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The interplay between macrophages and angiogenesis in development, tissue injury and regeneration

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ABSTRACT During organ development and remodeling, macrophages support angiogenesis, not only by secreting proangiogenic growth factors and matrix-remodeling proteases, but also by physically interacting with the sprouting vasculature to assist the formation of complex vascular networks. Recent data further indicate that embryonic and tumor-associated macrophages express similar genetic programs, possibly suggesting convergent functions in organogenesis and tumorigenesis. In this article, we review the role of macrophages in development, tissue injury and regeneration, by focusing on the mechanisms used by subsets of these cells, such as the TIE2-expressing macrophages, to regulate angiogenesis and lymphangiogenesis in both fetal and post-natal life.

KEY WORDS: *monocyte, lymphangiogenesis, wound healing, tumor, tumor-associated macrophage*

The monocyte/macrophage system

In adulthood, monocytes are short-lived cells that derive from bone marrow (BM) hematopoietic stem cells (HSCs) and circulate in the peripheral blood. In humans, monocytes represent 5-8% of all circulating leukocytes. Under inflammatory conditions, monocytes extravasate and migrate into tissues, where they differentiate into macrophages and dendritic cells (DCs). It is now clear that circulating monocytes are a heterogeneous cell population, which may comprise distinct subsets with distinguishing phenotypic and functional features (Geissmann *et al.*, 2010). Circulating monocytes can be conveniently identified by their physical properties (light scattering) and expression of colony-stimulating factor-1 receptor (CSF-1R, or CD115). Based on their phenotype (surface marker profile and gene expression) and functions, both mouse and human monocytes can be divided into at least two main subsets, which have been termed "inflammatory" or "classical" monocytes (Gr1$^+$/Ly-6C^{high} in the mouse; CD14$^+$CD16$^-$ in the human), and "resident", "patrolling" or "non-classical" monocytes (Gr1$^-$/Ly6C$^{low/-}$ in the mouse; CD14$^{low/-}$CD16$^+$ in the human), respectively (Geissmann *et al.*, 2010; Ziegler-Heitbrock *et al.*, 2010). A third subset has been described that displays intermediate features between the two main subsets (Geissmann *et al.*, 2010; Ziegler-Heitbrock *et al.*, 2010). Whereas "inflammatory" monocytes participate in inflammatory responses induced by pathogens or tissue damage, and can rapidly differentiate into inflammatory macrophages or DCs upon their extravasation, the role of "resident" monocytes is still poorly understood. Recent studies have suggested that "resident" monocytes patrol blood vessels (hence "patrolling" monocytes) and extravasate in response to vascular damage to facilitate tissue healing and revascularization (Auffray *et al.*, 2007; Nahrendorf *et al.*, 2007).

Both in development and adulthood, tissue macrophages phagocytose pathogens, dead cells, cellular debris, and various components of the extra-cellular matrix (ECM). Ilya Ilyich Mechnikov was the first to describe the process of phagocytosis in 1882, and macrophages were named after this feature as "big eaters" (from ancient Greek, *makros* "large" + *phagein* "eat"). However, it is now clear that macrophages are not only big eaters of pathogens and dead cells, but also important components of the stromal architecture of several tissues and organs, where they regulate organ homeostasis and remodeling (Pollard, 2009). For instance, Kupffer cells are specialized macrophages that line hepatic capillaries (termed sinusoids) in the liver, where they scavenge senescent erythrocytes, a process referred to as hemocatheresis. During development and tissue healing or regeneration, macrophages

Abbreviations used in this paper: BM, bone marrow; CSF, colony-stimulating factor; DC, dendritic cell; EC, endothelial cell; HSC, hematopoietic stem cell; IL, interleukin; MMP, matrix-metalloproteinase; NK, natural killer; TAM, tumor-associated macrophage; TEM, Tie2-expressing macrophage; VEGF, vascular endothelial growth factor.

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stimulate angiogenesis and facilitate tissue remodeling by secreting a number of proteases and growth factors (Pollard, 2009). In many tissues, macrophages can be identified by their expression of a few distinguishing markers, such as F4/80 (in the mouse) and CD68, an endosomal glycoprotein also known as macrosialin (both in mouse and human).

The ontogeny of macrophages

The first macrophages appear very early during mouse development, precisely at 7.5 days *post-coitum* (*dpc*). Bertrand and co-workers proposed a three-wave model for macrophage ontogeny, which takes place in the extra-embryonic yolk sac before the onset of definitive hematopoiesis (Bertrand *et al.*, 2005). The first macrophages that are found in the yolk sac have been reported to be of maternal origin; these maternally-derived macrophages might constitute a transient population and rapidly decrease at 8.5-9 *dpc*. The second and third macrophage populations appear in the yolk sac between 8 and 9 *dpc* and are derived from monopotent and erythromyeloid macrophage precursor cells, respectively (Bertrand *et al.*, 2005). Starting from 8.5 *dpc*, macrophages can be readily identified in both extra-embryonic and embryonic tissues, including yolk sac, neural, inter-somitic and inter-digital tissues (Pucci *et al.*, 2009). Definitive hematopoiesis (i.e., the generation of hematopoietic cells from multipotent HSCs) starts at 9.5-10 *dpc* in the Aorto-Gonado-Mesonephron (AGM) region of the embryo to continue in the fetal liver, spleen and BM at later stages of development. From the onset of definitive hematopoiesis throughout adult life, macrophages are thought to originate from HSCs through circulating monocyte intermediates. However, the precise origin of tissue-resident (e.g., the long-lived liver Kupffer cells and brain microglia) as well as inflammatory macrophages (i.e., macrophages that acutely infiltrate inflamed tissues) is still a matter of debate (Geissmann *et al.*, 2003; Geissmann *et al.*, 2010). For instance, it is not clear whether Kupffer cells and microglia originate from adult HSC-derived monocytes or self-renewing, nonHSC-derived macrophage precursors of yolk-sac origin (Chan *et al.*, 2007; Geissmann *et al.*, 2010). A recent study showed that postnatal HSCs do not significantly contribute to microglia homeostasis in the adult mouse brain (Ginhoux *et al.*, 2010). *In vivo* lineage tracing studies indeed showed that brain microglial cells derive from primitive myeloid progenitors that arise before 8 *dpc* (Ginhoux *et al.*, 2010). There is also evidence that, during inflammation, tissue macrophages undergo rapid *in situ* proliferation in order to increase population density (Jenkins *et al.*, 2011). These recent studies therefore indicate that resident macrophages can self-renew and expand *in situ* under the influence of appropriate stimuli and/or at selected anatomical sites (Ginhoux *et al.*, 2010; Jenkins *et al.*, 2011). Nevertheless, it is also clear that total body irradiation can enhance tissue-resident macrophage turnover from BM-derived, circulating monocytes (Biffi *et al.*, 2004; De Palma *et al.*, 2003).

The role of macrophages in development

Several transcription factors have been implicated in monocyte/macrophage development and commitment. PU.1 regulates both embryonic and adult hematopoiesis by interacting with many other transcription factors, including GATA1. PU.1$^{-/-}$ mice die at a late embryonic stage or shortly after birth and have impaired myelo- and lymphopoiesis (Iwasaki and Akashi, 2007). The conditional deletion of PU.1 in the adult BM results in a complete loss of common myeloid (CMP) and lymphoid (CLP) progenitors, but not megakaryocyte-erythroid progenitors (MegE), indicating that PU.1 is necessary for multipotent progenitors to proceed to the CMP/CLP but not MegE stage (Iwasaki and Akashi, 2007). Among the differentiated hematopoietic cells, PU.1 is expressed in the myeloid but not lymphoid, megakaryocyte and erythroid lineages, indicating a role for PU.1 in myeloid cell commitment and differentiation. PU.1 modulates the expression of several myeloid-specific genes, including granulocyte-colony stimulating factor (G-CSF) and granulocyte/monocyte-colony stimulating factor (GM-CSF) receptors (Burda *et al.*, 2010). Because PU.1$^{-/-}$ mice display only minor erythropoietic and megakariocytopoietic defects and remain vital until perinatal developmental stages, they represent a widely used model to study the effects of macrophage deficiency on organogenesis.

PU.1-deficient mice display an osteopetrotic phenotype (Tondravi *et al.*, 1997). Osteopetrosis is a metabolic bone disease characterized by the lack of BM cavities and defects in bone resorption. These defects are due to the lack of osteoclasts, a form of highly specialized macrophage that resides in the bone and regulates its remodeling. Similar to PU.1$^{-/-}$ mice, *Csf-1*$^{op/op}$ mice (which lack CSF-1, a key growth factor responsible for the proliferation, differentiation, survival, and chemotaxis of monocytes/macrophages and their precursors) and *Csf-1r*$^{-/-}$ mice also display an osteopetrotic phenotype. Interestingly, both *Csf-1*$^{op/op}$ and *Csf-1r*$^{-/-}$ mutant mice are severely depleted of most, but not all macrophage populations (Pollard, 2009). Although *Csf-1*$^{op/op}$ mice have many developmental abnormalities that can be attributed to the lack of individual macrophage populations (Pollard, 2009), they are viable and can be used as a model of adult macrophage deficiency in several experimental settings, including tumor progression (Lin *et al.*, 2001). The phenotype of *Csf-1r*$^{-/-}$ mice is similar to that of *Csf-1*$^{op/op}$ mice, but the former are more severely affected and rarely live beyond a few weeks of age.

Csf-1$^{op/op}$ mice have reduced numbers of macrophages in their tissues, particularly in the kidney, mammary and pancreatic glands. In the developing mammary gland, CSF-1 secreted by epithelial cells recruits CSF-R1^{+} (CD115^{+}) macrophages to the terminal end buds (Pollard, 2009). At this anatomical site, macrophages produce many growth factors and proteases that facilitate epithelial cell growth and invasion of the surrounding connective tissue; in fact, *Csf-1*$^{op/op}$ mice show reduced complexity of mammary ductal branching due to impaired recruitment of infiltrating macrophages at the terminal end buds (Pollard, 2009).

Rae and co-workers studied embryonic macrophages in *Csf-1r*-GFP transgenic mice, which express GFP preferentially in myelomonocytic cells (Rae *et al.*, 2007). *Csf-1r*-GFP–positive macrophages represented 3-10% of all embryo-derived cells at 11.5 *dpc* and were particularly abundant in developing limbs, lens, thymus and olfactory bulb. The authors then isolated GFP-positive macrophages from the brain, lung and kidney of 12 *dpc* embryos and compared their gene expression with that of macrophages isolated from adult organs. Microarray analyses indicated that embryonic macrophages, as compared to their adult macrophage counterpart, were enriched in transcripts associated with "wound-healing" and "angiogenesis" signatures. Among the up-regulated genes were mannose receptor (*Mrc1*), the scavenger receptors *Msr1* and *Mrs2*, the chemokine *Ccl24*, and the complement protein

C1q (Rae *et al.*, 2007). These genes are involved in recognition and engulfment of dying cells and components of the ECM, and are also up-regulated in tumor-associated macrophages (TAMs), as discussed below.

In the kidney, organogenesis involves the infiltration of the metanephric mesenchyme by the ureteric bud and subsequent branching morphogenesis. Rae and co-workers also reported that macrophages associate with developing renal tubules and blood vessels, perhaps facilitating their invasion and expansion into the surrounding connective tissue (Rae *et al.*, 2007). The authors further showed that embryonic macrophages promote tissue growth in metanephric explant cultures of 11.5 *dpc* embryos, partially in a CSF-1–dependent manner (Rae *et al.*, 2007). These data corroborate the notion that macrophages orchestrate tissue and organ morphogenesis by producing chemokines that in turn attract and/or activate other cell types (e.g., fibroblasts) in the tissue microenvironment. Thus, the CSF-1 / CSF-1R axis – via its effects on myelo-monocytopoiesis – represents a molecular hub that is important for physiologic tissue growth and remodeling, both in development and adulthood.

Macrophage regulation of angiogenesis in development and post-natal tissue remodeling

The formation of new, functional blood vessels requires the sprouting of pre-existing blood vessels and their subsequent fusion with other blood vessels, a process known as vascular anastomosis (Ribatti, 2010; Eichmann *et al.*, 2004; Carmeliet, 2005). Little is known about the mechanisms by which the endothelial cells (ECs) present at the leading edge of vascular sprouts (named endothelial "tip" cells) integrate directional cues from the environment and fuse to form new functional blood vessels. Fantin and co-workers have recently shown that yolk sac-derived macrophages mediate the fusion of endothelial tip cells and increase vascular complexity in the developing hindbrain (Fantin *et al.*, 2010).

In wild-type embryos, yolk sac-derived macrophages were often observed in close association with sprouting blood vessels (Fantin *et al.*, 2010). In the subventricular zone of the hindbrain, macrophages accumulated between 10-11.5 *dpc*, a time point during which blood vessels branch to form the subventricular vascular plexus. Once the subventricular vascular plexus had formed, macrophages decreased in this area while accumulated in deeper brain layers, concomitantly to the branching of radial arteries (Fantin *et al.*, 2010). These data suggest that macrophages associate with sites of active vascular branching in the developing hindbrain. To further study the role of macrophages in vascular patterning, the authors used macrophage-deficient PU.1$^{-/-}$ embryos (Fantin *et al.*, 2010). They found that the hindbrain of PU.1 mutants displayed normal numbers of vascular sprouts but fewer intersections among the blood vessels, indicating reduced vascular network complexity. Of note, the vascular phenotype of PU.1$^{-/-}$ embryos was less severe than that of *Vegfa*$^{120/120}$ mutants, which have impaired angiogenesis due to the absence of the master proangiogenic factor, vascular endothelial growth factor (VEGF)-A. Together, these findings suggest that macrophages support blood vessel formation downstream to VEGF-induced angiogenesis (Fantin *et al.*, 2010). The authors further showed that yolk sac-derived but not circulating monocyte-derived macrophages are important for brain angiogenesis in the developing brain. This may indicate that yolk sac macrophages infiltrate the brain before a functional circulation is established (Fantin *et al.*, 2010), in agreement with other studies (Ginhoux *et al.*, 2010).

From a mechanistic standpoint, the study of Fantin and co-workers suggested that macrophages promote vascular anastomosis in the developing hindbrain by physically assisting endothelial tip cell fusion through direct cell-to-cell contacts (Fantin *et al.*, 2010). A similar mechanism has been described recently by Rymo and co-workers, who showed that macrophages/microglial cells associate with endothelial tip cells also in the developing retina (Rymo *et al.*, 2011). Retinal macrophages were frequently found at sites where endothelial tip cells contact each other through their filopodia, and were present at 1:2 numeric ratio with the endothelial tip cells. These observations suggest that each macrophage/microglial cell can theoretically contact two neighboring endothelial tip cells. Interestingly, genetic ablation of microglia in *Csf-1*$^{op/op}$ mice determined a sparser vascular network associated with reduced numbers of filopodia-bearing sprouts. Moreover, the retinal vascular sprouts of macrophage-deficient mice were fewer and mostly radially-oriented, whereas those of wild-type mice displayed much higher complexity and were both radially- and forward-oriented, thus forming more intersections (Rymo *et al.*, 2011). The authors then asked whether microglial cells stimulate vessel branching through direct contacts with the endothelial network. To address this question, they used mouse aortic rings cultured with or without embedded microglia, and found that microglia induced significant vascular sprouting long before the cells had made physical contact with the growing vessel network (Rymo *et al.*, 2011). These data indicate that direct macrophage/EC cell-to-cell contacts are not strictly essential for vascular sprouting in the mouse aortic ring assay. Nevertheless, it cannot be excluded that cell-to-cell contacts are essential for directional, vascular morphogenesis *in vivo*, as implied by imaging data (Fantin *et al.*, 2010; Rymo *et al.*, 2011).

The requirement of macrophage/EC cell-to-cell contacts for vascular network formation is also suggested by studies showing that certain TAMs, namely the TIE2-expressing macrophages (TEMs), physically interact with tumor blood vessels and promote angiogenesis in mouse tumor models (De Palma *et al.*, 2003; De Palma *et al.*, 2005; De Palma *et al.*, 2008; De Palma and Naldini, 2009; Pucci *et al.*, 2009; Mazzieri *et al.*, 2011; Welford *et al.*, 2011; Squadrito and De Palma, 2011). Interestingly, both TEMs and embryonic macrophages that assist vascular morphogenesis express the angiopoietin receptor, TIE2 (De Palma *et al.*, 2005; Pucci *et al.*, 2009; Mazzieri *et al.*, 2011; Fantin *et al.*, 2010; Gordon *et al.*, 2010). TIE2 is a tyrosine kinase receptor broadly expressed by ECs; it regulates angiogenesis and vascular morphogenesis both in development and adulthood (Augustin *et al.*, 2009). Activated ECs (such as those of angiogenic blood vessels) secrete high levels of the TIE2 ligand, angiopoietin-2 (ANG2), which binds TIE2 both autocrinally on ECs and paracrinally on perivascular monocytes/macrophages to induce them to become highly proangiogenic (Coffelt *et al.,* 2010b). Such reciprocal signaling might regulate macrophage/EC interactions and facilitate macrophage-assisted vascular patterning both in developing organs and, as recently shown, in mouse tumor models (Mazzieri *et al.*, 2011).

Macrophages have also been implicated in the post-natal remodeling of the retinal vasculature. Retinal development requires the formation of a temporary vascular plexus (the hyaloid vessels), which is subsequently remodeled through a vascular regression phase.

Following remodeling, the dense primary vasculature is replaced by a narrower, smaller caliber secondary vasculature (Ishida *et al.*, 2003; Lobov *et al.*, 2005). In the mouse, retinal vascular regression occurs after birth and involves the activity of macrophages. Lobov and co-workers showed that the temporary hyaloid vessels can develop normally but fail to undergo regression in PU.1$^{-/-}$ mice, which lack macrophages (Lobov *et al.*, 2005). In the developing mouse retina, macrophages are the main source of WNT7B, a molecule that induces cell-cycle progression and can predispose target cells to subsequent apoptosis (Lobov *et al.*, 2005). Rao and co-workers further showed that macrophage-mediated vascular regression involves two distinct signals, WNT7B and ANG2 (Rao *et al.*, 2007). ANG2 has long been regarded as a natural antagonist for ANG1 (Augustin *et al.*, 2009), although recent studies have indicated that it may also function as a context-dependent TIE2 agonist (Daly *et al.*, 2006; Coffelt *et al.*, 2010b). In the presence of ANG2 produced by the hyaloid blood vessels, resident macrophages upregulate WNT7B, which induces the proliferation of vascular ECs and sensitizes them to ANG2-induced apoptosis. In the absence of ANG2, ANG1 signaling prevails and EC death is suppressed (Rao *et al.*, 2007). Together, these data suggest that macrophages directly regulate retinal vascular remodeling, but not initiation of retinal angiogenesis. Because a sizable fraction of the embryonic macrophages express the TIE2 receptor or a *Tie2*-GFP reporter gene in transgenic mice (Fantin *et al.*, 2010; Pucci *et al.*, 2009; Gordon *et al.*, 2010), it can be envisioned that ANG2 directly acts on macrophages via TIE2 to regulate WNT7B secretion (Rao *et al.*, 2007). It should be noted, however, that one study suggested that cytotoxic T cells – rather than macrophages – promote vascular regression and remodeling of the primary retinal plexus by inducing Fas ligand-mediated EC apoptosis (Ishida *et al.*, 2003).

During pregnancy, angiogenesis and vascular remodeling at the maternal-fetal interface ensure continuous blood flow to the fetus. Inadequate angiogenesis and vascular remodeling limit extraembryonic trophoblast invasion of the decidua (the maternal uterine mucosa during pregnancy) and may cause pre-eclampsia and miscarriage (Harris, 2010). Both in mice and humans, macrophages and natural killer (NK) cells infiltrate the human decidua and regulate spiral artery remodeling at the maternal-fetal interface. Decidual NK cells, but not peripheral blood-derived NK cell subsets, were shown to promote trophoblast invasion and angiogenesis by secreting interleukin-8 (IL-8) and many other proangiogenic factors (Hanna *et al.*, 2006). Interestingly, both macrophages and NK cells are absent from intact arteries, but accumulate in significant numbers in and around remodeling arteries (Harris, 2010). According to Hazan and co-workers, spiral artery remodeling may depend on matrix-metalloproteinase (MMP)-9, which is essentially produced by the macrophages and NK cells in the decidua (Hazan *et al.*, 2010). These MMP9-expressing leukocytes were shown to be in close association with blood vessels during the early phases of remodeling, and MMP2/9 inhibition in human placental-decidual explants resulted in failure of vascular remodeling (Hazan *et al.*, 2010). These data therefore suggest that macrophage-derived MMPs facilitate cell migration, invasion and vascular remodeling during pregnancy.

In summary, there is now compelling evidence for macrophages to regulate vascular morphogenesis during development and postnatal tissue/organ remodeling. In addition to expressing classic proangiogenic and tissue-remodeling factors, which may initiate angiogenesis, macrophages appear to support the formation of a functional vascular system by (i) assisting directional vessel growth via cell-to-cell contacts and/or their production of guidance factors that act iuxtacrinally on vascular sprouts after the induction of EC proliferation and angiogenesis; (ii) pruning primitive blood vessels (via secretion of proapoptotic factors) to remodel the vascular network.

Macrophage regulation of angiogenesis in tissue injury and repair

Macrophages likely represent the preeminent cells in the body endowed with the ability to migrate within tissues – even in hypoxic conditions – and with the capacity to modify the ECM and amplify paracrine signals. For example, in a transgenic model of ischemic cardiomyopathy in which monocytes are attracted to the myocardium by the targeted overexpression of CCL2 (a CC-chemokine formerly known as monocyte chemoattractant protein-1, MCP-1), monocytes/macrophages were found to drill tunnels in the ECM to facilitate the subsequent in-growth of new capillaries (Moldovan *et al.,* 2000). Macrophages may thus provide temporary scaffolds or paracrine support for the expansion and maturation of vascular networks, both in development (see above) and in pathophysiological conditions.

Wound healing is a complex biological process that occurs frequently during adulthood and requires the interplay among different cell types, including keratinocytes, fibroblasts and immune cells (Martin, 1997; Rodero *et al.*, 2011). Early studies suggested that macrophages and neutrophils play a crucial role during skin wound healing (Leibovich and Ross, 1975). Macrophages are generally believed to orchestrate the repair process by acting both as phagocytes (to clear cellular debris) and as a major source of growth factors (e.g., proangiogenic factors; epithelial-cell growth factors; etc…). Martin and co-workers however showed that macrophage-deficient PU.1$^{-/-}$ mice are able to repair skin wounds with similar time course to wild-type siblings (Martin *et al.*, 2003). Yet, healed skin wounds in macrophage-deficient mice showed minimal scar formation, a likely consequence of the reduced inflammatory response and lower rate of keratinocyte cell death occurring in the absence of proinflammatory macrophages (Martin *et al.*, 2003). More recent studies however showed that macrophages recruited during the diverse phases of skin repair (i.e., the inflammatory; tissue formation; and tissue maturation phases) exert distinct functions (Lucas *et al.*, 2010). By crossing transgenic mice that express the CRE recombinase from the *lysozyme M* (*LysM*) promoter, with transgenic mice that express the human diphtheria toxin (DT) receptor upon CRE-mediated recombination, the authors generated mice in which depletion of macrophages and other myeloid cells can be induced during specific stages of the repair response. Depletion of myeloid cells during the early stages of the repair response (inflammatory phase) significantly reduced the formation of vascularized granulation tissue, impaired epithelialization, and resulted in minimized scar formation (Lucas *et al.*, 2010), the latter finding in agreement with the study of Martin and co-workers (Martin *et al.*, 2003). In contrast, depletion of macrophages during the subsequent phase of the repair response (tissue formation) caused severe hemorrhage in the wound tissue and prevented tissue maturation and wound closure. Finally, macrophage depletion during the late stage of repair (tissue maturation) did not significantly impact the outcome of the

repair response (Lucas *et al.*, 2010). These interesting observations suggest that macrophages exert distinct functions during the diverse phases of skin repair. In particular, macrophages appear to play an important role in promoting (i) angiogenesis in the granulation tissue during the early phase of skin repair; (ii) vascular maturation and stabilization in the subsequent phases (Lucas *et al.*, 2010).

Tissue macrophages can display different activation states according to the stimuli present in the local microenvironment. In several microbial infections and other inflammatory conditions, the products of activated T helper-1 (Th1) lymphocytes and NK cells (e.g., interferon-gamma, IFNγ) can induce macrophages to undergo a "classical activation" (or "M1") program that enhances their cytotoxic and antimicrobial activity. Conversely, signals produced by Th2 polarized lymphocytes (e.g., IL4, IL10 or IL13) can induce macrophages to acquire an "alternative activation" (or "M2") status in various pathophysiological conditions, such as wound healing and granuloma formation, or in certain disease conditions, such as fibrosis, atherosclerosis, stroke, and cancer (Gordon, 2003; Gordon and Martinez, 2010; Mantovani *et al.*, 2002).

Distinct "types" of wound healing macrophages have been described to date; their phenotypic and functional characterization is, however, not yet exhaustive (Rodero *et al.*, 2011). Okuno and co-workers recently showed that the majority of BM-derived cells infiltrating both acute (dorsal excisional ear punch) and chronic (decubitus ulcer) skin wounds are macrophages (Okuno *et al.*, 2011). Most of these macrophages display an "alternatively" activated or M2-like phenotype (Gordon, 2003; Gordon and Martinez, 2010), e.g., express high levels of MRC1 and low levels of nitric oxide synthase-2 (NOS2) and IL-6. Interestingly, the authors found that the majority of these macrophages were located in perivascular areas at the wound healing site (Okuno *et al.*, 2011).

Recruitment of macrophages to the wound healing site may rely on different signals, such as CCL2/MCP1, macrophage inflammatory protein-1alpha (MIP1α), but also stromal cell-derived factor 1 (SDF1)/CXCL12 and G-CSF (Wu *et al.*, 2010). Okuno and co-workers showed that CSF-1 is early up-regulated at the wound healing site and plays an important role in recruiting macrophages (Okuno *et al.*, 2011). This was confirmed by inhibition of CSF-1R or CSF-1, which resulted in 80% reduction of the macrophage infiltrate. Moreover, the authors observed delayed wound healing in *Csf-1*$^{op/op}$ mice, and this correlated with decreased vascular density. However, the overall amounts of classic proangiogenic factors produced at the wounded site did not differ between *Csf-1*$^{op/op}$ and wild-type mice, whereas MMPs were significantly reduced. Thus, wound healing macrophages may exert their proangiogenic programs by physically interacting with sprouting blood vessels, facilitating their growth by degrading the ECM, and/or promoting vascular remodeling via non-canonical (e.g., VEGF-independent) angiogenic pathways (Okuno *et al.*, 2011).

Sindrilaru and co-workers recently reported that human chronic venous ulcers contain increased proportions of macrophages expressing M1-type markers (e.g., NOS2 and tumor necrosis factor-alpha (TNFα)) than healing wounds (Sindrilaru *et al.*, 2011). As mentioned above, "classically" activated or M1-polarized macrophages sustain tissue inflammation and actively phagocytose dead cells, cellular debris and microbes (Gordon, 2003; Gordon and Martinez, 2010). The authors found that iron deposits and their subsequent uptake by macrophages in chronic wounds impaired macrophage switching from the M1-like to the M2-like phenotype, a phenotypic switch that instead occurs in healing wounds (Sindrilaru *et al.*, 2011). Together with previous reports (Okuno *et al.*, 2011; Rodero *et al.*, 2011), this study suggests that M1-like macrophages prevail in early or chronic wounds, where they prevent infection and help clearance of necrotic tissue (Sindrilaru *et al.*, 2011). Conversely, proficient wound healing requires the participation of M2-like macrophages, which may either originate from M1-like macrophages or from different monocyte precursors.

Macrophages have also been implicated in the healing/regeneration of the nervous tissue. Barrette and co-workers showed that the depletion of CD11b$^+$ myeloid cells in *CD11b*-HSV-thymidine kinase transgenic mice impaired recovery of sciatic nerve function after injury (Barrette *et al.*, 2008). Depletion of myeloid cells was associated with a significant decrease in vascular density and delayed neural cell proliferation (Barrette *et al.*, 2008). Kigerl and co-workers further observed that M2-like macrophages transiently increase at the spinal cord injury site (Kigerl *et al.*, 2009). In this study, the authors also showed that neurons co-cultured with M2-like macrophages displayed more neuritis, and their axons projected over longer distances, compared to neurons co-cultured with M1-like macrophages (Kigerl *et al.*, 2010). These interesting observations suggest that M2-like macrophages participate in both vascular network formation and neural development. In this regard, it is increasingly appreciated that blood vessels and neurons share cell-surface receptors and guidance cues, such as several members of the VEGF and Semaphorin/Plexin families, to control their behavior during embryogenesis and, possibly, also in selected pathophysiological conditions during post-natal life (Fantin *et al.*, 2009).

The role of macrophages in lymphangiogenesis

Lymphatic vessels regulate tissue fluid homeostasis and play a crucial role in immune cell trafficking. The molecular pathways involved in lymphangiogenesis have been elucidated more in detail in the context of development. In the adult, lymphangiogenesis occurs in inflammation, tumors, and during the process of wound healing. In the mouse embryo, lymphangiogenesis begins at 9.5-10.5 *dpc*, after the cardiovascular system is formed (Tammela and Alitalo, 2010). Lymphatic ECs arise from specialized subpopulations of ECs in the anterior cardinal vein; these specialized ECs are positive for prospero-related homeodomain transcription factor (PROX-1), which is the master molecular switch for lymphatic EC determination, and hyaluronan receptor-1 (LYVE1).

In addition to angiogenesis, macrophages also appear to regulate lymphangiogenesis. For example, CSF-1 deficiency in *Csf-1*$^{op/op}$ mice reduces the abundance of macrophages in the developing trachea, resulting in defects in both vascular and lymphatic development (Kubota *et al.*, 2009). Bohmer and co-workers recently described a population of monocyte-lineage cells, defined by the expression of Syk tyrosine kinase (Bohmer *et al.*, 2010). These cells were characterized by their robust expression of proangiogenic factors, such as fibroblast growth factor-2 (FGF2), platelet-derived growth factor-B (PDGF-B), MMP2 and MMP9, as well as lymphangiogenic growth factors (VEGF-C and VEGF-D). Interestingly, the skin of *Syk*-decifient embryos showed increased accumulation of these proangiogenic monocyte-lineage cells, which expressed a more pronounced proangiogenic profile than their wild-type counterpart (Bohmer *et al.*, 2010). The enhanced infiltration by

lymphangiogenic monocytes in *Syk*-deficient embryos caused lymphatic hyperplasia, vessel dilation leading to blood-lymphatic shunts, and lethality during mid-gestation. Interestingly, the Syk-expressing monocytes also express TIE2 (Bohmer *et al.*, 2010) and display many similarities with the TIE2-expressing monocytes/macrophages found in tumors and embryos (Pucci *et al.*, 2009). Another recent study showed that clodronate-liposome–mediated depletion of VEGF-C–expressing, dermal macrophages counteracts hyperplasia of lymphatic capillaries in response to high-salt diet (Machnik *et al.*, 2009). Taken together, these findings (Bohmer *et al.*, 2010; Machnik *et al.*, 2009) indicate that, in addition to angiogenesis, monocyte/macrophages may also regulate lymphangiogenesis. Furthermore, they suggest that the enhanced recruitment of monocyte/macrophages that occurs in certain pathological conditions (e.g., salt-sensitive hypertension) may induce lymphatic hyperplasia and pathological lymphangiogenesis.

Another recent report implicated macrophages in lymphangiogenesis (Gordon *et al.*, 2010). In this study, the authors showed that 14.5-18.5 *dpc* PU.1$^{-/-}$ and *Csf-1r*$^{-/-}$ mice display hyperplastic lymphatic vessels in the skin. This phenotype appears to be due to the specific lack of myeloid-lineage cells, since lymphocyte-deficient *Rag2*$^{-/-}$ mice did not show hyperplastic lymphatic vessels in the skin (Gordon *et al.*, 2010). Although skin macrophages appeared dispensable for lymphatic vessel sprouting and branching, they influenced lymphatic vessel caliber, possibly by controlling either the proliferation or apoptosis of lymphatic ECs in the developing skin. In apparent contradiction with other studies (Bohmer *et al.*, 2010), the findings of Gordon and co-workers (Gordon *et al.*, 2010) also imply that macrophages may not comprise the principal source of pro-lymphangiogenic growth factors (including VEGF-C and VEGF-D) in the embryonic dermal microenvironment, and also suggest that the sources of proliferative and patterning signals driving embryonic lymphangiogenesis are likely to be distinct (Gordon *et al.*, 2010). The authors further characterized the dermal macrophages and identified a subset of LYVE1^{+} cells that intimately associated with the developing lymphatic vasculature. Interestingly, the gene expression profile of these LYVE1^{+} macrophages overlaps with that of both embryonic and tumor-derived TEMs (Pucci *et al.*, 2009). Because these LYVE1^{+} macrophages provide only 10% of the pro-lymphangiogenic molecules present in the dermal microenvironment (Gordon *et al.*, 2010), it is tempting to speculate that, in analogy with developmental (Fantin *et al.*, 2010) and tumor (Mazzieri *et al.*, 2011) angiogenesis, TEM-like embryonic macrophages are not essential for lymphatic vessel sprouting but play an important role in lymphatic vessel expansion and remodeling (Gordon *et al.*, 2010).

Similarities between embryonic and tumor-associated macrophages (TAMs)

In order to grow over a few millimeters in size, incipient tumors need to generate their own vasculature to enable oxygen and nourishment delivery to the expanding tumor cells (Carmeliet, 2005). It is now well established that tumor-associated stromal cells such as macrophages (TAMs), neutrophils and fibroblasts cooperate with tumor cells to facilitate angiogenesis in tumors (Squadrito and De Palma, 2011; Coffelt *et al.*, 2010a; Egeblad *et al.*, 2010; Murdoch *et al.*, 2008).

In several mouse tumor models, macrophages represent the most abundant tumor-infiltrating myeloid cells (Squadrito and De Palma, 2011). The early finding that high macrophage counts correlate with higher tumor angiogenesis in some human tumor types (Leek *et al.*, 1996) suggested that these cells might exert a direct proangiogenic function in tumors. However, compelling evidence for the ability of macrophages to promote tumor angiogenesis was only provided using mouse models of *in vivo* monocyte/macrophage deficiency or adoptive transfer. De Palma and co-workers showed that the genetic depletion of TEMs was sufficient to robustly inhibit angiogenesis in various subcutaneous tumor models (De Palma *et al.*, 2003; De Palma *et al.*, 2005). Moreover, by crossing *Csf1op/op* mice with mice that develop oncogene-induced mammary

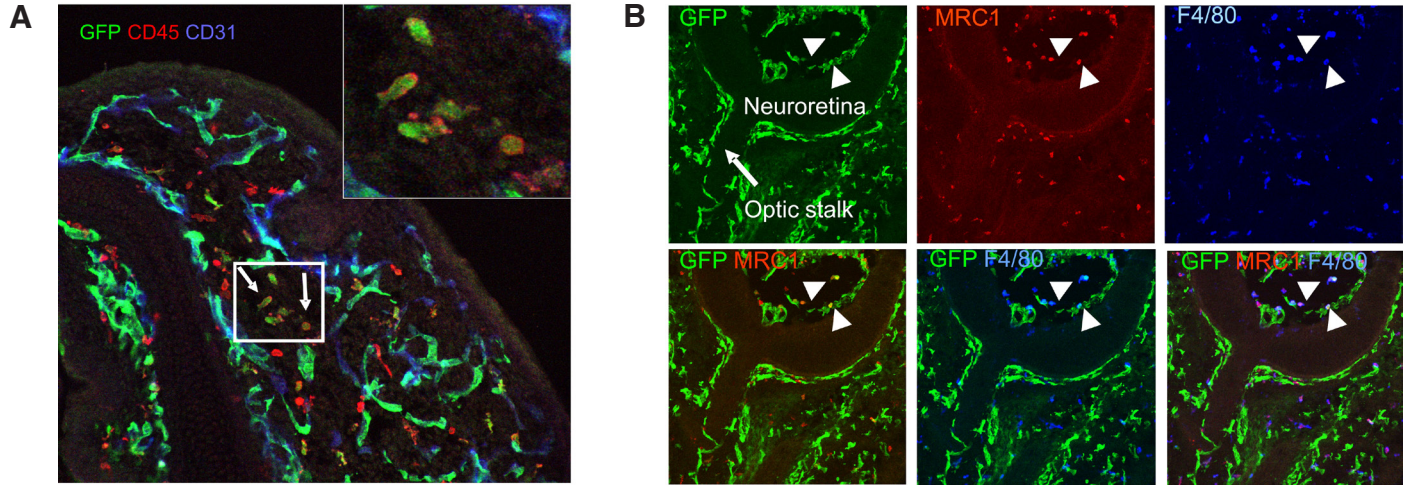

Fig. 1. Presence of *Tie2*-GFP^{+} macrophages in the mouse embryo. (A) *Several* Tie2-*GFP*$^{+}$*CD45*$^{+}$ *Tie2-expressing macrophages (TEMs; white arrows and inset on top right) are present in the developing brain of a Tie2-GFP transgenic embryo. Note that in* Tie2-*GFP transgenic mice, both vascular endothelial cells and TEMs express GFP from the* Tie2 *promoter/enhancer. TEMs are labeled in green (GFP); hematopoietic cells in red (CD45); blood vessels in blue (CD31).* **(B)** Tie2-*GFP*$^{+}$*F4/80*$^{+}$*MRC1*$^{+}$ *TEMs (arrowheads) are present in the developing eye of a* Tie2-*GFP transgenic embryo. Note the proximity of GFP*$^{+}$ *TEMs to the GFP*$^{+}$ *hyaloids blood vessels. TEMs are labeled in green (GFP); macrophages in red (MRC1) and blue (F4/80). Sections were obtained from late-stage embryos.*

tumors (MMTV-PyMT), Lin and co-workers found that the tumors of these macrophage-deficient mice had reduced angiogenesis and delayed progression to malignancy (Lin *et al.*, 2006). Finally, the depletion of tissue macrophages by clodronate-liposomes resulted in delayed angiogenesis and tumor growth in various tumor models (Zeisberger *et al.*, 2006). Together, these studies have highlighted the important role played by macrophages in tumor angiogenesis. Several mechanisms may be involved, such as macrophage production of a plethora of proangiogenic growth factors and chemokines. Moreover, TAMs can mobilize angiogenic growth factors embedded in the ECM thanks to their secretion of many proteases and other matrix-remodeling enzymes (Murdoch *et al.*, 2008). As mentioned above, similar to embryonic macrophages (Fantin *et al.*, 2010), subsets of TAMs – such as TEMs – can also interact directly with tumor ECs to facilitate blood vessel growth (De Palma *et al.*, 2005; Mazzieri *et al.*, 2011).

Ojalvo and co-workers performed gene expression analyses of TAMs isolated from late-stage, MMTV-PyMT mammary tumors, and compared their gene expression signature with that of splenic and embryonic macrophages (Ojalvo *et al.*, 2009). The data highlighted the tissue trophic functions of TAMs and their similarity with embryonic but not splenic macrophages. Pucci and co-workers further showed that TIE2$^+$ embryonic/fetal macrophages, which appear at 8.5 *dpc*, show common features with tumor-infiltrating TEMs (Pucci *et al.*, 2009). In the embryo, TEMs are particularly enriched in the mesenchyme surrounding neural organs; these TIE2$^+$ macrophages are often found in close proximity to blood vessels at distinct anatomical sites, including the hyaloid cavity of the eye (Fig. 1). Gene expression analyses further indicated that embryonic/fetal TEMs are molecularly similar to tumor-associated TEMs (Pucci *et al.*, 2009). Indeed, embryonic TEMs express the typical tumor TEM markers MRC1, haptoglobin/hemoglobin scavenger receptor (CD163), neuropilin-1 (NP1), stabilin-1 (STAB1), and LYVE1. Interestingly, tumor TEMs express lower VEGF levels that classic, TIE2-negative (inflammatory) TAMs (Pucci *et al.*, 2009). This may be consistent with the observation that TEMs preferentially reside around newly formed tumor blood vessels in viable tumor areas (note that VEGF is strongly upregulated in cells that reside in hypoxic or necrotic microenvironments). The finding of lower VEGF expression in TEMs than classic TAMs also suggests that the former are likely to promote tumor angiogenesis mostly in a VEGF-independent manner. Our recent studies suggest that tumor TEMs, similar to TIE2$^+$ embryonic macrophages (Fantin *et al.*, 2010), promote the formation of a functional tumor-associated vasculature by associating with sprouting tumor blood vessels, and that this function is regulated by the ANG2/TIE2 axis in macrophages (Mazzieri *et al.*, 2011).

It should be emphasized that embryonic and tumor macrophages have different origins: whereas fetal macrophages are derived from the extra-embryonic mesoderm (yolk sac), tumor macrophages likely originate from HSC-derived monocytes (De Palma *et al.*, 2003). Thus, it can be speculated that embryonic macrophages and specific TAM subsets, such as TEMs, express similar gene signatures as a result of convergent functional adaptation. Whereas TEMs generally constitute a minor macrophage subset in mouse tumors (Squadrito and De Palma, 2011), the majority of macrophages seem to express a TEM-like phenotype in the developing embryo (Fantin *et al.*, 2010; Pucci *et al.*, 2009). This may reflect the fact that variably inflamed, hypoxic, necrotic, hemorrhagic, as well as avascular or highly vascularized microenvironments are present in any given tumor. Such degree of histological heterogeneity is accompanied by a remarkable heterogeneity of macrophage phenotypes (Lewis and Pollard, 2006; Mantovani *et al.*, 2008; Qian and Pollard, 2010; Squadrito and De Palma, 2011). It appears that tumor TEMs mostly reside in viable and angiogenic tumor areas, whereas they are mostly excluded from necrotic, inner tumor areas (De Palma and Naldini, 2009). Developing embryos do not contain inflamed, necrotic or hemorrhagic microenvironments, but require sustained angiogenesis and tissue remodeling to support organ growth. These microenvironmental features may skew macrophage recruitment/differentiation toward a TEM-like (proangiogenic and pro-remodeling) phenotype, and even prevent the generation of cytotoxic (or proinflammatory) macrophages.

A recent study reported the generation of TEM-like, proangiogenic monocytes/macrophages from human embryonic stem cells (ESCs) (Klimchenko *et al.*, 2011). These TIE2$^+$ ESC-derived monocytic cells displayed enhanced expression of genes encoding matrix-degrading enzymes, proangiogenic factors and scavenger receptors, as was previously reported for tumor TEMs and other M2-like macrophages (Pucci *et al.*, 2009). Furthermore, they promoted vascular remodeling in xenotransplanted human tumors (Klimchenko *et al.*, 2011). The generation of TEM-like cells from ESCs may offer a valuable experimental tool for *in vitro* studies and, possibly, provide a source of proangiogenic cells for the treatment of ischemia, injured tissues and chronic wounds. Finally, it is conceivable that better characterizing the developmental roles of macrophages – including their genetic programs facilitating angiogenesis and tissue morphogenesis in developing organs – may provide clues to TAM function in cancer and identify molecular targets that could be exploited to inhibit their proangiogenic and protumoral activities in human neoplasia and in other diseases.

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