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Macrophages: An Inflammatory Link between Angiogenesis and Lymphangiogenesis

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Abstract

Angiogenesis and lymphangiogenesis often occur in response to tissue injury or in the presence of pathology (e.g. cancer), and it is these types of environments in which macrophages are activated and increased in number. Moreover, the blood vascular microcirculation and the lymphatic circulation serve as the conduits for entry and exit for monocyte-derived macrophages in nearly every tissue and organ. Macrophages both affect and are affected by the vessels through which they travel. Therefore, it is not surprising that examination of macrophage behaviors in both angiogenesis and lymphangiogenesis has yielded interesting observations that suggest macrophages may be key regulators of these complex growth and remodeling processes. In this review, we will take a closer look at macrophages through the lens of angiogenesis and lymphangiogenesis, examining how their dynamic behaviors may regulate vessel sprouting and function. We present macrophages as a cellular link that spatially and temporally connects angiogenesis with lymphangiogenesis, in both physiological growth and in pathological adaptations, such as tumorigenesis. As such, attempts to therapeutically target macrophages in order to affect these processes may be particularly effective, and studying macrophages in both settings will accelerate the field's understanding of this important cell type in health and disease.

Keywords

monocyte; macrophage; angiogenesis; lymphangiogenesis; endothelial cell; pericyte; tumor-associated macrophage

INTRODUCTION

Macrophages were discovered one hundred and thirty years ago [276], and they have long been regarded as an important part of the body's immune system for their phagocytic capabilities. For the last thirty years, we have appreciated their ability to secrete growth factors, chemokines, and extracellular matrix (ECM)-modifying enzymes that modify both

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the structure and function of the local environment [83,244,269]. However, only recently and because of technological advances in imaging, *in vivo* cell tracking, and flow cytometry, have we begun to learn about their phenotypic flexibility and the broad range of dynamic cell behaviors that macrophages exhibit during development, following injury, and in disease. Angiogenesis and lymphangiogenesis are prominent in these tissue remodeling settings, so it is perhaps not surprising that roles for -- and questions about -- macrophage identity, phenotype, and function in the context of blood vessel and lymphatic vessel growth are surfacing in the literature at a rapid rate. Amidst this “renaissance of the macrophage” our review aims to summarize the current understanding of macrophages, with a specific emphasis on their presumptive common roles in angiogenesis and lymphangiogenesis (Figure 1). Our focus on the current literature attempts to highlight some of the ongoing debates and unresolved questions that may have therapeutic relevance. First, we discuss macrophage origin and phenotypic diversity from an immunology perspective. Then, we highlight different macrophage behaviors and their interactions with vascular cells that have been shown to be important in regulating angiogenesis and lymphangiogenesis. Throughout our review, we point out some of the newly identified roles for macrophages, and we speculate about how macrophages could provide a mechanistic bridge between these two vessel remodeling processes. Finally, we discuss strategies for therapeutically targeting macrophages in the context of disease, and cancer in particular.

MACROPHAGE ORIGINS, LINEAGES, AND PHENOTYPIC DIVERSITY

Macrophages are versatile cells that have phenotypic diversity and carry out complex functions in disease and homeostasis (Figure 2, Table 1). The literature suggests that different subpopulations of macrophages play key roles in directing the innate immune response during developmental processes, as well as in initiation of injury, resolution of injury, and in chronic inflammatory conditions [5,65]. Furthermore, tissue resident macrophages in many organs have unique gene expression profiles [22,158] and fulfill special roles necessary for healthy function of the organ [122]. Technological breakthroughs in antibody design, lineage tracing, flow cytometry, and *in vivo* imaging have enabled immune cell phenotyping at an unprecedented level of detail. This has led to the identification of different sub-sets of macrophages and a deeper understanding of their diverse origins. In order to understand – and be able to question – the role of macrophages in angiogenesis and lymphangiogenesis, it is first important to summarize their origins, lineages, and phenotypic diversity.

Macrophage origins

Since the identification of the mononuclear phagocytic system in the 1960s, it was believed that macrophages found in the peripheral tissues were continuously replenished by hematopoietic stem cells in the bone marrow that differentiate through a series of intermediate progenitor cells to monocytes (Figure 2A) [283]. Monocytes are a phagocytic white blood cell of the innate immune system that ingest pathogens and cellular debris, present antigen to T cells, and have the capacity to differentiate into macrophages when they extravasate from the vasculature [222]. However, emerging evidence suggests that macrophages have alternative origins, wherein various tissue-resident macrophage

subpopulations undergo self-renewal within their tissues. This may have been overlooked until recently because of the reliance on bone marrow chimeric models to elucidate macrophage ontogeny [282,304]. In bone marrow chimeric models, mice are exposed to gamma irradiation, which destroys bone marrow stem cells. Then, irradiated mice receive an injection of donor stem cells that are typically modified to express a fluorescent marker, such as GFP, and reconstitute the bone marrow so as to enable visual tracking of monocyte-derived macrophages based on their fluorescence [107]. A limitation with this approach, however, is that the radiation used to deplete stem cells in the bone marrow also inflicts whole body injury, causing increased vascular permeability [69,140] and other non-physiologic behaviors [146,170]. This may artificially inflate the number of fluorescent monocyte-derived macrophages that are observed in injured tissues, especially in studies of the central nervous system [146,176,215,216,258]. Hence, the recent use of cell-type specific, endogenous fluorescent lineage reporters is revealing, for the first time, the abundance of tissue-resident macrophages that are not monocyte derived [105] but that instead originate from embryonic tissues, as discussed below.

Macrophage lineages

Macrophages can generally be divided into three groups: 1) recruited macrophages, 2) tissue-resident macrophages, and 3) perivascular macrophages. Recruited macrophages, also called “adult monocyte derived macrophages” or “infiltrating macrophages”, only transiently remain in the tissue compartment during inflammation to aid with the immune response [5], and then are hypothesized to either undergo apoptosis [122], emigrate from the tissue to the lymph nodes [253], reenter the bloodstream [96], or in some cases differentiate into a tissue-resident macrophage [122]. Recruited macrophages are derived primarily from classical monocytes (also called inflammatory monocytes), the dominant monocyte population that during homeostatic conditions can extravasate from blood vessels and patrol peripheral tissues with minimal differentiation [127]. Classical monocytes can differentiate to nonclassical monocytes (anti-inflammatory monocytes), a small subpopulation that remains in the bloodstream for immune surveillance and patrolling the vasculature [277]. During inflammatory conditions, classical monocytes are thought to often favor differentiating into recruited macrophages with an inflammatory phenotype, while nonclassical monocytes favor an anti-inflammatory phenotype [303]. Recently, a third subpopulation of monocytes has been proposed, intermediate monocytes, that have highly elevated expression of the angiogenic Tie-2 receptor and secretion of angiogenic cytokines like VEGF-A and MMP-9 [316]. While little is known about this subgroup, even whether it represents a transient switch between other subpopulations, one study found that intermediate monocytes were the only blood mononuclear cell that could induce capillary formation in a model of tumor angiogenesis, making this monocyte population a particularly interesting effector cell to examine for future investigations [60].

The majority of tissue resident macrophage populations are now recognized to be of embryonic origin and renew themselves primarily through mechanisms that are independent of hematopoiesis [105]. Tissue resident macrophages arise from the yolk sac hemogenic endothelium, a subpopulation of developmental pluripotent endothelial cells that exist in early embryogenesis [310]. These cells give rise to erythro-myeloid progenitors (EMPs),

which are split into two subpopulations based on time of formation (Figure 2B). Early EMPs give rise to yolk sack macrophages that eventually populate the brain to become microglia, the tissue resident macrophage for that organ [114]. Late EMPs migrate to the fetal liver and produce fetal liver monocytes, which enter the bloodstream, migrate to the peripheral tissues, and differentiate into the various tissue resident macrophages. Notable exceptions are the resident macrophage populations of the skin and gut, which are replenished continuously from classical monocytes [175,189]. While studies have shown that some subpopulations of recruited macrophages, predominantly of anti-inflammatory phenotype, can take up long term residence in tissues, there is evidence in some tissues that they do not transition to tissue resident macrophages and perform their specialized functions [5]. It is important to note that with the recent call to stringently classify macrophage subpopulations based on ontogeny [101], microglia will likely receive a distinct classification that separates them from all other tissue resident macrophages if their unique lineage is verified.

In both quiescent and inflammatory conditions, perivascular macrophages closely associate with the post-capillary venules in peripheral organs including skin, muscles, heart [98], central nervous system [149,297]. Whether or not this macrophage subpopulation exists in other tissues during quiescence is not known. Several studies indicate that perivascular macrophages play a key role in immune cell recruitment and extravasation [2,106]. However, the uniqueness of perivascular macrophages is unclear because an exclusive lineage marker has yet to be identified for this subpopulation and many studies simply merge this group with monocyte derived macrophages or tissue resident macrophages [57,100]. Perivascular macrophages are hypothesized to originate from adult monocytes [23,217], but this evidence is based on bone marrow chimera experiments, which, as discussed above, can create a nonphysiologic environment [140]. Regardless of whether this subgroup is derived from the tissue resident niche or from monocytes, we believe that this population should remain distinct from other tissue-resident macrophages and from recruited monocyte-derived macrophages: perivascular macrophages possess a unique morphology and localization along the vasculature compared to other tissue resident macrophages. Therefore, they may experience different metabolic stimuli and cytokine profiles as a result of being more proximal to the circulating blood pool. Moreover, perivascular macrophages in quiescent tissues may be uniquely pre-conditioned by the tissue environment in which they have resided in contrast to the circulating monocyte-derived macrophages that are recruited during inflammation [158]. Importantly, the potential presence of perivascular macrophages in the same local environments (or “niches”) as tissue resident and inflammatory macrophage sub-types motivates future investigations aimed at determining whether the three sub-types are more similar than different when it comes to their functionality in the tissue -- especially in developing or injured tissues, such as those where angiogenesis and/or lymphangiogenesis are occurring.

Macrophage phenotypic diversity

Macrophages exhibit a vast and dynamic range of different phenotypes. Within the field of immunology, macrophage classification schemes have emerged and are undergoing redefinition [178], with several new, competing schemes proposed to incorporate recent biological insights. The prevailing macrophage phenotype classification scheme is inspired

by T helper cell nomenclature. Classically activated macrophages, known as the M1 phenotype, are stimulated by IFN-γ, TNF-α, and LPS, and provoke secretion of cytotoxic agents, such as nitric oxide, and pro-inflammatory cytokines, including IL-1, IL-6, IL-12, IL-23, and TNF-α [178]. Alternatively activated macrophages, known as the M2 phenotype, are stimulated by IL-4 and IL-10, and these cells secrete the growth promoting molecule, orthinine [183], as well as anti-inflammatory cytokines that promote angiogenesis, such as IL-10 and TGF-β [235]. In terms of their metabolism, M1 macrophages obtain energy primarily through glycolysis and metabolize arginine though nitric oxide synthase, while M2 macrophages obtain energy through oxidative pathways and metabolize arginine through arginase to fuel their longer-term functions associated with inflammation resolution [90,183,224].

An expanded version of this classification scheme renamed M2 to “M2a”, and added several additional groups, including: M2b, which is characterized by immune complex formation and toll-like receptor activation, M2c, which represents deactivated macrophages that suppress proinflammatory cytokines, and M2d, which represents a regulatory macrophage [236] that is often grouped with tumor associated macrophages (TAMs) [27,51,104]. However, this classification scheme has possible nomenclature pitfalls, such as the fact that M2 macrophages behave as pro-inflammatory macrophages in certain situations [21,116] and do not necessary exhibit clear-cut activation states *in vivo* [178].

An alternative classification scheme proposes a three-category “color wheel” of macrophage activation that categorizes the cells according to specific functions they perform: classically activated macrophages, wound-healing macrophages, and regulatory macrophages [187]. Yet another classification scheme parses macrophage phenotype based on *in vitro* stimulation of macrophages using individual cytokines and paired cytokine combinations [193]. It may also be possible to characterize macrophages *in vivo* based on the paracrine factors that they secrete, which can be determined using fluorescent *in situ* hybridization (FISH) probes against mRNA transcripts for cytokines [309], such as IL-1 and IL-10. FISH can be multiplexed with standard immunostaining, and conducted on whole mount or tissue sections [214].

While each macrophage classification scheme is unique, we attempt to relate them to one another by providing a suggested mapping of macrophage subpopulations and activation states across these different classification schemes (Table 2). It is unclear which of these macrophage classification schemes will gain the most widespread acceptance in the long term. In the meantime, we recommend that studies evaluating macrophage phenotype should: 1) use panels of expression markers that are consistent with the literature, 2) reflect on the latest insights in immunology to aid in the interpretation of findings, and 3) consider the possibility that phenotype determinations may be accurate only for transient windows of time, especially in settings of dynamic tissue remodeling involving angiogenesis and/or lymphangiogenesis.

MACROPHAGE DYNAMICS IN ANGIOGENESIS

Trafficking of monocyte-derived macrophages in angiogenesis

Recruited macrophages can be derived from monocytes that are activated in the bone marrow by chemokines like MCP-1 to enter the bloodstream and traffic to sites of tissue injury [252]. This process follows a step-wise sequence of events that is characteristic for most leukocytes, which has been termed the “leukocyte adhesion cascade” [165]. Many of the hematopoietic cytokines (e.g. GM-CSF, [91]) and cell adhesion molecules (e.g. P-selectin, [18]) implicated in monocyte trafficking have historically been associated with circulating “progenitor cell” trafficking during injury-induced vascular growth and remodeling. Indeed, shared trafficking mechanisms between bone marrow-derived, pro-angiogenic “progenitor cells”, such as the endothelial progenitor cells (EPCs) identified in the late 1990’s [15], may have both contributed to the confusion and helped to resolve the controversy over EPC identity. Critical reassessment during the last five years has suggested that EPCs are linked to the plasticity of monocyte–derived macrophages [75], and their participation in adult angiogenesis is more accurately described by macrophage dynamics, which will be discussed in more detail below.

The classical view is that monocyte trafficking into injured tissue occurs from post-capillary venules, and recent studies have confirmed that post-capillary venules are important sites for monocyte entry into the tissue during microvascular growth and remodeling [32]. Post-capillary venules have also long been regarded as the vessels in the microcirculation that give rise to new capillaries during angiogenesis [192,212]. Moreover, it has been suggested that monocytes preferentially transmigrate through post-capillary venules at locations where ECM protein levels in the basement membrane are decreased [293]. Because the early steps of capillary sprouting angiogenesis are associated with ECM remodeling in the basement membrane [237], and monocyte-derived macrophages are known to secrete ECM modifying proteins (e.g. MMP-9) that may further enhance a tissue’s ability to support capillary sprouting [62], the spatial-temporal relationships between monocyte-derived macrophage trafficking and angiogenesis in post-capillary venules may have important consequence. Indeed, over twenty-five years ago, it was suggested that macrophage influx and angiogenesis were spatially correlated and likely causally intertwined in tumorigenesis [196], although the details of this interrelationship are still being clarified.

Images of macrophages at single time-points in different remodeling microvascular networks (Figure 3) allude to how these cells traffic to tissues and interact with vascular cells following extravasation. Micrographs such as these also motivate the need for dynamic imaging approaches that capture the time-course of monocyte-derived macrophage trafficking. What if there are other routes of monocyte extravasation during angiogenesis, for example, through new capillary sprouts [85]? Could alternative trafficking dynamics offer new insights about the role of monocytes and macrophages in endothelial tip-cell specification? Or is it possible that monocyte-derived macrophages that have trafficked to/through capillary sprouts differentiate directly into blood endothelial cells because of their spatial positioning in the neovessel wall? These are just a few of the currently unanswered questions that may benefit from the new experimental tools and models described in the

final section of this review. Importantly, at the present time the dynamics of macrophages in tissues (including tumors) are incompletely characterized, and there is evidence to support that macrophage flux, both into and out of tissues, can be affected by the tissue environment and whether there is an acute or chronic disruption to tissue homeostasis.

Paracrine roles for macrophages in angiogenesis

Macrophages have been shown to secrete a variety of pro- and anti-angiogenic growth factors (Table 3). Secretion of VEGF-A by monocyte-derived macrophages, in particular, appears to be critical for inducing angiogenesis in tissue repair [266]. Using a CCR2-eGFP reporter mouse model, Willenborg et al. demonstrated that VEGF-A expression by monocyte-derived $CCR2^{+}Ly6C^{+}$ macrophages is required for the induction of capillary sprouting in the setting of skin wound healing [172,300]. In full-thickness cutaneous wounds made in CCR2 knockout mice, where the recruitment of $Ly6C^{+}$ circulating blood monocytes was impaired, reduced VEGF-A expression levels in granulation tissue was accompanied by significantly decreased angiogenesis [280]. In this study, VEGF-A -expressing macrophages that were recruited to the wound site exhibited characteristics of M1 and M2 macrophage sub-sets, including expression of the proinflammatory mediators IL-6 and iNOS, as well as Tie-2, which has been shown to be expressed by tumor-associated, M2-like macrophages [300]. Moreover, evaluation of wounds in mice with myeloid cell–specific VEGF-A deletion revealed that macrophage-derived VEGF-A during the early stage of tissue growth was critical to achieving physiologic tissue vascularization and healing.

It has also been suggested that the polarization state of macrophages can affect the profile of growth factors that they secrete, so this may imply that different macrophage subset affect different stages of angiogenesis – from capillary sprout initiation to neovessel maturation [259]. By analyzing the expression of genes and the angiocrine secretion profiles of different subsets of macrophages, Spiller et al. showed that M1 macrophages express genes involved in the initiation of angiogenesis, including VEGF-A and FGF2. They also showed that M2a macrophages express and secrete high levels of PDGF-BB, while M2c macrophages secrete high levels of MMP-9. By analyzing macrophage infiltration and the extent of angiogenesis in collagen scaffolds that were implanted subcutaneously in a murine model for 10 days, these authors found that both M1 and M2 macrophages were required to achieve vascularization. Uncrosslinked collagen scaffolds were surrounded by a fibrous capsule containing predominantly M2 macrophages, and they underwent minimal angiogenesis. In contrast, glutaraldehyde-crosslinked collagen scaffolds recruited both M1 and M2 macrophages and experienced more robust angiogenesis. Based on the different growth factor secretion profiles and their apparent synergistic behaviors in bioimplant angiogenesis, the authors suggested that M1 macrophages stimulate capillary sprouting, while M2 macrophages may aid in vessel stabilization through pericyte recruitment.

Overall, these studies imply that tissue engineering and regenerative medicine strategies that take steps to control macrophage dynamics may achieve more effective angiogenesis, particularly in the context of bioimplant integration into the recipient tissue bed. Along these lines, the inclusion of monocyte and macrophage chemo-attractants in bioimplant designs has been pursued as a strategy to enhance macrophage recruitment and the induction of

angiogenesis. In a recent study, co-delivery of CSF1 with VEGF-A in a hydrogel implant induced more robust angiogenesis and pericyte-recruitment to neovessels in the murine corneal pocket angiogenesis model than VEGF-A alone [118]. Such a finding exemplifies the value of elucidating the relative contribution of macrophages to angiogenesis versus other factors or cellular players. Interestingly, Kobyashi et al. demonstrated in 2002 that the direct injection of leukocytes induces angiogenesis, yet since this time more attention has been given to the use of various stem cell populations as cell-based stimulators of growth [13,50,182]. Hence, it is important to consider which cell type dominates in a dynamic growth and remodeling setting and how different cell types influence one another (e.g. mesenchymal stem cells and macrophages). This point was recently highlighted by a study showing that the therapeutic effects of adipose-derived progenitor cells was dependent on the presence of CD11b+ macrophages [186].

Macrophage interactions with endothelial cells, pericytes, and vascular smooth muscle cells

Macrophages engage in a number of heterotypic cell-to-cell interactions with other cell types, including endothelial cells, pericytes, and vascular smooth muscle cells, and these interactions help to regulate angiogenesis during embryonic development and in adult responses to injury. Macrophages were first characterized by their phagocytic capabilities, so it is not surprising that they have been shown to orchestrate neovessel pruning during the developmental maturation of microvessel networks. Macrophage pruning of vessels via phagocytosis of endothelial cell and pericytes has been identified in a number of different vascular patterning processes during development and post-natal remodeling. During fetal organogenesis of the testes, for example, macrophages arise from primitive yolk sac-derived hematopoietic progenitors, and nearly all of them express CD206, a marker consistent with an M2 macrophage polarization state [61]. Macrophage depletion in this tissue results in disrupted vascular patterning due to inadequate to vascular pruning -- a macrophage behavior that is also known to be important in the murine postnatal eye during postnatal pruning of the hyaloid and pupillary membrane vasculatures [171,213]. In adulthood, macrophage phagocytic behaviors may be important in pathological microvascular remodeling. In rats made diabetic by streptozotocin injection, CCL2 levels were upregulated and there was a two-fold increase in the number of $CX3CR1^{+}/CD11b^{+}$ macrophages in the retina [223]. Whether the elevated levels of macrophages observed in this diabetic rodent model portend phagocytosis as a mechanism underpinning retinal vessel pruning and edema in patients with diabetic retinopathy remains to be determined.

In addition to their phagocytic capabilities, macrophages have been shown to make cell-to-cell contacts with endothelial cells in an orientation that “bridges” two or more angiogenic sprouts. In studies of hindbrain development in mouse and zebrafish, macrophage bridging of endothelial cells enhanced endothelial cell tip cell fusion, or anastomosis [78]. Similarly, in the developing retina, tissue-resident macrophages, or microglia, have also been identified associating with endothelial tip cells. Genetic ablation of microglia in the $Csf1^{op/op}$ mouse has been shown to result in a sparser retinal vascular network with less branching [153], and in one study also resulted in endothelial sprouts with fewer filopodia [239]. To what extent macrophage bridging enacts control over endothelial cell sprout anastomosis during

angiogenesis in the adult, and whether the effects of macrophage bridging are mediated by paracrine and/or juxtracrine signaling with endothelial cells, have yet to be determined.

Macrophages have been shown to interact directly with microvascular pericytes during tissue injury in response to pericyte-derived chemokines, such as MIF [264]. In the inflamed mouse ear stimulated by TNF or LPS, dynamic intravital two-photon microscopy has revealed that pericytes instruct macrophages with pattern-recognition and motility programs via ICAM-1 dependent contact after they have extravasated from post-capillary venules [264]. Very recent evidence also suggests that the recruitment of TAMs promotes pericyte-endothelial interactions in the brain, and in the absence of macrophages, pericytes lose their ability to associate with endothelial cells [307]. Together, these studies imply that pericytes and macrophages have two-way communication with one another that could be important in directing angiogenesis and microvascular remodeling.

Macrophages also affect vascular smooth muscle cell behaviors that are relevant to growth and remodeling of the microcirculation. For example, monocyte-derived macrophages play an important role in ligation-induced arteriogenesis by stimulating collateral artery diameter enlargement through smooth muscle cell hypertrophy and proliferation [32]. Recent studies by our group and by others have further suggested that macrophage recruitment and polarization may be a viable therapeutic target for enhancing collateral microvessel growth in settings of tissue ischemia [16,33,39,200].

Evidence for macrophage differentiation into vascular cells

An emerging body of evidence alludes to the fact that macrophages may possess the ability to trans-differentiate into vascular wall cells, including vascular smooth muscle cells, pericytes, and endothelial cells. Recent work by Shankman et al., has suggested that vascular smooth muscle cells residing in the medial layer of large vessels can give rise to macrophages and mesenchymal stem cells in response to injury [250]. Vascular smooth muscle cell-specific conditional knockout of Krüppel-like factor 4 (Klf4) reduced the numbers of SMC-derived macrophage and mesenchymal stem cells, as well as the size of atherosclerotic lesions. Whether the converse is true – i.e. that macrophages possess the ability to differentiate into SMCs -- has sparked recent debate [273]. And, whether the transdifferentiation of macrophages to SMCs or SMCs to macrophages is a phenomenon of relevance to growth and remodeling of the microcirculation remains an open but interesting question, particularly due to its potential implications in arteriogenesis [14,108,123].

In the setting of tumor angiogenesis, bone marrow-derived cells have been shown to differentiate into both pericytes and macrophages [68,207]. The evidence for macrophages and pericytes expressing overlapping sets of surface proteins [143] can make delineating their identity and defining their respective lineages a bit confusing. Additionally, pericytes have a suggested link to fibroblast origins [143], but fibroblasts are not well defined and may represent an ambiguous population of interstitial cells that has overlapping lineage with macrophages and/or pericytes. Macrophage-to-pericyte or macrophage-to-fibroblast transdifferentiation could have important consequences in the regulation of angiogenesis, particularly in settings where high levels of pericyte coverage are essential for maintaining

barrier function of the tissue, like in the blood-brain barrier and the blood-retinal barrier, as well as in pathological situations where fibrosis is a contributing factor.

As mentioned above, whether or not endothelial cells (or circulating “endothelial progenitor cells”) arise from the transdifferentiation of monocytes or macrophages has been a topic of great controversy in recent years. Leukocyte sub-populations and circulating progenitor cells have been shown to have overlapping cell surface marker expression profiles [115,225], leading to the suggestion that monocytes/macrophages are capable of incorporating into neovessels as endothelial cells [148]. In 2002, work by Moldovan et al. supported this paradigm by suggesting that macrophages drill interstitial tunnels that can be occupied by endothelial cells, which are themselves derived from macrophages [184]. However, the expression of characteristic endothelial markers (CD31, VEGFR2, and Tie-2) by a subset of monocytes, termed “myeloid angiogenic cells” (MACs) [38], offers a different explanation for the observed phenotypic overlap. While MACs have been shown to be highly angiogenic and resemble alternatively activated M2 macrophages, their inability to differentiate into endothelial cells has been demonstrated in murine models of ischemia [181,280]. The notion of phenotypic overlap between endothelial cells and monocyte-derived macrophages has also been challenged by studies showing that monocytes uptake platelet microparticles bearing endothelial surface markers [218]. While there is now more recent evidence to support the potential for bone marrow myeloid cell differentiation into endothelial progenitor cells, the frequency of this occurrence and the specificity of the eliciting stimuli need to be defined in order to appreciate the relative contribution of this process in different angiogenesis settings. Highly specific genetic reporter-based lineage-tracing studies coupled with dynamic imaging are needed to unequivocally prove or disprove the potential for phenotypic transitions to occur between macrophages and vascular wall cells (i.e. endothelial cells, pericytes, and smooth muscle cells) during angiogenesis.

CONNECTIONS BETWEEN ANGIOGENESIS AND LYMPHANGIOGENESIS

The review of macrophage dynamics during angiogenesis motivates the recognition that macrophages also play similar roles in lymphangiogenesis. Knowing that lymphatic microvascular network coordination with the blood microvasculature plays a key role in normal physiological functions, such as local tissue fluid balance, tissue perfusion, and immune surveillance [56,167,233], and given the importance of lymphangiogenesis during tumor growth and cancer metastasis, it is surprising that more laboratories do not study both angiogenesis and lymphangiogenesis at the same time. Recent work identifying the temporal and spatial relationships between these two processes highlights the importance of investigating both processes at the same time using the same model systems [20,24,272]. Considering the close relationships that macrophages have with blood and lymphatic endothelial cells, studying macrophage behaviors in this context offers a potentially fruitful approach to unite angiogenesis and lymphangiogenesis research.

During embryonic development, Prox1+ endothelial cells bud off of the cardinal vein to form the lymphatic vascular system [299], and in adults it is this Prox1 identity that is required for lymphatic endothelial cell function and identity maintenance. While lymphatic endothelial cells express many of the same markers as blood endothelial cells (von

Willebrand factor, anti-thrombin 3, and CD31 [3]), they can be distinguished from blood endothelial cells by lymphatic-specific markers including Prox1, VEGFR3, Podoplanin, and lymphatic endothelial hyaluronan receptor-1 (LYVE-1) [298]. The traditional paradigm that the lymphatic and blood circulations are distinct from one another was first challenged over 30 years ago with the identification of connections between these two systems in the microcirculation of dog hearts after lymphatic obstruction [73]. More recently, research focused on blood/lymphatic vessel mispatterning based on direct lymphatic blood perfusion has supported the existence of direct connections between lymph and blood vessels. Injections of FITC-labeled dextran or BSI-lectin identified blood-to-lymphatic perfusion in mice lacking SLP-76 [1], Rac1 [54], O-glycan [88], or Fiaf [17]. Similar results were documented in Prox1 conditional-mutant mice [132]. There is also support that environmental factors might be sufficient to influence lymphatic identity and mispatterning. Lin et al. demonstrated that LPA causes HUVECs to upregulate lymphatic marker expression *in vitro* [168]. Cooley et al. showed that HUVECs cultured in three versus two dimensions also are capable of transitioning to a lymphatic identity [48] and Jensen et al., [56] suggested that nitric oxide could induce hypoxia induced lymphatic blood perfusion in zebrafish at sites of arterial-lymphatic anastomoses. So, what we know now is that lymphatic vessels can connect directly to blood vessels and that lymphatic and blood endothelial cells exist as two states along a shared phenotype spectrum[42]. The similarities (rather than differences) between lymphatic and blood endothelial cells offer an opportunity to apply our knowledge about angiogenesis to advance our understanding about lymphangiogenesis, and vice versa. Moreover, the potential link that macrophages may provide -- either physically or by providing paracrine signaling -- between these two circulatory systems should be explored in this context.

As an example, we recently discovered that vascular island incorporation, characterized as the proliferation and connection of disconnected endothelial segments with nearby networks, as a new mode of growth associated with angiogenesis [142,144,263]. Support of this dynamic is provided by the evidence that migration and re-connection of isolated lymphatic endothelial cells segments was already characterized as an accepted mode of lymphangiogenesis [24,141,210,238]. Now questions regarding how vascular islands are guided to connect to either blood or lymphatic networks and, for that matter, how angiogenesis and lymphangiogenesis are interdependently regulated remain to be answered. Interestingly, the observation of lymphatic island formation and fusion to nearby networks during recurrent inflammation-induced lymphangiogenesis correlated with an increase in CD11b positive macrophage accumulation [142]. The possible role of macrophages in what we have termed vascular island incorporation, however, remains to be explored.

We speculate that macrophage dynamics may offer putative mechanistic linkages between angiogenesis and lymphangiogenesis. Just like in angiogenesis, macrophages stimulate lymphangiogenesis via paracrine mechanisms and have been suggested to possess the ability to differentiate into lymphatic endothelial cells [72,102,179]. A future challenge is to determine if macrophages merely are independent players in these vessel growth processes or if they orchestrate coordination between the two.

MACROPHAGE DYNAMICS IN LYMPHANGIOGENESIS

While lymphangiogenesis research is benefiting from its own renaissance, our knowledge still lags what we know about angiogenesis. Compare a recent PubMed search for “macrophages and angiogenesis” resulting in ~3700 articles versus a search for “macrophages and lymphangiogenesis” resulting in ~230. This discrepancy emphasizes the need for more lymphangiogenesis studies focused on the assessment of macrophage dynamics that have been identified during angiogenesis in regulating various aspects of lymphangiogenesis. Snapshots of CD11b+ and LYVE-1+ interstitial cells near and apparently interacting with lymphatic sprouts (Figure 4) suggests that macrophages are involved in lymphangiogenesis in many of the same ways that they are in involved in angiogenesis. In this section, we attempt to synthesize two motivating themes for macrophages dynamics in lymphangiogenesis, paracrine signaling and endothelial cell differentiation, that parallel what have already presented for angiogenesis. For more comprehensive lists of the overlapping roles for macrophages in both processes we suggest the following reviews [3,203,227].

Lymphangiogenesis occurs in lymphatic capillaries, referred to as initial lymphatic vessels and consisting of only endothelial cells. In contrast, upstream collecting, or contractile lymphatic vessels, are covered with smooth muscle cells. So, while the roles of macrophages, mast cells, and other immune cell types have been implicated as modulators of smooth muscle cell pumping along collecting lymphatics [40,41,152], macrophage interactions during lymphangiogenesis are limited to the endothelium with their major role attributed to paracrine signaling. Interestingly, observation of pericytes along LYVE-1, Prox1 negative, and Podoplanin-negative endothelial extensions off initial lymphatics implicates a possible transient role for pericytes in some form of lymphangiogenesis [232], yet for now any macrophage-pericyte interaction during this process remains unknown.

Paracrine roles for macrophages in lymphangiogenesis

The main commonality that macrophages exhibit in lymphangiogenesis and angiogenesis is their secretion of paracrine signaling molecules [203,227]. And, as could be expected, macrophages during lymphangiogenesis secrete many of the same molecules that they have been shown to secrete during angiogenesis (VEGF-A, VEGF-C, PDGF, bFGF, TNF-α, MMP-2, MMP-9, Ang2, TGF-β and others (Table 3; [221]), with the most important factor in lymphangiogenesis considered to be VEGF-C. While VEGF-C can also stimulate angiogenesis, it is more often associated with lymphangiogenesis [24,137,260,271]. The recruitment and dramatic accumulation of macrophages and production of VEGF-C during inflammation induced lymphangiogenesis is supported by numerous studies [72,135,138,141,148]. In recognition of its importance, an opportunity exists to link how other molecular signals involved in lymphangiogenesis intersect with VEGF-C. As an example, work by Suzuki et al. suggests that macrophage upregulation of VEGF-C expression leading to lymphangiogenesis is induced by TGF-β [270]. Another example is seen with the tumor nuclear factor-alpha (TNF-α) signaling pathway, which has been reported to depend completely on VEGFR3 activation. Tumor necrosis factor receptor 1 (TNFR1) activation in macrophages by tumor necrosis factor-alpha (TNF-α), one of the

prominent pro-inflammatory cytokines, stimulates the secretion of VEGF-C, which in turn activates VEGFR3 on lymphatic endothelial cells [95].

Evidence for macrophage differentiation into lymphatic endothelial cells

In addition to paracrine mechanisms, macrophages have also been suggested contribute to lymphangiogenesis by transdifferentiating into lymphatic endothelial cells [72,102,179]. Most of this evidence is based on the co-labeling of macrophage and lymphatic endothelial cell makers and cell-lineage studies similar to the analogous studies showing macrophage-to-blood endothelial transition [221]. Hall et al., demonstrated via time-lapse analysis of LPS stimulated lymphangiogenesis in the mouse diaphragm that macrophage-derived lymphatic endothelial progenitor cells incorporated into lymphatic vessels and that cultured macrophages can be induced to express lymphatic markers [102]. Similarly, in the study by El-Chemaly et al., Cd11b+ macrophages were shown to form lymphatic like structures *in vitro* in Matrigel that were positive for LYVE-1 and podoplanin [72]. In an experimental autoimmune orchitis (EAO) model, a model for studying chronic inflammation of the testes, F4/80+ macrophages were found to migrate towards and get incorporated into the lymphatic capillary walls and, intriguingly, not the walls of blood capillaries [111]. In a mouse model of tail lymphedema, Shimizu et al. injected adipose-derived regenerative cells into the skin flap surgically created on the tail and concluded that M2 macrophages could be a source of lymphatic endothelial progenitor cells [255]. Compelling evidence is also provided in work by Maruyama et al., which demonstrates that lymphatic endothelial cells in corneal lymphangiogenesis arise from CD11b+ cells [179].

In light of the convincing evidence for injected lymphatic endothelial progenitor cells to also transdifferentiate into lymphatic endothelial cells and contribute to lymphangiogenesis in various models of injury, inflammation and cancer [47,161,219,226,274,281], critical questions exist regarding how macrophages fit in. Are macrophages a subset of these lymphatic progenitor cell populations or should they be considered a different cell type all together? Lymphatic endothelial cell progenitor cell populations include, for example, adipose derives stem cells, bone marrow derived lymphatic EPCs, CD34+/VEGFR3+ lymphatic/vascular EPCs, and multi-potent mesenchymal stem cells. Similar to the analogous scenario discussed for angiogenesis, future conclusions about macrophage transdifferentiation into vascular wall cells warrant a better understanding of the importance and differences between macrophages and the other progenitor cell types. The relatively low transdifferentiation percentages also raise questions about how much this process contributed to the overall lymphangiogenic effect versus paracrine mechanisms. Regardless, convincing evidence suggests that macrophages can become lymphatic endothelial cells during development and in adult tissues. Additional relevant follow-on questions in the context of lymphangiogenesis and angiogenesis include: do subpopulations of macrophages become lymphatic versus blood endothelial cells, can lymphatic or blood endothelial cells de-differentiate back into macrophages, and how is a macrophage guided to one endothelial cell fate verse the other?

TUMOR-ASSOCIATED MACROPHAGES IN ANGIOGENESIS AND LYMPHANGIOGENESIS AND THERAPEUTIC IMPLICATIONS

Macrophages are known to play important roles in a number of major diseases, including hypertension [174], neurodegenerative disease [29], diabetes [151], and cancer [220]. In the remainder of this section, we will focus on cancer because the roles of macrophages in both tumor angiogenesis and lymphangiogenesis have been a topic of study for quite some time, and recent evidence depicts how influential macrophages can be in these vessel remodeling settings and how macrophages might serve as efficacious therapeutic targets in cancer.

Cancer is an inflammatory process, as the seemingly foreign cells grow and invade healthy tissue, the immune system mounts a response to remove the disease [49]. Due to the ability of cancer cells to adapt to avoid immunity and eventually induce tolerance, this inflammatory process is perpetual. As part of this process, there is aberrant induction of angiogenic and lymphangiogenic processes, which are closely linked to growth and metastasis [36]. However, unlike the vascularization that occurs during development, tumor-associated blood vessels are poorly formed structures with blind ends and large gaps between the endothelial cells, leading to irregular and often increased fluid flux into the tumor. There are numerous outstanding reviews chronicling the structure [79], function [125], mechanistic regulation [296], and inhibition [81,126] of tumor associated blood vessels. Due to an increased interstitial fluid flux into the tumor, along with an increase in solid stress from the tumor cells and ECM, the total interstitial pressure increases 10–100 fold compared to healthy tissue [191]. This change in biophysical forces leads to increased drainage to surrounding healthy tissue and tissue lymphatics. These changes, along with chemical signaling from the tumor microenvironmental cells to the lymphatics, can lead to pathological alterations in the lymph vasculature [261]. Tumor-associated lymphatics (TALs) are not as well-studied as tumor-associated blood vessels, but increasing evidence suggests their importance to cancer progression, particularly metastasis [9]. Lymphangiogenesis in cancer can occur intratumorally (e.g., in melanoma [55], colorectal cancer [8], and inflammatory cancers [188]), peritumorally (e.g., in invasive breast carcinoma [247,248] [67]), or in the draining lymph nodes [155,208]. TAMs are thought to play important roles in both angiogenesis and lymphangiogenesis, through their secretion of pro-angiogenic and pro-lymphangiogenic molecules (VEGF-C, VEGF-D, VEGF-A, TNF-α[45]). It is important to point out that many of the chemokines and growth factors that macrophages secrete, such as VEGF-A and VEGF-C, are also known to elicit mitogenic and chemotactic behaviors in macrophages. Hence, it is important to consider the relative roles of paracrine vs. autocrine signaling within a local tissue environment, and these signaling pathways may be dynamic and spatially heterogeneous – and at this point, difficult to assess *in vivo* using the tools that are currently available.

Tumor-associated macrophage phenotypes in cancer

TAMs have been implicated in almost every facet of tumor progression to promote malignancy [46]. Macrophages have been implicated in every cancer as an indicator of poor prognosis, except in colorectal where they have been correlated with good prognosis [312]. This apparent contradiction stems from the polarization state of these macrophages [11].

Traditional delineations of classically and alternatively-activated macrophages have been used to identify the subpopulations within cancers. Historically, TAMs have been described as primarily M2-like macrophages, with high expression levels of TGF-β, IL-10, M-CSF, VEGFs, MMPs, TNF-α, and chemokines such as CCL17 and CCL22 [177]. They can be identified primarily by the expression of CD163 and CD68 (which is expressed by all myeloid cells), but can vary in expression of a number of other macrophage differentiation and tissue-specific markers [27]. Recently, the generalization of TAMs as M2-like alternatively activated cells has been challenged with new evidence that a spectrum of macrophages exists in tumors, and the ratio of M2-like/M1 macrophages may in fact be more predictive of poor prognosis in place of total infiltration [136,177]. Current evidence suggests that these populations may vary based on localization within the tumor, with pro-angiogenic M2-like macrophages congregating in areas of hypoxia, and pro-inflammatory M1 macrophages in regions of tissue inflammation [164]. Recent comprehensive reviews highlight the role of TAMs in cancer progression [202] and their therapeutic response [59]. It is important to be cautious when interpreting the existing macrophage classifications in the context of tumors because they are built on emerging evidence, and none of them is universally accepted.

TAMs in tumor angiogenesis

The role of TAMs in the induction of tumor angiogenesis has been observed for decades, both *in vitro* and *in vivo* [162]. In patients, a high number of TAMs has been correlated with increased microvessel density in multiple tumor types. Pro-angiogenic Tie2+/CD31+ TAMs produce a number of angiogenic growth factors, namely VEGF-A, bFGF, TGF-α, IL-1β, PDGF-BB, and ECM remodeling molecules, such as the MMPs[59,147,288]. Crossbreeding of the transgenic murine model of breast carcinoma (MMTV-PyMT) with a CSF-1 null mouse results in increased macrophage infiltration, enhanced angiogenesis, and quicker cancer progression[169]. Experiments in Lewis lung carcinoma using clodronate liposomes to deplete macrophages similarly show reductions in both TAMs and angiogenesis[317]. Thus, there is a direct link between macrophages and tumor angiogenesis that is likely due to TAM-mediated modulation of the local growth factor environment and/or, perhaps, by differentiating into tumor endothelial cells.

TAMs in tumor lymphangiogenesis

More recently, the role of TAMs in lymphangiogenesis has been explored in the context of the tumor microenvironment. TAMs have been implicated in encouraging tumor cell migration towards lymphatics and lymph nodes, modulating the inflammatory microenvironment to induce lymphangiogenesis, and priming tumor draining lymph nodes. VEGF-C-expressing TAMs correlate with high lymphatic vessel density in pancreatic carcinoma [155], gastric cancer [8], skin carcinoma, [188] and lymph node positive breast carcinoma [248]. Artificially increasing VEGF-A and VEGF-C in fibrosarcoma cells implanted into the avascular mouse cornea showed increases in inflammatory macrophages in addition to increases in intratumoral lymphangiogenesis (for VEGF-C) and peritumoral lymphangiogenesis (for VEGF-A) [28]. Soluble VEGFR3 delivered to orthotopic models of bladder cancer reduce lymphangiogenesis and metastasis. These effects were primarily

mediated by macrophages which were identified as the primary contributors to VEGFC in the tumors [302].

In vitro, macrophages migrate towards VEGF-A and VEGF-C, and in vivo, tumors with high levels of these growth factors have increased macrophage infiltration. However, macrophages also secrete VEGF-A and VEGF-C, increasing the angiogenic microenvironment prior to lymphangiogenesis [227]. Recently Cao and colleagues showed that TAMs secrete VEGF-C in response to TNF-α activation, acting to replace previously reported direct angiogenic and lymphangiogenic effects of TNF-α in Lewis lung carcinoma and ovarian cancer models [131]. In a number of clinical cohorts and murine models of cancers, subsets of TAMs have also been shown to express lymphatic endothelial cell markers, including the VEGF-C receptor, VEGFR3 [256], LYVE1 [245]. VEGFR3+ macrophages are found in multiple types of cancers, including inflammatory skin and breast cancers [10,256], and Lyve1+/CD11b+ macrophages associate with aberrant lymphatics throughout the mesentery in murine models of ovarian cancer [130]. It has been suggested that these particular populations of macropahges may serve as lymphatic endothelial progenitor cells (for a recent review see [221]). Coukos and colleagues harvested bone-marrow derived cells from patients and differentiated these towards a lymphatic endothelial progenitor phenotype which was CD11b+/C14+/VEGFR3+/GP38+/Lyve1+[281]. These cells could be differentiated into lymphatic endothelial cells and secrete VEGFC when exposed to breast cancer patient-derived serum. Interestingly, these populations of lymphatic vascular endothelial progenitor cells can be found circulating in the bloodstream of patients with lung cancer and elevated populations correlate with poor prognosis [30]. These populations of progenitors are just being elucidated in the context of cancer for their role in both angiogenesis and lymphangiogenesis. It is interesting to speculate how the parallel roles that TAMs play in angiogenesis and lymphangiogenesis – in secreting pro-angiogenic and pro-lymphangiogenic growth factors and in potentially serving as a source of endothelial progenitor cells – might serve to link these two vessel growth and remodeling processes and offer a therapeutic target with a double-edged sword.

Targeting TAMs to treat cancer

The landscape of cancer therapy is becoming more complex as new immunotherapies are introduced and chemotherapy regimens become personalized. Evidence suggests that the tumor vasculature [126] and TAMs [59] are involved in the success or failure of most therapeutic strategies and may offer intriguing targets for adjuvant treatment. Therefore, pharmacological blockade of TAMs, angiogenesis, and lymphangiogenesis are ongoing approaches for cancer therapy. Targeting angiogenesis via VEGFR2 blocking antibodies, such as bevacizumab, in murine models of pancreatic cancer reduces the population of intratumoral TAMs [66]. Similarly, inhibition of VEGFR3 with a small molecule inhibitor in the 4T1 murine model of breast carcinoma and Rip1-Tag2 model of pancreatic cancer reduces number of infiltrating TAMs [7]. Conversely, TAM inhibition via CSF1R knockout leads to alterations in angiogenesis in lymphangiogenesis in multiple cancer models [153]. Pharmacological inhibition using anti-M-CSF1R therapeutic antibodies decreases angiogenesis and lymphangiogenesis [230] and can enhance the benefit of immunotherapy in murine models of pancreatic cancer [315]. Inhibition of TAMs leads to an overall

reduction in growth and metastasis in cancers, and thus is a promising strategy for therapy alone or in conjunction with traditional approaches.

Interestingly, current standard of care therapies have been tied to increased angiogenesis, lymphangiogenesis and macrophage infiltration. Radiation therapy, common in most cancers, induces infiltration of VEGFC+ CD68+ macrophages and blood vessel expansion 2–8 weeks post therapy but increased lymphatic vessel density one year after therapy [124]. Paclitaxel, another common therapy, induces intratumoral lymphangiogenesis in murine models of breast cancer, potentially through recruitment of myeloid cells, leading to chemoresistant tumors [289]. Alteration of the activation state of TAMs towards an immunogenic phenotype augments the benefit of immunotherapy in murine models of lung cancer [87]. These complex therapeutic interactions, coupled with the complex interactions of macrophages with blood vessels, lymphatic vessels, and other stromal cells in the tumor environment, create unique therapeutic opportunities that lie at the union of tumor angiogenesis and lymphangiogenesis.

SUMMARY AND FUTURE PERSPECTIVES

From development to adult tissue regeneration and disease scenarios, the macrophage is an inflammatory cell “integrator” that links different areas of vascular biology, including angiogenesis and lymphangiogenesis. While their origin, identity, and sub-type classifications are active areas of study in the field of immunology, macrophage presence and involvement in multiple facets of angiogenesis and lymphangiogenesis are becoming studied more widely by the vascular biology community. A better understanding of macrophages and their interactions with endothelial cells, stem cells, pericytes, and other interstitial cells may inspire new therapeutic strategies for regenerating tissues and curing diseases where deregulated angiogenesis and lymphangiogenesis are culprits, such as in cancer.

While this review is an attempt to highlight the current state of knowledge with regards to macrophage dynamics in angiogenesis and their overlapping roles in lymphangiogenesis, more questions remain unanswered than answered. For example, we focused on macrophage involvement in blood and lymphatic capillary sprouting. Almost nothing remains known about their involvement in other modes of microvessel growth, such as intussusception or vascular island incorporation. The extent to which developmental programs regarding macrophage dynamics are reinstituted during adult microvessel remodeling is an also open question, and the genetic and epigenetic regulatory controls that govern macrophage phenotype (and phenotype switching) in remodeling tissues are not well defined. There may exist opportunities to advance our understanding of interactions between macrophages and lymphatic endothelial cells based on our knowledge of how macrophages interact with blood endothelial cells. Last but not least, the relative contribution of macrophages to angiogenesis and lymphangiogenesis compared to other cell types, and their suitability for therapeutic targeting are only beginning to be evaluated [16].

Recognition of the importance of macrophages in microvessel wall growth is not new [269]; however, macrophages are re-emerging as a cell type of interest in angiogenesis and

lymphangiogenesis. As the spotlight turns to macrophages in these dynamic processes, there is a growing appreciation that we cannot necessarily rely on single time-point "snapshots" of macrophages in tissues that use phenotypic markers to delineate them from other cell types. Because of their spectrum of dynamic behaviors, we submit there is an opportunity to apply *in vivo* lineage tracing and depletion models to gain further insights (Table 4). Furthermore, there have been significant advances in targeted drug delivery to allow researchers to pharmacologically manipulate certain pathways within macrophages, using agents such as liposomes conjugated with macrophage targeting antibodies or ligands (anti-VCAM-1 and CD169 ligand), lectins (mannosylated, oligmannosylated), or peptides (muramyl tripeptides) [139,145]. Alternatively, new delivery systems such as microspheres, nanovesicles, and niosomes can target macrophages in the absence of additional active targeting moieties [154,291]. These tools, when used in combination with standard *in vivo* assays of angiogenesis and lymphangiogenesis, may prove to answer many of the provocative questions that we have put forth throughout this review. Finally, we submit that the development and deployment of new computational modeling approaches [19,211] will enable *in silico* hypothesis testing, provide unparalleled quantitative descriptions of the interactions between macrophages and vascular wall cells that may be important in regulating angiogenesis and lymphangiogenesis, and facilitate the design of "smart" therapies that account for and/or harness macrophage dynamics to control microvessel growth.

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ABBREVIATIONS

Ang-2	angiopoietin-2
BSI	Bandeiraea simplicifolia (Griffonia simplicifolia)
CCL2/MCP-1	chemokine (C-C motif) ligand 2
CCL17	chemokine (C-C motif) ligand 17
CCL22	chemokine (C-C motif) ligand 22
CCR2	chemokine (C-C motif) receptor type 2
CD11b	cluster of differentiation 11b
CD163	cluster of differentiation 163
CD206	cluster of differentiation 206
CD31	cluster of differentiation 31
CD68	cluster of differentiation 68
CSF1	colony stimulating factor 1

CSF1R	colony stimulating factor 1 receptor
CX3CR1	chemokine (C-X3-C motif) receptor 1
ECM	extracellular matrix
EMPs	erythro-myeloid progenitors
EPC	endothelial progenitor cell
FACS	fluorescence activated cell sorting
bFGF	basic fibroblast growth factor
FGF2	basic fibroblast growth factor 2
F4/80	EGF-like module-containing mucin-like hormone receptor-like 1
Fiaf	fasting-induced adipose factor
FISH	fluorescent *in situ* hybridization
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor
HPMCs	human peritoneal mesothelial cells
HUVEC	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon gamma
iNOS	inducible nitric oxide synthase
IL-1	interleukin-1
IL-4	interleukin-4
IL-6	interleukin-6
IL-10	interleukin-10
IL-12	interleukin-12
IL-23	interleukin-23
Klf4	krüppel-like factor 4
LPA	lysophosphatidic acid
LPS	lipopolysaccharide
Ly6C	lymphocyte antigen 6 complex
LYVE-1	lymphatic endothelial hyaluronan receptor-1
MAC	myeloid angiogenic cells
Mif	macrophage migration inhibitory factor

M-CSF	macrophage colony-stimulating factor
MMP-2	matrix metaolprotinase-2
MMP-9	matrix metaolprotinase-9
MMTV	mouse mammary tumor virus promoter
PDGF	platelet derived growth factor
PDGFβ	platelet derived growth factor beta
Prox1	Prospero Homeobox 1
PyMT	polyoma virus middle T antigen transgene
Rac1	RAS-related C3 botulinum substrate 1
Rip1	rat insulin promoter 1
SIX1	sine oculis homeobox homeobox 1
SLP-76	lymphocyte cytosolic protein 2
SMC	smooth muscle cell
Tag2	SV40 large t antigen transgene
TAL	tumor-associated lymphatic
TAM	tumor-associated macrophage
Tie-2	receptor tyrosine kinase of the Tie family
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor-alpha
TNFR1	Tumor necrosis factor receptor 1
VEGF	vascular endothelial growth factor family
VEGFR2	vascular endothelial growth factor receptor-2
VEGFR3	vascular endothelial growth factor receptor-3

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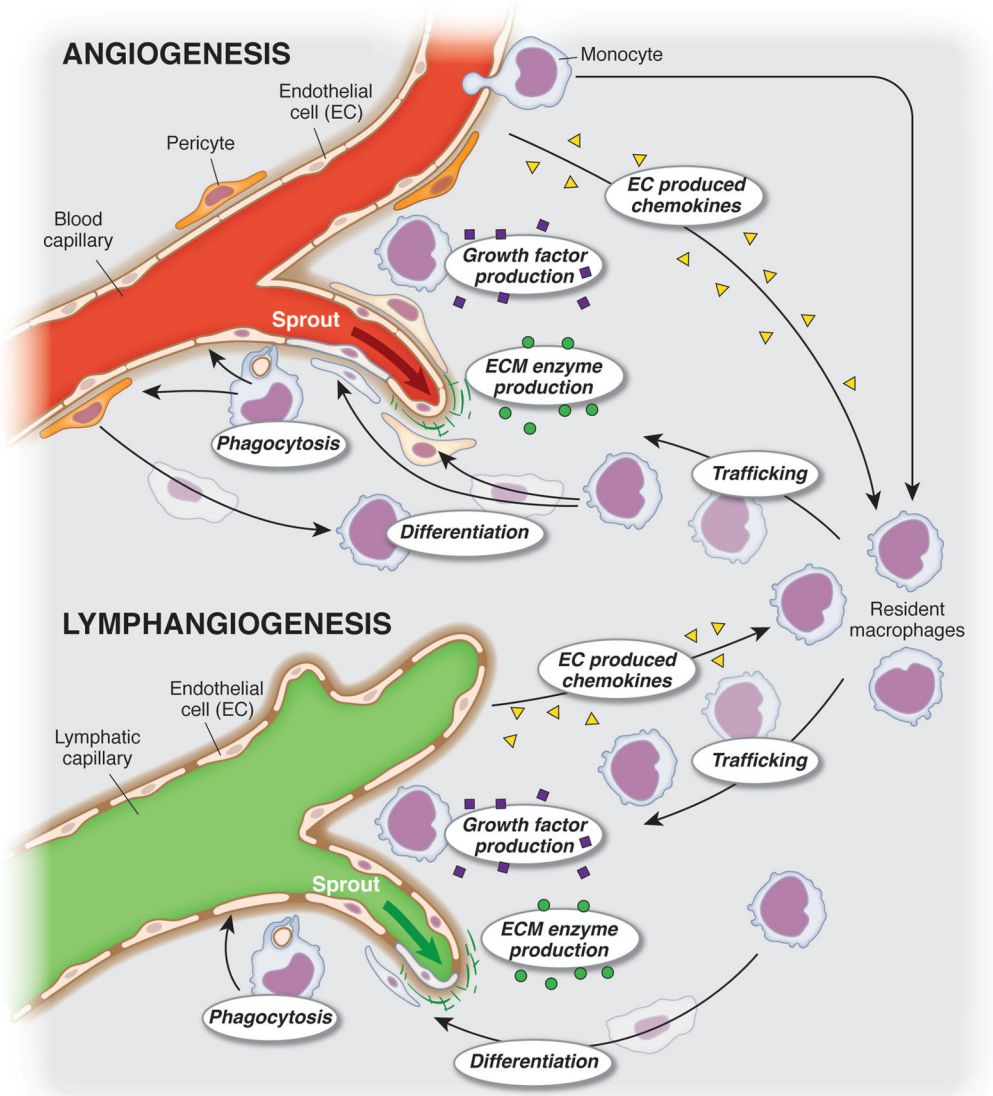

Figure 1. Macrophage dynamics during angiogenesis and lymphangiogenesis

Macrophages exhibit a variety of dynamic cell behaviors, including phagocytosis, trafficking, differentiation, proliferation, and secretion of growth factors, enzymes, and chemokines, that are potentially important to the growth and remodeling of the microvasculature during angiogenesis and lymphangiogenesis. The common dynamics implicates macrophages as an attractive cellular target for therapies pro- and against both processes a cellular link that can be targeted.

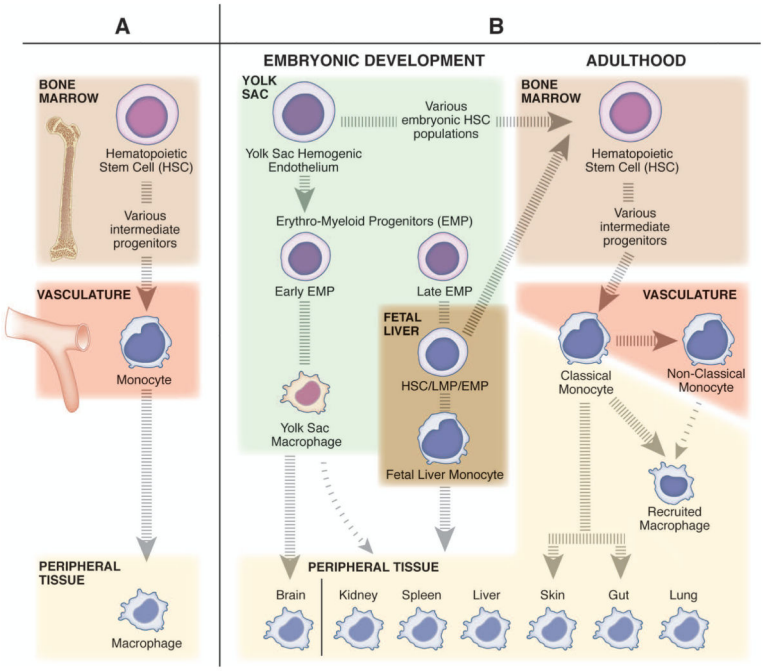

Figure 2. The diverse origins of macrophages and monocytes

(A) The classic belief that tissue resident macrophages are populated from monocytes through adult hematopoiesis. In the bone marrow, hematopoietic stem cells (HSCs) differentiate into monocytes through a series of intermediate progenitors, extravasate from the vasculature, and differentiate into macrophages to maintain the tissue resident niches. (B) The more contemporary view that tissue resident macrophages undergo self-renewal, and have a distinct embryonic origin sourced from populations of erythro-myeloid progenitors (EMPs) derived from the yolk sac hemogenic endothelium. The early population of EMPs differentiate into yolk sac macrophages and then migrate to the tissues and contribute to the tissue resident macrophage population. The late population of EMPs gives rise to fetal liver monocytes through an unknown intermediate embryonic progenitor cell type (maybe EMP, HSC, or lympho-myeloid progenitor (LMP)). Fetal liver monocytes then go on to seed the tissue resident macrophage populations for all peripheral tissues other than the brain. Only the skin and gut resident macrophage populations are recognized to be replenished in homeostatic conditions by bone marrow derived cells, specifically classical monocytes. Classical monocytes (inflammatory monocytes) patrol the peripheral tissues in homeostatic conditions, while nonclassical (anti-inflammatory monocytes) surveil the vasculature. In inflammatory conditions, classical monocytes, and to a lesser extent nonclassical monocytes, can leave the vasculature and differentiate into recruited macrophages to transiently regulate the immune response.

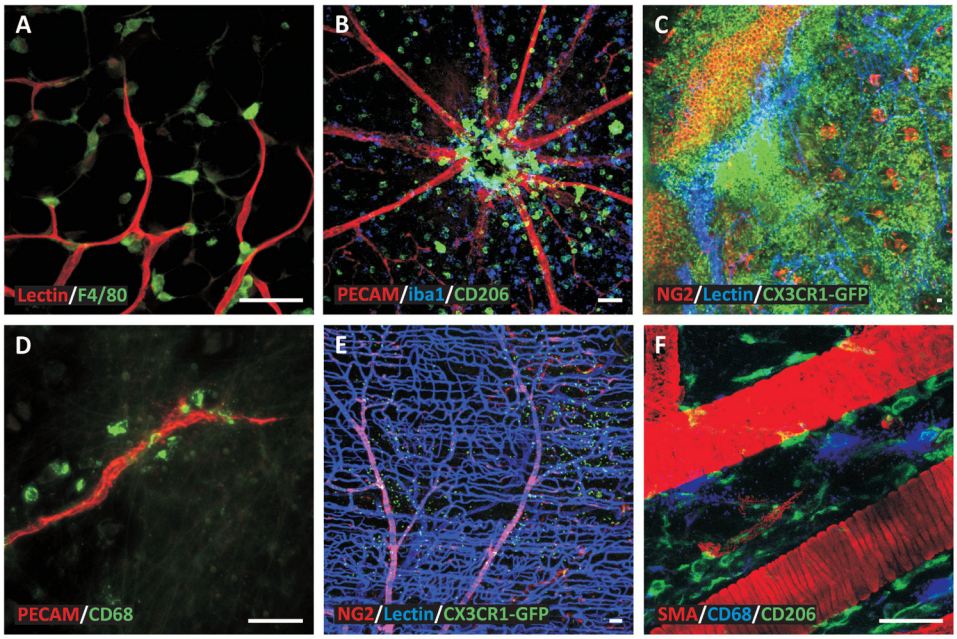

Figure 3. Examples of macrophage spatial distributions during angiogenesis in various tissues

The heterogeneity in macrophage localization emphasizes the need to identify how and why macrophages are recruited to specific locations within microvascular networks. A) Mouse adipose tissue stimulated by a ligation in a feeding arter (Red: Blood vessels, Green: Macrophages). B) Mouse retina stimulated by IL-1β. (Red: Blood vessels, Blue: M2a Macrophages, Green: Macrophages). C) Mouse ear tissue stimulated by a burn injury (Red: Perivascular cells, Blue: Blood vessels, Green: Macrophages). D) Rat mesentery tissue stimulated by chronic hypoxia (Red: Blood vessels, Green: Macrophages). E) Mouse spinotrapezius muscle (Red: Perivascular cells, Blue: Blood vessels, Green: Macrophages). F) Mouse spinotrapezius muscle (Red: Smooth muscle cells, Blue: Mast cells, Green: Macrophages). Scale bars = 50 microns.

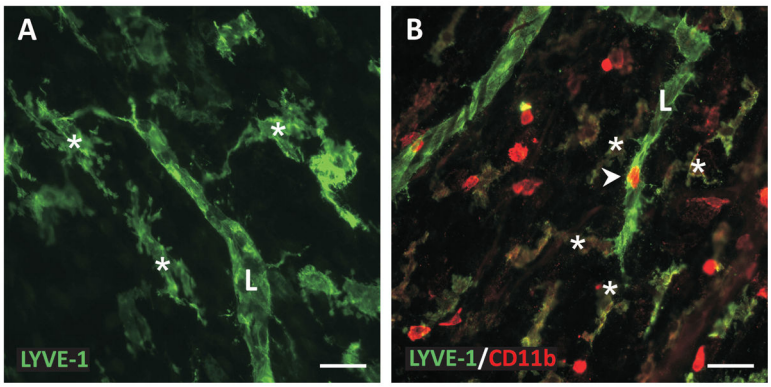

Figure 4. Examples of macrophages nearby lymphatic sprouts during lymphangiogenesis

A) LYVE-1 positive interstitial cells nearby a LYVE-1 positive lymphatic vessel (L) in cultured rat mesenteric tissues stimulated with VEGF-C. B) LYVE-1/CD11b co-labeling during lymphangiogenesis in the rat mesentery tissue stimulated by chronic inflammation. Cellular extensions from interstitial cells (asterisks) toward the lymphatic vessel support the ability for macrophages to interact with and become lymphatic endothelial cells. Arrowhead identifies a CD11b positive cell along the lymphatic sprout. Scale bars = 50 microns.

Table 1

Subpopulations of macrophages and monocytes relevant to angiogenesis and lymphangiogenesis

Name	Tissue Compartment	Origin in Adult	Notable Functions and Characteristics	Reference
Classical Monocyte	Bloodstream, Spleen, Tissue	Adult Bone Marrow	Patrol peripheral tissue in homeostasis.	[303]
Intermediate Monocyte	Bloodstream, Spleen	Circulation	High expression of Tie2, VEGF-A and MMP-9.	[316]
Nonclassical Monocyte	Bloodstream, Spleen	Circulation	Patrol bloodstream for immune surveillance.	[303]
Recruited Macrophage	All Peripheral Tissues	Adult Bone Marrow	Transiently bolster immune response and recovery in peripheral tissues during inflammatory conditions.	[93,303]
Perivascular Macrophage	All Peripheral Tissue	Adult Bone Marrow (?)	Mediate neutrophil recruitment during inflammation.	[2,100]
Kupffer Cells	Liver	Fetal Liver	Prevent inflammatory response of nonpathogenic stimuli such as blood borne food and commensal bacteria antigens.	[65]
Alveolar Macrophages	Lung	Fetal Liver	Clearance of excess and degraded alveolar surfactant layer components: insures optimum gas exchange through alveoli.	[70,74]
Red Pulp Macrophages	Spleen	Adult Bone Marrow	Phagocytosis of injured and senescent erythrocytes and blood-borne particulates.	[74,156]
Marginal Zone Macrophages	Spleen	Unknown	Early infection response by trapping blood borne antigens, rapidly removing them from circulation.	[4,74]
Intestinal Macrophages	Intestines	Adult Bone Marrow	Regulates peristalsis of the colon.	[74,190]
Dermal Macrophages	Skin	Adult Bone Marrow	Specialized roles in scavenging and killing microbes. Unable to migrate to and activate T cells.	[74,275]
Cardiac Macrophages	Heart	Various[a]	Protect heart muscle from hypertensive stress response.	[74,89]
Kidney Macrophages	Kidney	Adult Bone Marrow (?)	Regulation of tubule cell apoptosis through nitric oxide.	[74,150]
Peritoneal Macrophages	Peritoneal Cavity	Fetal Liver	Massive disappearance of these cells under inflammatory conditions (function unknown).	[37]
Microglia	Brain	Yolk Sac	Necessary for neuronal pruning during development, and important for neuronal homeostasis, synaptic remodeling, regulating blood brain barrier.	[198]
Adipose Associated Macrophages	Adipose Tissue	Unknown	Regulate insulin sensitivity and body temperature.	[58,199]
Osteoclast	Bone Marrow	Adult Bone Marrow	Bone remodeling, support for hematopoiesis.	[122]

[a]Heart macrophages are split into two groups: CCR2- macrophages originate from yolk sack and fetal liver, CCR2+ macrophages originate from adult bone marrow.

Abbreviations: CCR2: chemokine (C-C motif) receptor 2, Tie2: receptor tyrosine kinase of the Tie family, VEGF: vascular endothelial growth factor family, MMP-9: matrix metaolprotinase-9.

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Table 2

Mapping of M1/M2 macrophage polarization states to other published macrophage classification schemes.

Scheme	**Phenotype**				
Bipolar					
Extended Bipolar	**M1**	**M2b**	**M2c**	**M2a**	**M2d**
Surface Markers	*CD80*	*CD86*	*CD163*	*CD206*	*CD163*
	CD86	*MHCII*	*TLR1*	*CD163*	
	TLR2		*TLR8*	*MHCII*	
	TLR4			*IL-1R II*	
	MHCII			*Fizz1*	
Secreted Cytokines	*TNF-α*	*IL-1*	*IL-10*	*IL-10*	*IL-10*
	IL-1β	*IL-6*	*TGF-β*	*TGF-β*	*IL-12*
	IL-6	*IL-10*		*IL-1rα*	*TNF-α*
	IL-12	*TNF-α*			*TGF-β*
Stimulus Based	**M(IFN-γ) M(LPS+INF-γ)**	**M(LPS+INF-γ) M(LPS)**	**M(−) M(GC)**	**M(GC+TGF-β) M(IL-10)**	**M(Ic) M(IL-4)**
Color Wheel	**Inflammatory**		**Regulatory**		**Wound Healing**
Secreted Cytokines	*TNF-α*		*IL-10*		*CCL18*
	iNOS		*SPHK1*		*CCL18*
	CCL15		*LIGHT*		*CCL18*
	CCL20		*CCL1*		*CCL18*

Abbreviations: CD80: cluster of differentiation 80, CD86: cluster of differentiation 86, TLR2: toll-like receptor 2, TLR4: toll-like receptor 4, TNF-α: tumor necrosis factor α, IL-1β: interleukin-1 beta, IL-6: interleukin-6, IL-12: interleukin-12, CD163: cluster of differentiation 163, TLR1: toll like receptor 1, TLR8: toll-like receptor 8, IL-10: interleukin-10, TGF-β: transforming growth factor beta, CD86: cluster of differentiation 86, MHCII: major histocompatibility complex II, SR: scavenger receptor CD206: cluster of differentiation 206, IL-1: interleukin-1, IL-1ra: interleukin-1 receptor a, CD14: IL-12: interleukin-12, iNOS: inducible nitric oxide synthase, CCL15: chemokine (c-c motif) ligand 15, CCL20: Chemokine (c-c motif) ligand 20, SPHK1: sphingosine kinase 1, LIGHT: TNF superfamily 14, CCL1: chemokine (C-C motif) ligand 1, CCL18: chemokine (c-c motif) ligand 18, FACTOR XIII-A: fibrin stabilizing factor, RELMα: Resistin-Like Molecules alpha, CCL17: chemokine (c-c motif) ligand 17.

Table 3

Pro- and anti-angiogenic/lymphangiogenic cytokines and other paracrine signals secreted by monocytes and macrophages

Cytokine Name	Effect	Reference
Acidic Firbroblast Growth Factor (aFGF)	Pro-Angiogenic	[31,295]
Angiopoietin-2 (Ang2)	Pro-Angiogenic, Pro-Lymphangiogenic	[80,119,314]
Angiostatin	Anti-Angiogenic, Anti-Lymphangiogenic	[76,180,251]
Angiotensin converting enzyme (ACE)	Anti-Angiogenic	[278,287,306]
Angiotropin	Pro-Angiogenic	[112,113]
Basic Fibroblast Growth Factor (bFGF)	Pro-Angiogenic, Pro-Lymphangiogenic	[206,268]
Chemokine (C-C motif) Ligand 2 (CCL2)	Pro-Angiogenic, Pro-Lymphangiogenic	[63,133,262]
Epithelial Neutrophil Activating peptide-78 (ENA-78/CXCL5)	Pro-Angiogenic	[99,166]
Granulocyte colony stimulating factor (G-CSF)	Pro-Angiogenic, Anti-Angiogenic	[160,197,234,279]
Granulocyte-macrophage colony stimulating factor (GM-CSF)	Pro-Angiogenic, Pro-Lymphangiogenic	[157,254,313]
Insulin-like Growth Factor 1 (IGF-1)	Pro-Angiogenic, Pro-Lymphangiogenic	[28,268]
Insulin-like Growth Factor 2 (IGF-2)	Pro-Angiogenic, Pro-Lymphangiogenic	[28,267]
Interleukin-1 Alpha (IL-1α)	Pro-Angiogenic, Pro-Lymphangiogenic	[290,294]
Interleukin-1 Beta (IL-1β)	Pro-Angiogenic; Pro-Lymphangiogenic	[290,294]
Interleukin-10 (IL-10)	Anti-Angiogenic	[301]
Interleukin-19 (IL-19)	Pro-Angiogenic	[229]
Interleukin-20 (IL-20)	Pro-Angiogenic, Anti-Angiogenic, Pro-Lymphangiogenic	[103,110,117]
Interleukin-6 (IL-6)	Pro-Angiogenic, Pro-Lymphangiogenic	[34,77]
Macrophage-derived Endothelial Cell Inhibitor (MD-ECI)	Anti-Angiogenic	[26]
Matrix Metalloproteinase-2 (MMP-2)	Pro-Angiogenic, Pro-Lymphangiogenic	[64,246]
Matrix Metalloproteinase-9 (MMP-9)	Pro-Angiogenic, Pro-Lymphangiogenic	[25,64,308]
N-acetyl-Ser-Asp-Lys-Pro (Ac-SDKP)	Pro-Angiogenic	[195,265]
Platelet Derived Growth Factor (PDGF)	Pro-Angiogenic, Pro-Lymphangiogenic	[35,243]
Platelet-derived Endothelial Cell Growth Factor (PD-ECGF)	Pro-Angiogenic	[86]
Thrombospondin-1 (TSP-1)	Anti-Angiogenic, Anti-Lymphangiogenic	[53,84,159]
Thrombospondin-2 (TSP-2)	Anti-Angiogenic	[159,209]
Transforming Growth Factor-α (TGF-α)	Pro-Angiogenic	[268]
Transforming Growth Factor-β (TGF-β)	Pro-Angiogenic, Anti-Lymphangiogenic, Pro-Lymphangiogenic	[82,128,163,205]
Tumor Necrosis Factor Alpha (TNF-α)	Pro-Angiogenic, Pro-Lymphangiogenic	[162,227]
Urokinase Plasminogen Activator (uPA)	Pro-Angiogenic, Pro-Lymphangiogenic	[12,204]
Vascular Endothelial Growth Factor A xxx ($VEGFA_{xxx}$)	Pro-Angiogenic, Pro-Lymphangiogenic	[52,201,301]
Vascular Endothelial Growth Factor A xxx b ($VEGFA_{xxx}b$)	Anti-Angiogenic	[201]

Cytokine Name	Effect	Reference
Vascular Endothelial Growth Factor C	Pro-Angiogenic, Pro-Lymphangiogenic	[24,257]
Vascular Endothelial Growth Factor D	Pro-Angiogenic, Pro-Lymphangiogenic	[129,133,231]

Table 4

Small animal models that have been used to study monocyte/macrophage trafficking and function

In Vivo Macrophage Tracking Models in Mice			
Name	**Cell Types[a]**	**Considerations**	**Reference**
CD68-GFP[1]	All Mϕ	Cannot distinguish Mϕ subpopulations. Not known if perivascular Mϕ included.	[121]
CX3CR1-GFP[2]	Monocytes, RMϕ, *NK, DC, Microglia.*	Degraded Mϕ chemotaxis in homozygous knock-in, alters macrophage behavior. Minor effect with heterozygous.	[134]
CCR2-RFP[3]	*Monocytes*, RMϕ, *NK, T Cells*	Can track recently extravasated macrophages: CCR2 expression is lost over time. Heterozygous mouse has reduced migration from BM and extravasation, severe for homozygous.	[240,284,311]
DPE-GFP	PVMϕ, αβ-T Cells	Only demonstrated in dermal tissue, not known if constitutive, nor proportion of PVMϕ population included.	[2]
CSF1R-EGFP	TRMϕ, All myeloid cells.	No ability to trace lineage in inflammation. Although used to study TRMϕ, many cell types marked according to other sources.	[120,185,242]
CX3CR1^{CreER}: R26-YFP	Monocytes, RMϕ, TRMϕ	TMX induction can be tuned for lineage tracing of different cells. Adult cell types with particular TMX induction protocol must be validated.	[94,305]
Gamma Irradiation and GFP BMT	All adult BM-derived cells	Extensively used model. Non-physiologic whole body injury increases RMϕ contribution to tissues, partial depletion of TRMϕ populations.	[292]
Csf1r$^{Cre/WT}$: Rosa26^{eYFP}	Mϕ derived from YS-Mϕ, *TRMϕ*	Single TMX injection leads to incomplete induction.	[114]
S100a4$^{Cre/WT}$: Rosa26^{eYFP}	Mϕ derived from fetal liver monocytes, *TRMϕ*	Single TMX injection leads to incomplete induction.	[114]
Runx1$^{Cre/WT}$: Rosa26^{eYFP}	Fetal lymphoid progenitors and adult HSCs	Single TMX injection leads to incomplete induction. Unclear which cells in adult included.	[241]
CAG-GFP Parabiosis with γ Irradiation	All myeloid lineage except erythrocytes.	Avoid bone marrow transplantation to track myeloid cells. Irradiation causes whole body injury, non-physiologic conditions.	[6]
BM Transplant with Busulfan	All myeloid lineage	Bone marrow chimera that avoids irradiation and whole body injury, but tracks a multitude of cells types.	[146]
GFP Parabosis	All myeloid lineage	Labels minority of cell populations (30%).	[5,105]

Models of Macrophage Depletion			
Name	**Depleted Cell Types[a]**	**Considerations**	**Reference**
CD11b-DTR	Monocytes, Mϕ, Neutrophils	Effective acute depletion of target cells, possible to acquire immunity to DT treatments, produces low grade inflammation, multiple cell types [43]	[71,194]
CD169-DTR	RMϕ, *TRMϕ*	Possible to acquire immunity to DT treatments, produces low grade inflammation [43]	[44]
CD68-DTA	All Mϕ	Only 50% depletion of CD68+ cells.	[92]
CD11b-HSVTK	Monocytes, Mϕ, Neutrophils	Unknown efficacy for TRMϕ. Can produce low grade inflammation from apoptosis [43].	[109]
Chlodronate Liposomes	Monocyte, Mϕ	Can deplete TRMϕ or Monocytes/Mϕ depending on method of administration. Any leakage of compound from cells can directly inhibit angiogenesis [228]. Low grade inflammation from apoptotic cells [43].	[285,286]

Models of Macrophage Depletion			
Name	**Depleted Cell Types[a]**	**Considerations**	**Reference**
LysM-Cre/DTR	All myeloid lineage	Constitutive cre expression of DTR improves ablation efficacy. Depletion of multiple cell types.	[97]
$LysM^{Cre}x$ $CSF1R^{LsL\text{-}DTR}$	Monocyte, *Mϕ*	Elegant genetic approach to target cell populations. Inconsistent TRMϕ depletion between tissues, perhaps only monocyte-derived subgroups.	[249]
Neutralizing CSF1R Antibody	TRMϕ	Targets tissue resident macrophages. Variable and incomplete ablation efficacy (~80%) in peripheral tissue, not fully validated.	[173]

Abbreviations: Mϕ: macrophages, RMϕ: recruited macrophages, TRMϕ: tissue resident macrophages, PVMϕ: perivascular macrophages, YS-Mϕ: yolk sack macrophages, TMX: tamoxifen. GFP: green fluorescent protein, EGFP: enhanced green fluorescent protein, RFP: red fluorescent protein, YFP: yellow fluorescent protein, CRE: cre recombinase, CRE-ER: cre recombinase fused with human estrogen receptor 2, WT: wild type. CD68: cluster of differentiation 68, CX3CR1: chemokine (C-X3-C motif) receptor 1, CCR2: chemokine (C-C motif) receptor 2, DPE, CSF1R: Colony Stimulating Factor 1 Receptor, BMT: bone marrow transplant, S100a4: S100 Calcium Binding Protein A4, Runx1: Runt-Related Transcription Factor 1, CAG: CMV early enhancer/chicken beta actin, DTR: diptheria toxin receptor. DTA: diptheria toxin fragment A. HSVTK: herpes simplex virus thymidine kinase type 1 gene, CD11b: Integrin, Alpha M (Complement Component 3 Receptor 3 Subunit), CD169: Sialic Acid Binding Ig-Like Lectin 1, LysM: Putative Peptidoglycan-Binding, Domain Containing 4.