential targets for intervention. Here, we review experimental approaches employed in the study of macrophage interactions with carcinoma cells with an emphasis on imaging invasive and metastatic cell motility in breast carcinomas.

Keywords

Imaging; metastasis; macrophages; tumour cells

Introduction

Macrophages play important roles as resident cells in tissues and during inflammation, where they are typically recruited from the circulation as monocytic precursors and coordinate the inflammatory response through phagocytosis and other effector functions. However, increased macrophage density in the stroma of several tumours, including breast cancer, has been correlated with poor patient prognosis and has been associated with invasive phenotypes. In particular, macrophages appear to be critical for tumourigenesis in several types of inflammation-induced cancer and participate in almost all aspects of malignancy (Bingle *et al.*, 2002; Talmadge *et al.*, 2007). Importantly, the roles of these tumour-associated macrophages (TAMs) in breast cancer have been substantiated experimentally in mouse models of cancer [reviewed in (Qian & Pollard, 2010)]. One of the first definitive experimental examples of TAM involvement in malignancy came from studies of mice lacking the pleiotropic cytokine colony stimulating factor-1 (CSF-1), which is required for normal monocyte lineage development, including the generation of macrophages. These mice, when expressing the Polyoma middle T (PyMT) antigen under a mammary-specific promoter (MMTV) to drive spontaneous mammary carcinogenesis, showed decreased levels of circulating tumour cells, lung metastases and decreased angiogenesis, despite unaltered primary tumour growth (Lin *et al.*, 2001; Lin *et al.*, 2006). These findings established a firm causal relationship between TAM functions and cancer malignancy and metastasis. A number of studies have subsequently confirmed the role of TAMs in malignancy and identified further intrinsic and microenvironmental mechanisms that skew TAMs towards performing protumour functions [reviewed in (Qian & Pollard, 2010)].

In spite of these data, TAMs do not comprise a homogenous collection of cells. Rather, they play diverse roles in tumour progression, depending both on the function of the infiltrating monocytic precursor cell and on instructions derived from the local microenvironment. These protumour functions may thus include angiogenesis, modulation of immune responses towards the tumour including suppressive and tolerogenic activities and assisting invasion and metastasis (Qian & Pollard, 2010; Laoui *et al.*, 2011; Ruffell *et al.*, 2012). It would therefore be interesting to determine whether distinct TAM subpopulations have predetermined functions in malignancy as recent transcript expression analyses suggest (Ojalvo *et al.*, 2009; Pucci *et al.*, 2009). As such, direct visualization of TAM-carcinoma cell and TAM-microenvironment interactions has emerged as a powerful tool in better understanding the diverse roles of macrophages in the primary tumour and beyond. In combination with analyses of cell signalling pathways and gene expression profiles (Ojalvo *et al.*, 2009; Ojalvo *et al.*, 2010), we are now able to dissect the many roles of macrophages inside the primary tumour stroma as well as in organs where metastases are formed, and assign specific TAM-mediated functions during the course of metastasis.

Intravital imaging studies using multiphoton laser-scanning microscopes together with a combination of labelling techniques to specifically identify tumour cells and macrophages have provided a true breakthrough in our understanding of distinct TAM functions (Beerling *et al.*, 2011; Wyckoff *et al.*, 2011). Specifically, TAMs facilitate the invasion of tumour cells into the surrounding stroma and towards blood vessels, where they also promote the

intravasation of cancer cells into the bloodstream (Fig. 1A). These events are achieved by chemotactic cues that stimulate the directional movement of cancer cells as well as factors that alter the stiffness and composition of the extracellular matrix (ECM) thus facilitating passage through nonpermissive scaffolds (e.g. via the secretion of matrix degrading enzymes or ECM molecules) (Roussos *et al.*, 2011b; Friedl & Alexander, 2011). In addition, perivascular macrophages form complexes with migratory tumour cells in the primary tumour and complex formation is required for intravasation to begin systemic dissemination to distant sites. The perivascular macrophage–tumour cell complex observed in living mouse mammary tumours has been identified in tissue of breast cancer patients and correlated with distant metastasis (Robinson *et al.*, 2009). Furthermore, a distinct macrophage population termed metastasis-associated macrophages (MAMs) has been identified in the lung where it actively participates in the metastatic seeding of extravasating tumour cells (Qian *et al.*, 2009). Therefore, rather than employing limiting endpoints in the analyses of factors that contribute to tumour cell dissemination, we are now able to observe in real time each step and assign temporally and spatially unique roles to molecules, genes, cell signalling pathways and cell interactions of interest.

This extraordinary complexity and range of TAM functions has forced researchers to develop *in vitro* systems for modelling and visualizing each step of the metastatic cascade. Recreation of the tumour microenvironment *in vitro* provides a faster and more controlled environment for the elucidation of mechanisms of crosstalk between macrophages and carcinoma cells. Experimental settings may range from simple two-dimensional (2D) cocultures on planar surfaces to more complex three-dimensional (3D) matrices and multicellular assemblies to better mimic *in vivo* conditions. This way, screening for identification of contributing factors in each step is simplified, whereas at the same time the resolution and spatiotemporal regulation of the process are improved and complement *in vivo* findings.

Imaging macrophage–tumour cell interactions *in vivo*

Identification of TAMs in the primary tumour as imaged by intravital microscopy

Intravital multiphoton imaging in both transgenic mouse models of breast cancer (MMTV–PyMT) as well as in syngeneic and allogeneic grafts of rat MTLn3 mammary carcinoma cells has shown that macrophages are essential for tumour cell invasion in the primary tumour. *In vivo*, macrophages can be visualized by microscopy by the uptake of fluorescent-coupled dextrans following intravenous injection due to the phagocytic properties of macrophages (Wyckoff *et al.*, 2007; Egeblad *et al.*, 2008). Additionally, macrophages—and other cells of the myeloid lineage—can be imaged in transgenic mice, which express fluorescent proteins under myeloid-specific promoters (Wyckoff *et al.*, 2004; Wyckoff *et al.*, 2007; Hume, 2011). Using this multiphoton imaging technology, TAMs have been localized at the collagen-rich tumour margin, in association with tumour cells in regions of active cell migration and deep inside the tumour in association with blood vessels (Wyckoff *et al.*, 2007). The fact that no single myeloid marker can be used reliably to identify any one distinct myeloid population may contribute to confusion in the characterization of TAMs. Imaging their behaviour *in vivo* therefore is a better way to functionally characterize them

and assess how they affect tumour progression. For example, a recent intravital imaging study identified a population of myeloid cells that resided at the tumour margin and was capable of engaging in short- and long-term interactions with T cells thus leading the authors to classify them as antigen presenting dendritic cells (DCs; Engelhardt *et al.*, 2012). These DCs were positive for cell surface markers shared with TAMs, including F4/80 and the integrin CD11b and they were also capable of phagocytosis of tumour cell-derived material (Engelhardt *et al.*, 2012). This profile is similar to that of TAMs coinvasive with breast tumour cells *in vivo* (Ojalvo *et al.*, 2009), however their distinct morphology, localization and ability to interact with T cells supports their classification as *bona fide* DCs and distinct from TAMs involved in metastatic program of tumour cells (see later).

Chemotaxis, migration and invasion in the primary tumour

What had been previously unappreciated with the exclusive use of fixed tissue was that overall tumour cell motility is increased *in vivo* when in proximity to TAMs and that tumour cells and macrophages often comigrate. Specifically, breast tumour cells observed by intravital imaging move as single amoeboid cells in either random fashion or in coordinated streams, that is, single files of nonattached cells following one another (Wang *et al.*, 2002; Wyckoff *et al.*, 2007; Roussos *et al.*, 2011a). This streaming mode of migration consists of alternating tumour cells and macrophages (Fig. 1B) and depletion of macrophages (e.g. by injection of liposome encapsulated clodronate) leads to significantly decreased streaming motility, suggesting that macrophage function is essential for this process (Roussos *et al.*, 2011a). Overall, multicellular streaming migration of tumour cells with macrophages is more efficient than random cell movement *in vivo*, with cells in streams moving significantly faster, with increased path length and directionality compared to randomly moving single cells (Roussos *et al.*, 2011a).

At the molecular level, this efficient long-distance comigration of the two cell types is coordinated by an epidermal growth factor (EGF)–CSF-1 paracrine loop (Wyckoff *et al.*, 2004). In this loop, tumour cells secrete CSF-1, which stimulates chemotaxis of macrophages towards them and also stimulates the macrophages to secrete EGF. In turn, the EGF is sensed by the tumour cells, which respond by migrating towards the macrophages and also secreting more CSF-1 (Wyckoff *et al.*, 2004). These events lead to a relay chemotaxis scheme where the EGF–CSF-1 signals are amplified from one cell to the next (Fig. 1A), thus enabling the macrophage–tumour cell streams to travel over long distances inside the primary tumour. Confirmation of the function of the paracrine loop between tumour cells and macrophages *in vivo* has been demonstrated by genetic or pharmacological ablation of CSF-1 in transgenic mice and by intravital imaging in both mouse and rat mammary tumours (Wyckoff *et al.*, 2004; Roussos *et al.*, 2011a). In addition, by use of an *in vivo* invasion assay in which cells from live primary tumours can be induced to migrate towards microneedles and hydrogel capsules that generate chemotactic gradients of either EGF or CSF-1 (Wyckoff *et al.*, 2000; Wyckoff *et al.*, 2004; Raja *et al.*, 2010), both EGF and CSF-1 signalling were shown to be essential for the *in vivo* comigration of tumour cells and macrophages (Wyckoff *et al.*, 2004; Roussos *et al.*, 2011a). Other microenvironmental signals, such as CXCL12 or heregulin beta1, can also trigger this paracrine event in rat MTLn3 and MMTV–PyMT mammary tumours, however EGF–CSF-1 signalling is still

required, suggesting that this loop is the 'central engine' for macrophage–tumour cell comigration (Hernandez *et al.*, 2009). Finally, the paracrine loop can also be recapitulated *in vitro* with purified tumour cell and macrophage populations (Goswami *et al.*, 2005; DeNardo *et al.*, 2009), as is discussed later. Interestingly, invasive tumour cells that are collected from live mammary tumours in response to chemotaxis overexpress a unique isoform of the actin cytoskeleton regulatory protein Mena, Mena^{INV} (Goswami *et al.*, 2009), which has been shown to dramatically increase the sensitivity of tumour cells to respond to EGF by 25- to 50-fold (Philippar *et al.*, 2008; Roussos *et al.*, 2011a). Increased sensitivity to EGF enhances tumour cell ability to respond to shallow gradients of EGF gradients *in vivo*, and hence, amplifying the paracrine interaction with macrophages leading to increased primary tumour invasion (observed both by multiphoton intravital imaging and by immunohistochemistry of fixed tumour tissue (Roussos *et al.*, 2011a).

Tumor cell intravasation and hematogenous dissemination

The role of macrophages in tumour cell intravasation in mouse mammary tumours has been studied *in vivo* by intravital multiphoton imaging. In MMTV–PyMT transgenic mice, macrophages are located on the abluminal side of blood vessels, where they attract the tumour cells towards the vessels and assist tumour cell intravasation (Wyckoff *et al.*, 2007). Indeed, macrophage depletion by genetic ablation in transgenic mice or blocking the functions of either EGF or CSF-1 results in a decrease in the number of circulating tumour cells in the blood of mammary tumour-bearing mice (Wyckoff *et al.*, 2007; DeNardo *et al.*, 2009; Roussos *et al.*, 2011a). As in comigration and invasion, the paracrine EGF–CSF-1 interaction loop is essential in the macrophage-assisted intravasation of breast tumour cells.

According to the data described above from multiphoton intravital imaging, intravasation occurs at areas of the tumour where perivascular macrophages assist invasive tumour cells to enter the blood vessel. This suggests that a microanatomical structure of an invasive tumour cell, a macrophage and an endothelial cell (blood vessel) in direct contact should exist in metastatic tumours and such a structure may initiate metastasis. This tripartite structure has been denoted as a tumour microenvironment of metastasis (TMEM; Fig. 1C). Indeed, and in agreement with the mouse and rat data, Robinson *et al.* reported that TMEM structures exist in paraffin-embedded tumour tissue from breast cancer patients (Robinson *et al.*, 2009). In addition, TMEM count significantly correlates with the development of distant organ metastasis in breast cancer patients (Robinson *et al.*, 2009). Recently it was also shown that the number of TMEM scored in human breast tumours strongly correlates with Mena^{INV} expression in samples collected from the same tumours by fine needle aspiration (Roussos *et al.*, 2011c) indicating that macrophage–tumour cell streams that form in response to elevated EGF receptor (EGFR) sensitivity to EGF, as induced by expression of Mena^{INV}, are involved in TMEM assembly. These results suggest that macrophage-assisted tumour cell intravasation functions in patients with breast tumour to contribute to distant metastasis.

Extravasation and establishment of metastasis at secondary sites

Accumulation of myeloid derived cells at distant sites, such as the lung, has been shown to be triggered by the primary tumour, creating a 'premetastatic niche' that enhances successful establishment of secondary metastases at these sites (Kaplan *et al.*, 2005; Hiratsuka *et al.*,

2006; Erler *et al.*, 2009; Psaila & Lyden, 2009). These recruited myeloid cells express the VEGF receptor 1 (VEGFR1) and are CD11b-positive, a profile typical of cells of the mononuclear phagocytic lineage. However, the previous studies, although seminal in uncovering mechanisms of premetastatic niche formation, mainly used endpoint assays without high-resolution imaging to dissect the exact contribution of these cells to metastasis. In more recent studies, where the isolated process of extravasation was analysed in more detail, macrophages specifically were shown to be important at the metastatic site. A distinct subpopulation of macrophages (expressing the markers F4/80, CSF-1R, CD11b, VEGFR, CCR2 and lacking expression of Gr1), which differs from the resident lung macrophages, is recruited to the lungs of mammary tumour-bearing mice (Qian *et al.*, 2009; Qian *et al.*, 2011; Gil-Bernabe *et al.*, 2012). These macrophages are essential to establish macrometastases at the lung site. *Ex vivo* imaging of lungs with fluorescently labelled macrophages and tumour cells has shown that these MAMs assist tumour cell extravasation from the blood vasculature into the lung tissue, in a VEGF dependent manner (Qian *et al.*, 2011). In addition, macrophages support the initial growth of the extravasated tumour cells, as ablation of these recruited macrophages leads to a decrease in lung metastasis even after the initial dissemination has been established (Qian *et al.*, 2009). These data suggest that macrophages promote metastatic progression not only at the primary tumour site, but also at the metastatic niche.

Imaging macrophage–tumour cell interactions *in vitro*

2D cocultures

Coculturing macrophages with tumour cells on planar, 2D surfaces is an easy method for determining whether or not tumour cell migratory properties are affected by the presence of TAMs (Goswami *et al.*, 2005). Indeed, MTLn3 rat mammary carcinoma cells convert from a nonpolarized round cell morphology to an elongated one (Fig. 1D), with prominent directional protrusions when in the presence of macrophages (Goswami *et al.*, 2005). Concurrently, their migratory patterns also change from random to directional with an increase in the net distance migrated, indicative of directional cues provided by the cocultured macrophages. As with the *in vivo* setting, carcinoma cell motility *in vitro* depends on EGF secreted by macrophages, which is specifically upregulated following stimulation with tumour-derived CSF-1. Blocking either the CSF-1R in macrophages or the EGFR in carcinoma cells inhibits macrophage-induced carcinoma cell protrusions (Goswami *et al.*, 2005). A similar approach has identified macrophages as being important in affecting the motile properties of colon carcinoma cells and revealed SDF-1 and VEGF, but not EGF, as important factors secreted by macrophages (Green *et al.*, 2009). Likewise, microglia, specialized macrophages residing in the central nervous system, promote glioblastoma cell invasion via a similar EGF–CSF-1 paracrine interaction (Coniglio *et al.*, 2012). This approach, albeit reductionist, reveals the enhancement of cancer cell motility parameters by macrophages and may be used to identify major determining chemotactic factors in the motility of carcinoma cells of various origins.

3D invasion assays

Despite the ease and usefulness of a 2D morphological and motility assay, cell migration in 3D matrices, as is the case *in vivo*, is more complex requiring, apart from soluble guidance cues, different secreted factors that remodel the matrix in order to facilitate passage of cells, several of which are provided by TAMs (Joyce & Pollard, 2009; Friedl & Alexander, 2011; Magalhaes *et al.*, 2011). Furthermore, tumour cells adapt their motility strategy in changing microenvironments or when responding to therapeutic agents and inhibitors aiming at impeding their invasion (Alexander & Friedl, 2012). Consequently, imaging motility in 3D matrices could provide a more useful approach to complement *in vivo* experiments and to screen for agents that arrest tumour cell migration with limited bioavailability for *in vivo* experiments. Efforts are also under way to alter the composition, crosslinks and stiffness of the ECM components of these 3D matrices in an effort to better mimic the distinct extracellular environments encountered by carcinoma cells and TAMs *in vivo* (Levental *et al.*, 2009; Van Goethem *et al.*, 2010).

Invasion of MTLn3 carcinoma cells into an overlaying collagen I matrix, as assessed by confocal microscopy (Fig.1D), requires macrophages and the aforementioned EGF-CSF-1 paracrine loop (Goswami *et al.*, 2005). Human carcinoma cells (MDA-MB-231) also exhibit strong macrophage dependence for invasion using the same *in vitro* 3D invasion assay. By contrast, the macrophage dependence during invasion *in vivo* is less pronounced due to autocrine CSF-1 signalling by human cancer cells, which is initiated by additional microenvironmental inputs (Patsialou *et al.*, 2009). This example points to some of the inherent limitations of *in vitro* approaches and highlights the necessity to complement *in vitro* experiments with *in vivo* measurements.

Nevertheless, these *in vitro* assays still offer the ability to explore other pathways and experimental approaches in dissecting the contribution of macrophage-derived signals during invasion. For example, CSF-1 binds to the CSF-1 receptor (CSF-1R) tyrosine kinase on the cell surface, which then becomes autophosphorylated and activates a number of signalling pathways. Among the phosphorylated tyrosines on CSF-1R, Y721 links to PI3K and regulates macrophage motility (Pixley & Stanley, 2004). When macrophages deficient in CSF-1R were complemented with either wild-type CSF-1R or a receptor bearing the Y721F mutation, invasion of MTLn3 cells was significantly reduced in the presence of the Y721F macrophages, confirming the role of this residue in stimulating macrophage-dependent tumour cell invasion (Sampaio *et al.*, 2011). Such approaches therefore permit a more thorough dissection, down to single amino acid resolution, of macrophage-specific pathways that impart on tumour cell invasion. Equivalent results would be very difficult to obtain *in vivo* as it would require the generation of complex transgenic tumour animal models.

Additionally, 3D collagen matrix assays have been used to delineate the contribution of macrophage-derived proteases that facilitate invasion through ECM degradation and the formation of pores or tracks that facilitate tumour cell migration. For example, it was uncovered that macrophage-derived lysosomal cathepsin proteases B and S are required for efficient 3D invasion of breast cancer cells (Gocheva *et al.*, 2010). In a similar setting, it was

found that invasion of human breast carcinoma cells through matrigel, a dense matrix, required the proteolytic contribution of macrophages, which was depended on the phagocyte-specific nonreceptor tyrosine kinase Hck (Guiet *et al.*, 2011). Importantly, the later study revealed that the presence of macrophages influenced the mode of migration used by tumour cells, which switched from mesenchymal in the absence of macrophages, to ameboid in their presence, whereas at the same time shifting their requirement for proteases in order to invade the surrounding matrix. Indeed, whereas tumour cells alone required MMP activity for invasion, in the presence of macrophages invasion was independent of tumour-derived MMPs and proceeded via the use of macrophage-derived proteases (Guiet *et al.*, 2011).

In conclusion, modelling and imaging macrophage–carcinoma cell interactions in 3D matrices offers a unique scheme in the delineation of complex biochemical determinants that contribute to invasion and motility in scaffolds of defined protein composition and mechanical properties.

Migration in 1D—can it recapitulate TAM-cancer cell migration?

Two-photon intravital imaging has identified collagen fibres inside the primary tumour, which serve as tracks onto which cancer cells migrate (Wang *et al.*, 2002; Provenzano *et al.*, 2009; Roussos *et al.*, 2011a). Recent *in vitro* studies on micropatterned surfaces showed that mesenchymal cells migrating on thin '1D' tracks, mimicking the topography of matrix fibres, had faster migration speeds and showed persistent polarity on a single axis during cell movement, as compared to those on 2D surfaces (Doyle *et al.*, 2009). This opens a new experimental opportunity for researchers to explore cancer cell motility and interactions with macrophages. Indeed, our results using ECM protein-coated 1D substrates show that MTLn3 cells and macrophages spontaneously assemble into arrays of alternating tumour cells and macrophages resembling the streaming invasion observed in primary mammary tumours using intravital imaging (Sharma *et al.*, 2012). Similar to the *in vivo* observations, tumour cells demonstrate increased motility on 1D substrates in the presence of macrophages and this enhancement is dependent on an intact EGF–CSF-1 paracrine interaction (Sharma *et al.*, 2012). Collectively, these data suggest that 1D scaffolds can be used as a reliable approach to model and image macrophage–tumour cell interactions. It must be noted that so far these experiments have been performed in the absence of a directional source of chemoattractant and future incorporation of an external chemotactic gradient may allow for a more accurate evaluation of motility and relay chemotaxis mechanisms on fibrillar matrices.

Modelling intravasation of cancer cells and the contributions of perivascular macrophages

As discussed above, perivascular macrophages have been observed to play important roles in the intravasation of cancer cells *in vivo*. To recreate the TMEM and examine macrophage-dependent intravasation *in vitro*, an abluminal-to-luminal transendothelial migration (intravasation) assay has been developed (Fig. 1E). This *in vitro* assay recapitulates the macrophage-dependent intravasation observed *in vivo* while allowing for increased control in the variation of the presence of macrophages to investigate their necessity in tumour cell intravasation. In this assay, macrophages and carcinoma cells are plated on the abluminal side—an endothelial monolayer on a Matrigel coating. Cells then invade through the

Matrigel and endothelium to cross into the bottom chamber containing chemoattractant, mimicking physiological intravasation (Roussos *et al.*, 2011a). This *in vitro* assay recreates the paracrine interaction between tumour cells and macrophages observed at sites of tumour cell intravasation. Using this assay, MTLn3 rat mammary carcinoma cells cannot traverse the endothelium in the absence of macrophages. Crucially, in the presence of macrophages, only Mena^{INV}-expressing MTLn3 carcinoma cells can transmigrate efficiently (Roussos *et al.*, 2011a), consistent with the enhanced Mena^{INV} expression and TMEM structures observed in patient tissues described above. Therefore, this assay can be used to screen for cancer cell (autonomous and non-autonomous factors) that contribute specifically to intravasation. Application of other parameters that influence endothelial function, such as altered barrier function or the effect of luminal flow, may be incorporated into this assay in order to better mimic the behaviour of the tumour vasculature.

Whole body imaging of TAMs

With an increased appreciation of the role of TAMs in promoting tumour progression and metastasis new techniques for imaging macrophages are being developed for translational application using clinically relevant imaging modalities including magnetic resonance imaging (MRI), positron emission tomography (PET) and computed tomography (CT).

Macrophages have been tracked using *ex vivo* labelling with liposomal dyes (Sista *et al.*, 2009) or super paramagnetic iron oxide (SPIO) nanoparticles to describe the fate of macrophages in the tumour microenvironment (Valable *et al.*, 2007). Indeed studies of DiD-labelled monocytes introduced intravenously into MMTV-PyMT tumour-bearing mice demonstrated that these monocytes accumulate in the mammary tumours (Sista *et al.*, 2009). Although this approach allows for the delineation of the role of macrophage infiltration into a tumour, clinical applications are challenging.

SPIO nanoparticles used for MRI imaging or conjugated with fluorophores, or PET imaging agents have been shown to accumulate in tumour macrophages when injected *in vivo* (Moore *et al.*, 2000; Nahrendorf *et al.*, 2010; Daldrup-Link *et al.*, 2011; Keliher *et al.*, 2011). Preferential association of SPIO nanoparticles for myeloid cells has been determined by the colocalization of SPIO density with CD11b⁺, F4/80⁺, Ly6C^{hi} (Leimgruber *et al.*, 2009; Keliher *et al.*, 2011) or CD68⁺ cells (Daldrup-Link *et al.*, 2011). In these studies, few neutrophils are labelled (approximately 15% of SPIO labelled cells) indicating that the labelled cells are TAMs (Leimgruber *et al.*, 2009). TAM depletion via administration of either clodronate liposomes (Leimgruber *et al.*, 2009) or anti-CSF-1 antibody resulted in the reduction of SPIO contrast by MRI (Daldrup-Link *et al.*, 2011) confirming that macrophages are labelled with the SPIO nanoparticles. These studies also demonstrate that TAMs can be targeted in the tumour microenvironment using injectable contrast agents with clinical imaging methods.

Colocalization of SPIO with macrophages in tissues has been shown to occur several days after injection, indicative of monocyte and macrophage labelling in the blood, spleen or bone marrow with subsequent tissue infiltration (Wu *et al.*, 2006; Oweida *et al.*, 2007). This long-term labelling method has been used to longitudinally monitor macrophage infiltration

in a transplantable tumour model (Shih *et al.*, 2011). With a single systemic injection of SPIO 5 days after tumour implantation, TAMs were monitored up to 14 days after tumour implantation. At day 5 after tumour implantation, TAMs were determined to localize in new tumour areas that have low blood vessel density. By day 14 TAMs were primarily located adjacent to blood vessels as confirmed by immunohistochemistry (Shih *et al.*, 2011). These data demonstrate that TAM localization changes during tumour progression and that TAMs are associated with angiogenesis before the onset of rapid tumour growth (Shih *et al.*, 2011), supporting earlier observations on the role of TAMs in regulating angiogenesis (Lin *et al.*, 2006). This strategy of a single administration of SPIO can be used to track TAMs longitudinally and further elucidate TAM–carcinoma cell interactions in animal models as well as in human patients.

SPIO nanoparticles are clinically relevant as they are similar to the clinically approved SPIO contrast agents. Specifically, Ferumoxyl (Feraheme), as used by Daldrup-Link *et al.* (Daldrup-Link *et al.*, 2011) has been approved by the FDA for intravenous treatment of iron deficiency. These animal studies demonstrate that clinically relevant contrast agents and imaging modalities can be used to monitor TAMs during tumour progression.

Conclusions and perspectives

Intravital imaging of tumours has provided unparalleled insight in the understanding of the tumour microenvironment. In particular, it has highlighted the role of macrophages in cancer progression and identified topographically and functionally distinct populations, including TAMs that assist in invasion of tumour cells and perivascular macrophages that facilitate tumour cell intravasation. Complemented by *in vitro* imaging approaches, they have allowed further dissection and validation of key molecules and signalling pathways that are necessary in discrete steps during tumour cell metastasis.

Based on the above experimental data, isolation of invading tumour cells and coinvasive TAMs from patients could provide a significant step towards assessing the risk of metastatic disease in cancer patients. A more cell biology-oriented evaluation of each patient may allow for earlier decisions in therapy thereby reducing the time to treatment. In combination with clinical imaging approaches, these methods may also be used to monitor disease progression over time including the potential for identifying metastasis or tumour recurrence at the primary site. As our understanding of the different subtypes of TAMs in tumour progression such as angiogenesis, tumour cell intravasation and tumour cell extravasation in metastasis increases, the identification of TAMs in the primary tumour may have greater clinical impact.

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Fig. 1.

TAM–tumour cell interactions during invasion and intravasation. (A) Schematic representation of the interactions between TAMs and tumour cells. Tumour cells (green) leave the main tumour mass and invade into the stroma (i) facilitated by chemotactic factors secreted by TAMs (red) and amplified by a paracrine EGF–CSF-1 loop. The dashed arrow denotes the direction of movement. Once at the abluminal side of blood vessels (ii), tumour cells intravasate with the assistance of perivascular TAMs in the microanatomical structure termed tumour microenvironment of metastasis (TMEM). Both TAM-dependent steps can be imaged *in vivo/in situ* (B, C) and recreated *in vitro* (D, E). (B) *In vivo* migration can occur in streams as imaged by intravital multiphoton microscopy (upper panel) of MTLn3 cells (green) and Texas red dextran-labelled macrophages (orange outline). Bar: 25 μ m. The bottom panel shows immunohistochemical staining of mammary tumour fixed and paraffin-embedded MTLn3 tumours expressing Mena^{INV} (T; pink) and macrophages stained for F4/80 (M Φ ; grey). Images are reproduced from (Roussos *et al.*, 2011a). (C) TMEM assembly is correlated with tumour cell intravasation. Left panel: multiphoton microscopy image of MTLn3-EGFP-Mena^{INV} cells (green) adjacent to Texas red-labelled perivascular macrophages (red). Scale bar: 25 μ m. Arrow indicates TMEM, defined as perivascular macrophages and Mena^{INV}-expressing tumour cells in direct contact with each other and the vessel wall. Asterisk indicates the blood vessel. Image reproduced with permission from (Roussos *et al.*, 2011c). Right panels: TMEM from a human patient sample; tumour cell (red), macrophage (brown) and endothelial cell (blue) with a higher magnification of the inset shown on the right. Image reproduced from (Robinson *et al.*, 2009). (D) Coinvasion of macrophages and tumour cells can be recreated *in vitro*. Upper panels: In 2D cultures, MTLn3 cells (white outlines) are rounded but elongate and acquire a polarized morphology in the presence of macrophages (M Φ ; white asterisks), indicative of paracrine interaction between the two cell types. Lower panels: Z stack reconstruction of a 3D invasion assay where MTLn3 cells invade the collagen only in the presence of macrophages. MTLn3 cells (green) were plated alone or in the presence of macrophages (red) and overlaid with a collagen I gel. After incubation for 16 h, cells were fixed and imaged with a confocal microscope. Bars, 20 μ m. Images courtesy of Dan Ishihara, A.D. and D.C., Albert Einstein College of Medicine. (E) Schematic representation of the *in vitro* tumour cell intravasation assay. Macrophages

(red) and tumour cells (green) are allowed to cross an endothelial monolayer (pink) from the abluminal to the luminal direction through the aid of a chemotactic gradient. Image adapted from (Roussos *et al.*, 2011a).