



Innate effector cells in angiogenesis and lymphangiogenesis

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Angiogenesis and lymphangiogenesis are distinct and complex processes requiring a finely tuned balance between stimulatory and inhibitory signals. During adulthood, angiogenesis and lymphangiogenesis are activated at sites of tumor growth, tissue injury and remodeling, and chronic inflammation. Vascular endothelial growth factors (VEGFs), angiopoietin (ANGPTs) and a multitude of additional signaling molecules play distinct roles in the modulation of angiogenesis/lymphangiogenesis. VEGFs and ANGPTs activate specific tyrosine kinase receptor (e.g., VEGFR1, VEGFR-2, VEGFR-3 and TIE2 respectively), expressed on blood endothelial cells (angiogenesis) and lymphatic endothelial cells (lymphangiogenesis). Although tumor cells produce VEGFs and other proangiogenic mediators, tissue resident (e.g., macrophages, mast cells) and circulating immune cells (e.g., basophils, neutrophils, monocytes, eosinophils) are an important source of angiogenic/lymphangiogenic mediators in inflammation and in tumor microenvironment and at site of chronic inflammation. Certain immune cells can also release anti-angiogenic factors. Mast cells, basophils, neutrophils and presumably other immune cells are not only a source of angiogenic/lymphangiogenic molecules, but also their target. Cells of the immune system need consideration as major players and possible targets for therapeutic manipulation of angiogenesis/lymphangiogenesis in chronic inflammatory disorders and tumors.

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Introduction

Angiogenesis and lymphangiogenesis are distinct and complex processes requiring a finely tuned balance between stimulatory and inhibitory signals [1^{**},2^{*},3]. Analogous to blood vessels, the formation of new lymphatic vessels occurs vigorously during embryogenesis, but is restricted in adults [1^{**}]. During adulthood, angiogenesis/lymphangiogenesis is limited to sites of chronic inflammation [4], tissue injury or remodeling [5], and cancer [6^{**}].

Angiogenesis is initiated by activation of vascular endothelial growth factor receptor 2 (VEGFR2, a tyrosine kinase (TK) receptor expressed on blood endothelial cells (BECs), by soluble vascular endothelial growth factor-A (VEGF-A)). Although cancer cells are an important source of VEGF-A and other pro-angiogenic mediators [7–9], immune cells in tumor microenvironment (TME) increase VEGF-A availability during the angiogenic switch [10^{*}]. Angiogenesis requires the participation of additional signaling molecules, including angiopoietin 2 (ANGPT2) and delta ligand-like4 (DLL4), which respectively, destabilizes endothelial cells (EC) junctions and controls the tip-cell phenotype [11].

VEGFs and their endothelial TK receptors are central regulators of vasculogenesis, angiogenesis, and

lymphangiogenesis [12]. VEGF-A signaling through VEGFR2 is the major angiogenic pathway, and blockage of VEGF-A/VEGFR2 signaling is the major antiangiogenic strategy [13]. VEGF-C and VEGF-D, through the engagement of VEGFR3 on lymphatic endothelial cells (LECs) induce lymphangiogenesis in tumors and stimulate the formation of metastasis [14,15].

The vascular endothelial growth factor family and their receptors

The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF) [10]. VEGFs bind with different specificity to three mainly endothelial transmembrane receptors, VEGFR-1, VEGFR-2, and VEGFR-3 [16,17,18]. All VEGFRs have a series of immunoglobulin-like domains in the extracellular part and an intracellular split TK domain [12]. Neuropilins 1 (NRP1) and 2 (NRP2) function as co-receptors for specific VEGFs [3]. VEGF-A signaling through VEGFR2 activates angiogenesis by inducing the survival, proliferation, sprouting and migration of BECs, increases endothelial permeability [19,20] and induces inflammation [16,21,22].

There are several splicing isoforms of VEGF-A (121, 165, 189, and 206) which differ in their binding to matrix and to co-receptors; for example, VEGF-A₁₂₁ is acidic and freely diffusible whereas VEGF-A₁₆₅, VEGF-A₁₈₉, and VEGF-A₂₀₆ are basic and bind to heparin and heparin proteoglycans on cellular surfaces and extracellular matrices [23]. VEGF-A binding to VEGFR2 causes receptor dimerization and autophosphorylation at several TK residues [24]. Activation of the receptor leads to its internalization, degradation in the lysosomes or through the proteasome pathway. These are mechanisms for VEGFR2 signal downregulation and the rapid turnover of VEGF-A/VEGFR2 is important for the tight control of VEGFR signaling [17].

VEGF-A is primarily known for its essential role in physiologic and pathologic angiogenesis [25] and also retains lymphangiogenic properties [26]. The angiogenic activity of VEGF-A is mediated by interaction with VEGFR2, whereas the lymphangiogenic activity is promoted by binding to VEGFR2/VEGFR3 heterodimer receptor [12]. VEGF-A stimulates lymphangiogenesis also indirectly by recruiting immune cells (e.g., macrophages, mast cells) that produce VEGF-C and VEGF-D [21,27].

PlGF is expressed in the placenta, heart and lungs and has four isoforms (PlGF1–4) [28,29]. VEGF-B is highly expressed in heart, skeletal muscle and brown fat in adults and has two major isoforms in humans: VEGF-B₁₆₇ binds to heparin proteoglycans, whereas VEGF-B₁₈₆ does not bind heparin and is more soluble [30]. PlGF and VEGF-B bind with high affinity to VEGFR1 whose TK

activity is weak and downstream signaling poorly understood [17]. VEGFR1 is expressed on ECs, some immune cells and pericytes, and its TK activity is required for cell migration towards VEGFs or PlGF [16,22,31]. VEGF-B and PlGF have been shown to be angiogenic in certain pathophysiological settings [32]. VEGF-B can modulate coronary vessel growth and cardiac hypertrophy and lipid metabolism [29,33].

The VEGF-C/VEGFR3 signaling pathway is the main pathway implicated in lymphangiogenesis [18]. VEGF-C is produced as a precursor protein, which is activated by intracellular convertase [34]. The secreted disulfide-linked VEGF-C subunit only binds VEGFR3, but the factor is further proteolysed in the extracellular environment by proteases to generate homodimeric proteins with high affinity for both VEGFR2 and VEGFR3 [35]. VEGF-C is crucial for the survival, proliferation, and migration of LECs [36]. VEGF-D also binds VEGFR3 to promote lymphangiogenesis. Maturation of VEGF-D is similar to that of VEGF-C and occurs by the cleavage of N- and C- terminal regions. VEGF-D overexpression correlates with lymphatic vessels growth and lymphatic metastasis [37]. VEGFR3, expressed on LECs, is activated by both VEGF-C and VEGF-D [14]. The role of VEGFR2 in lymphangiogenesis is still not completely clarified. VEGFR2 is expressed in LEC at low levels, but VEGF-A overexpression in skin and in models of wound healing and skin tumors cause lymphatic vessels hyperplasia [38,39].

Angiopoietins (ANGPTs) play an important role in modulating angiogenesis and lymphangiogenesis. The ANGPT/Tie system consists of two cell-surface TK receptors (TIE1 and TIE2) and two ligands ANGPT1 and ANGPT2. TIE2 is primarily expressed on ECs and binds both ANGPTs, whereas TIE1 is an orphan receptor that can modulate TIE2 activity. ANGPT1 and ANGPT2 bind TIE2 on ECs, but elicit different responses. ANGPT1 is expressed by perivascular cells, such as pericytes and sustains EC survival of blood endothelial cells. By contrast, ANGPT2, secreted by ECs, acts autocrinally and paracrinally as TIE2 ligand to promote angiogenesis and lymphangiogenesis [40].

Several chemokines, produced by immune and non-immune cells, play an important role in the modulation of angiogenesis and anti-angiogenesis [41]. These molecules are also involved in the recruitment of tumor infiltrating immune cells [42].

Inflammatory angiogenesis and lymphangiogenesis

The association between angiogenesis and tumor growth attracted paramount interest during the last decades for the obvious implications of the nature of initiation of tumors and the possibility to inhibit cancer growth by

blocking angiogenesis [13]. By contrast, inflammation-associated angiogenesis and lymphangiogenesis was a rather neglected field until recently. The interest in inflammatory angiogenesis/lymphangiogenesis increased exponentially during last years for a number of reasons. First, chronic low grade inflammation is an essential hallmark of cancer [43^{••}]. Second, angiogenesis and lymphangiogenesis are components of several inflammatory disorders [10[•],44]. Third, there is compelling evidence that several immune cells can be involved, directly and indirectly, in the modulation of angiogenesis and lymphangiogenesis [4,8,10[•],16^{••},19^{••},21[•],27[•],41[•],44]. The latter observation led to the recognition that the interactions between immune cells and the vascular system are involved in a multitude of human inflammatory diseases in addition to cancer [45[•]]. Moreover, accumulating evidence suggests that inflammation-associated angiogenesis/lymphangiogenesis is not merely an endpoint phenotype of inflammation, but rather a dynamic reaction that can alter the process of inflammatory tissue repair and resolution [4]. Finally, there is evidence that circulating progenitor cells can be incorporated into the growing lymphatic vessels and trans-differentiate into LECs [4,46].

Human innate effector cells modulate angiogenesis and lymphangiogenesis

In 1971, Judah Folkman, the father of angiogenic research suggested that tumor growth is angiogenesis-dependent and inhibition of angiogenesis could be of therapeutic value [47]. He also suggested that immune cells, in particular macrophages and mast cells, could be a source of angiogenic factors modulating tumor growth. There is now compelling evidence that several cells of innate and adaptive immune system can play major roles in the complex processes of inflammatory and tumor angiogenesis/lymphangiogenesis [2[•],45[•]].

Monocytes

Transcriptomic, epigenetic, and functional studies have delineated three mononuclear cell lineages within myeloid network: monocytes, macrophages, and dendritic cells [48]. In the past it was assumed that monocytes merely constitute a transient precursor for tissue macrophages. Classical dendritic cells and macrophages originate independently of monocyte input. Conversely, monocytes have emerged as a highly plastic and dynamic cellular system. There are currently three main human monocyte subpopulations defined as CD14⁺ CD16⁺ (classical monocytes), CD14⁺ CD16⁺ (intermediate), and CD14⁻ CD16⁺ (non-classical monocytes) [49].

Several stimuli (e.g., M-CSF, ATP, cysteinyl-leukotriene D₄) can stimulate VEGF production from human monocytes [50–52]. These cells are also a target of angiogenic mediators. Human monocytes express VEGFR1 whose activation by VEGF-A induce migration [22]. VEGF-A-

induced chemotaxis of CD16⁺ monocytes is reduced compared to CD16⁻ monocytes due to a lower VEGFR1 expression [53].

De Palma and collaborators have identified a unique subset of human monocytes which express the TIE2 receptor [54[•]]. TIE2-expressing monocytes are angiogenic and lymphangiogenic and have been associated with different human tumors [55,56]. Moreover, ANGPT2 stimulates TIE2-expressing monocytes to suppress T cell activation [57] and it has been shown that targeting the ANGPT2/TIE2 axis inhibits tumor growth [58].

Macrophages

Macrophages play a pivotal role in chronic inflammation and tumor growth [59]. These cells are the predominant immune cells in inflamed tissues as well as in various solid tumors where they are referred to as tumor-associated macrophages (TAMs) [60]. Macrophages can promote tumor progression either directly, by stimulating the proliferation of cancer cells, or indirectly, by producing angiogenic and lymphangiogenic factors [61]. These programs are induced by TAMs in TME by signals resulting from metabolic conditions (e.g., hypoxia), low pH or from mediators (e.g., cytokines, LPS, adenosine) in TME [10[•],61].

Macrophages purified from human lung parenchyma (HLMs) activated by secreted phospholipases A₂ (sPLA₂) [27[•]] and lipopolysaccharide (LPS) expressed VEGF-A, VEGF-B, VEGF-C, and VEGF-D at both RNA and protein levels [62[•]]. Adenosine induced a synergistic increase of VEGF-A released from HLMs through the engagement of A_{2a} and A₃ receptors [27[•]]. These results demonstrate that human tissue resident macrophages can be a source of both proangiogenic and lymphangiogenic factors. Interestingly, HLMs express the cannabinoid receptors types 1 and 2 (CB1 and CB2 receptors) and the activation of these receptors by specific agonists inhibits LPS-induced production of VEGF-A, VEGF-C, ANGPT1 and ANGPT2. The latter findings suggest that activation of cannabinoid receptors on tissue resident macrophages may be a novel strategy to modulate vascular remodeling in cancer and chronic inflammation.

There is increasing evidence of the heterogeneity of the origin of monocytes and monocyte-derived macrophages in rodents and in humans [63[•]]. Interestingly, human monocyte-derived macrophages produce VEGF-A and VEGF-C, but the release is not modified by the CB1 and CB2 agonists [62[•]]. Macrophage-derived proteases including matrix metalloproteinases (MMPs) (e.g., MMP2, MMP9, MMP12) and serine or cysteine proteinases, such as cathepsins and plasminogen activator, facilitate the infiltrative growth of tumor cells in the TME [64[•],65].

Mast cells

Mast cells are tissue-resident immune cells, and their involvement in tumor angiogenesis has long been demonstrated [8,44]. Tissue mast cells and peripheral blood basophils are the only cells expressing the high-affinity receptors (FcεRI) for IgE and synthesizing a plethora of pro-inflammatory and immuno-regulatory factors. Although these cells share some similarities, they play different roles in several aspects of innate and adaptive immunity [66*].

Several immunologic and non-immunologic stimuli (e.g., anti-IgE, PGE₂, substance P, corticotropin-releasing hormone, adenosine) induce the production of VEGF-A from human mast cells (HMCs) [67–69]. We have demonstrated that human lung mast cells (HLMCs) constitutively express several isoforms of VEGF-A (121, 165, 189, and 206) and that the IgE-mediated activation of these cells can induce the release of VEGF-A [21*]. In addition to VEGF-A, HMCs express the two canonical isoforms of VEGF-B (167 and 186). Importantly, supernatants of immunologically activated HLMCs induced an angiogenic response in the chick embryo-chorioallantoic membrane (CAM). Interestingly, HLMCs expressed lymphangiogenic factors, such as VEGF-C and VEGF-D and adenosine increased VEGF-A, and VEGF-C production. Both VEGFR1 and VEGFR2 are expressed on HMCs and VEGF-A, VEGF-B, VEGF-C and VEGF-D induce mast cell chemotaxis *in vitro* [21*] and *in vivo* [7] through the activation of both VEGFR1 and VEGFR2. Collectively, these results indicate that HMCs are a major source of both angiogenic and lymphangiogenic factors. Moreover, VEGFs produced at sites of inflammation and tumors can amplify mast cell infiltration by interacting with VEGFRs. Mast cell-derived proteases, such as trypsin, MMP2 and MMP9 chymase, can modulate angiogenesis [66*]. Mast cells can reduce the efficacy of anti-angiogenic therapy by releasing granzyme B [70*]. It remains to be investigated whether human mast cells from different anatomic sites and subpopulations of these cells also express anti-angiogenic molecules when exposed to immunological stimuli.

Basophils

Basophils purified from peripheral blood of healthy donors constitutively express mRNA for three isoforms of VEGF-A (121, 165 and 189) and two isoforms of VEGF-B (167 and 186) [16**]. In addition, basophils infiltrating human nasal polyps contained VEGF-A stored in secretory granules. IgE-mediated activation of basophils induced the rapid release of VEGF-A. Human basophils also express on their surface VEGFR2 and the co-receptors NRP1 and NRP2. VEGF-A induces basophil chemotaxis through the engagement of VEGFR2. These results can have relevance in inflammatory disorders [10*,44] and in certain tumors [71] in which basophils can infiltrate the site of chronic inflammation

and TME. In contrast to mast cells, basophils do not express/release lymphangiogenic factors. An interesting cross-talk between HMCs and basophils is mediated by the ANGPT1/TIE2 system. ANGPT1 is present in cytoplasmic vesicles of human basophils and their activation rapidly release ANGPT1 that induce HMC chemotaxis through the activation of TIE2 on their surface [72]. Although basophils can play a role in tumor growth [8,66*] and have been detected in draining lymph nodes of cancer patients [71], the production of lymphangiogenic factors by these cells should be further evaluated.

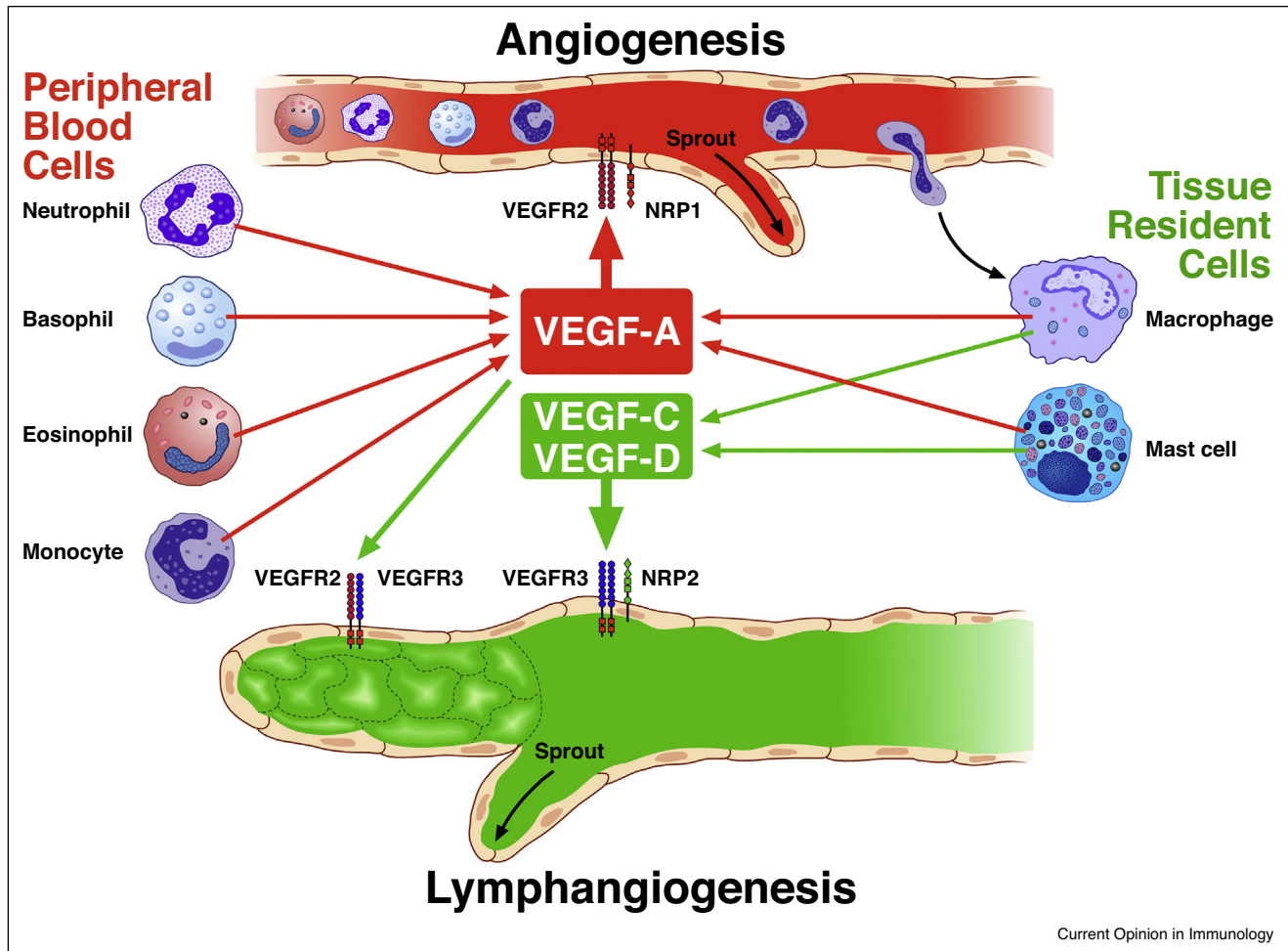
Neutrophils

Neutrophils (PMNs) are abundant granulocytes in human blood and account for a substantial proportion of immune cells in TME in experimental and human tumors [61,73]. Because of their terminally differentiated phenotype and short-half life, the role of neutrophils in tumor development has been erroneously considered marginal. Recent evidence indicates that tumor-associated neutrophils (TAN) can exert anti-tumoral as well as pro-tumoral functions and PMNs display unsuspected plasticity [74*]. Human PMNs release VEGF-A in response to various stimuli (e.g., fMLF, LPS) [75]. We found that several sPLA₂ induce the release of pro-angiogenic molecules such as VEGF-A, CXCL8/IL-8 and ANGPT1 [19**]. Interestingly, sPLA₂ induced the secretion of the anti-angiogenic VEGF-A isoform, namely VEGF-A_{165b}. While the release of VEGF-A, CXCL8/IL-8, and ANGPT1 was mediated by sPLA₂ enzymatic activity and/or binding to a cell membrane receptor(s), the release of VEGF-A_{165b} required the interaction with β integrins. These results suggest that the engagement of different receptors on human PMNs results in the release of different isoforms of VEGF-A exerting opposite effects (e.g., proangiogenic vs antiangiogenic). The translational relevance of these findings is supported by the observation that sPLAs are increased in human cancer [76] that are infiltrated by PMNs [73]. There is no evidence that human the activation of PMNs induces the production of lymphangiogenic factors. Neutrophil-derived MMP9 prompts the angiogenic switch in TME [77].

Eosinophils

Eosinophils represent a minor granulocytic cell infiltrate in experimental and human tumors and have been erroneously neglected for decades. Immunologists and Oncologists are now appreciating the role of these cells in experimental and human tumors [78**,79]. Eosinophils are present in TME of several human solid and hematologic tumors [78**] and their infiltration depends on a combination of cytokines/chemokines produced by tumors and by tumor-infiltrating immune cells. Human eosinophils produce several angiogenic factors such as VEGF-A [80,81*], fibroblast growth factors (FGF-2) [82,83], CXCL8/IL-8 [84], and osteopontin [85]. Human eosinophils also produce MMP9 [86–88]. Eosinophils

Figure 1



Several cells of innate and adaptive immune system play a role in the complex process of inflammatory and tumor angiogenesis and lymphangiogenesis. Activation of human peripheral blood neutrophils, basophils, eosinophils and monocytes induce the production of VEGF-A, which is the main proangiogenic factor. This molecule signals through the engagement of VEGFR2 on blood endothelial cells (BECs). VEGF-A also has pro-lymphangiogenic properties by binding to VEGFR2/VEGFR3 heterodimer receptor on lymphatic endothelial cells (LECs). By contrast, tissue resident macrophages and mast cells produce both proangiogenic (VEGF-A) and lymphangiogenic factors (VEGF-C and VEGF-D). The lymphangiogenic activity of VEGF-C and VEGF-D is mediated by binding to VEGFR3 on LECs. Neutropilins (NRP1 and NRP2) function as co-receptors and amplify the tyrosine kinase activity of VEGFRs.

have been detected in metastatic lymph nodes of cancer patients, but the production of lymphangiogenic factors by these cells should be further addressed.

Concluding remarks and perspectives

Compelling evidence indicates that cells of innate and adaptive immune system can participate in the complex processes of inflammatory angiogenesis and lymphangiogenesis by releasing a wide spectrum of factors which varies enormously among the various cells. Neutrophils, basophils, monocytes and eosinophils isolated from peripheral blood of healthy donors are a major source of several isoforms of proangiogenic molecules. By contrast, macrophages and mast cells isolated from human tissues produce both angiogenic and lymphangiogenic

factors (Figure 1). A growing list of immunologic and non-immunologic stimuli present at sites of inflammation and in TME activate *in vitro* cells of innate immunity to produce angiogenic/lymphangiogenic molecules. However, a note of caution should be mentioned because studies *in vitro* are performed in physiological conditions which are different from the hostile environment at sites of inflammation and tumor growth (e.g., low pH, high concentration of adenosine and oxygen radicals). All the above factors can profoundly influence the metabolic activities of immune cells.

Mast cells, basophils, neutrophils, monocytes and presumably other immune cells are not only a source of angiogenic/lymphangiogenic molecules, but also a target

[16^{••},21[•],22]. In fact, HMCs and basophils express specific receptors (VEGFR1, VEGFR2 and TIE2) and co-receptors (NRP1 and NRP2) for angiogenic and lymphangiogenic factors. These mediators can also exert pro-inflammatory effects (e.g., chemotaxis) by engaging receptors and co-receptors present on HMCs, basophils and presumably other immune cells. Thus, it is likely that angiogenic/lymphangiogenic mediators produced by immune cells at site of inflammation or in TME can contribute to chronic inflammation and cancer growth by recruiting further immune cells.

Human basophils, mast cells, macrophages, and PMNs are among the few cells that express both isoforms of VEGF-B [16^{••},19^{••},21[•],27[•]]. Although VEGF-B can modulate coronary vessels growth [33,89[•]] and lipid metabolism [90], it remains the most mysterious member of the VEGF family [91]. We have found that VEGF-B can play a role in inflammation by inducing immune cells chemotaxis [16^{••},21[•]]. After being neglected for years, the role of VEGF-B in inflammation and immunity deserves further investigations.

Angiogenesis and lymphangiogenesis are controlled by a delicate balance between a plethora of stimulating and inhibitory factors which are incompletely understood. We have found that the engagement of different receptors on human neutrophils can produce distinct isoforms of VEGF-A exerting pro-angiogenic and anti-angiogenic activity [19^{••}]. It will be important to verify whether the activation of subpopulations of PMNs (e.g., N1 vs N2) leads to the preferential production of pro-angiogenic or anti-angiogenic molecules. Moreover, additional studies are urgently needed to identify the release of anti-angiogenic factors from other human immune cells activated by different stimuli.

TAM phenotypes are highly plastic, and recent reports suggest that the canonical model distinguishing polarized anti-tumor M1 and alternatively polarized pro-tumor M2 incompletely accounts for the phenotypic diversity *in vivo* [92]. In addition, there is evidence, at least in rodents, of subpopulations of N1 and N2 neutrophils [74[•]]. Recent advances in single-cell genomics technologies are beginning to allow us the identification of a wide spectrum of TAM and T cell phenotypes [92]. The characterization of different phenotypes of cells of the adaptive and innate immune system will reveal subpopulations of immune cells producing distinct patterns of pro-angiogenic and anti-angiogenic/lymphangiogenic molecules.

Recent studies have shown that tumor regression or refractoriness after immune checkpoint blockade (ICB) with monoclonal antibodies targeting cytotoxic T lymphocytes associated antigen 4 (CTLA-4) or programmed cell-death protein 1 (PD-1) were associated with altered serum levels of ANGPT2 [93[•]]. Moreover, ICB activates

CD4⁺ T lymphocytes which favor tumor blood vessel normalization [94]. Thus, tumor response to immunotherapy may involve immune-mediated anti-angiogenic mechanisms. Cells of the immune system and their mediators need consideration for future therapeutic manipulation of angiogenesis/lymphangiogenesis in chronic inflammatory disorders and tumors.

Conflict of interest statement

Nothing declared.

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