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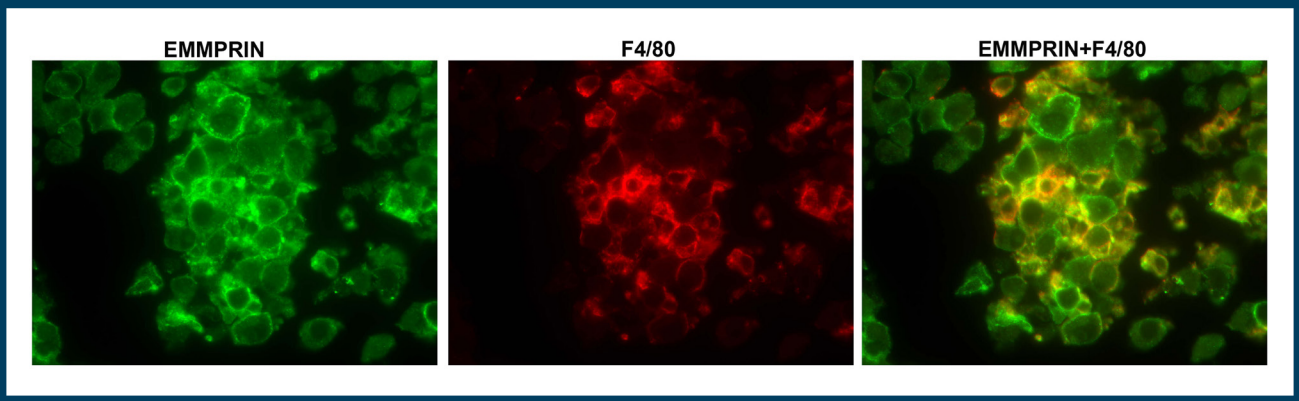

THE REGULATION OF ANGIOGENESIS BY TISSUE CELL-MACROPHAGE INTERACTIONS

Topic Editors
Michal A. Rahat, Bernhard Hemmerlein and Vijaya Iragavarapu-Charyulu

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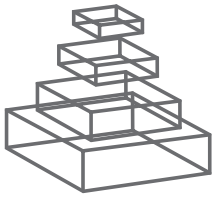

frontiers

ISSN 1664-8714
ISBN 978-2-88919-317-2
DOI 10.3389/978-2-88919-317-2

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THE REGULATION OF ANGIOGENESIS BY TISSUE CELL-MACROPHAGE INTERACTIONS

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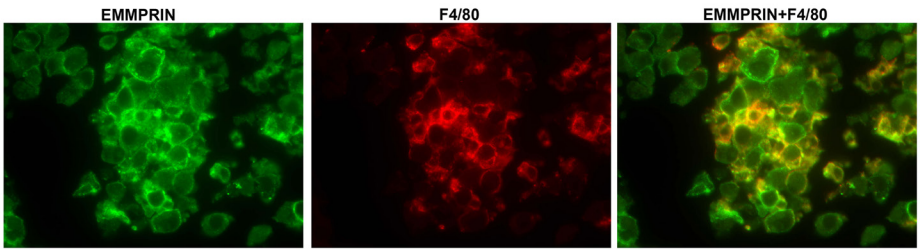

EMMPRIN mediates tumor cells-macrophage interactions. Formalin-fixed RENCA tumor sections (5μm thick) were immunofluorescently stained for EMMPRIN (green) and macrophages (the pan-macrophage marker F4/80, red), showing that tumor cells and macrophages express membranal EMMPRIN, and their co-localization (yellow) suggests that EMMPRIN mediates their interaction. Magnification X400.
Image provided by Michal A. Rahat and Bernhard Hemmerlein.

Angiogenesis is the physiological process where new blood vessels grow from existing ones, in order to replenish tissues suffering from inadequate blood supply. Perhaps the most studied angiogenic process occurs in solid tumors whose growing mass and expanding cells create a constant demand for additional supply of oxygen and nutrients for survival. However, other physiological and clinical conditions, such as wound healing, ischemic events, autoimmune and age-related diseases also involve angiogenesis. Angiogenesis is a well-structured process that begins when oxygen and nutrients are depleted, leading to the release of chemokines and growth factors that attract immune cells, particularly macrophages and endothelial cells to the site. Macrophages that are recruited to the site, as well as tissue cells and endothelial cells, secrete pro-angiogenic mediators that affect endothelial cells and promote angiogenesis. These mediators include growth factors such as vascular endothelial cell growth factor (VEGF), matrix metalloproteinases (MMPs), as well as low levels of mediators that are usually seen as pro-inflammatory but are pro-angiogenic when secreted in low levels (e.g. nitric oxide (NO) and TNFα). Thus, macrophages play a major role in angiogenesis.

Macrophages exhibit high plasticity and are capable of shifting between different activation modes and functions according to their changing microenvironment. Small differences in the composition of activating factors (e.g. TLR ligands such as LPS, anti-inflammatory cytokines, ECM molecules) in the microenvironment may differently activate macrophages to yield classically activated macrophages (or M1 macrophages) that can kill pathogen and tumor cells, alternatively activated macrophages (or M2 macrophages) that secrete anti-inflammatory cytokines, resolution macrophages (rMφ) that are involved in the resolution of inflammation, or regulatory macrophages (e.g. Myeloid-Derived Suppressor Cells - MDSCs) that control the function of other immune cells. In fact, macrophages may be activated in a spectrum of subsets that may differently contribute to angiogenesis, and in particular non-classically activated macrophages such as tumor-associated macrophages (TAMs) and Tie2-expressing monocytes (TEMs) can secrete high amounts of pro-angiogenic factors (e.g. VEGF, MMPs) or low levels of pro-inflammatory mediators (e.g. NO or TNFα) resulting in pro-angiogenic effects.

Although the importance of macrophages as major contributors and regulators of the angiogenic process is well documented, less is known about the interactions between macrophages and other cell types (e.g. tumor cells, normal epithelial cells, endothelial cells) that regulate angiogenesis. We still have only limited understanding which proteins or complexes mediate these interactions and whether they require cell-cell contact (e.g. through integrins) or soluble factors (e.g. the EGF-CSF-1 loop), which signaling pathways are triggered in each of the two corresponding cell types, and how this leads to secretion of pro- or anti-angiogenic factors in the microenvironment. The regulation of such interactions and through them of angiogenesis, whether through post-translational modifications of proteins or via the involvement of microRNA, is still unclear. The goal of this Research Topic is to highlight these interactions and their regulation in the context of both physiological and pathological conditions.

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frontiers in
PHYSIOLOGY

EDITORIAL
published: 09 July 2014
doi: 10.3389/fphys.2014.00262

The regulation of angiogenesis by tissue cell-macrophage interactions

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Keywords: tumor cells, chemokines, semaphorins, chitinases, EMMPRIN, nitric oxide, hypoxia, radiation

Angiogenesis, the sprouting of new blood vessels from existing ones, is a process important both in physiological and pathogenic conditions. During angiogenesis, endothelial cells proliferate, migrate and organize into new, functional blood vessels. Macrophages play an important role in this process as they process microenvironmental cues, and directly secrete or stimulate other cell types to secrete pro-angiogenic mediators, including chemokines, cytokines and growth factors that together stimulate endothelial cell proliferation, degrade the extracellular matrix (ECM), and attract leukocytes to further enhance angiogenesis. Since macrophages are extremely plastic and can be differently activated by various stimuli, they exert many regulatory effects on angiogenesis, although many aspects of this regulation are still unclear.

This volume highlights several examples of macrophages-driven regulation of angiogenesis. We begin with an introductory review which describes the role of tumor-associated macrophages (TAMs) in angiogenesis and lymphangiogenesis. Riabov et al. (2014) describe the crosstalk between tumor cells and TAMs in hypoxic and cytokine-rich microenvironment, resulting in the induction of pro-angiogenic behavior in both cell types.

We then chose to highlight the role of the chitinases, a family of proteins with newly discovered roles in angiogenesis. In this review by Shao (2013), the roles and mode of action of YKL-40/chitinase-3-like-1 (CHI3L1) are described focusing on its angiogenic signature pertaining to tumor vascularization and development. YKL-40 regulates tumor vascularization mediated by endothelial cells and promotes vascular integrity supported by smooth muscle cells. Shao also reports that while YKL-40-induced angiogenic response in endothelial cell is VEGF-independent, YKL-40 itself is induced when VEGF is inhibited.

Next, an original paper by Libreros et al. (2013) suggests that increased levels of CHI3L1 produced by pulmonary macrophages set up the pre-metastatic niche in breast cancer. CHI3L1 enhances angiogenesis by inducing chemokines (CCL2 and CXCL2) and MMP-9 expression, and attraction of macrophages into the lung. The importance of CHI3L1 as a regulator of angiogenesis, as well as possible target of treatment, is demonstrated by the addition of chitin microparticles that can inhibit CHI3L1, chemokines and MMP-9 production in the pre-metastatic lung.

Several pro-inflammatory mediators are long known to be involved in angiogenesis, and the next three reviews elaborate on these. Owen and Mohamadzadeh (2013) describe the potential roles of M2 TAMs in modulating angiogenesis through production of chemokines. The role of the pro-angiogenic ELR+ chemokines that attract neutrophils, and the angiostatic ELR- chemokines, with the exception of CXCL12, are discussed in the context of tumor progression. M1 vs. M2 secreted profiles of cytokines/chemokines which may play roles in immune regulation, angiogenesis, tumor progression and metastasis are highlighted.

Voronov et al. (2014) describe the roles of the IL-1 family of proteins in angiogenesis, and especially IL-1β that can directly drive endothelial cells to proliferate and generate tube-like structures, and to secrete pro-angiogenic cytokines and chemokines. Additionally, IL-1α attracts macrophages and collaborates with VEGF to enhance angiogenesis. Knockout mice for each of the different members of the family help reveal that IL-1β, and to a lesser extent IL-1α, are responsible for the pro-angiogenic effects, and therefore IL-1β depletion could reduce angiogenesis.

The dual role of nitric oxide in tumor biology, as a cytotoxic factor for tumor cells, and as a pro-angiogenic factor, is summarized by Rahat and Hemmerlein (2013). The authors caution against the common use of iNOS immunohistochemical staining as a prognostic factor, as no correlation between survival rates, invasiveness or tumor recurrence after therapy is found. This is due to the ability of the microenvironment (e.g. hypoxia) to inhibit iNOS activity, even when it is highly expressed. Furthermore, tumor cells can manipulate their own expression of iNOS through the expression of microRNA-146a, to control their fate and evade macrophage-induced cell death, suggesting new possible therapeutic approaches.

Two original papers demonstrate the pro-angiogenic role of additional, less studied proteins. Garcia-Areas et al. (2014) suggest that semphorin 7A (SEMA7A), a member of the semaphorin family involved in neuronal axonal guidance, is also

a pro-angiogenic factor whose expression is increased in tumor-bearing mice. When triggered by SEMA7A, macrophages elevate secretion of angiogenic CXCL2/MIP-2 while silencing SEMA7A resulted in decreased tumor angiogenesis, and lower levels of CXCL2/MIP-2, CXCL1 and MMP-9.

Amit-Cohen et al. (2013) report that EMMPRIN, a transmembranal protein that is overexpressed in tumor cells and have a pro-angiogenic activity, can induce the expression of MMP-9 and VEGF from macrophages in a secreted form instead of membranal form. Tumor cells and macrophages must be co-cultured for this effect, emphasizing the importance of their interaction. Secreted EMMPRIN levels were elevated through shedding-off of the membranal protein by the activity of a serine protease that was not yet identified.

The final paper sheds light on the clinical aspect of vasculature re-growth after radiation therapy. Russell and Brown (2013) argue that because radiation destroys endothelial cells and tumor vasculature, tumor hypoxia is markedly increased resulting in induction of HIF-1 and HIF-2. These factors enhance the secretion of pro-angiogenic cytokines, chemokines and growth factors and recruit bone marrow-derived monocytes and macrophages which differentiate into TAMs and TEMs in the tumor. Thus, targeting the infiltration process after radiation therapy may improve the efficiency of treatment, prevent re-establishment of new vasculature and reduce tumor recurrence.

Collectively, the articles in this topic highlight the importance and complexity of the interactions between macrophages and tumor cells in angiogenesis. Macrophages emerge as key regulators of the process because of their ability to communicate with tumor or stromal cells, sense the microenvironment and respond by inducing secretion of potent pro-angiogenic mediators. Some of these pro-angiogenic mediators and the triggers for their enhanced expression and secretion are at the focus of the current topic. Although more research is required, it is clear that tumor cell-macrophage interactions must precede and regulate the secretion phase—a point that was emphasized throughout this volume. This regulatory ability of the macrophages, and the fact that many mediators are both pro-inflammatory and pro-angiogenic, strongly links inflammation and angiogenesis together. Many inflammatory diseases, including cancer, are characterized by enhanced angiogenesis and a prominent macrophage component. The findings described here may be relevant for understanding macrophage-angiogenesis networks and potential therapy designs.

REFERENCES

Amit-Cohen, B. C., Rahat, M. M., and Rahat, M. A. (2013). Tumor cell-macrophage interactions increase angiogenesis through secretion of EMMPRIN. *Front. Physiol.* 4:178. doi: 10.3389/fphys.2013.00178

Garcia-Areas, R., Libreros, S., Amat, S., Keating, P., Carrio, R., Robinson, P., et al. (2014). Semaphorin7A promotes tumor growth and exerts a pro-angiogenic effect in macrophages of mammary tumor-bearing mice. *Front. Physiol.* 5:17. doi: 10.3389/fphys.2014.00017

Libreros, S., Garcia-Areas, R., Keating, P., Carrio, R., and Iragavarapu-Charyulu, V. L. (2013). Exploring the role of CHI3L1 in "pre-metastatic" lungs of mammary tumor-bearing mice. *Front. Physiol.* 4:392. doi: 10.3389/fphys.2013.00392

Owen, J. L., and Mohamadzadeh, M. (2013). Macrophages and chemokines as mediators of angiogenesis. *Front. Physiol.* 4:159. doi: 10.3389/fphys.2013.00159

Rahat, M. A., and Hemmerlein, B. (2013). Macrophage-tumor cell interactions regulate the function of nitric oxide. *Front. Physiol.* 4:144. doi: 10.3389/fphys.2013.00144

Riabov, V., Gudima, A., Wang, N., Mickley, A., Orekhov, A., and Kzhyshkowska, J. (2014). Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis. *Front. Physiol.* 5:75. doi: 10.3389/fphys.2014.00075

Russell, J. S., and Brown, J. M. (2013). The irradiated tumor microenvironment: role of tumor-associated macrophages in vascular recovery. *Front. Physiol.* 4:157. doi: 10.3389/fphys.2013.00157

Shao, R. (2013). YKL-40 acts as an angiogenic factor to promote tumor angiogenesis. *Front. Physiol.* 4:122. doi: 10.3389/fphys.2013.00122

Voronov, E., Carmi, Y., and Apte, R. N., (2014). The role IL-1 in tumor-mediated angiogenesis. *Front. Physiol.* 5:114. doi: 10.3389/fphys.2014.00114

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 June 2014; accepted: 23 June 2014; published online: 09 July 2014.
Citation: Rahat MA, Hemmerlein B and Iragavarapu-Charyulu V (2014) The regulation of angiogenesis by tissue cell-macrophage interactions. Front. Physiol. ***5****:262. doi: 10.3389/fphys.2014.00262*
This article was submitted to Vascular Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 28 May 2013
doi: 10.3389/fphys.2013.00122

YKL-40 acts as an angiogenic factor to promote tumor angiogenesis

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A secreted glycoprotein YKL-40 also named chitinase-3-like-1 is normally expressed by multiple cell types such as macrophages, chondrocytes, and vascular smooth muscle cells. However, a prominently high level of YKL-40 was found in a wide spectrum of human diseases including cancers and chronic inflammatory diseases where it was strongly expressed by cancerous cells and infiltrating macrophages. Here, we summarized recent important findings of YKL-40 derived from cancerous cells and smooth muscle cells during tumor angiogenesis and development. YKL-40 is a potent angiogenic factor capable of stimulating tumor vascularization mediated by endothelial cells and maintaining vascular integrity supported by smooth muscle cells. In addition, YKL-40 induces FAK-MAPK signaling and up-regulates VEGF receptor 2 in endothelial cells; but a neutralizing antibody (mAY) against YKL-40 inhibits its angiogenic activity. While YKL-40 is essential for angiogenesis, little is known about its functional role in tumor-associated macrophage (TAM)-mediated tumor development. Therefore, significant efforts are urgently needed to identify pathophysiological function of YKL-40 in the dynamic interaction between tumor cells and TAMs in the tumor microenvironment, which may offer substantial mechanistic insights into tumor angiogenesis and metastasis, and also point to a therapeutic target for treatment of cancers and other diseases.

Keywords: YKL-40, angiogenesis, VEGF, tumor cells, vascular endothelial cells, tumor-associated macrophages, tumor microenvironment, neutralizing anti-YKL-40 antibody

INTRODUCTION

YKL-40 is a 40-kDa secreted glycoprotein discovered as a heparin-binding protein and belongs to the chitinase gene family that binds to chitin-like oligosaccharides (Shackelton et al., 1995; Hu et al., 1996; Fusetti et al., 2003). However, it does not have chitinase/hydrolase activity because of the substitution of an essential glutamic acid with leucine in the chitinase-3-like catalytic domain (Renkema et al., 1998; Fusetti et al., 2003). YKL-40 is normally expressed by a number of different cell types including chondrocytes (Hu et al., 1996), synoviocytes (Nyirkos and Golds, 1990), vascular smooth muscle cells (Shackelton et al., 1995), macrophages (Rehli et al., 1997), and neutrophils (Kzhyshkowska et al., 2007), and it has been recognized as a growth factor capable of stimulating connective tissue cell growth and endothelial cell migration, and inhibiting mammary epithelial cell differentiation (Malinda et al., 1999; De Ceuninck et al., 2001; Recklies et al., 2002; Scully et al., 2011). However, the pathophysiological function of YKL-40 is still not fully understood.

Growing evidence has indicated that expression levels of YKL-40 are elevated in multiple human diseases including type 2 diabetes (Persson et al., 2012), obesity and insulin resistance in children (Kyrgios et al., 2012), Alzheimers' diseases (Perrin et al., 2011), heart failure (Harutyunyan et al., 2012), and other cardiovascular disorders (Kjaergaard et al., 2010). In addition, elevated YKL-40 was found in a vast array of inflammatory diseases that contain bacterial infections (Kronborg et al., 2002), rheumatoid arthritis (Nielsen et al., 2011), osteoarthritis (Volck et al., 2001), hepatic fibrosis (Pizano-Martinez et al., 2011), and hepatitis (Johansen et al., 2000; Fontana et al., 2010), asthma and chronic obstructive pulmonary diseases (Park et al., 2010), neuroinflammation (Bonneh-Barkay et al., 2010), and bowel lesion (Vind et al., 2003). In the chronic inflammatory diseases, YKL-40 is appreciated to mediate infiltration, differentiation, and maturation of macrophages, the primary leukocytes in response to inflammation (Boot et al., 1995; Rehli et al., 1997; Renkema et al., 1998; Rehli et al., 2003). The cytokines colony-stimulating factor-1 and granulocyte macrophage colony-stimulating factor, essential for macrophage recruitment, displayed the ability to induce 180–200 fold higher levels of YKL-40 mRNA transcripts in macrophages, thus rendering infiltrating macrophages mature (Hashimoto et al., 1999; Suzuki et al., 2000). Studies with YKL-40 deficient mice offered strong evidence supporting the role of

Abbreviations: VEGF, vascular endothelial growth factor; Flk-1, VEGF receptor 2; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; bFGF, basic fibroblastic growth factor; mAY, neutralizing anti-YKL-40 antibody; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; Erk, Extracellular signal-regulated kinase; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; JNK, c-Jun N-terminal kinase; TAM, tumor-associated microphage; GBM, glioblastoma; GSDC, glioblastoma stem-differentiated cells; HMVEC, human microvascular endothelial cells; VE-cad, vascular endothelial cadherin; N-cad, neural cadherin; ER, estrogen receptor; PR, progesterone receptor; Her2/*neu*, human epidermal growth factor receptor 2; MMP, metalloproteinase; shRNA, mall hairpin; HS, heparan sulfate.

YKL-40 in macrophage activity, as these mice exhibited markedly diminished antigen-induced Th2 inflammation and impaired macrophage activation and differentiation (Lee et al., 2009).

Over the past decades, multiple independent studies have demonstrated that high serum levels of YKL-40 are correlated with metastasis and poor survival in a variety of human carcinomas such as breast cancer (Jensen et al., 2003), colorectal cancer (Cintin et al., 1999), ovarian cancer (Hogdall et al., 2003), leukemia (Bergmann et al., 2005), lymphoma (Hottinger et al., 2011), and glioblastoma (GBM) (Pelloski et al., 2005), suggesting that serum levels of YKL-40 serve as a diagnostic and prognostic cancer biomarker. YKL-40 is expressed by both tumor cells and their surrounding tumor infiltrating macrophages also named tumor-associated macrophages (TAM) that produce various tumor-promoting factors including angiogenic factors [vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblastic growth factor (bFGF), platelet-derived growth factor (PDGF)] (Chong et al., 1999; Ganapathy et al., 2010), cytokines (IL-1, IL-6) (Wang et al., 2009; Pini et al., 2012), and chemokines (CCL-2, CCL-18, CXCL-12) (Dubinett et al., 2010; Chen et al., 2011a,b,c; Fridlender et al., 2011; Boimel et al., 2012). Although the overall pathological role and molecular mechanisms of YKL-40 in tumorigenesis remain to be established, an angiogenic feature has been reported to regulate tumor development in breast cancer, colon cancer, and GBM (Shao et al., 2009; Francescone et al., 2011; Kawada et al., 2012). Here, this review primarily focused on the angiogenic signature of YKL-40 derived from tumor cells and smooth muscle cells, as a model is illustrated in **Figure 1**, while a potential distinct role of YKL-40 in TAM-mediated tumor development warrants further investigation.

AN ANGIOGENIC SIGNATURE OF YKL-40

Due to lack of its chitinase activity, the pathological role of YKL-40 in cancer development has not been substantially explored yet. Gp38k, a YKL-40 homolog, was found to induce endothelial cell migration, indicative of angiogenic activity (Nishikawa and Millis, 2003). To evaluate if YKL-40 possesses the same angiogenic activity in cancer, a breast cancer line MDA-MB-231 and colon cancer lines HCT-116 and SW480 were engineered to express ectopic YKL-40 (Shao et al., 2009; Kawada et al., 2012). Xenotransplantation of YKL-40-expressing tumor cells gave rise to 4–8 fold larger tumors than ones formed from their corresponding control cells, while acquired expression of

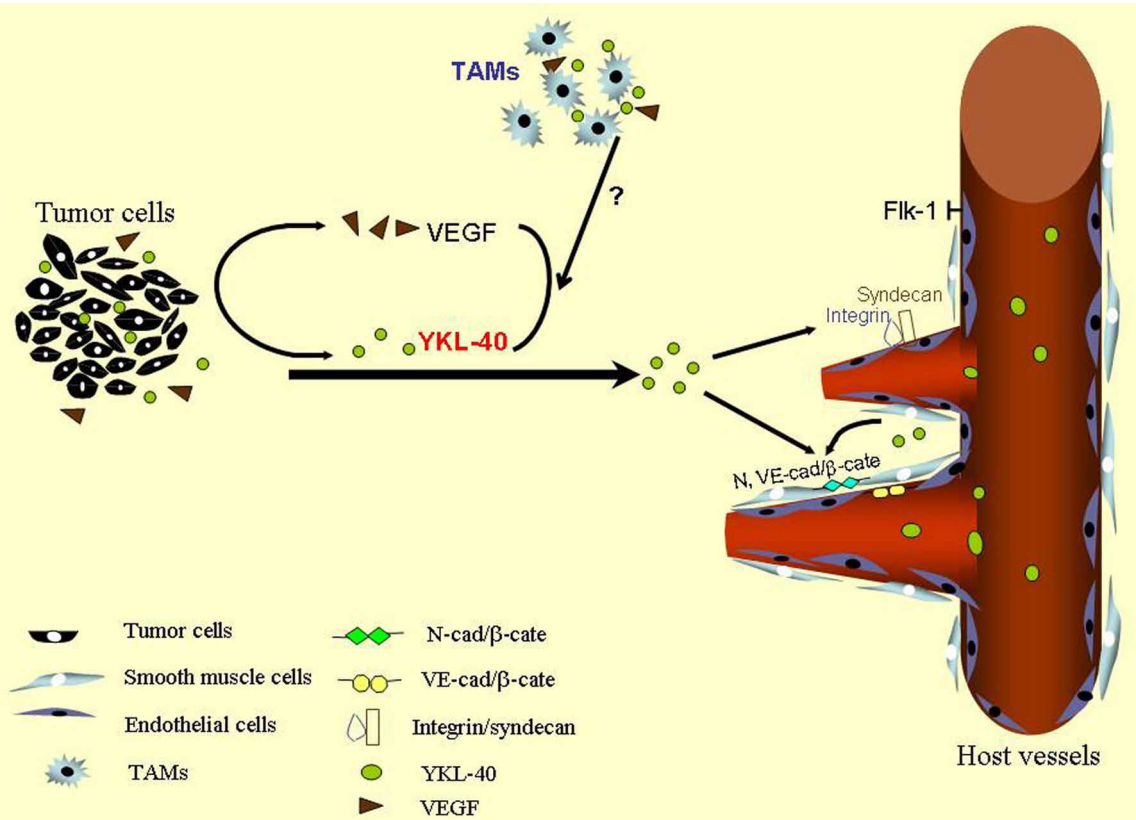

FIGURE 1 | A scheme for YKL-40-induced tumor angiogenesis. YKL-40 secreted from tumor cells stimulates vascular endothelial cell activation to induce tumor angiogenesis through membrane receptor coupling of syndecan-1 with integrin. YKL-40 regulates VEGF in tumor cells and both may synergistically promote endothelial cell angiogenesis. YKL-40 derived from smooth muscle cells also controls vessel stability and permeability via inducing association of N- and VE-cad with β-catenin (β-cate) expressed by smooth muscle cells and endothelial cells, respectively. TAMs participate in the vascular development probably through YKL-40, which warrants further investigation. Green balls indicate secreted YKL-40 and brown triangles represent VEGF.

YKL-40 did not predispose these cells toward increased proliferation in the cultured condition. Immunohistochemical studies indicated that levels of blood vasculature formed in YKL-40-expressing MDA-MB-231, HCT-116, and SW480 tumors were 1.8–2.0 fold greater than those in control tumors, suggesting that YKL-40 acts as an angiogenic factor to promote vessel formation and tumor growth. Such an angiogenic capability of YKL-40 was also validated in GBM (Francescone et al., 2011), the most lethal primary brain tumor characterized by vigorous vascularization (Wen and Kesari, 2008). For example, YKL-40-directed gene knockdown in GBM-derived U87 cells notably suppressed tumor angiogenesis, as the vessel density of YKL-40 shRNA tumors was decreased to 44% of vasculature relative to control tumors and tumor volume was accordingly reduced to approximately 30% of control counterparts (Shao et al., 2009). All of these multiple *in vivo* approaches demonstrate the angiogenic signature of YKL-40 in the tumor development, based on these xenografts carrying different levels of YKL-40. However, this angiogenic phenotype may also involve tumor-promoting function of host-derived cells in the tumor microenvironment, as increased infiltrating macrophages were observed in the YKL-40-expressing tumors, but not in the control tumors (Kawada et al., 2012). It will be interesting to know if these macrophages also increase to produce YKL-40 that enhances the angiogenesis induced by tumor-derived YKL-40.

To monitor its direct effects on vascular endothelial cells, conditioned media derived from both MDA-MB-231 and HCT-116 cells ectopically expressing YKL-40 or vector were introduced to human microvascular endothelial cells (HMVEC) and tested for endothelial cell angiogenic activity *in vitro*. Analogous to the findings in animals, both YKL-40-producing tumor cells induced endothelial cell migration and tube formation (Shao et al., 2009). Likewise, SW480 over-expressing YKL-40 also enhanced migration and tube formation of human umbilical vein endothelial cells by 1.4–2 fold greater than the control cells expressing vector (Kawada et al., 2012). YKL-40 gene knockdown abrogated these angiogenic activities. In addition, conditioned medium of U87 cells expressing YKL-40 shRNA inhibited the angiogenic activities of HMVEC vs. control cell medium (Francescone et al., 2011). To further support these *in vitro* data and firmly establish the angiogenic signature for YKL-40, recombinant YKL-40 was created and characterized for the angiogenic activity. YKL-40 stimulated endothelial cell migration and tube formation approximately 3–4 fold greater than control cells, the angiogenic capability identical to VEGF, one of the most potent angiogenic factors (Shao et al., 2009). It was noted that most of these cultured concentrations of YKL-40 between 100 and 200 ng/ml were based on serum levels of YKL-40 observed in cancer patients (Jensen et al., 2003; Johansen et al., 2003). However, it is unclear if these concentrations indeed reflect YKL-40 levels in the local tumor, because the serum levels are probably derived from multiple organs and also involve the dilution effect. Therefore, a cautious interpretation from these cultured systems should be considered in stimulating YKL-40's action *in vivo*. Nevertheless, all these animal and cultured data suggest that YKL-40 acts as an angiogenic factor to trigger tumor vascular development.

RELATIONSHIP BETWEEN YKL-40 AND VEGF

In the tumor microenvironment, a significant amount of angiogenic factors are secreted from tumor cells and activate adjacent vascular endothelial cells to induce angiogenic responses by means of a paracrine loop (Hanahan and Weinberg, 2010). YKL-40 and VEGF are believed to be mainly derived from tumor cells and both display strong angiogenic activities in tumor development, but their regulatory relationship has not been revealed until recently. YKL-40-induced endothelial cell angiogenic responses in culture were VEGF-independent, as an anti-VEGF neutralizing antibody failed to impede YKL-40-induced migration and tube formation of HMVECs (Shao et al., 2009). This data suggests that YKL-40 and VEGF individually promote endothelial cell angiogenesis. U87 brain tumor cells were found to express high levels of YKL-40 and VEGF (Francescone et al., 2011). When YKL-40 expression was inhibited via small hairpin RNA (shRNA), a reduction of VEGF was subsequently obtained in these tumor cells, indicative of a regulatory role of YKL-40 in VEGF production. In light of a potential similar role of VEGF in YKL-40 expression, transient neutralization of VEGF using a neutralizing anti-VEGF antibody for 24 h did not have impacts in YKL-40 production. Interestingly, inhibition of VEGF for 1 week noticeably induced expression of YKL-40, the unexpected event identical to the documented evidence using VEGF shRNA in U87 cells (Saidi et al., 2008). These results imply that VEGF does not regulate YKL-40, but a long-term blockade of VEGF may result in angiogenic compensative activities of tumor cells by inducing YKL-40. It is most likely that a long course of the stress caused by blockade of one growth factor and/or angiogenic factor commits the cells to induce expression of other potent angiogenic factors in order for cell survival and function. It was noted that these tumor cells such as brain tumor cells express a high level of angiogenic factors able to promote vascular development (Junker et al., 2005a,b; Francescone et al., 2011). This phenomenon was identically observed in a number of tumor models treated chronically with a single anti-angiogenic drug, the event known as angiogenic rebound (see below). However, it needs to determine if this angiogenic switch is unique for highly angiogenic tumors, but not for other non-angiogenic tumors.

Apart from their relationship defined earlier in cultured cancer cell lines, studies on human cancers also suggest the similar association of YKL-40 with VEGF in tumor angiogenesis. Tumor specimens from 12 cases of patients with GBM were used to test the relationship between YKL-40 and VEGF (Francescone et al., 2011). Expression of YKL-40 and VEGF in tumor samples displayed a trend toward positive correlation ($p = 0.062$), but a larger sample pool sufficient to establish their relationship is required. In context with the findings *in vitro*, all the evidence suggests that YKL-40 regulates VEGF in tumor cells and both may exert a synergistic impact in tumor vascularization (**Figure 1**).

A chronic course of angiogenic blockade in either YKL-40 or VEGF may not receive a full elimination of tumor angiogenesis; instead, an unexpected compensation by the other factor may lead to an opposite outcome including resistance to the single-factor treatment and angiogenic rebound. Indeed, the theme of this anti-angiogenic bypass upon a chronic single

treatment has been supported by a number of strong evidence documented in pre-clinical and clinical trials. For instance, individual anti-angiogenic treatment with bevacizumab (anti-VEGF antibody, Avastin), DC101 (anti-VEGF receptor antibody), or sunitinib (anti-VEGF receptor kinase inhibitor) can elicit vascular rebound and tumor cell invasiveness and metastasis in several animal models (Casanovas et al., 2005; Ebos et al., 2009; Paez-Ribes et al., 2009). In clinical trials, the benefit of anti-angiogenic agents (e.g., sunitinib, bevacizumab) appears to be transitory in the treatment of several types of advanced cancers, as drug resistance, tumor regrowth, and extensive vascular recovery rapidly develop, once the therapy is terminated (Bergers and Hanahan, 2008; Burstein et al., 2008; Verhoeff et al., 2009; Wick et al., 2010). In addition, it is noteworthy that bevacizumab has been removed by the Food and Drug Administration from monotreatment of metastatic breast cancers, based on insufficient amelioration of patient overall survival. While it is emerging that a monotherapy against a single factor could unexpectedly result in conflicting outcomes, it is still enigmatic if YKL-40 acts as a major factor to contribute to the angiogenic rebound in these patients that are treated with one drug such as bevacizumab. Nevertheless, to prevent either anti-VEGF or possible anti-YKL-40 resistance, it should be taken into account for a combined regimen with anti-VEGF and anti-YKL-40 therapies in cancer patients.

MOLECULAR MECHANISMS OF YKL-40 IN ENDOTHELIAL CELLS AND TUMOR CELLS

Although membrane receptors specific for YKL-40 binding remain to be identified, heparin-binding affinity of YKL-40 seems to be essential for its activity, resembling the heparin-binding property of other secreted proteins such as extracellular matrix protein vitronectin and angiogenic factors bFGF and VEGF (Bernfield et al., 1999; Beauvais et al., 2004). The heparin binding affinity is at least approximately 100-fold lower (disassociation constant Kd $\sim 10^{-8} - 10^{-9}$ M) than their specific receptor binding (Kd $\sim 10^{-11} - 10^{-12}$ M), but this binding can facilitate their adjacent specific receptor binding (Baird et al., 1988; Park et al., 2000; Prince et al., 2010). Syndecan-1, a transmembrane receptor, is the major source of cell surface heparan sulfate (HS). There is compelling evidence demonstrating that endowed with the HS chain on its ectodomains, syndecan-1 acts as a matrix co-receptor with adjacent membrane-bound receptors such as integrins to mediate cell adhesion and/or spreading (McQuade et al., 2006). This co-membrane receptor model of syndecan-1 with integrin was found to play an indispensable role in mediating YKL-40-induced angiogenic responses (Shao et al., 2009). YKL-40 can induce coupling of syndecan-1 with integrin $\alpha_v\beta_3$ through binding to HS and then activate intracellular signaling effectors focal adhesion kinase (FAK^{861}) and mitogen-activated protein kinase (MAPK) that regulate endothelial cell adhesion and motility (**Figure 2**). In addition, treatment of HMVEC with recombinant YKL-40 increases protein expression and active form of both VEGF receptor 2 (Flk-1) and intracellular extracellular signal-regulated kinase (Erk 1 and 2) that in turn enhance angiogenic signaling pathways (Faibish et al., 2011; Lee et al., 2011). Furthermore, an additional phosphoinositide 3-kinase-protein kinase B (PI3K-AKT) pathway responsible for YKL-40's action in vascular endothelial cells is proposed, but no date has confirmed it yet.

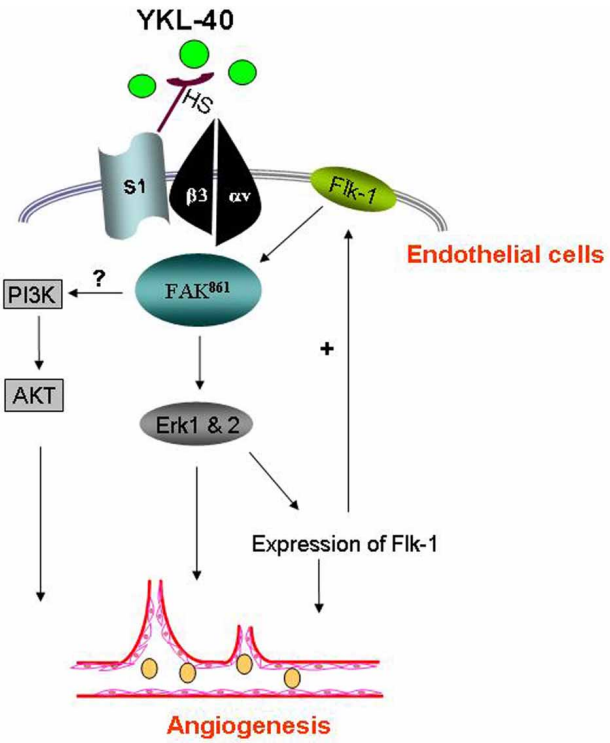

FIGURE 2 | YKL-40 induces angiogenic signaling in endothelial cells. YKL-40 induces the coordination of syndecan-1 (S1) and integrin $\alpha_v\beta_3$ through binding heparan sulfate chains (HS) of S1 on cell surface. The intracellular signaling pathway includes $pFAK^{861}$ and downstream MAP kinase Erk 1 and 2, leading to angiogenic responses and angiogenic gene expression as well (e.g., Flk-1). Flk-1 up-regulation in turns activates the signal transduction cascade, constituting a positive feedback loop to enhance angiogenic responses. Elevated Flk-1 may also sensitize angiogenic responses to VEGF. An additional PI3K-AKT pathway participating in YKL-40-induced angiogenesis in endothelial cells warrants further investigation.

The signaling activation by YKL-40 in endothelial cells was similarly identified in the tumor line U87 cells, in which YKL-40 induces strong association of syndecan-1 with different integrin $\alpha_v\beta_5$ and downstream activation of FAK^{397} and Erk 1 and 2, thus targeting VEGF expression that evokes endothelial cell angiogenesis (Francescone et al., 2011). In apoptotic responses, YKL-40 was found to prevent U87 cell death from γ-irradiation through activation of PI3K-AKT pathways, the signal transduction identical to the cascade that mediates YKL-40-induced mitogenesis in connective tissue cells (Recklies et al., 2002). In SW480 cells, YKL-40 also regulated MAPK including Erk 1, 2, and JNK that induce expression of IL-8 and monocyte chemoattractant protein-1, facilitating angiogenesis (Kawada et al., 2012). Therefore, YKL-40 acts as an angiogenic factor and a growth factor to induce distinct signaling cascades in endothelial cell angiogenesis and tumor cell survival, respectively.

ACTIVITY OF YKL-40 IN VASCULAR SMOOTH MUSCLE CELLS

As discussed earlier, neo-vascular development is mainly ascribed to the activation of vascular endothelial cells, the primary component of blood vessels. However, a functional role of YKL-40 in smooth muscle cells or vascular pericytes, another subset of vascular cell populations that support vessel integrity and stability, is poorly understood, even those cells express YKL-40. Using a smooth muscle cell model named glioblastoma stem-differentiated cells (GSDC), we found that YKL-40 enhances both GSDC and HMVEC contacts, restricts vascular leakage, and stabilizes vascular networks (Francescone et al., 2013). Furthermore, the vascular sprouting and stability mediated by smooth muscle-like cells are dependent on signaling activation induced by YKL-40, which includes interaction of membrane adhesion molecules neural cadherin (N-cad) with β-catenin and downstream intracellular cytoskeleton smooth muscle alpha actin (**Figure 1**). Likewise, adhesion and permeability of HMVECs regulated by YKL-40 rely on the interaction of vascular endothelial cadherin (VE-cad) with β-catenin and downstream effector actin. YKL-40 gene knockdown in GSDCs leads to disruption of association of VE-cad with β-catenin and increases endothelial cell permeability via a paracrine manner. In GSDCs, YKL-40 shRNA also inhibits interaction of N-cad with β-catenin and reduces GSDC-mediated vessel stability, suggesting that both vascular cell populations regulated by YKL-40 coordinately contribute to the angiogenesis. Furthermore, xenotransplantation of GSDCs expressing YKL-40 shRNA in mice gives rise to impaired blood vessel integrity with collapsed vessel lumens and diminished smooth muscle-like cell coverage; whereas control GSDCs develop extensive and stable blood vessels covered with more smooth muscle-like cells, highlighting a unique role of YKL-40 derived from smooth muscle-like cells in the maintenance of vascular permeability, stability, and angiogenesis. Although the interaction between cadherins and catenin is vital for YKL-40's function, it is still unknown whether or not this interaction is dependent on pre-activation of syndecan-1 that is for YKL-40 binding on the membrane.

FUNCTIONAL BLOCKADE OF YKL-40—A POTENTIAL TOOL FOR ANTI-ANGIOGENIC THERAPY

A neutralizing anti-YKL-40 antibody (named mAY) from mice immunized against recombinant YKL-40 was recently established (Faibish et al., 2011). HMVEC migration and tube formation induced by YKL-40 in a dose-dependent fashion were markedly suppressed by mAY. mAY was also found to abolish YKL-40-induced activation of Flk-1 and intracellular signaling MAP kinase Erk 1 and Erk 2 in HMVEC. In addition, mAY facilitated death responses of the U87 glioblastoma cell line to γ-irradiation through decreased expression of pAKT and AKT (Faibish et al., 2011). Consistent with these data from cultured cells, tumor angiogenesis developed from xenografted U87 cells expressing YKL-40 was abrogated in mice treated with mAY, whereas vigorous angiogenesis was observed in mIgG-treated control tumors. Similar studies focusing on YKL-40 neutralization in the angiogenesis of colon cancer unveiled the identical importance for the anti-YKL-40 antibody (Kawada et al., 2012). Therefore, the evidence from such pre-clinical trials has hold therapeutic promise for formulating a humanized anti-YKL-40 antibody in the treatment of cancer patients as well as other possible diseases.

Chitin can bind to both chitinases that have hydrolase activity and chitinase-like proteins that lack the enzymatic activity such as YKL-40 (Lee et al., 2008). Size difference of chitin exhibits distinct capabilities of inducing host immune responses, as small particles ($<10\,\mu m$) can induce TH1 type immune responses whereas large ones ($>50\,\mu m$) activate TH2 type responses (Shibata et al., 1997; Lee et al., 2008). Recently, Iragavarapu-Charyulu's group has utilized small chitin to test a hypothesis that saturation of YKL-40's binding can alleviate its direct tumor-promoting effects on tumors (Libreros et al., 2012). Chitin ($1–10\,\mu m$) has strong binding affinity with YKL-40 and is associated with activation of M1 type macrophages. This binding between chitin and YKL-40 may induce immune response shift from pro-tumorigenic TH2 type (M2 macrophage activation) to anti-tumorigenic TH1 type (M1 macrophage activation). They found that the treatment of mammary tumor-bearing mice with chitin significantly decreased serum levels and splenic macrophages of YKL-40, CXCL2, and MMP-9, thereof impeding lung metastasis. It remains to be determined if the reduction of YKL-40 expression and subsequent inhibition of tumor progression are different from treatment with large chitin. Other alternative possible approaches that block YKL-40 signaling pathways may also suffice to prevent YKL-40 activity or be synergistic in conjunction therapies with YKL-40-directed inhibitors. Nonetheless, the recent multiple animal approaches to blocking YKL-40 function offer therapeutic value potential for modalities of clinical patients.

YKL-40 IN HUMAN TUMOR ANGIOGENESIS

A multitude of clinical studies have revealed that serum levels of YKL-40 were elevated in patients with a series of carcinomas including breast (Jensen et al., 2003), colorectum (Cintin et al., 1999), ovary (Hogdall et al., 2003), prostate (Kucur et al., 2008), brain (Pelloski et al., 2005), and blood (Bergmann et al., 2005). These increased levels were correlated with poorer survival of cancer patients (Cintin et al., 1999, 2002; Hogdall et al., 2003; Jensen et al., 2003; Johansen et al., 2003; Bergmann et al., 2005; Pelloski et al., 2005), suggesting that serum levels of YKL-40 serve as a prognostic cancer biomarker (Johansen et al., 2009).

While amounting evidence was documented in the study of serum levels of YKL-40, there is relatively limited evidence focusing on YKL-40 expression in cancers, particularly for its association with angiogenesis. Thirty-eight cases of breast infiltrating ductal carcinomas were surveyed for relationship of YKL-40 with vessel formation using immunohistochemistry of CD34, a vascular endothelial cell marker (Shao et al., 2009). Of those 38 cancers, 23.7% (9 cases) contained high expression levels of YKL-40 and 23.7% (9 cases) displayed medium levels of YKL-40; whereas 52.6% (20 cases) were negative or low. These three groups with different expression levels of YKL-40 were found to be significantly correlated with different degrees of vascularization with CD34-positive vessels in tumor sections ($p = 0.006$), in which the blood vessel density of the two groups that demonstrated high and medium levels of YKL-40 were 2.1 and 1.6-fold greater than the group expressing low YKL-40, respectively. Consistent

with this finding, of 61 colorectal cancer samples, 37 and 24 cases expressing strong YKL-40 and weak YKL-40 exhibited 2.0 and 1.6-fold higher microvessel density than did 12 normal subjects, respectively (Kawada et al., 2012). In addition, studying 11 cases of patients with GBM revealed that the higher the YKL-40 expression, the more extensive the vessels appeared to be (Francescone et al., 2011). All of the evidence demonstrates that YKL-40 expression in cancer is associated with vascular network development, underscoring the angiogenic property of YKL-40 identified in pre-clinical (cultured cells and xenografted animal models) and clinical studies.

In the study of YKL-40 expression and clinical outcomes, several independent studies with large breast cancer cohorts from different laboratories including ours demonstrate that YKL-40 expressed by breast cancer is associated with estrogen receptor (ER^-), progesterone receptor (PR^-), and human epidermal growth factor receptor 2 (Her2/*neu*) (Kim et al., 2007; Roslind et al., 2007b; Shao et al., 2011). Unexpectedly, cancer tissue expression, contrary to its levels in the blood, was not correlated with patient overall survival or disease-free survival in 8-year follow-up studies (Shao et al., 2011). This finding was reinforced by the others surveying 630 breast cancer patients (Roslind et al., 2007b). Interestingly, strong expression levels of YKL-40 were identified in TAMs in both breast cancer and lung cancer, as these TAMs surrounding tumor cells co-expressed YKL-40 and CD68, a marker of macrophages (Junker et al., 2005a,b; Roslind et al., 2007a; Stearman et al., 2008). It is well established that infiltrating macrophages play an essential role for angiogenesis in both inflammatory diseases and tumor development, because increased infiltration of macrophages leads to accumulation of multiple growth factors (TGF-β, EGF, bFGF, VEGF, and PDGF) that modulate tissue repair and angiogenesis (Chong et al., 1999; Ganapathy et al., 2010). Furthermore, increased macrophage density in cancers correlates with tumor angiogenesis and poorer patient survival (Leek et al., 1996, 2000; Bingle et al., 2002; Tsutsui et al., 2005). However, it remains to be clarified if the expression of YKL-40 by TAMs is associated with cancer metastasis and patient survival. Validating their relationship may provide a key role of TAMs in the contribution to cancer malignancy.

UNANSWERED QUESTIONS

It has been established that chronic inflammation is a key component of cancer development and metastasis (Coussens and Werb, 2002). TAMs, the primary infiltrating leukocytes, act as a core mediator to regulate inflammatory responses that exacerbate the pathogenesis of cancers (Coussens and Werb, 2002; Pollard, 2004; Lewis and Pollard, 2006). Although tumor-derived YKL-40 was reported to be associated with macrophage recruitment and angiogenesis in colorectal cancer (Kawada et al., 2012), we still lack sufficient knowledge regarding the functional role and molecular mechanisms of YKL-40 in TAM-mediated tumorigenesis. TAMs have the ability to render tumor cells invasive through up-regulation of multiple inflammatory factors such as cytokines, growth factors, chemokines, and metalloproteinases (MMPs). It is noted that YKL-40 is essential for macrophage differentiation and maturation (Rehli et al., 1997, 2003). Thus, it is intriguing to interrogate if the inflammatory responses mediated by these factors in the tumor microenvironment are dependent on TAM-derived YKL-40. For example, little is known if YKL-40 up-regulates inflammatory cytokines in TAMs, even though YKL-40 is recognized as an inflammatory factor and can induce IL-8 from tumor cells (Rathcke and Vestergaard, 2006; Qin et al., 2007; Kawada et al., 2012). YKL-40 can induce VEGF in tumor cells as discussed earlier, but the similar relationship in TAMs remains to be established. In addition, it is still unclear regarding the molecular mechanisms by which YKL-40 regulates macrophage recruitment, differentiation, and maturation. Identification of potential signaling mediators in TAMs may provide alternative approaches to block activities of TAMs, thus impeding tumor progression. Besides TAMs, other YKL-40-producing cells surrounding tumor cells and TAMs (e.g., neutrophils) should be not neglected in the tumor microenvironment, as these cell populations likely coordinate with tumor cells, TAMs, and vessel cells to facilitate tumor cell ectopic dissemination. The function of cell-associated YKL-40 in tumor may be different from free form of YKL-40 in the blood, because we currently do not know receptors or ligands for YKL-40 binding. This may also explain the difference between serum levels and cancer cell levels of YKL-40 in association with tumor malignancy. Thus, characterization of their relationship will aid in establishing a new therapeutic target for treatment.

YKL-40 harbors chitinase-3-like catalytic domains, but does not possess chitinase activities. Therefore, its functional domain(s) are still unclear. Chen at al., recently reported that a chitin-binding motif located between 325 and 339 amino acid residues at the C terminus of YKL-40 is critical for YKL-40 inflammatory activities including AKT-mediated cytokine production (IL-8 and TNF-α) in colonic epithelial cells (Chen et al., 2011a,b). This motif may be also vital for other activities of YKL-40 such as angiogenic function, tumor cell survival, and inflammatory responses of TAMs, all of which need to be proven in individual cell types. Moreover, if a single amino acid residue within this motif is found to mainly contribute to YKL-40's function, this could help screen new therapeutic agents aiming at this specific element. Finally, one of the most challenging research approaches is to identify the membrane receptor(s) specific for YKL-40 binding, which would not only provide new mechanistic insights into YKL-40's action, but also establish proof-of-principle for offering a novel mechanistically-directed target in treatment of a wide spectrum of cancers as well as other types of diseases. Therefore, gaining such important knowledge about pathological activities and molecular mechanisms of YKL-40 will unequivocally pave a fundamental way toward an advanced platform able to notably improve the current diagnosis, prognosis, and therapy of multiple human diseases that are associated with increased levels of YKL-40.

ACKNOWLEDGMENTS

This work was supported by NCI R01 CA120659, DOD W81XWH-06-1-0563, and Collaborative Biomedical Research Program, Baystate Medical Center/University of Massachusetts at Amherst.

REFERENCES

Baird, A., Schubert, D., Ling, N., and Guillemin, R. (1988). Receptor- and heparin-binding domains of basic fibroblast growth factor. *Proc. Natl. Acad. Sci. U.S.A.* 85, 2324–2328.

Beauvais, D. M., Burbach, B. J., and Rapraeger, A. C. (2004). The syndecan-1 ectodomain regulates alphavbeta3 integrin activity in human mammary carcinoma cells. *J. Cell Biol.* 167, 171–181. doi: 10.1083/jcb.200404171

Bergers, G., and Hanahan, D. (2008). Modes of resistance to anti-angiogenic therapy. *Nat. Rev. Cancer* 8, 592–603. doi: 10.1038/nrc2442

Bergmann, O. J., Johansen, J. S., Klausen, T. W., Mylin, A. K., Kristensen, J. S., Kjeldsen, E., et al. (2005). High serum concentration of YKL-40 is associated with short survival in patients with acute myeloid leukemia. *Clin. Cancer Res.* 11, 8644–8652. doi: 10.1158/1078-0432.CCR-05-1317

Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., et al. (1999). Functions of cell surface heparan sulfate proteoglycans. *Annu. Rev. Biochem.* 68, 729–777. doi: 10.1146/annurev.biochem.68.1.729

Bingle, L., Brown, N. J., and Lewis, C. E. (2002). The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J. Pathol.* 196, 254–265. doi: 10.1002/path.1027

Boimel, P. J., Smirnova, T., Zhou, Z. N., Wyckoff, J., Park, H. I., Coniglio, S. J., et al. (2012). Contribution of CXCL12 secretion to invasion of breast cancer cells. *Breast Cancer Res.* 14, R23. doi: 10.1186/bcr3108

Bonneh-Barkay, D., Wang, G., Starkey, A., Hamilton, R. L., and Wiley, C. A. (2010). *In vivo* CHI3L1 (YKL-40) expression in astrocytes in acute and chronic neurological diseases. *J. Neuroinflamm.* 7:34. doi: 10.1186/1742-2094-7-34

Boot, R. G., Renkema, G. H., Strijland, A., van Zonneveld, A. J., and Aerts, J. M. (1995). Cloning of a cDNA encoding chitotriosidase, a human chitinase produced by macrophages. *J. Biol. Chem.* 270, 26252–26256. doi: 10.1074/jbc.270.44.26252

Burstein, H. J., Elias, A. D., Rugo, H. S., Cobleigh, M. A., Wolff, A. C., Eisenberg, P. D., et al. (2008). Phase II study of sunitinib malate, an oral multitargeted tyrosine kinase inhibitor, in patients with metastatic breast cancer previously treated with an anthracycline and a taxane. *J. Clin. Oncol.* 26, 1810–1816. doi: 10.1200/JCO.2007.14.5375

Casanovas, O., Hicklin, D. J., Bergers, G., and Hanahan, D. (2005). Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. In Cancer Cell, pp. 299–309. doi: 10.1016/j.ccr.2005.09.005

Chen, C. C., Llado, V., Eurich, K., Tran, H. T., and Mizoguchi, E. (2011a). Carbohydrate-binding motif in chitinase 3-like 1 (CHI3L1/YKL-40) specifically activates Akt signaling pathway in colonic epithelial cells. *Clin. Immunol.* 140, 268–275. doi: 10.1016/j.clim.2011.04.007

Chen, C. C., Pekow, J., Llado, V., Kanneganti, M., Lau, C. W., Mizoguchi, A., et al. (2011b). Chitinase 3-like-1 expression in colonic epithelial cells as a potentially novel marker for colitis-associated neoplasia. *Am. J. Pathol.* 179, 1494–1503. doi: 10.1016/j.ajpath.2011.05.038

Chen, J., Yao, Y., Gong, C., Yu, F., Su, S., Chen, J., et al. (2011c). CCL18 from tumor-associated macrophages promotes breast cancer metastasis via PITPNM3. *Cancer Cell* 19, 541–555. doi: 10.1016/j.ccr.2011.02.006

Chong, H., Vodovotz, Y., Cox, G. W., and Barcellos-Hoff, M. H. (1999). Immunocytochemical localization of latent transforming growth factor-beta1 activation by stimulated macrophages. *J. Cell. Physiol.* 178, 275–283. doi: 10.1002/(SICI)1097-4652(199903)178:3<275::AID-JCP1>3.0.CO;2-Q

Cintin, C., Johansen, J. S., Christensen, I. J., Price, P. A., Sorensen, S., and Nielsen, H. J. (1999). Serum YKL-40 and colorectal cancer. *Br. J. Cancer* 79, 1494–1499. doi: 10.1038/sj.bjc.6690238

Cintin, C., Johansen, J. S., Christensen, I. J., Price, P. A., Sorensen, S., and Nielsen, H. J. (2002). High serum YKL-40 level after surgery for colorectal carcinoma is related to short survival. *Cancer* 95, 267–274. doi: 10.1002/cncr.10644

Coussens, L. M., and Werb, Z. (2002). Inflammation and cancer.[see comment]. *Nature* 420, 860–867. doi: 10.1038/nature01322

De Ceuninck, F., Gaufillier, S., Bonnaud, A., Sabatini, M., Lesur, C., and Pastoureau, P. (2001). YKL-40 (cartilage gp-39) induces proliferative events in cultured chondrocytes and synoviocytes and increases glycosaminoglycan synthesis in chondrocytes. *Biochem. Biophys. Res. Commun.* 285, 926–931. doi: 10.1006/bbrc.2001.5253

Dubinett, S. M., Lee, J. M., Sharma, S., and Mule, J. J. (2010). Chemokines: can effector cells be redirected to the site of the tumor? *Cancer J.* 16, 325–335. doi: 10.1097/PPO.0b013e3181eb33bc

Ebos, J. M., Lee, C. R., Cruz-Munoz, W., Bjarnason, G. A., Christensen, J. G., and Kerbel, R. S. (2009). Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis.[see comment]. *Cancer Cell* 15, 232–239. doi: 10.1016/j.ccr.2009.01.021

Faibish, M., Francescone, R., Bentley, B., Yan, W., and Shao, R. (2011). A YKL-40–neutralizing antibody blocks tumor angiogenesis and progression: a potential therapeutic agent in cancers. *Mol. Cancer Therap.* 10, 742–751. doi: 10.1158/1535-7163.MCT-10-0868

Fontana, R. J., Dienstag, J. L., Bonkovsky, H. L., Sterling, R. K., Naishadham, D., Goodman, Z. D., et al. (2010). Serum fibrosis markers are associated with liver disease progression in non-responder patients with chronic hepatitis C. *Gut* 59, 1401–1409. doi: 10.1136/gut.2010.207423

Francescone, R. A., Ngernyuang, N., Yan, W., Bentley, B., and Shao, R. (2013). Tumor-derived mural-like cells coordinate with endothelial cells: role of YKL-40 in mural cell-mediated angiogenesis. *Oncogene*. doi: 10.1038/onc.2013.160. [Epub ahead of print].

Francescone, R. A., Scully, S., Faibish, M., Taylor, S. L., Oh, D., Moral, L., et al. (2011). Role of YKL-40 in the angiogenesis, radioresistance, and progression of glioblastoma. *J. Biol. Chem.* 286, 15332–15343. doi: 10.1074/jbc.M110.212514

Fridlender, Z. G., Kapoor, V., Buchlis, G., Cheng, G., Sun, J., Wang, L. C., et al. (2011). Monocyte chemoattractant protein-1 blockade inhibits lung cancer tumor growth by altering macrophage phenotype and activating CD8+ cells. *Am. J. Res. Cell Mol. Biol.* 44, 230–237. doi: 10.1165/rcmb.2010-0080OC

Fusetti, F., Pijning, T., Kalk, K. H., Bos, E., and Dijkstra, B. W. (2003). Crystal structure and carbohydrate-binding properties of the human cartilage glycoprotein-39. *J. Biol. Chem.* 278, 37753–37760. doi: 10.1074/jbc.M303137200 pp.37753-60

Ganapathy, V., Ge, R., Grazioli, A., Xie, W., Banach-Petrosky, W., Kang, Y., et al. (2010). Targeting the Transforming Growth Factor-beta pathway inhibits human basal-like breast cancer metastasis. *Mol. Cancer* 9:122. doi: 10.1186/1476-4598-9-122

Hanahan, D., and Weinberg, R. A. (2010). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013

Harutyunyan, M., Christiansen, M., Johansen, J. S., Kober, L., Torp-Petersen, C., and Kastrup, J. (2012). The inflammatory biomarker YKL-40 as a new prognostic marker for all-cause mortality in patients with heart failure. *Immunobiology* 217, 652–656. doi: 10.1016/j.imbio.2011.11.003

Hashimoto, S., Suzuki, T., Dong, H. Y., Yamazaki, N., and Matsushima, K. (1999). Serial analysis of gene expression in human monocytes and macrophages. *Blood* 94, 837–844.

Hogdall, E. V., Johansen, J. S., Kjaer, S. K., Price, P. A., Christensen, L., Blaakaer, J., et al. (2003). High plasma YKL-40 level in patients with ovarian cancer stage III is related to shorter survival. *Oncol. Rep.* 10, 1535–1538.

Hottinger, A. F., Iwamoto, F. M., Karimi, S., Riedel, E., Dantis, J., Park, J., et al. (2011). YKL-40 and MMP-9 as serum markers for patients with primary central nervous system lymphoma. *Ann. Neurol.* 70, 163–169. doi: 10.1002/ana.22360

Hu, B., Trinh, K., Figueira, W. F., and Price, P. A. (1996). Isolation and sequence of a novel human chondrocyte protein related to mammalian members of the chitinase protein family. *J. Biol. Chem.* 271, 19415–19420. doi: 10.1074/jbc.271.32.19415

Jensen, B. V., Johansen, J. S., and Price, P. A. (2003). High levels of serum HER-2/neu and YKL-40 independently reflect aggressiveness of metastatic breast cancer. *Clin. Cancer Res.* 9, 4423–4434.

Johansen, J. S., Christensen, I. J., Riisbro, R., Greenall, M., Han, C., Price, P. A., et al. (2003). High serum YKL-40 levels in patients with primary breast cancer is related to short recurrence free survival. *Breast Cancer Res. Treat.* 80, 15–21.

Johansen, J. S., Christoffersen, P., Moller, S., Price, P. A., Henriksen, J. H., Garbarsch, C., et al. (2000). Serum YKL-40 is increased in patients with hepatic fibrosis. *J. Hepatol.* 32, 911–920.

Johansen, J. S., Schultz, N. A., and Jensen, B. V. (2009). Plasma

YKL-40: a potential new cancer biomarker? *Future Oncol.* 5, 1065–1082. doi: 10.2217/fon.09.66

Junker, N., Johansen, J. S., Andersen, C. B., and Kristjansen, P. E. (2005a). Expression of YKL-40 by peritumoral macrophages in human small cell lung cancer. *Lung Cancer* 48, 223–231. doi: 10.1016/j.lungcan.2004.11.011

Junker, N., Johansen, J. S., Hansen, L. T., Lund, E. L., and Kristjansen, P. E. (2005b). Regulation of YKL-40 expression during genotoxic or microenvironmental stress in human glioblastoma cells. *Cancer Sci.* 96, 183–190. doi: 10.1111/j.1349-7006.2005.00026.x

Kawada, M., Seno, H., Kanda, K., Nakanishi, Y., Akitake, R., Komekado, H., et al. (2012). Chitinase 3-like 1 promotes macrophage recruitment and angiogenesis in colorectal cancer. *Oncogene* 31, 3111–3123. doi: 10.1038/onc.2011.498

Kim, S. H., Das, K., Noreen, S., Coffman, F., and Hameed, M. (2007). Prognostic implications of immunohistochemically detected YKL-40 expression in breast cancer. *World J. Surg. Oncol.* 5:17. doi: 10.1186/1477-7819-5-17

Kjaergaard, A. D., Bojesen, S. E., Johansen, J. S., and Nordestgaard, B. G. (2010). Elevated plasma YKL-40 levels and ischemic stroke in the general population. *Ann. Neurol.* 68, 672–680. doi: 10.1002/ana.22220

Kronborg, G., Ostergaard, C., Weis, N., Nielsen, H., Obel, N., Pedersen, S. S., et al. (2002). Serum level of YKL-40 is elevated in patients with *Streptococcus pneumoniae* bacteremia and is associated with the outcome of the disease. *Scand. J. Infect. Dis.* 34, 323–326. doi: 10.1080/00365540110080233

Kucur, M., Isman, F. K., Balci, C., Onal, B., Hacibekiroglu, M., Ozkan, F., et al. (2008). Serum YKL-40 levels and chitotriosidase activity as potential biomarkers in primary prostate cancer and benign prostatic hyperplasia. *Urol. Oncol.* 26, 47–52. doi: 10.1016/j.urolonc.2007.07.020

Kyrgios, I., Galli-Tsinopoulou, A., Stylianou, C., Papakonstantinou, E., Arvanitidou, M., and Haidich, A. B. (2012). Elevated circulating levels of the serum acute-phase protein YKL-40 (chitinase 3-like protein 1) are a marker of obesity and insulin resistance in prepubertal children. *Metabolism.* 61, 562–568. doi: 10.1016/j.metabol.2011.09.004

Kzhyshkowska, J., Gratchev, A., and Goerdt, S. (2007). Human chitinases and chitinase-like proteins as indicators for inflammation and cancer. *Biomark. Insights* 2, 128–146.

Lee, C. G., Da Silva, C. A., Dela Cruz, C. S., Ahangari, F., Ma, B., Kang, M. J., et al. (2011). Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Annu. Rev. Physiol.* 73, 479–501. doi: 10.1146/annurev-physiol-012110-142250

Lee, C. G., Da Silva, C. A., Lee, J. Y., Hartl, D., and Elias, J. A. (2008). Chitin regulation of immune responses: an old molecule with new roles. *Curr. Opin. Immunol.* 20, 684–689. doi: 10.1016/j.coi.2008.10.002

Lee, C. G., Hartl, D., Lee, G. R., Koller, B., Matsuura, H., Da Silva, C. A., et al. (2009). Role of breast regression protein 39 (BRP-39)/chitinase 3-like-1 in Th2 and IL-13-induced tissue responses and apoptosis. *J. Exp. Med.* 206, 1149–1166. doi: 10.1084/jem.20081271

Leek, R. D., Hunt, N. C., Landers, R. J., Lewis, C. E., Royds, J. A., and Harris, A. L. (2000). Macrophage infiltration is associated with VEGF and EGFR expression in breast cancer. *J. Pathol.* 190, 430–436. doi: 10.1002/(SICI)1096-9896(200003)190:4<430::AID-PATH538>3.0.CO;2-6

Leek, R. D., Lewis, C. E., Whitehouse, R., Greenall, M., Clarke, J., and Harris, A. L. (1996). Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res.* 56, 4625–4629.

Lewis, C. E., and Pollard, J. W. (2006). Distinct role of macrophages in different tumor microenvironments. *Cancer Res.* 66, 605–612. doi: 10.1158/0008-5472.CAN-05-4005

Libreros, S., Garcia-Areas, R., Shibata, Y., Carrio, R., Torroella-Kouri, M., and Iragavarapu-Charyulu, V. (2012). Induction of proinflammatory mediators by CHI3L1 is reduced by chitin treatment: decreased tumor metastasis in a breast cancer model. *Int. J. Cancer* 131, 377–386. doi: 10.1002/ijc.26379

Malinda, K. M., Ponce, L., Kleinman, H. K., Shackelton, L. M., and Millis, A. J. (1999). Gp38k, a protein synthesized by vascular smooth muscle cells, stimulates directional migration of human umbilical vein endothelial cells. *Exp. Cell Res.* 250, 168–173. doi: 10.1006/excr.1999.4511

McQuade, K. J., Beauvais, D. M., Burbach, B. J., and Rapraeger, A. C. (2006). Syndecan-1 regulates alphavbeta5 integrin activity in B82L fibroblasts. *J. Cell Sci.* 119, 2445–2456. doi: 10.1242/jcs.02970

Nielsen, K. R., Steffensen, R., Boegsted, M., Baech, J., Lundbye-Christensen, S., Hetland, M. L., et al. (2011). Promoter polymorphisms in the chitinase 3-like 1 gene influence the serum concentration of YKL-40 in Danish patients with rheumatoid arthritis and in healthy subjects. *Arth. Res. Therapy* 13, R109. doi: 10.1186/ar3391

Nishikawa, K. C., and Millis, A. J. (2003). gp38k (CHI3L1) is a novel adhesion and migration factor for vascular cells. *Exp. Cell Res.* 287, 79–87.

Nyirkos, P., and Golds, E. E. (1990). Human synovial cells secrete a 39 kDa protein similar to a bovine mammary protein expressed during the non-lactating period. *Biochem. J.* 269, 265–268.

Paez-Ribes, M., Allen, E., Hudock, J., Takeda, T., Okuyama, H., Vinals, F., et al. (2009). Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis.[see comment]. *Cancer Cell* 15, 220–231. doi: 10.1016/j.ccr.2009.01.027

Park, J. A., Drazen, J. M., and Tschumperlin, D. J. (2010). The chitinase-like protein YKL-40 is secreted by airway epithelial cells at base line and in response to compressive mechanical stress. *J. Biol. Chem.* 285, 29817–29825. doi: 10.1074/jbc.M110.103416

Park, P. W., Reizes, O., and Bernfield, M. (2000). Cell surface heparan sulfate proteoglycans: selective regulators of ligand-receptor encounters. *J. Biol. Chem.* 275, 29923–29926. doi: 10.1074/jbc.R000008200

Pelloski, C. E., Mahajan, A., Maor, M., Chang, E. L., Woo, S., Gilbert, M., et al. (2005). YKL-40 expression is associated with poorer response to radiation and shorter overall survival in glioblastoma. *Clin. Cancer Res.* 11, 3326–3334. doi: 10.1158/1078-0432.CCR-04-1765 pp.3326-34

Perrin, R. J., Craig-Schapiro, R., Malone, J. P., Shah, A. R., Gilmore, P., Davis, A. E., et al. (2011). Identification and validation of novel cerebrospinal fluid biomarkers for staging early Alzheimer's disease. *PLoS ONE* 6:e16032. doi: 10.1371/journal.pone.0016032

Persson, F., Rathcke, C. N., Gall, M. A., Parving, H. H., Vestergaard, H., and Rossing, P. (2012). High YKL-40 levels predict mortality in patients with type 2 diabetes. *Diabetes Res. Clin. Pract.* 96, 84–89. doi: 10.1016/j.diabres.2011.12.008

Pini, M., Rhodes, D. H., Castellanos, K. J., Hall, A. R., Cabay, R. J., Chennuri, R., et al. (2012). Role of IL-6 in the resolution of pancreatitis in obese mice. *J. Leukoc. Biol.* 91, 957–966. doi: 10.1189/jlb.1211627

Pizano-Martinez, O., Yanez-Sanchez, I., Alatorre-Carranza, P., Miranda-Diaz, A., Ortiz-Lazareno, P. C., Garcia-Iglesias, T., et al. (2011). YKL-40 expression in CD14 liver cells in acute and chronic injury. *World J. Gastroenterol.* 17, 3830–3835. doi: 10.3748/wjg.v17.i33.3830

Pollard, J. W. (2004). Tumour-educated macrophages promote tumour progression and metastasis. *Nat. Rev. Cancer* 4, 71–78. doi: 10.1038/nrc1256

Prince, R. N., Schreiter, E. R., Zou, P., Wiley, H. S., Ting, A. Y., Lee, R. T., et al. (2010). The heparin-binding domain of HB-EGF mediates localization to sites of cell-cell contact and prevents HB-EGF proteolytic release. *J. Cell Sci.* 123, 2308–2318. doi: 10.1242/jcs.058321

Qin, W., Zhu, W., Schlatter, L., Miick, R., Loy, T. S., Atasoy, U., et al. (2007). Increased expression of the inflammatory protein YKL-40 in precancers of the breast. *Int. J. Cancer* 121, 1536–1542. doi: 10.1002/ijc.22881

Rathcke, C. N., and Vestergaard, H. (2006). YKL-40, a new inflammatory marker with relation to insulin resistance and with a role in endothelial dysfunction and atherosclerosis. *Inflamm. Res.* 55, 221–227. doi: 10.1007/s00011-006-0076-y

Recklies, A. D., White, C., and Ling, H. (2002). The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and activates both extracellular signal-regulated kinase- and protein kinase B-mediated signalling pathways. *Biochem. J.* 365, 119–126. doi: 10.1042/BJ20020075

Rehli, M., Krause, S. W., and Andreesen, R. (1997). Molecular characterization of the gene for human cartilage gp-39 (CHI3L1), a member of the chitinase protein family and marker for late stages of macrophage differentiation. *Genomics* 43, 221–225. doi: 10.1006/geno.1997.4778

Rehli, M., Niller, H. H., Ammon, C., Langmann, S., Schwarzfischer, L., Andreesen, R., et al. (2003).

Transcriptional regulation of CHI3L1, a marker gene for late stages of macrophage differentiation. *J. Biol. Chem.* 278, 44058–44067. doi: 10.1074/jbc.M306792200

Renkema, G. H., Boot, R. G., Au, F. L., Donker-Koopman, W. E., Strijland, A., Muijsers, A. O., et al. (1998). Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl hydrolases secreted by human macrophages. *Eur. J. Biochem.* 251, 504–509.

Roslind, A., Johansen, J. S., Junker, N., Nielsen, D. L., Dzaferi, H., Price, P. A., et al. (2007a). YKL-40 expression in benign and malignant lesions of the breast: a methodologic study. *Appl. Immunohistochem. Mole. Morphol.* 15, 371–381. doi: 10.1097/01.pai.0000213146.77772.6a

Roslind, A., Knoop, A. S., Jensen, M. B., Johansen, J. S., Nielsen, D. L., Price, P. A., et al. (2007b). YKL-40 protein expression is not a prognostic marker in patients with primary breast cancer. *Breast Cancer Res. Treat.* 112, 275–285.

Saidi, A., Javerzat, S., Bellahcene, A., De Vos, J., Bello, L., Castronovo, V., et al. (2008). Experimental anti-angiogenesis causes upregulation of genes associated with poor survival in glioblastoma. *Int. J. Cancer* 122, 2187–2198. doi: 10.1002/ijc.23313

Scully, S., Yan, W., Bentley, B., Cao, Q. J., and Shao, R. (2011). Inhibitory activity of YKL-40 in mammary epithelial cell differentiation and polarization induced by lactogenic hormones: a role in mammary tissue involution. *PLoS ONE* 6:e25819. doi: 10.1371/journal.pone.0025819

Shackelton, L. M., Mann, D. M., and Millis, A. J. (1995). Identification of a 38-kDa heparin-binding glycoprotein (gp38k) in differentiating vascular smooth muscle cells as a member of a group of proteins associated with tissue remodeling. *J. Biol. Chem.* 270, 13076–13083. doi: 10.1074/jbc.270.22.13076

Shao, R., Cao, Q. J., Arenas, R. B., Bigelow, C., Bentley, B., and Yan, W. (2011). Breast cancer expression of YKL-40 correlates with tumour grade, poor differentiation, and other cancer markers. *Br. J. Cancer* 105, 1203–1209. doi: 10.1038/bjc.2011.347

Shao, R., Hamel, K., Petersen, L., Cao, Q. J., Arenas, R. B., Bigelow, C., et al. (2009). YKL-40, a secreted glycoprotein, promotes tumor angiogenesis. *Oncogene* 28, 4456–4468. doi: 10.1038/onc.2009.292

Shibata, Y. Metzger, W. J., and Myrvik, Q. N. (1997). Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan: mannose receptor-mediated phagocytosis initiates IL-12 production. *J. Immunol.* 159, 2462–2467.

Stearman, R. S., Dwyer-Nield, L., Grady, M. C., Malkinson, A. M., and Geraci, M. W. (2008). A macrophage gene expression signature defines a field effect in the lung tumor microenvironment. *Cancer Res.* 68, 34–43. doi: 10.1158/0008-5472.CAN-07-0988

Suzuki, T., Hashimoto, S., Toyoda, N., Nagai, S., Yamazaki, N., Dong, H. Y., et al. (2000). Comprehensive gene expression profile of LPS-stimulated human monocytes by SAGE. *Blood* 96, 2584–2591.

Tsutsui, S., Yasuda, K., Suzuki, K., Tahara, K., Higashi, H., and Era, S. (2005). Macrophage infiltration and its prognostic implications in breast cancer: the relationship with VEGF expression and microvessel density. *Oncol. Rep.* 14, 425–431.

Verhoeff, J. J., van Tellingen, O., Claes, A., Stalpers, L. J., van Linde, M. E., Richel, D. J., et al. (2009). Concerns about anti-angiogenic treatment in patients with glioblastoma multiforme. *BMC Cancer* 9:444. doi: 10.1186/1471-2407-9-444

Vind, I., Johansen, J. S., Price, P. A., and Munkholm, P. (2003). Serum YKL-40, a potential new marker of disease activity in patients with inflammatory bowel disease. *Scand. J. Gastroenterol.* 38, 599–605.

Volck, B., Johansen, J. S., Stoltenberg, M., Garbarsch, C., Price, P. A., Ostergaard, M., et al. (2001). Studies on YKL-40 in knee joints of patients with rheumatoid arthritis and osteoarthritis. Involvement of YKL-40 in the joint pathology. *Osteoarth. Cart.* 9, 203–214. doi: 10.1053/joca.2000.0377

Wang, H., Lathia, J. D., Wu, Q., Wang, J., Li, Z., Heddleston, J. M., et al. (2009). Targeting interleukin 6 signaling suppresses glioma stem cell survival and tumor growth. *Stem Cells* 27, 2393–2404. doi: 10.1002/stem.188

Wen, P. Y., and Kesari, S. (2008). Malignant gliomas in adults. *New Eng. J. Med.* 359, 492–507. doi: 10.1056/NEJMra0708126

Wick, W., Weller, M., van den Bent, M., and Stupp, R. (2010). Bevacizumab and recurrent malignant gliomas: a European perspective. *J. Clin. Oncol.* 28, e188–189; author reply e190–182. doi: 10.1200/JCO.2009.26.9027

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 18 March 2013; paper pending published: 01 April 2013; accepted: 10 May 2013; published online: 28 May 2013.

*Citation: Shao R (2013) YKL-40 acts as an angiogenic factor to promote tumor angiogenesis. Front. Physiol. **4**:122. doi: 10.3389/fphys.2013.00122*

This article was submitted to Frontiers in Vascular Physiology, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 25 December 2013
doi: 10.3389/fphys.2013.00392

Exploring the role of CHI3L1 in "pre-metastatic" lungs of mammary tumor-bearing mice

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Elevated levels of chitinase-3-like-1 (CHI3L1) are associated with poor prognosis, shorter recurrence-free intervals and low survival in breast cancer patients. Breast cancer often metastasizes to the lung. We hypothesized that molecules expressed in the "pre-metastatic" lung microenvironment could support the newly immigrant tumor cells by providing growth and angiogenic factors. Macrophages are known to play an important role in tumor growth by releasing pro-angiogenic molecules. Using mouse mammary tumor models, we have previously shown that during neoplastic progression both the mammary tumor cells and splenic macrophages from tumor-bearing mice express higher levels of CHI3L1 compared to normal control mice. However, the role of CHI3L1 in inducing angiogenesis by macrophages at the pulmonary microenvironment to support newly arriving breast cancer cells is not yet known. In this study, we determined the expression of CHI3L1 in bronchoalveolar lavage macrophages and interstitial macrophages in regulating angiogenesis that could support the growth of newly immigrant mammary tumor cells into the lung. Here we show that *in vitro* treatment of pulmonary macrophages with recombinant murine CHI3L1 resulted in enhanced expression of pro-angiogenic molecules including CCL2, CXCL2, and MMP-9. We and others have previously shown that inhibition of CHI3L1 decreases the production of angiogenic molecules. In this study, we explored if *in vivo* administration of chitin microparticles has an effect on the expression of CHI3L1 and pro-angiogenic molecules in the lungs of mammary tumor-bearing mice. We show that treatment with chitin microparticles decreases the expression of CHI3L1 and pro-angiogenic molecules in the "metastatic" lung. These studies suggest that targeting CHI3L1 may serve as a potential therapeutic agent to inhibit angiogenesis and thus possibly tumor growth and metastasis.

Keywords: CHI3L1, tumor growth, angiogenesis, macrophages, chitin microparticles, pulmonary environment

INTRODUCTION

Metastasis is the major cause of death in breast cancer patients. It is well established that breast cancer often metastasizes to the lung. Preferential colonization of specific tissues by breast cancer cells could be partially determined by the nature of microenvironment within the target organ (Steeg, 2006). Recent studies have implicated specific cellular elements in the lungs that contribute to tumor growth. These include airway epithelial cells and immune cells, such as interstitial and alveolar macrophages, among others. Studies have demonstrated functional, morphological, and phenotypic differences between these interstitial and alveolar macrophages (Sebring and Lehnert, 1992; Prokhorova et al., 1994; Johansson et al., 1997). However, there are limited studies on interstitial macrophages in human lungs compared to alveolar macrophages, which can be easily obtained by bronchoalveolar lavage (BAL). The role of either interstitial or alveolar macrophages in the pre-metastatic lung in breast cancer metastasis has not yet been elucidated. We hypothesize that interstitial macrophages, alveolar macrophages, or both, may alter the pre-metastatic landscape of the lung.

Pulmonary macrophages may exert anti-tumor effects to suppress the growth of newly-immigrated breast cancer cells, or alternatively exert pro-tumor effects by producing growth factors that support their establishment in the lung. By release of proteases, growth factors and cytokines, activated macrophages have the potential to influence each phase of the angiogenic process. This includes stimulating remodeling of the local extracellular matrix, inducing endothelial cells to migrate or proliferate, and inhibiting formation of differentiated capillaries.

We and others have recently shown that a glycoprotein known as chitinase-3-like-1 protein is produced by macrophages from tumor-bearing hosts (Kawada et al., 2012; Libreros et al., 2012). Chitinase-3-like-1 glycoprotein (aka BRP-39, YKL-40) is a secreted protein that is upregulated in various types of cancers, including breast (Johansen et al., 2003). This molecule is synthesized under inflammatory conditions, including bronchial

asthma, inflammatory bowel disease, and cancer, but is not highly expressed under physiological conditions (Johansen et al., 2006; Mizoguchi, 2006; Chupp et al., 2007; Coffman, 2008; Lee et al., 2011; Libreros et al., 2013). CHI3L1 is a chitin-binding glycoprotein that belongs to the family of chitinase-like proteins, but is lacking in enzymatic activity (Henrissat and Bairoch, 1993). This glycoprotein is expressed and secreted by a variety of cell types including articular chondrocytes, synoviocytes, osteoblasts, macrophages, neutrophils, and epithelial cells (Johansen et al., 2001; Rehli et al., 2003; Mizoguchi, 2006; Rathcke and Vestergaard, 2006; Lee et al., 2009). In examining the function of CHI3L1, we and others have found that CHI3L1 stimulates the production of pro-angiogenic molecules (Shao et al., 2009; Kawada et al., 2012; Libreros et al., 2012). Conversely, (Shao et al., 2009) and (Libreros et al., 2012), have shown that inhibiting CHI3L1 with neutralizing antibodies or administration of chitin microparticles, decreases the expression of pro-angiogenic molecules (Shao et al., 2009; Libreros et al., 2012).

Currently there is little known regarding mechanistic links between CHI3L1 expression by macrophages in the "pre-metatastic" lung, and tumor-related angiogenesis. We therefore examined the possibility that macrophage-derived CHI3L1 in the lung, upregulated by exposure to circulating CHI3L1 produced by tumor cells, "conditions" this organ to favor establishment of newly-arrived, metastasizing cancer cells. Thus, in this study we tested the role of CHI3L1 expression by bronchoalveolar and interstitial macrophages in regulating angiogenesis to promote the growth of new mammary tumor cells in the lung. We demonstrate here that: (1) CHI3L1 is secreted by interstitial and alveolar macrophages from mammary tumor bearers; (2) CHI3L1 induces expression of pro-angiogenic molecules in interstitial and alveolar macrophages; (3) *in vivo* treatment with chitin microparticles reduces angiogenesis; and (4) this treatment also decreases expression of CHI3L1, CCL2, CXCL2 and MMP-9. Our findings suggest that CHI3L1 may play a role in preparing the "soil" in the "pre-metastatic" lung. Therefore, CHI3L1 may be an attractive therapeutic target to inhibit breast cancer metastasis.

MATERIALS AND METHODS

MICE AND CELL LINES

Female BALB/c mice were used in all studies (Charles River Laboratories, 8–12 week-olds), and were housed and used according to the National Institutes of Health guidelines, under protocols approved by Florida Atlantic University Institutional Animal Care and Use Committee. Mammary tumor cells (4T1-luc-A4; Caliper Life Sciences, Hopkinton, MA) were maintained in RPMI with 10% FCS (Kim et al., 2010). Tumor cells were implanted in mice by subcutaneous injection of 1×10^5 4T1 tumor cells in the lower right ventral quadrant. These tumors metastasize to the lung ∼4–5 weeks post-tumor cell implantation. Normal mice and tumor bearers were assessed at ∼2 weeks- ("pre-metastatic") and 5 weeks post-tumor implantation ("metastatic") for the expression of cytokines. Tumor bearers treated with chitin microparticles (see below) were assessed at 5 weeks post-tumor cell implantation for metastasis, cytokine expression and tumor angiogenesis using AngioSense probe.

ISOLATION OF EPITHELIAL CELLS AND MACROPHAGES FROM THE LUNGS

Bronchoalveolar macrophage isolation

Tracheas of euthanized mice were cannulated and lavaged with 1 mL of saline. The bronchoalveolar lavage fluid (BALF) was recovered to obtain alveolar macrophages as described by Kogiso et al. (2011). Plastic-adherent macrophages were then enriched by incubating BALF cells (10^6 cells/mL) in complete media (RPMI 1640 with 10% FBS, 10 units/mL penicillin and 10 μg/mL streptomycin) for 1 h in a 5% CO_2 incubator at 37°C. Non-adherent cells were removed by washing with media, and the BALF macrophages were then isolated by labeling with CD11b magnetic microbeads (Miltenyi Biotec, Cambridge, MA). $CD11b^+$ cells were purified by positive selection using AutoMACs (Miltenyi). Alveolar $CD11b^+$ macrophages isolated from all animals revealed >90% purity as determined by flow cytometric analysis (FACS Calibur).

Isolation of alveolar epithelial cells and interstitial macrophages

Immediately after collecting BALF specimens, pulmonary epithelial cells and interstitial macrophages were isolated as previously described, with modifications [35]. Media (1 mL, RPMI 1640 with 2% FBS and 1 mg/mL dispase (Invitrogen, Grand Island, NY)) was perfused into the airways and alveoli of the lungs. The lungs were then removed and incubated at 37°C for 1 h in the same media with 2 mg/mL collagenase type II (Invitrogen) added, followed by tissue mincing. Cells were filtered through Teflon mesh of 40 μM pores and washed with the media with no centrifugation at 0°C. Macrophages were isolated by plastic adherence, and were then purified by magnetic beads as described. The purity of the cells was >90% for CD11b expression as determined by flow cytometric analysis. Non-adherent cells (alveolar epithelial cells) were labeled with CD146 microbeads (MiltenyiBiotec) and purified by AutoMACs (MiltenyiBiotec). The purity was confirmed to be >90% as assessed by flow cytometric analysis.

Cell culture

Purified alveolar epithelial cells, and alveolar and interstitial macrophages, were cultured at 2×10^6 cells/mL for 18 h in complete media as described previously (Owen et al., 2005). All cells were stimulated with either 500 ng/mL LPS (Sigma Chemical Co., St. Louis, MO), or in combination with 1 ng/mL or 5 ng/mL of endotoxin-free rmCHI3L1 (R&D systems, Minneapolis, MN). Growth media (GM) was included as a control condition for some of the cultures. Cell-free supernatants were then collected and stored at −80°C.

FLOW CYTOMETRY

Total lung homogenates and BALF cells were isolated and resuspended in FACS buffer (PBS with 1% BSA and 0.1% sodium azide) and stained for 30 min at 4°C with FITC-conjugated antibodies against CD11b (BDBiosciences, San Jose, CA), APC-conjugated Ly6C, PerCP-conjugated Ly6G, PerCPF4/80 (all from BDBiosciences) or CD146 (Miltenyi Biotec). Isotype-matched IgG was used for internal controls. For intracellular cytokine staining, BD cytofix/cytoperm + fixation/permeabilization kit were used according to manufacturer's instructions and this was

followed by labeling for CHI3L1. CHI3L1 antibody (generously provided by Dr. Alison Humbles, MedImmune, MD) was conjugated to a fluorescent dye using Lightning-Link PE conjugation kit (Novus, Littleton, CO) following manufacturer's instructions. Samples were acquired in a FACSCalibur flow cytometer (BD Biosciences) and analyzed by Flow Jo software (Tree Star, Inc., Ashland, OR).

CYTOKINE ELISA

BALF samples and culture supernatants from cells obtained from control and mammary tumor bearers were analyzed for CHI3L1, CCL2, CXCL2 and MMP-9 levels by ELISA (all from R&D Systems) according to manufacturer's instructions. Absorbance at 450 nm with wavelength correction at 570 nm was measured with a Tecan SLT Rainbow Reader (Lab Instruments, Research Triangle Park, NC) and optical density (OD) values of samples were converted to picograms against a standard curve plotted from known quantities of recombinant murine cytokines.

WESTERN BLOT ANALYSIS

Cells from total lung homogenates were lysed with sample buffer (20 mM dithiothreitol, 6% SDS, 0.25 M Tris, pH 6.8, 10% glycerol, 10 mM NaF and bromophenyl blue) and used to extract total protein. Total protein (20 μg) was resolved on 4–20% Mini-Protean SDS-PAGE gradient gels (BioRad Life Sciences, Hercules, CA) and transferred to PVDF membrane (Pierce) using a semi-dry transfer transblotter (BioRad). The membranes were blocked overnight at 4°C in SeaBlock (Calbiochem), and subsequently incubated at room temperature for 1 h with anti-mouse CHI3L1 polyclonal antibody (1 μg/mL) (Quidel, San Diego, CA) and anti-mouse beta actin polyclonal antibody (0.25 μg/mL) (Li-Cor Biosciences, Lincoln, NE). Immunoblots were washed with 0.5% Tween-PBS followed by 1 h incubation at room temperature with appropriate infrared dye-conjugated secondary antibodies (Li-Cor Biosciences). Blots were washed again with 0.5% Tween-PBS and then dried at 37°C for 20 min. The membranes then were imaged and bands quantified using the Li-Cor Odyssey imaging system. Levels of proteins of interest were normalized to beta-actin.

CONFOCAL MICROSCOPY

Lungs from control mice and 2-week mammary tumor bearers were perfused with a mixture of Optimal Tissue Cutting (OCT) embedding compound and PBS (70%/30% respectively). The lungs were then removed and snap frozen on dry ice. 5 μM cryostat sections were mounted on SuperFrost Plus slides (Fisher Scientific, Fair Lawn, NJ), fixed in 4% paraformaldehyde, and labeled with goat anti-mouse CC10 (1:100, Santa Cruz Biotech, Santa Cruz, CA) and rabbit anti-mouse CHI3L1 (1 μg/μL, Quidel) as described below. Alveolar and interstitial macrophages were plated (0.5 x 10^6 cells) on coverslips, fixed in 4% paraformaldehyde, and labeled with goat anti-mouse CD68 (1:100, macrophage marker) (Santa Cruz Biotech) and rabbit anti-mouse CHI3L1 (Quidel). Both cryostat sections and cells were blocked in 10% normal horse serum in PBS prior to staining with primary antibodies overnight at 4°C. After washing in PBS, cells and sections were incubated in the following secondary antibodies: FITC-donkey anti-rabbit and PE-donkey anti-goat (1:2000, both from Invitrogen, Life Technologies). To visualize nuclei, material was mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA), and examined by confocal microscopy (Carl Zeiss LSM 700, Microimaging, Thornwood, NY).

In vivo TREATMENT WITH CHITIN MICROPARTICLES

Chitin microparticles (1–10 μM, kindly provided by Dr. Yoshimi Shibata, Florida Atlantic University, FL) were prepared as described previously (Shibata et al., 1997; Strong et al., 2002; Nishiyama et al., 2006). Tumor-bearing mice were treated by intraperitoneal injection with chitin microparticles (1 mg/mouse) starting 3 days post-tumor implantation and continuing every third day for 5 weeks (Libreros et al., 2012).

ANGIOGENESIS DETERMINATION

To assess vascularization *in vivo*, the near-infrared blood pool agent AngioSense 680 probe (2 nmol/mouse in 150 μL volume; Perkin Elmer, Waltham, MA) was injected via tail vein 24 h before imaging. Mice were imaged using a bioluminescence optical imager (IVIS Lumina LTE, Perkin Elmer). Maximal near infrared signals were quantified using Living Image 2.5 image analysis software (Xenogen, Perkin Elmer). Infrared signals are reported as photons/sec.

STATISTICAL ANALYSES

Results are expressed as group means ± *SD*. Statistical analyses were performed using GraphPad Prism 3 software (LaJolla, CA). Statistical comparisons of paired groups were determined by Student's *t* tests. Values of $p < 0.05$ were considered statistically significant.

RESULTS

CHI3L1 EXPRESSION IS INCREASED IN BALF AND TOTAL LUNG FROM MAMMARY TUMOR-BEARING MICE

Increased levels of CHI3L1 in the sera of breast cancer patients are associated with poor prognosis (Johansen et al., 2003). We have previously reported higher circulating levels of CHI3L1 in 4T1 mammary tumor-bearing mice compared with normal mice (Libreros et al., 2012). This tumor model shares similar characteristics with human breast cancer patients as mice bearing 4T1 mammary tumors exhibit spontaneous tumor cell metastasis to the lung. The levels of CHI3L1 in the lungs are increased during pulmonary inflammation, and inflammation is known to contribute to tumor growth and metastasis. Since it is known that breast tumor cells metastasize to the lung, we determined if CHI3L1 expression is specifically altered in lungs of mammary tumor bearers compared to control mice.

The "pre-metastatic" and "metastatic" stages were described by Yan et al. using the 4T1 mammary tumor model, with the pre-metastatic stage occurring at 14 days post-tumor cell inoculation, and the metastatic stage at 4 weeks (Yan et al., 2010). We therefore assessed CHI3L1 expression in the lungs of mice inoculated with 4T1 mammary tumor cells at 2 weeks post-cell implantation, a time point at which no visible micrometastasis is observed in the lungs (data not shown), and at

5 weeks when metastasis of 4T1 cells is known to be well-established (Yan et al., 2010; Libreros et al., 2012). We first measured circulating levels of CHI3L1, which increased from 25×10^3 ng/mL at 2 weeks, to 125×10^3 ng/mL at 5 weeks (**Figure 1A**). ELISA measurements demonstrated that significantly higher levels of CHI3L1 were also present in both BALF samples (**Figure 1B**) and total lung homogenates (**Figure 1C**) at 5 weeks post-tumor cell implantation, compared to the 2-week time point. These higher levels of CHI3L1 could be due to expression by the pulmonary tissue itself and/or the tumor cells that have infiltrated by 5 weeks (Libreros et al., 2013). Samples from the "pre-metastatic" stage would help differentiate between these possibilities, as tumor cells have not yet infiltrated, and we performed additional analyses at this stage. At 2 weeks post-inoculation, significantly higher levels of CHI3L1 were measured by ELISA in BALF samples from tumor bearers compared to control mice (**Figure 2A**). Western blot analysis of whole lungs from pre-metastatic tumor-bearers confirmed higher levels of pulmonary CHI3L1 (**Figure 2B**), and ELISA assays of total lung homogenates quantified this increase at 2 weeks (**Figure 2C**). CHI3L1 is secreted by a variety of cell types, including macrophages, neutrophils, colonic epithelial cells, and chondrocytes (Nyirkos and Golds, 1990; Hakala et al., 1993; Renkema et al., 1998; Volck et al., 1998; Mizoguchi, 2006), and recent studies by Lee et al. (2009) have shown that CHI3L1 (aka BRP-39) is upregulated in inflamed airway epithelium, and that it plays an active role in pulmonary inflammation (Lee et al., 2009). We therefore determined if CHI3L1 expression is specifically altered in lung epithelial cells isolated from mammary tumor bearers at 2 weeks post-inoculation, compared to those from control mice. Production of CHI3L1 was increased more than 5-fold in pulmonary epithelial cells from tumor bearers, as measured by ELISA at 18 h post-plating (**Figure 2D**). To promote "inflammatory" conditions, cultures were treated with LPS to stimulate cytokine production, which exacerbated the increase in CHI3L1 levels displayed by cells from tumor bearers (**Figure 2D**). Localization of CHI3L1 in lung tissue samples by immunofluorescence showed that CHI3L1 was expressed by lung epithelial cells ($CC10^+$ cells), and that this expression was increased in the airways of mammary tumor bearing mice compared to controls (**Figure 2E**).

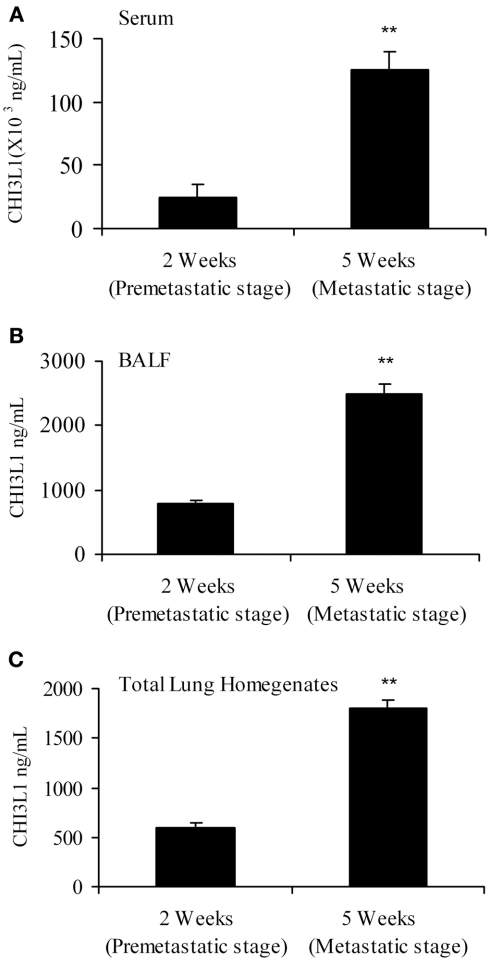

FIGURE 1 | CHI3L1 is expressed in serum and pulmonary tissue of mammary tumor-bearing mice at 2 weeks ("pre-metastatic") and 5 weeks (metastatic) post-tumor cell implantation. (A) Serum **(B)** BALF and **(C)** Total lung homogenates from 2 week and 5 week mammary tumor bearers were analyzed for CHI3L1 expression by ELISA. $N = 10$ mice/group; $^{**}p \leq 0.001$.

CHI3L1 EXPRESSION IS INCREASED IN $CD11B^+GR1^+$ CELLS OF MAMMARY TUMOR BEARERS

Myeloid-derived cells have been shown to be important in promoting tumor growth, metastasis, and angiogenesis (van Kempen and Coussens, 2002; Yang et al., 2004). The lungs of 4T1 mammary tumor bearers show infiltration by myeloid-derived suppressor cells, and in particular by $CD11b^+Gr1^+$ cells that establish a pre-metastatic niche by secreting proinflammatory mediators (Yan et al., 2010; Younos et al., 2011). We have previously shown that splenic myeloid cells from mammary tumor-bearing mice express CHI3L1 (Libreros et al., 2012). To clearly delineate myeloid populations of cells in pulmonary tissue that could contribute to CHI3L1 expression, single cell suspensions prepared from total lungs of normal and 2-week mammary tumor-bearers were analyzed by flow cytometry. As shown in **Figures 3A–C** (one representative assay out of five), $CD11b^+Ly6C^+$ cells from mammary tumor bearers express CHI3L1 at higher levels compared to normals. $CD11b^+Ly6G^+$ cells from tumor bearers express CHI3L1 but these levels are lower compared to the levels observed in $CD11b^+Ly6C^+$ cells (**Figures 3D–F**).

Since BALF was shown to contain elevated levels of CHI3L1, we determined which cell populations in the lavage contribute to the expression of CHI3L1 at 2 weeks post-tumor cell inoculation. Toward this we assessed $CD11b^+Ly6C^+$ and $CD11b^+Ly6G^+$ cells in the lavage. $CD11b^+Ly6C^+$ cells from mammary tumor bearers express higher levels of CHI3L1 compared to normals (**Figures 4A–C**). Similar to what was observed in total lung homogenates, $CD11b^+Ly6G^+$ cells from tumor bearers express CHI3L1 at lower levels compared to the $CD11b^+Ly6C^+$ cells (**Figures 4D–F**).

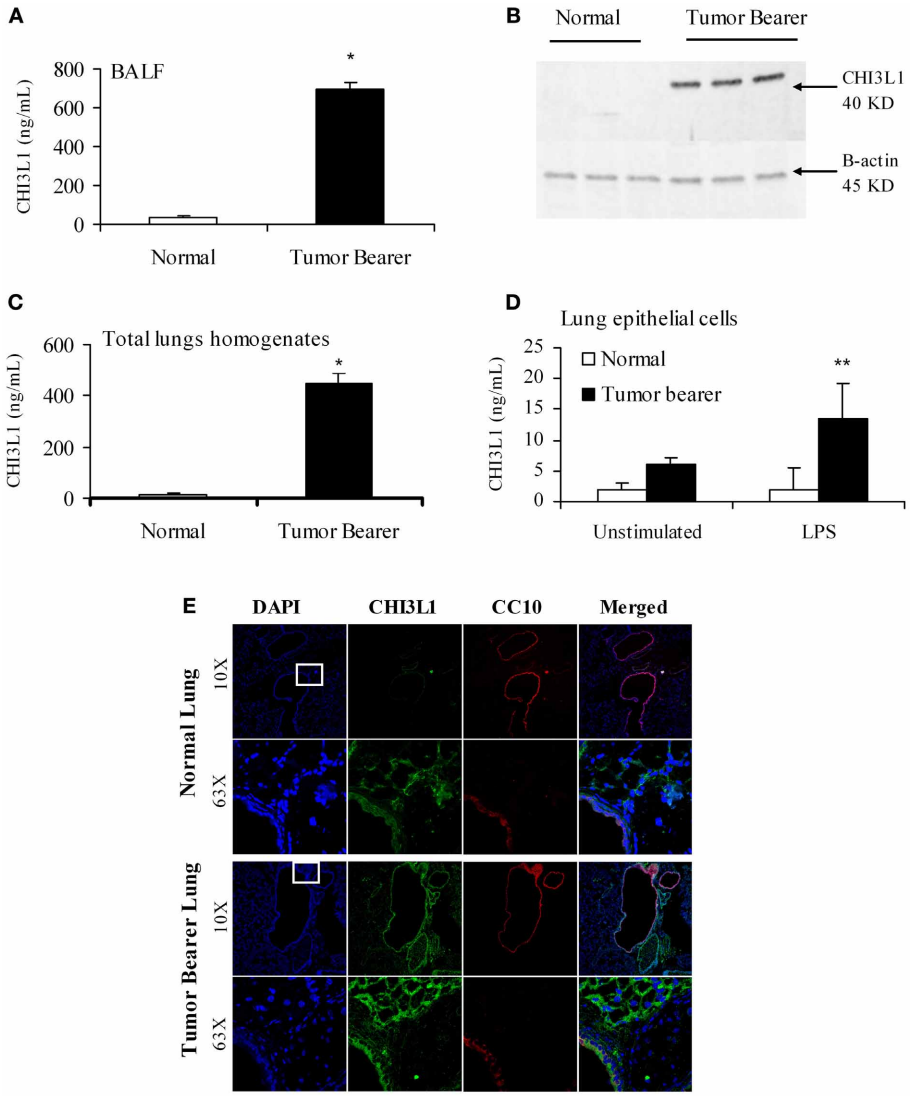

FIGURE 2 | CHI3L1 is expressed at higher levels in the pulmonary tissue of 2-week 4T1 mammary tumor bearers. (A) CHI3L1 expression in bronchoalveolar lavage fluid by ELISA; **(B)** Western blot analysis of total lung homogenates for CHI3L1 expression; (**C,D**) CHI3L1 expression by ELISA of total lung homogenates **(C)** and lung epithelial cells **(D)**. **(E)** Cellular co-localization of CHI3L1 with CC10, an airway-epithelial cell marker, in cryostat sections visualized by confocal microscopy. For all experiments, $N = 10$/group; $^{*}p \leq 0.05$; $^{**}p \leq 0.001$.

CHI3L1 EXPRESSION IS INCREASED IN MACROPHAGES FROM THE LUNGS OF MAMMARY TUMOR-BEARING MICE

We have previously shown that CHI3L1 is expressed at higher levels in splenic macrophages of mammary tumor-bearing mice (Libreros et al., 2012). In this study, we determined the expression levels of CHI3L1 in macrophages from the "pre-metastatic" lungs. Two broad subsets of macrophages are found in the lungs of mice and humans, i.e., alveolar macrophages which line the surface of alveoli, and interstitial macrophages that are localized in the space between alveolar epithelium and vascular endothelium (Schneberger et al., 2011). Thus, alveolar and interstitial macrophages from normal and 2-week tumor bearers were purified as described in the Methods section, and cultured in either the absence or presence of LPS. Interstitial macrophages from mammary tumor-bearing mice secrete CHI3L1 and these levels were further increased by stimulation with LPS as determined by ELISA (**Figure 5A**). Localization of CHI3L1 in interstitial macrophages was then confirmed by immunofluorescent labeling. Confocal images revealed higher intensity of CHI3L1 expression in $CD68^{+}$interstitial macrophages from tumor bearers, relative to normal mice (**Figure 5B**). Purified alveolar macrophages were also analyzed. Similar to what was observed in the interstitial macrophage population, there were higher than normal levels of CHI3L1 present in culture supernatants of alveolar macrophages from 2 week tumor-bearing mice (**Figure 5C**). Intensity of CHI3L1 staining in alveolar macrophages similarly was greater in tumor bearers' macrophages, as determined by confocal microscopy (**Figure 5D**). Interestingly, the expression

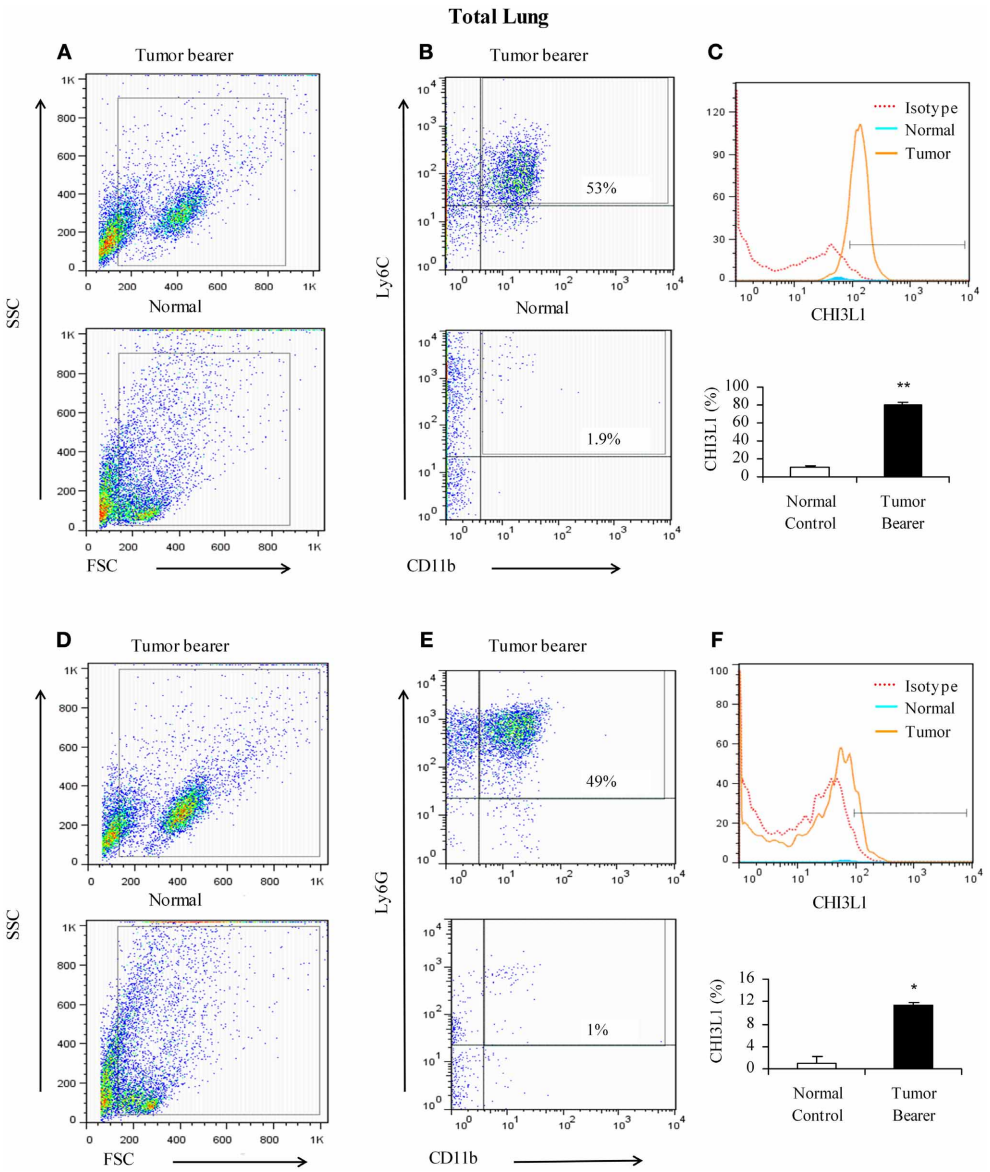

FIGURE 3 | CHI3L1 is expressed at higher levels in CD11b$^+$Ly6C$^+$ total lung cells in 2-week mammary tumor bearers. (A,D) Representative scatter plots of forward scatter vs. side scatter in total lung from normal and tumor bearers; **(B)** CD11b$^+$Ly6C$^+$ cells from total lungs of normal and tumor bearers and assessed for CHI3L1 expression; **(C)** Representative overlay histogram plot gated on CD11b$^+$ and Ly6C$^+$ cells for CHI3L1 expression; **(E)** CD11b$^+$Ly6G$^+$ cells from total lungs of normal and tumor bearers and assessed for CHI3L1 expression; **(F)** Representative overlay histogram plot gated on CD11b$^+$ and Ly6G$^+$ cells for CHI3L1 expression. For all experiments, $N = 10$/group; $^*p \leq 0.05$; $^{**}p \leq 0.001$.

levels of CHI3L1 were higher in interstitial macrophages compared to alveolar macrophages.

CHI3L1 EXACERBATES THE PRODUCTION OF PRO-ANGIOGENIC MOLECULES IN LPS-STIMULATED PULMONARY MACROPHAGES

Yan et al. (2010) found that the expression of MMP-9 and CCL-2 is upregulated in the lungs of 2 week tumor-bearing mice compared to normal mice (Yan et al., 2010). We and others have shown that CHI3L1 induces the expression of CCL2, CXCL2 and MMP-9 in splenic macrophages (Mizoguchi, 2006; Letuve et al., 2008; Kawada et al., 2012; Libreros et al., 2012), but there are few studies to date on the biological role of CHI3L1 in pulmonary macrophages. In this study we tested the effects of CHI3L1 on interstitial and alveolar macrophages isolated from normal mice, and analyzed the production of the pro-angiogenic molecules CCL2, CXCL2 and MMP-9 by ELISA. Cells were treated with CHI3L1 in combination with LPS, which is necessary for expression of angiogenic molecules by *ex vivo* macrophages. Treatment of either interstitial or alveolar macrophages with LPS alone or in combination with rmCHI3L1 (1 ng/mL or 5 ng/mL) resulted in a dose-dependent increase in the production of CCL2 (**Figures 6A,B**), CXCL2 (**Figures 6C,D**) and MMP-9

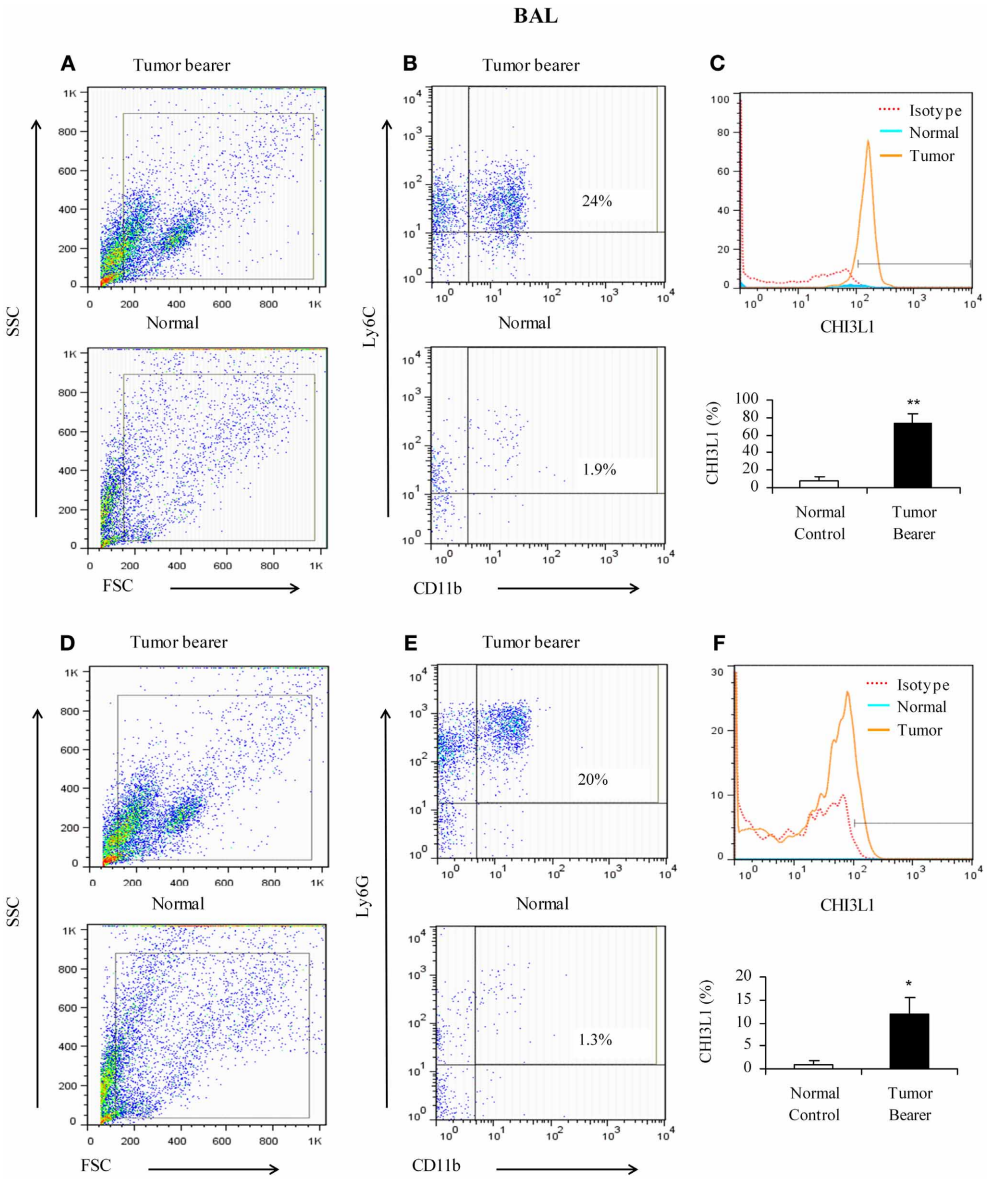

FIGURE 4 | CHI3L1 is expressed at higher levels in CD11b$^+$Ly6C$^+$ bronchoalveolar lavage cells in 2-week mammary tumor bearers. (A,D) Representative scatter plots of forward scatter vs. side scatter in alveolar lavage from normal and tumor bearers; **(B)** CD11b$^+$Ly6C$^+$ cells from alveolar lavage of normal and tumor bearers and assessed for CHI3L1 expression; **(C)** Representative overlay histogram plot gated on CD11b$^+$ Ly6C$^+$ cells for CHI3L1 expression; **(E)** CD11b$^+$Ly6G$^+$ cells from alveolar lavage of normal and tumor bearers and assessed for CHI3L1 expression; **(F)** Representative overlay histogram plot gated on CD11b$^+$Ly6G$^+$ cells for CHI3L1 expression. For all experiments, $N = 10$/group; $^*p \leq 0.05$; $^{**}p \leq 0.001$.

(**Figures 6E,F**) in both interstitial and alveolar macrophages. Culturing with rmCHI3L1 alone in the absence of LPS revealed a similar trend to the one observed in cultures containing both LPS and rmCHI3L1, but the levels of the proinflammatory mediators secreted were lower (data not shown). The combined effects of LPS and rmCHI3L1 produced the greatest increase in the expression of pro-angiogenic molecules.

In vivo TREATMENT WITH CHITIN MICROPARTICLES DECREASES ANGIOGENESIS

Intraperitoneal treatment of mammary tumor bearers with chitin microparticles, a substrate for CHI3L1, results in decreased tumor growth and pulmonary metastasis (Libreros et al., 2012). We and others established that 4T1 mammary tumors begin to infiltrate the lungs by 3 weeks post-tumor cell implantation, and observable metastatic foci are seen in the lungs at 5 weeks post-tumor cell implantation (Yan et al., 2010; Libreros et al., 2012). Therefore, we used 5 week tumor bearers to assess the effects of *in vivo* chitin microparticle treatment. To test if early treatment with chitin microparticles affects angiogenesis and tumor growth, an *in vivo* AngioSense probe was used. Mice treated with chitin microparticles after tumor cell inoculation had significantly reduced angiogenic fluorescent signals in *in vivo* imaged tumors compared to the tumors from untreated

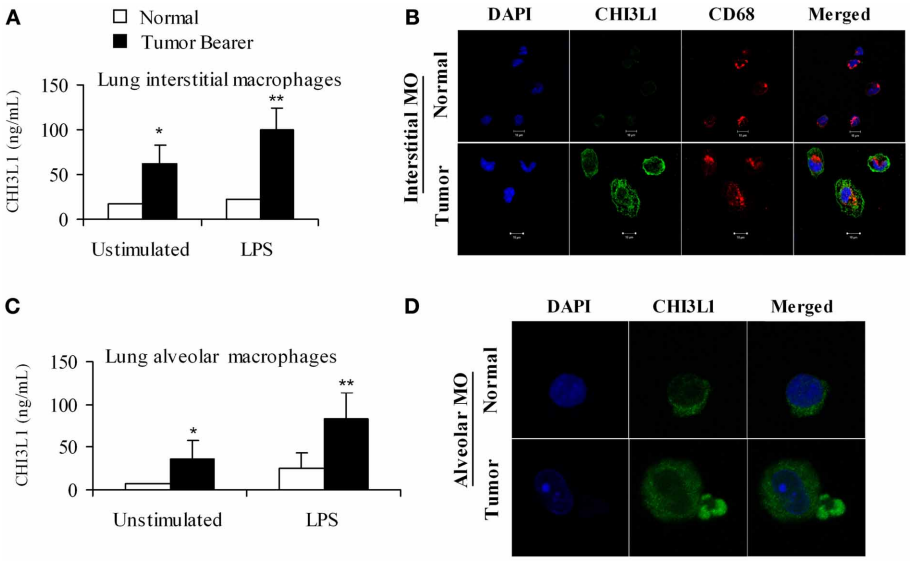

FIGURE 5 | CHI3L1 is expressed at higher levels in pulmonary macrophages from 2-week mammary tumor bearing mice. (A) CHI3L1 ELISA of interstitial macrophages from normal and mammary tumor bearers; **(B)** Cellular co-localization of CHI3L1 with CD68, a macrophage marker was visualized by confocal microscopy, scale bar 10 μM; **(C)** CHI3L1 ELISA of alveolar macrophages from normal and mammary tumor bearers; **(D)** Cellular localization of CHI3L1 was visualized by confocal microscopy, scale bar 10 μM. For all experiments, $N = 10$/group; $^{*}p \leq 0.05$; $^{**}p < 0.001$.

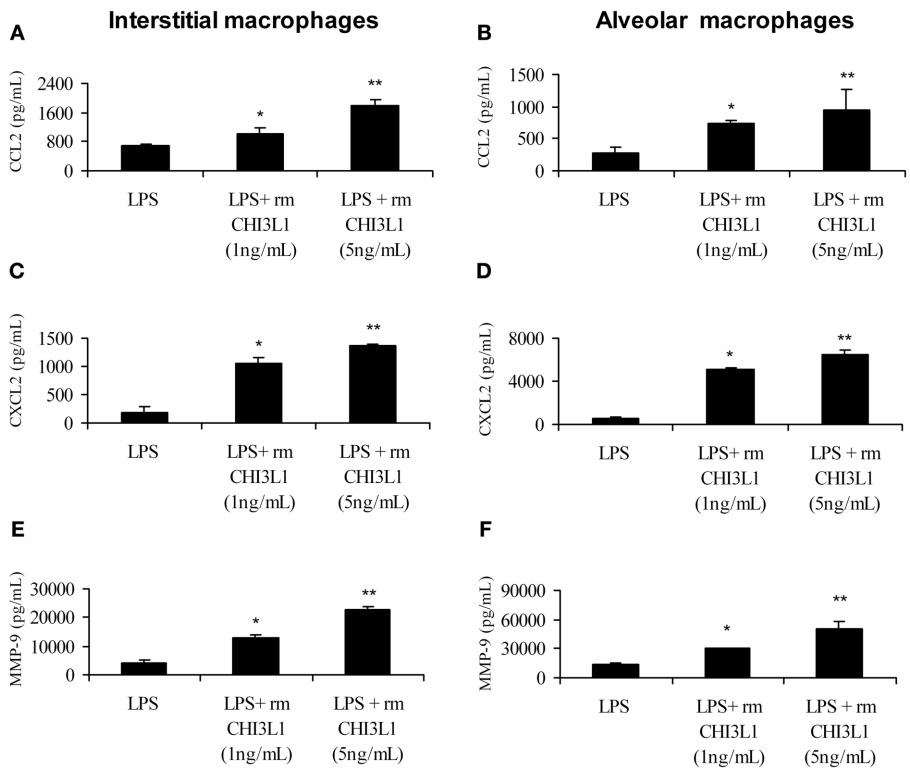

FIGURE 6 | CHI3L1 increases the expression of CCL2, CXCL2 and MMP-9. Pulmonary macrophages from normal mice were cultured overnight with LPS (500 ng/mL) alone and with either 1 ng/mL or 5 ng/mL of rmCHI3L1. Cell free supernatants were then analyzed for CCL2 in: **(A)** interstitial or **(B)** alveolar macrophages; CXCL2 in: **(C)** interstitial or **(D)** alveolar macrophages; and MMP-9 in: **(E)** interstitial or **(F)** alveolar macrophages. For all experiments, $N = 10$/group; $^{*}p \leq 0.05$; $^{**}p \leq 0.001$.

mice (**Figure 7A**). Quantification of fluorescent signals confirmed these results (**Figure 7B**). Previously we noted that tumor size in chitin-treated, 5 week tumor-bearing mice was significantly smaller than normal. We now show with the AngioSense probe that there was a significant decrease in angiogenic fluorescent signals in the excised tumors of chitin-treated mice, compared to untreated tumor bearers (**Figure 7C**). Quantification of fluorescent signals is depicted in **Figure 7D**.

Following metastasis, decreased tumor growth in the target organ also could be due to decreased angiogenesis. Since treatment with chitin microparticles decreased angiogenesis in the primary tumor, we tested to see if *in vivo* treatment with chitin microparticles also had an effect on angiogenesis in the metastatic lung. Excised lungs from chitin-treated and untreated mice at 5 weeks post-tumor cell inoculation were imaged as above to evaluate angiogenesis. Similar to what was observed in the tumor tissue, there was a significant reduction in fluorescent AngioSense signals, indicating decreased angiogenesis in the lungs of mice treated with chitin microparticles, compared to untreated controls (**Figure 7E**). Quantification of these fluorescent signals is shown in **Figure 7F**.

In vivo TREATMENT WITH CHITIN MICROPARTICLES DECREASES THE PRODUCTION OF PRO-ANGIOGENIC MOLECULES BY PULMONARY MACROPHAGES

Faibish et al. (2011) found that neutralizing antibody to YKL-40 blocks tumor angiogenesis by inhibiting endothelial cell tube formation (Faibish et al., 2011) while (Kawada et al., 2012) demonstrated increased microvessel density in CHI3L1-transfected colon cancer cells (Kawada et al., 2012). Since CHI3L1 expression by splenic macrophages is altered *in vivo* by chitin microparticle treatment (Libreros et al., 2012) we next determined if CHI3L1 expression in lung tissue is similarly affected. Bronchoalveolar lavage fluid was analyzed for the expression of pro-angiogenic

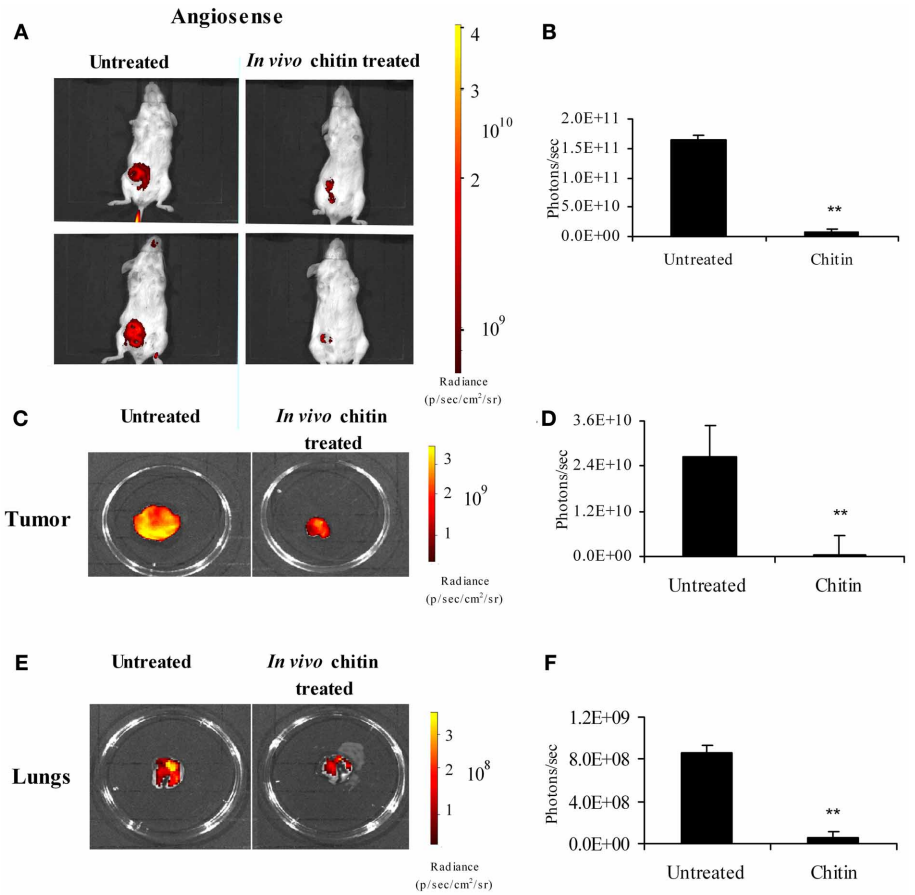

FIGURE 7 | *In vivo* treatment with chitin microparticles decreases angiogenesis. 4T1 mammary tumor bearers at 5 weeks post-tumor implantation were treated *in vivo* with chitin microparticles (1mg/mL) and analyzed for angiogenesis using AngioSense probe by *in vivo* imaging system. **(A)** Ventral image of untreated and chitin treated mammary tumor bearing mice showing 2 out 5 mice/treatment group; **(B)** Quantification (photons/s) of AngioSense specific fluorescent signal indicating decreased angiogenesis at 21 days post-tumor implantation in the chitin treated group; **(C)** Excised tumors from untreated and chitin treated mice indicating decreased fluorescent signal in the chitin treated group; **(D)** Quantification (photons/s) of AngioSense specific fluorescent signal indicating decreased angiogenesis at 21 days post-tumor implantation in the chitin treated group; **(E)** Excised lungs from untreated and chitin treated mice indicating decreased fluorescent signal in the chitin treated group; **(F)** Quantification (photons/s) of AngioSense specific fluorescent signal indicating decreased angiogenesis in the lungs at 21 days post-tumor implantation in the chitin treated group compared to untreated group. For all experiments, $N = 5$/group; $^{**}p \leq 0.001$.

molecules. *In vivo* treatment with chitin microparticles resulted in decreased CHI3L1 expression in BALF collected form 5 week tumor-bearers (**Figure 8A**). As CHI3L1 promotes expression of pro-angiogenic molecules, we reasoned that decreased CHI3L1 levels in chitin-treated mice should result in decreased expression of pro-angiogenic molecules. We found that the levels of CCL2 (**Figure 8B**), CXCL2 (**Figure 8C**) and MMP-9 (**Figure 8D**) in BALF samples were indeed decreased by *in vivo* chitin treatment. We also found that interstitial and alveloar macrophages from chitin-treated mammary tumor bearers exhibited reduced expression of CHI3L1, and these same pro-angiogenic molecules, at 5 weeks post-tumor cell inoculation (**Figure 9**).

DISCUSSION

Circulating tumor cells must invade and proliferate in a target organ to establish metastasis. It is well established that preferential tissue colonization is determined not only by features intrinsic to the type of tumor cell, but also in part by the unique nature of each target organ (Steeg, 2006). The microenvironment, the "soil" or the "pre-metastatic niche," in the target organ contributes to the survival of these cells. To our knowledge, there are no studies to date defining the role of CHI3L1 in pulmonary tissue in terms of promoting survival and growth of invading breast cancer cells. In this study, we examined the role of pulmonary macrophages in preparing the "soil" or the "pre-metastatic niche" for establishing breast cancer metastasis. We used an *in vivo* mouse mammary tumor model mimicking CHI3L1 expression in breast cancer patients to examine the role of CHI3L1 and CHI3L1-induced angiogenic molecules in the pulmonary microenvironment during the emergence of metastasis.

We show here that CHI3L1 levels are increased in both the "pre-metastatic" lung and "metastatic" lung of mammary tumor-bearing mice. Higher levels of CHI3L1 were observed not only in the serum, but also in BALF and lung tissue homogenates. We found that expression of CHI3L1 is upregulated in lung epithelial cells, as well as in alveolar and interstitial macrophages of mammary tumor-bearing mice. Importantly, CHI3L1 was found to induce the production of angiogenic molecules, CCL2, CXCL2 and MMP-9 in both alveolar and interstitial macrophages from normal mice. We also demonstrate that *in vivo* treatment with chitin microparticles, a substrate for CHI3L1, resulted in decreased production of CHI3L1, CCL2, CXCL2, and MMP-9 in BALF, and more specifically by interstitial and alveolar macrophages of mammary tumor-bearing mice. Decreased production of these molecules has been correlated with decreased levels of angiogenesis in tumors (Arenberg et al., 1998; Mehrad et al., 2007; Gerber et al., 2009). Transfection of HCT116 tumor cells with CHI3L1 enhances tumor growth, while *in vivo* treatment with anti-CHI3L1 neutralizing antibodies decreases angiogenesis (Shao et al., 2009; Kawada et al., 2012). Using administration of chitin microparticles, a molecule that binds to chitinases and chitin-like molecules (Ober and Chupp, 2009), we have shown previously that splenic macrophages from treated mice produce lower levels of pro-angiogenic molecules compared to untreated mammary tumor bearers, and that tumor growth and metastasis are reduced by this treatment (Libreros et al., 2012). To monitor angiogenesis during the "metastatic" stage,

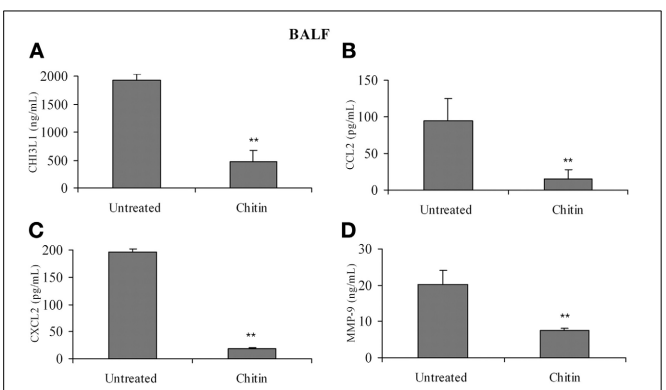

FIGURE 8 | *In vivo* treatment with chitin microparticles decreases CHI3L1, CCL2, CXCL2, and MMP-9 expression in BALF. BALF from untreated and chitin treated mice at 5 weeks post-tumor implantation was analyzed for: **(A)** CHI3L1; **(B)** CCL2; **(C)** CXCL2; and **(D)** MMP-9 expression by ELISA. For all experiments, $N = 10$/group; $^{**}p \leq 0.001$.

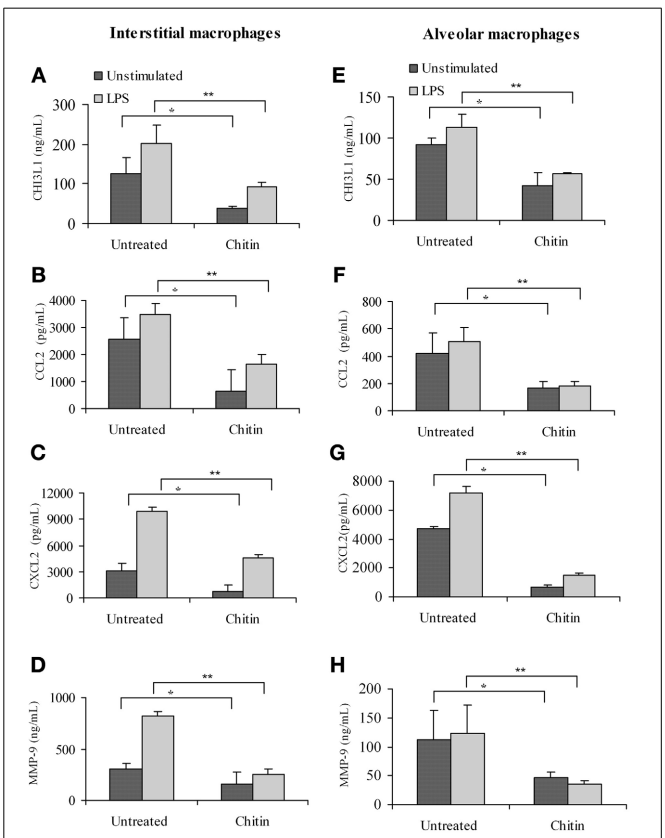

FIGURE 9 | *In vivo* treatment with chitin microparticles decreases CHI3L1, CCL2, CXCL2, and MMP-9 expression in pulmonary macrophages from 5 week mammary tumor bearers. Interstitial macrophages from untreated and chitin treated mice was analyzed for: **(A)** CHI3L1; **(B)** CCL2; **(C)** CXCL2; and **(D)** MMP-9 expression by ELISA, and alveolar macrophages from untreated and chitin treated mice was analyzed for: **(E)** CHI3L1; **(F)** CCL2; **(G)** CXCL2; and **(H)** MMP-9 expression by ELISA. For all experiments, $N = 10$/group; $^{*}p \leq 0.05$; $^{**}p \leq 0.001$.

in this study we used the AngioSense 680 probe, a marker for blood vessel density, to directly assess *in vivo* tumor angiogenesis. *In vivo* imaging clearly demonstrated the effectiveness of chitin microparticle treatment on angiogenesis. Chitin-treated mice had lower fluorescent signals compared to the untreated controls, and more importantly, excised tumors, as well as lungs from treated mice, had lower levels of AngioSense signals compared to the untreated group. It is well-established that tumors do not grow $>1\,mm^3$ in size without an adequate blood supply (Folkman, 1971), and our results indicate that decreased angiogenesis in mammary tumors correlates well with the smaller size of these tumors in chitin-treated mice. In this study, tumor size as determined by assessing for luciferase signals revealed $<0.5 \times 10^6$ photons in treated group vs. 3×10^6 in untreated group ($p < 0.005$). Reduced angiogenesis in the lungs also correlates well with the lower levels of pro-angiogenic molecules expressed by alveolar and interstitial macrophages in the chitin-treated group.

Macrophages have been described to be one of the key players in many types of cancers by producing a variety of factors that can either promote or inhibit tumor growth and metastasis (Mantovani et al., 2002). Numerous studies have reported on the role of tumor-infiltrating macrophages (TAMs) on tumor growth. There are very few reports on the role of macrophages at a metastatic site in terms of supporting the growth of infiltrating tumor cells. As the metastatic site offers new challenges for circulating tumor cells in terms of their survival, our focus has been to characterize macrophages in the lung microenvironment of mammary tumor bearers in terms of how they may support invading breast cancer cells. Although tumor cells and activated splenic macrophages express CHI3L1, a molecule associated with poor prognosis in breast cancer patients (Lal et al., 1999; Lau et al., 2006; Coffman, 2008; Libreros et al., 2012), few studies have analyzed CHI3L1 expression by alveolar and interstitial macrophages in tumor bearing models. In comparing the different cell types present in the lung, i.e., epithelial cells and macrophages, we found that both interstitial and alveolar macrophages from 2 week mammary tumor-bearing mice express much higher levels of CHI3L1 compared to epithelial cells. Prior work by Chupp et al., reported higher levels of CHI3L1 expression in biopsied lung tissue from patients with severe asthma, compared to those with a milder form, and that it localizes to the subepithelium of pulmonary tissue (Chupp et al., 2007). Using ova-sensitized and challenged mice, Lee et al. (2009) showed that CHI3L1 is expressed by airway epithelial cells and F4/80-positive macrophages during antigen-induced inflammation (Lee et al., 2009). Our results suggest that at early, pre-metastatic stages, CHI3L1 expression by either the airway epithelium or by "activated" lung macrophages may be induced by circulating tumor-derived factors including CHI3L1, and that this in turn promotes conditions that favor the later establishment of infiltrating tumor cells.

The biological roles of CHI3L1 have been recently characterized in terms of cell proliferation, angiogenesis, chemotaxis, and cell adhesion (Coffman, 2008; Shao et al., 2009; Kawada et al., 2012). We have previously shown that splenic macrophages from mammary tumor-bearing mice secrete higher levels of the pro-angiogenic molecules CCL2, CXCL2, and MMP-9 (compared to non-tumor bearers) and that CHI3L1 stimulates this increased production (Libreros et al., 2012). There are only few studies to date that have compared the production of angiogenic molecules by alveolar and interstitial macrophages in response to CHI3L1 in the context of inflammation. Letuve et al., showed that smokers with chronic obstructive pulmonary disease (COPD) had elevated serum levels of CHI3L1, and BALF samples contained a greater proportion of alveolar macrophages expressing CHI3L1 than smokers without COPD or non-smokers (Letuve et al., 2008). Inflammation associated with pulmonary sarcoid granulomas is also accompanied by expression of CHI3L1protein by both mononuclear cells/macrophages and giant cells of the granuloma (Johansen et al., 2005). Expression of CHI3L1 in the inflamed pulmonary environment may affect the function of local lung macrophages, and thereby favor the production of pro-angiogenic substances that promote tumor establishment and growth. Our evidence suggests that expression of CCL2, CXCL2, and MMP-9 by LPS-treated interstitial and alveolar macrophages from normal mice is enhanced by rmCHI3L1. These results are in agreement with those of Letuve et al., and Kawada et al., in that CHI3L1 stimulates macrophage production of IL-8 (homolog of mouse CXCL2), MCP-1 (CCL2) and MMP-9 (Letuve et al., 2008; Kawada et al., 2012). In addition to its angiogenic function, CCL2 acts as a chemoattractant molecule that recruits not only tumor cells, but also leukocytes that provide growth factors for the immigrant population of tumor cells (Carr et al., 1994; Craig and Loberg, 2006). We have previously shown that T lymphocytes from mammary tumor-bearing mice produce CCL2 and that T cell-derived CCL2 could also contribute to tumor growth directly via its pro-angiogenic activity and indirectly by attracting monocytes that secrete growth-promoting factors (Owen et al., 2005). Decreased levels of CCL2 therefore may have growth inhibitory activity on tumor cells. Additionally MMP-9, through its extracellular remodeling activities, may facilitate the immigration of tumor cells into the pulmonary environment (Coussens and Werb, 1996; Werb et al., 1999). Prior studies demonstrating that CHI3L1 promotes both macrophage recruitment and angiogenesis in colorectal cancer (Kawada et al., 2012) lend support to the idea that CHI3L1 expressed by interstitial and alveolar lung macrophages in the mammary tumor-bearing mice may likewise promote the migration and growth of metastasizing tumor cells.

At metastatic sites, specific populations of myeloid cells, i.e., $CD11b^+Gr1^+$ cells, have been found to promote tumor cell extravasation, seeding and persistent growth (Qian et al., 2009, 2011; Yan et al., 2010). The effect of a primary tumor affecting a distant organ such as the lung was previously investigated by (Yan et al., 2010), and it was found that $CD11b^+Gr1^+$ cells are increased in number in the "pre-metastatic" lungs of mice with mammary tumors (Yan et al., 2010). We have previously shown that splenic $CD11b^+Gr1$ cells express CHI3L1. Since Gr1 marker is a composite epitope between Ly6C and LyG antigens, we assessed the expression on CHI3L1 in $CD11b^+Ly6C^+$ vs. $CD11b^+Ly6G^+$ populations of cells from total lung and the lavage. We demonstrate that $CD11b^+Ly6C^+$ populations in the lungs of tumor bearers produce high levels of CHI3L1. In the study by Yang et al. (2004) $CD11b^+Gr1$ cells in the pre-metastatic lung down-regulated IFN-γ, contributing to the

immunosuppressive stage, and we have shown previously that CHI3L1 downregulates IFN-γ expression by splenic T cells (Libreros et al., 2012). Additionally, use of CHI3L1 knockout mice in an allergic pulmonary model was shown to increase IFN-γ in comparison to levels in wild type mice with normal CHI3L1 expression (Lee et al., 2009). It may be speculated that CHI3L1 in the pulmonary tissue may have similar effects on local IFN-γ expression, and thus contribute toward the establishment of metastases in our mammary tumor bearers. Chitin microparticle suppression of CHI3L1 could counteract this, and our prior findings of increased IFN-γ levels in chitin microparticle-treated tumor-bearers is consistent with this hypothesis, as these mice show decreased mammary tumor growth (Libreros et al., 2012). Decreased tumor burden is known to affect the success of tumor cell metastasis to peripheral organs. Binding of chitin to CHI3L1 may neutralize the adverse effects of CHI3L1 on tumor growth and metastasis both by decreasing angiogenesis and increasing IFN-γ expression. Thus, understanding how molecules like CHI3L1, expressed in the target organ at "pre-metastatic" stages, can promote the establishment of cancer cells at these target sites, may provide insights about how to disrupt these mechanisms therapeutically. Chitin microparticles may represent one possible method to neutralize the adverse effects of endogenous CHI3L1 on cancer cell growth, particularly in an inflammatory tissue environment.

ACKNOWLEDGMENTS

The authors thank Drs. Yoshimi Shibata and Kathleen Guthrie for critical review of the manuscript. Support was provided by grants from the NIH/National Cancer Institute to V. I-C. (R15CA135513 and R15CA135513-S1) and contribution from Mr. Jack Laub.

REFERENCES

Arenberg, D. A., Keane, M. P., DiGiovine, B., Kunkel, S. L., Morris, S. B., Xue, Y. Y., et al. (1998). Epithelial-neutrophil activating peptide (ENA-78) is an important angiogenic factor in non-small cell lung cancer. *J. Clin. Invest.* 102, 465–472. doi: 10.1172/JCI3145

Carr, M. W., Roth, S. J., Luther, E., Rose, S. S., and Springer, T. A. (1994). Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc. Natl. Acad. Sci. U.S.A.* 91, 3652–3656. doi: 10.1073/pnas.91.9.3652

Chupp, G. L., Lee, C. G., Jarjour, N., Shim, Y. M., Holm, C. T., He, S., et al. (2007). A chitinase-like protein in the lung and circulation of patients with severe asthma. *N. Engl. J. Med.* 357, 2016–2027. doi: 10.1056/NEJMoa073600

Coffman, F. D. (2008). Chitinase 3-Like-1 (CHI3L1): a putative disease marker at the interface of proteomics and glycomics. *Crit. Rev. Clin. Lab. Sci.* 45, 531–562. doi: 10.1080/10408360802334743

Coussens, L. M., and Werb, Z. (1996). Matrix metalloproteinases and the development of cancer. *Chem. Biol.* 3, 895–904. doi: 10.1016/S1074-5521(96)90178-7

Craig, M. J., and Loberg, R. D. (2006). CCL2 (Monocyte Chemoattractant Protein-1) in cancer bone metastases. *Cancer Metastasis Rev.* 25, 611–619. doi: 10.1007/s10555-006-9027-x

Faibish, M., Francescone, R., Bentley, B., Yan, W., and Shao, R. (2011). A YKL-40-neutralizing antibody blocks tumor angiogenesis and progression: a potential therapeutic agent in cancers. *Mol. Cancer Ther.* 10, 742–751. doi: 10.1158/1535-7163.MCT-10-0868

Folkman, J. (1971). Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.* 285, 1182–1186. doi: 10.1056/NEJM197111182852108

Gerber, P. A., Hippe, A., Buhren, B. A., Muller, A., and Homey, B. (2009). Chemokines in tumor-associated angiogenesis. *Biol. Chem.* 390, 1213–1223. doi: 10.1515/BC.2009.144

Hakala, B. E., White, C., and Recklies, A. D. (1993). Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family. *J. Biol. Chem.* 268, 25803–25810.

Henrissat, B., and Bairoch, A. (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* 293(Pt 3), 781–788.

Johansen, J. S., Christensen, I. J., Riisbro, R., Greenall, M., Han, C., Price, P. A., et al. (2003). High serum YKL-40 levels in patients with primary breast cancer is related to short recurrence free survival. *Breast Cancer Res. Treat* 80, 15–21. doi: 10.1023/A:1024431000710

Johansen, J. S., Jensen, B. V., Roslind, A., Nielsen, D., and Price, P. A. (2006). Serum YKL-40, a new prognostic biomarker in cancer patients? *Cancer Epidemiol. Biomarkers Prev.* 15, 194–202. doi: 10.1158/1055-9965.EPI-05-0011

Johansen, J. S., Milman, N., Hansen, M., Garbarsch, C., Price, P. A., and Graudal, N. (2005). Increased serum YKL-40 in patients with pulmonary sarcoidosis–a potential marker of disease activity? *Respir. Med.* 99, 396–402. doi: 10.1016/j.rmed.2004.09.016

Johansen, J. S., Olee, T., Price, P. A., Hashimoto, S., Ochs, R. L., and Lotz, M. (2001). Regulation of YKL-40 production by human articular chondrocytes. *Arthritis Rheum.* 44, 826–837. doi: 10.1002/1529-0131(200104)44:4<826::AID-ANR139>3.0.CO;2-U</ArticleId>

Johansson, A., Lundborg, M., Skold, C. M., Lundahl, J., Tornling, G., Eklund, A., et al. (1997). Functional, morphological, and phenotypical differences between rat alveolar and interstitial macrophages. *Am. J. Respir. Cell Mol. Biol.* 16, 582–588. doi: 10.1165/ajrcmb.16.5.9160840

Kawada, M., Seno, H., Kanda, K., Nakanishi, Y., Akitake, R., Komekado, H., et al. (2012). Chitinase 3-like 1 promotes macrophage recruitment and angiogenesis in colorectal cancer. *Oncogene* 31, 3111–3123. doi: 10.1038/onc.2011.498

Kim, J. B., Urban, K., Cochran, E., Lee, S., Ang, A., Rice, B., et al. (2010). Non-invasive detection of a small number of bioluminescent cancer cells *in vivo*. *PLoS ONE* 5:e9364. doi: 10.1371/journal.pone.0009364

Kogiso, M., Nishiyama, A., Shinohara, T., Nakamura, M., Mizoguchi, E., Misawa, Y., et al. (2011). Chitin particles induce size-dependent but carbohydrate-independent innate eosinophilia. *J. Leukoc. Biol.* 90, 167–176. doi: 10.1189/jlb.1110624

Lal, A., Lash, A. E., Altschul, S. F., Velculescu, V., Zhang, L., McLendon, R. E., et al. (1999). A public database for gene expression in human cancers. *Cancer Res.* 59, 5403–5407.

Lau, S. H., Sham, J. S., Xie, D., Tzang, C. H., Tang, D., Ma, N., et al. (2006). Clusterin plays an important role in hepatocellular carcinoma metastasis. *Oncogene* 25, 1242–1250. doi: 10.1038/sj.onc.1209141

Lee, C. G., Da Silva, C. A., Dela Cruz, C. S., Ahangari, F., Ma, B., Kang, M. J., et al. (2011). Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Annu. Rev. Physiol.* 73, 479–501. doi: 10.1146/annurev-physiol-012110-142250

Lee, C. G., Hartl, D., Lee, G. R., Koller, B., Matsuura, H., Da Silva, C. A., et al. (2009). Role of breast regression protein 39 (BRP-39)/chitinase 3-like-1 in Th2 and IL-13-induced tissue responses and apoptosis. *J. Exp. Med.* 206, 1149–1166. doi: 10.1084/jem.20081271

Letuve, S., Kozhich, A., Arouche, N., Grandsaigne, M., Reed, J., Dombret, M. C., et al. (2008). YKL-40 is elevated in patients with chronic obstructive pulmonary disease and activates alveolar macrophages. *J. Immunol.* 181, 5167–5173.

Libreros, S., Garcia-Areas, R., and Iragavarapu-Charyulu, V. (2013). CHI3L1 plays a role in cancer through enhanced production of pro-inflammatory/pro-tumorigenic and angiogenic factors. *Immunol. Res.* 57, 99–105. doi: 10.1007/s12026-013-8459-y

Libreros, S., Garcia-Areas, R., Shibata, Y., Carrio, R., Torroella-Kouri, M., and Iragavarapu-Charyulu, V. (2012). Induction of proinflammatory mediators by CHI3L1 is reduced by chitin treatment: decreased tumor metastasis in a breast cancer model. *Int. J. Cancer*, 131, 377–386. doi: 10.1002/ijc.26379

Mantovani, A., Sozzani, S., Locati, M., Allavena, P., and Sica, A. (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* 23, 549–555. doi: 10.1016/S1471-4906(02)02302-5

Mehrad, B., Keane, M. P., and Strieter, R. M. (2007). Chemokines as mediators of angiogenesis. *Thromb. Haemost.* 97, 755–762. doi: 10.1160/TH07-01-0040

Mizoguchi, E. (2006). Chitinase 3-like-1 exacerbates intestinal inflammation by enhancing bacterial adhesion and invasion in colonic epithelial cells. *Gastroenterology* 130, 398–411. doi: 10.1053/j.gastro.2005.12.007

Nishiyama, A., Tsuji, S., Yamashita, M., Henriksen, R. A., Myrvik, Q. N., and Shibata, Y. (2006) Phagocytosis of N-acetyl-D-glucosamine particles, a Th1 adjuvant, by RAW 264.7 cells results in MAPK activation and TNF-alpha, but not IL-10, production. *Cell Immunol.* 239, 103–112. doi: 10.1016/j.cellimm.2006.04.003

Nyirkos, P., and Golds, E. E. (1990). Human synovial cells secrete a 39 kDa protein similar to a bovine mammary protein expressed during the non-lactating period. *Biochem. J.* 269, 265–268.

Ober, C., and Chupp, G. L. (2009). The chitinase and chitinase-like proteins: a review of genetic and functional studies in asthma and immune-mediated diseases. *Curr. Opin. Allergy Clin. Immunol.* 9, 401–408. doi: 10.1097/ACI.0b013e3283306533

Owen, J. L., Lopez, D. M., Grosso, J. F., Guthrie, K. M., Herbert, L. M., Torroella-Kouri, M. et al. (2005). The expression of CCL2 by T lymphocytes of mammary tumor bearers: role of tumor-derived factors. *Cell Immunol.* 235, 122–135. doi: 10.1016/j.cellimm.2005.08.032

Prokhorova, S., Lavnikova, N., and Laskin, D. L. (1994). Functional characterization of interstitial macrophages and subpopulations of alveolar macrophages from rat lung. *J. Leukoc. Biol.* 55, 141–146.

Qian, B., Deng, Y., Im, J. H., Muschel, R. J., Zou, Y., Li, J., et al. (2009). A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. *PLoS ONE* 4:e6562. doi: 10.1038/nature10138

Qian, B. Z., Li, J., Zhang, H., Kitamura, T., Zhang, J., Campion, L. R., et al. (2011). CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature* 475, 222–225. doi: 10.1146/annurev-physiol-012110-142250

Rathcke, C. N., and Vestergaard, H. (2006). YKL-40, a new inflammatory marker with relation to insulin resistance and with a role in endothelial dysfunction and atherosclerosis. *Inflamm. Res.* 55, 221–227. doi: 10.1007/s00011-006-0076-y

Rehli, M., Niller, H. H., Ammon, C., Langmann, S., Schwarzfischer, L., Andreesen, R., et al. (2003). Transcriptional regulation of CHI3L1, a marker gene for late stages of macrophage differentiation. *J. Biol. Chem.* 278, 44058–44067. doi: 10.1074/jbc.M306792200

Renkema, G. H., Boot, R. G., Au, F. L., Donker-Koopman, W. E., Strijland, A., Muijsers, A. O., et al. (1998). Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl hydrolases secreted by human macrophages. *Eur. J. Biochem.* 251, 504–509. doi: 10.1046/j.1432-1327.1998.2510504.x

Schneberger, D., Aharonson-Raz, K., and Singh, B. (2011). Monocyte and macrophage heterogeneity and Toll-like receptors in the lung. *Cell Tissue Res.* 343, 97–106. doi: 10.1007/s00441-010-1032-2

Sebring, R. J., and Lehnert, B. E. (1992). Morphometric comparisons of rat alveolar macrophages, pulmonary interstitial macrophages, and blood monocytes. *Exp. Lung Res.* 18, 479–496. doi: 10.3109/01902149209064341

Shao, R., Hamel, K., Petersen, L., Cao, Q. J., Arenas, R. B., Bigelow, C., et al. (2009). YKL-40, a secreted glycoprotein, promotes tumor angiogenesis. *Oncogene* 28, 4456–4468. doi: 10.1038/onc.2009.292

Shibata, Y., Metzger, W. J., and Myrvik, Q. N. (1997). Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan: mannose receptor-mediated phagocytosis initiates IL-12 production. *J. Immunol.* 159, 2462–2467.

Steeg, P. S. (2006). Tumor metastasis: mechanistic insights and clinical challenges. *Nat. Med.* 12, 895–904. doi: 10.1038/nm1469

Strong, P., Clark, H., and Reid, K. (2002). Intranasal application of chitin microparticles down-regulates symptoms of allergic hypersensitivity to Dermatophagoides pteronyssinus and Aspergillus fumigatus in murine models of allergy. *Clin. Exp. Allergy* 32, 1794–1800. doi: 10.1046/j.1365-2222.2002.01551.x

van Kempen, L. C., and Coussens, L. M. (2002). MMP9 potentiates pulmonary metastasis formation. *Cancer Cell* 2, 251–252. doi: 10.1016/S1535-6108(02)00157-5

Volck, B., Price, P. A., Johansen, J. S., Sorensen, O., Benfield, T. L., Nielsen, H. J., et al. (1998). YKL-40, a mammalian member of the chitinase family, is a matrix protein of specific granules in human neutrophils. *Proc. Assoc. Am. Physicians* 110, 351–360.

Werb, Z., Vu, T. H., Rinkenberger, J. L., and Coussens, L. M. (1999). Matrix-degrading proteases and angiogenesis during development and tumor formation. *APMIS* 107, 11–18. doi: 10.1111/j.1699-0463.1999.tb01521.x

Yan, H. H., Pickup, M., Pang, Y., Gorska, A. E., Li, Z., Chytil, A., et al. (2010). Gr-1+CD11b+ myeloid cells tip the balance of immune protection to tumor promotion in the premetastatic lung. *Cancer Res.* 70, 6139–6149. doi: 10.1158/0008-5472.CAN-10-0706

Yang, L., DeBusk, L. M., Fukuda, K., Fingleton, B., Green-Jarvis, B., Shyr, Y., et al. (2004). Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* 6, 409–421. doi: 10.1016/j.ccr.2004.08.031

Younos, I., Donkor, M., Hoke, T., Dafferner, A., Samson, H., Westphal, S., et al. (2011). Tumor- and organ-dependent infiltration by myeloid-derived suppressor cells. *Int. Immunopharmacol.* 11, 816–826. doi: 10.1016/j.intimp.2011.02.021

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 July 2013; accepted: 12 December 2013; published online: 25 December 2013.

*Citation: Libreros S, Garcia-Areas R, Keating P, Carrio R and Iragavarapu-Charyulu VL (2013) Exploring the role of CHI3L1 in "pre-metastatic" lungs of mammary tumor-bearing mice. Front. Physiol. **4**:392. doi: 10.3389/fphys.2013.00392*

This article was submitted to Vascular Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 05 July 2013
doi: 10.3389/fphys.2013.00159

Macrophages and chemokines as mediators of angiogenesis

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Accumulating evidence attests to the important roles of both macrophages and chemokines in angiogenesis. Tumor-associated macrophages or TAMS constitute the major fraction of tumor-infiltrating leukocytes and are recruited by a number of chemoattractants that are produced by the tumor and tumor-associated stroma. This heterogeneous cell population is activated by a variety of stimuli and becomes polarized to result in functionally different phenotypes regarding tumor progression. As opposed to classically activated or M1 macrophages that exhibit anti-tumor functions, most TAMS are considered to be of the alternatively activated or M2 phenotype, and express multiple cytokines, proteases, and chemokines that promote tumor angiogenesis. Chemokines also have disparate effects on angiogenesis regulation, as several members of the CXC and CC chemokine families are potent inducers of angiogenesis, while a subset of CXC chemokines are angiostatic. This review summarizes the current literature regarding the roles and modes of action of macrophage-derived chemokines as mediators of angiogenesis.

Keywords: angiogenesis, chemokines, chemokine receptors, macrophages, tumor-associated macrophages, tumors

INTRODUCTION

Human chemokines are a superfamily of 48 small (approximately 8–14 kDa) chemoattractant cytokines that are divided into four subfamilies, CXC, CC, (X)C, and CX_3C, based on the arrangement of the conserved N-terminal cysteine residues, where "X" represents any amino acid (Zlotnik and Yoshie, 2000). Systematic chemokine nomenclature is based on the cysteine subfamily, followed by an "L" for ligand and a numerical designation (Zlotnik and Yoshie, 2000; Mukaida and Baba, 2012). Since some chemokines only exist in humans or in mice, human chemokines may be designated with capital letters, while the murine chemokines are written in lower-case letters (Zlotnik and Yoshie, 2012). The biologic effects of these proteins are mediated by a superfamily of 19 seven transmembrane G protein-coupled receptors (7TM GPCRs). There is also a set of "atypical" chemokine receptors that do not mediate cell migration, but rather, regulate inflammation by acting as decoy or scavenger receptors (Mantovani et al., 2010). Several chemokine decoy receptors have been identified (D6, DARC/Duffy antigen receptor for chemokines, CCXCKR); all have mutations that prevent G protein-coupling and thus, intracellular signaling, acting instead to alter local concentrations of chemokines within a microenvironment and influencing subsequent immune responses (Collins et al., 2010; Yoshimura and Oppenheim, 2011).

Chemokines can be thought of as "inflammatory" or "homeostatic" depending on whether they are induced during inflammation or constitutively expressed in certain tissues (Zlotnik et al., 2011). Homeostatic chemokines are expressed in lymphoid or other organs, are involved in leukocyte homing and trafficking, and are well conserved between species (Zlotnik and Yoshie, 2012). Inflammatory chemokines are primarily involved in the recruitment of leukocytes to areas of inflammation and can have marked differences in function between species (Islam et al., 2011; Zlotnik et al., 2011). Inflammatory chemokines play key roles in tumor progression, as they determine the immune cell infiltrate in the tumor microenvironment, modulate the immune response, and participate in angiogenesis and dissemination of the tumor.

In this review, we discuss the potential roles of macrophages and their production of chemokines in modulating angiogenesis.

ANGIOGENESIS

There are two major processes involved in the formation of blood vessels, vasculogenesis and angiogenesis. Vasculogenesis typically describes the generation of blood vessels *de novo* from mesenchymal blood islands that develop into blood cells and vascular endothelium (Lu et al., 2011). Angiogenesis is defined as the sprouting of new vessels from pre-existing ones (Risau, 1997). Physiologic angiogenesis occurs during embryonic development, wound healing, and female reproductive cycling, and involves vessel destabilization, endothelial cell migration and proliferation, and sprouting. These processes are followed by a resolution phase with reduced endothelial cell proliferation and vessel stabilization (Motz and Coukos, 2011). In the adult organism, angiogenesis is typically associated with pathologic processes such as cancer, stroke, diabetes, and other inflammatory diseases such as psoriasis and arthritis (Kiefer and Siekmann, 2011); unlike physiologic angiogenesis, pathologic angiogenesis does not have a resolution phase and results in a highly disorganized vascular network (Motz

and Coukos, 2011). Hypoxia or low oxygen tension is the primary factor in the induction of angiogenesis. Inflammatory cells are recruited to ischemic tissues and extravasate to these areas via tethering to P-selectin expressed on activated endothelial cells and platelets (Egami et al., 2006). Once there, these inflammatory cells release cytokines, vasoactive molecules, and chemokines in response to the hypoxia. The resulting vascular networks of tumors are leaky and hemorrhagic, with abnormal endothelial cell proliferation and apoptosis; they are poorly functional with excessive convoluted branching that results in oxygen depletion and extracellular acidosis (Nagy et al., 2010; Fokas et al., 2012). These chaotic vessels lack distinct venules, capillaries, or arterioles, and are lined by endothelial cells that differ from normal endothelial cells both molecularly and functionally and are supported by abnormal pericytes that are loosely attached and do not provide full coverage to the vessel (Bussolati et al., 2011).

Since they were first isolated from adult peripheral blood in 1997 (Asahara et al., 1997), emerging data have revealed a role for endothelial progenitor cells (EPCs) in the process of tumor neovascularization. Circulating EPCs or angioblasts comprise a very minor subpopulation in the blood that is most likely derived from hemangioblast precursors (Asahara et al., 2011). They were first characterized by their expression of CD31, Flk-1/ vascular endothelial growth factor receptor (VEGFR)-2, Tie-2, and their release of nitric oxide (Asahara et al., 1997; Ahn and Brown, 2009). These progenitor cells home to sites of neovascularization, differentiate into endothelial cells, and have been reported to compose anywhere from <0.01% in B16 melanoma (Purhonen et al., 2008) to >80% of the tumor vasculature in B6RV2 lymphoma (Lyden et al., 2001). Using a preclinical model of murine Lewis lung carcinoma metastasis, investigators found that these cells comprised 12% of the neovasculature in the metastatic lesions, and more importantly, demonstrated that blocking their mobilization significantly inhibited angiogenesis and decreased the formation of lethal macrometastases, implicating these cells in "the angiogenic switch" (Gao et al., 2008). Despite the discrepancies in their reported contributions to the composition of tumor vasculature, these cells can contribute to neovascularization by virtue of their production of pro-angiogenic mediators including VEGF, insulin-like growth factor (IGF)-1, angiopoitin (Ang)-1 and -2, and **CXCL12**/stromal cell-derived factor-1/SDF-1 (Ahn and Brown, 2009).

Vascular endothelial growth factor (VEGF), also known as VEGF-A, is the prototypical pro-angiogenic cytokine secreted by hypoxic tumor cells, tumor-associated macrophages (TAMs), and endothelial cells within the tumor microenvironment. It was originally demonstrated to be an endothelial growth factor and a potent inducer of vascular permeability (Claesson-Welsh and Welsh, 2013). VEGF has also been shown to be chemotactic for monocytes *in vitro* via VEGF receptor 1/FLT1 and VEGFR2/KDR (Murdoch et al., 2008). Thus, this molecule is an obvious target for anti-angiogenic therapy in cancer patients. However, a clinical study using laser capture microdissection (LCM) and gene expression profiling in rectal carcinoma patients using bevacizumab (Genentech), an anti-VEGF antibody, found that CXCL12, CXCR4, and **CXCL6**/granulocyte chemoattractant protein-2/GCP-2 expression were induced in rectal cancer cells with bevacizumab administration, while neuropilin 1 was increased in TAMs (Xu et al., 2009). Furthermore, increased plasma levels of CXCL12 in these patients after treatment were associated with rapid disease progression and metastasis. The authors speculated that the CXCL12–CXCR4 pathway may be a tumor resistance or escape mechanism with anti-VEGF monotherapy, as this pathway is also strongly implicated in angiogenesis (Xu et al., 2009).

CXC CHEMOKINES AND THE ELR MOTIF

CXC chemokines can be further be classified as ELR$^+$ or ELR$^-$, based on the presence of a glutamate-leucine-arginine amino acid sequence at the N-terminus of the protein. The **ELR$^+$** chemokines, **CXCL1**/growth-regulated oncogene α/GROα, **CXCL2**/GROβ, **CXCL3**/GROγ, **CXCL5**/epithelial cell-derived neutrophil activating peptide-78/ENA-78, **CXCL6**/granulocyte chemoattractant protein-2/GCP-2, **CXCL7**/neutrophil-activating protein-2/NAP-2, and **CXCL8**/ interleukin (IL)-8/IL-8, are chemotactic for neutrophils, and are pro-angiogenic (Raman et al., 2011). All of the murine ELR$^+$ CXC chemokines signal via the CXCR2 receptor, while human ELR$^+$ CXC chemokines signal primarily through CXCR2, but can also signal through CXCR1 (Keeley et al., 2011). It is important to note that there is no homologue of the human *CXCL8/IL-8* gene in mice or rats (Nomiyama et al., 2010). Additionally, humans have both *CXCR1* and *CXCR2* genes for the ELR$^+$ chemokine receptors, whereas *CXCR1* has not been found in mice or rats (Moepps et al., 2006; Mukaida and Baba, 2012). CXCL8 is the prototypic ELR$^+$ pro-angiogenic chemokine in its promotion of endothelial cell migration, invasion, and proliferation, all of which result in the formation of capillary-like structures within tumors (Ben-Baruch, 2012). In addition to its secretion by tumor cells, CXCL8 has also been shown to be produced by monocytes when cultured with supernatants from freshly excised breast cancer tissue and mammary tumor cell lines. Moreover, the cultured monocytes also secreted the pro-angiogenic chemokines, CXCL1, CXCL2, CXCL3, CXCL5, and CXCL7, resulting in micro vessel formation, with no production of angiostatic chemokines (Toulza et al., 2005).

CXCL8 may also play a role in angiogenesis as a potent neutrophil chemoattractant (Mantovani et al., 2010; Tazzyman et al., 2013). In multistep pancreatic islet carcinogenesis in RIP1-Tag2 transgenic mice, it was determined that neutrophils were required for the "angiogenic switch" due to their production of matrix metalloproteinase (MMP)-9 that activates VEGF (Nozawa et al., 2006). Using this model, investigators demonstrated that MMP-9 was expressed by neutrophils infiltrating the angiogenic islets and the tumors, while MMP-9-expressing macrophages were located along the periphery of the tumors, where tumor growth and angiogenesis occur (Nozawa et al., 2006). The ELR$^+$ chemokine, CXCL6, may also contribute to angiogenesis via its recruitment of neutrophils. For instance, it has been shown that CXCL6, CXCL8, and CCL2 are co-induced in micro vascular endothelial cells after stimulation with pro-inflammatory stimuli (Gijsbers et al., 2005). Using immunohistochemistry (IHC) on patient biopsies, it was shown that endothelial cells from various gastrointestinal tumors (e.g., adenocarcinomas of the esophagus, stomach,

colon, and pancreas) expressed CXCL6, which strongly correlated with leukocyte infiltration of the tumors and MMP-9 expression. While CXCL6 only weakly induced the proliferation of endothelial cells, it did synergize with CCL2 in neutrophil chemotaxis, allowing for neutrophil-derived proteases to degrade extracellular matrix and promote neovascularization (Gijsbers et al., 2005). Other mediators implicated in the "angiogenic switch" include, fibroblast growth factor (FGF), PDGFs, lysophosphatic acid (LPA), and angiopoitins (Hanahan and Weinberg, 2011; Fagiani and Christofori, 2013).

The **ELR**$^-$ chemokines, **CXCL4**/platelet factor-4/PF-4, **CXCL4L1**/CXCL4 variant, **CXCL9**/monokines induced by interferon-γ/Mig, **CXCL10**/interferon-γ inducible protein-10/IP-10, **CXCL11**/IFN-inducible T cell chemoattractant/I-TAC, **CXCL13**/B cell attracting chemokine-1/BCA-1, and **CXCL14**/breast- and kidney-expressed chemokine/BRAK, are chemotactic for lymphocytes and natural killer (NK) cells and are angiostatic (Raman et al., 2011). CXCL4, CXCL4L1, CXCL9, CXCL10, and CXCL11 are all reported to be ligands for CXCR3 (Struyf et al., 2011). These angiostatic chemokines play important roles in tumor progression, as over-expression of CXCL4 inhibits angiogenesis, tumor growth, and metastasis (Yamaguchi et al., 2005). In fact, CXCL4 was the first described angiostatic chemokine, which was found to inhibit endothelial migration and proliferation, and the binding of fibroblast growth factor (FGF)-2 and VEGF to their receptors (Maione et al., 1990; Airoldi and Ribatti, 2011). CXCL4 was once thought to only be expressed by megakaryocytes and platelets, however, human monocytes, mast cells, and activated T cells are now known to secrete this chemokine (Vandercappellen et al., 2011). CXCL4L1 is a homologue of CXCL4 and differs by three amino acid residues at the C-terminus of the protein. Both genes are located on human chromosome 4, and *CXCL4L1*, which is only present in humans and some primates, likely arose from recent duplication of the *CXCL4* gene (Dubrac et al., 2010). These proteins are not identical in function, as CXCL4L1 is a more potent inhibitor of endothelial cell migration and angiogenesis than its homologue, as well as being more diffusible, having a longer half-life, and acting in a paracrine manner, as opposed to CXCL4's juxtacrine activity (Dubrac et al., 2010).

THE CXCL12/CXCR4 AXIS

An important exception to the **ELR**$^-$ rule is **CXCL12**/stromal cell-derived factor-1/SDF-1, which is ELR$^-$, but promotes angiogenesis via binding to its receptor, CXCR4 (Singh et al., 2013). Currently, CXCR4 is one of the most studied chemokine receptors and is over-expressed in over 20 different human tumors, including prostate, breast, ovarian, lung, pancreatic, colorectal, and melanoma (Balkwill, 2004; Singh et al., 2013). It has been shown that CXCL12 binding to CXCR4 induces Akt phosphorylation and increases production of the major angiogenic factor, VEGF, in the human breast cancer cell line, MDA-MB-23 (Liang et al., 2007). Experimental models of melanoma, colon, pancreatic, thyroid, and prostate cancer have demonstrated that organ directed metastasis is mediated by CXCR4$^+$ tumor cells migrating to CXCL12$^+$ organs such as the liver and the lungs (Domanska et al., 2013). Studies of glioblastomas and neuroblastomas have also shown that CXCR4$^+$ monocytes recruited to tumors promote new vascular formations within the neoplasms; the monocytes first establish themselves within perivascular areas of the tumor and then release pro-angiogenic factors such as VEGF and angiopoitins, with subsequent recruitment of bone marrow-derived endothelial and perivascular progenitors that will compose the vasculature (Jodele et al., 2005; Domanska et al., 2013).

CXCL12 is normally expressed by the mesenchymal stroma of the lungs, liver, lymphatic tissues, and bone marrow (Domanska et al., 2013). Despite its documented role in tumorigenesis, CXCL12 is considered a homeostatic chemokine by virtue of its pivotal role in the retention and homing of hematopoietic stem cells in the bone marrow and in lymphocyte trafficking (Teicher and Fricker, 2010). In addition to its expression on most leukocyte subsets, CXCR4$^+$ cells that can directly or indirectly participate in angiogenesis include, smooth muscle cell progenitors, endothelial cell precursors, and immature and mature hematopoietic cells (Petit et al., 2007; Teicher and Fricker, 2010). CXCL12 directly mediates angiogenesis through its binding to CXCR4 on endothelial cells and by recruiting endothelial progenitor cells, while indirectly it induces the secretion of pro-angiogenic factors such as VEGF, CXCL8, and CXCL1 by leukocytes, tumor cells, and endothelial cells that express CXCR4 (Verbeke et al., 2011).

CXCR7 is another receptor with high affinity for CXCL12, and its recent discovery has complicated the understanding of the CXCL12/CXCR4 axis. While CXCR4 and CXCR7 are both moderately expressed on normal endothelial cells, the expression of CXCR7 on endothelial cells within the tumor microenvironment is markedly up-regulated and has recently been suggested as a marker of tumor vasculature in various tumors such as renal carcinoma and gliomas (Singh et al., 2013). Conversely, other researchers demonstrated that co-expression of both CXCR4 and CXCR7 resulted in decreased CXCL12-mediated intravasation of mammary carcinoma cells and fewer metastases of these tumors to the lungs (Hernandez et al., 2011; Singh et al., 2013). Moreover, CXCR7 is also able to bind with low affinity to the angiostatic ELR$^-$ chemokine, CXCL11, which itself can also bind to CXCR3 (Lasagni et al., 2003). Clearly, additional research is necessary to better understand the crosstalk between these chemokines and their multiple receptors.

THE LONE CX_3C CHEMOKINE

The single member of the CX_3C subfamily, **CX_3CL1**/fractalkine, signals through its chemokine receptor, CX_3CR1, and is unique in that it is a cell surface transmembrane protein that functions as an adhesion molecule that can be proteolytically cleaved by the metalloproteinases, ADAM10 and ADAM 17, to form the active soluble chemoattractant (White and Greaves, 2009). This chemokine recruits lymphocytes, NK cells, and monocytes, and has been shown to participate in angiogenesis through several different mechanisms. For example, Kumar *et al.* have shown that a bone marrow derived CX_3CR1^+ monocyte subpopulation is capable of differentiating into smooth muscle-like cells subsequent to CX_3CL1–CX_3CR1 interactions in blood vessel walls after vessel injury (Kumar et al., 2010). Furthermore, a competent CX_3CL1–CX_3CR1 interaction is necessary for

nascent microvessel formation, maturation, and vascular structural integrity in two models of neovascularization, and for the differentiation of CX_3CR1^+ monocytes into smooth muscle-like cells *in vivo* (Kumar et al., 2013). Loss of this chemokine/receptor interaction resulted in the development of smaller, leaky, poorly developed, and hemorrhagic microvessels in Matrigel and experimental plaque models of neovascularization (Kumar et al., 2013).

Utilizing inducible fibroblast growth factor receptor 1 (FGFR1) in a murine mammary tumor cell line and its endogenous expression in the breast cancer cell line, HS578T, another laboratory has recently shown that activation of this tyrosine kinase receptor leads to CX_3CL1 production by tumor cells and subsequently, enhanced macrophage recruitment to mammary cells during the early stages of tumorigenesis *in vitro* and *in vivo* (Reed et al., 2012). By blocking CX_3CR1 *in vivo*, these researchers demonstrated decreased macrophage infiltration into the mammary epithelium of MMTV-iFGFR1 mice and decreased angiogenesis (Reed et al., 2012). It is important to note that CX_3CL1 is typically up-regulated on inflamed endothelium via the pro-inflammatory cytokines, IL-1, tumor necrosis factor (TNF-α), and interferon (IFN-γ) (Lee et al., 2013).

As lipid-laden macrophages or "foam cells" are the defining feature of early atherosclerosis, laboratories focusing on this disease process have also studied the molecules that direct monocyte migration from the peripheral blood to vessel walls. Accordingly, Saederup *et al.* created $CX_3CL1^{-/-}CCR2^{-/-}ApoE^{-/-}$ mice, and demonstrated independent roles for CCR2 and CX_3CL1 in the accumulation of macrophages in the artery walls of mice deficient in apolipoprotein E (ApoE) (Saederup et al., 2008). *CCR2* and *CX_3CR1* are located too close together on murine chromosome 9 for the generation of mice deficient in both receptors. Deletion of both *CX_3CL1* and *CCR2* resulted in dramatically reduced macrophage accumulation in artery walls compared to deletion of only one of those genes, suggesting that these molecules work additively, and that they recruit different monocyte subsets from the circulating $Ly6C^{hi}$ population in atherogenesis (Saederup et al., 2008; Lee et al., 2013).

MACROPHAGES

Analogous to the T helper cell Th1/Th2 classification, macrophages can be broadly divided into a classically activated M1 phenotype or alternatively activated M2 macrophages (Hao et al., 2012). M1 pro-inflammatory macrophages are activated by IFN-γ, TNF-α, and engagement of Toll-like receptors (TLRs) by microbial stimuli such as lipopolysaccharide (LPS), and release inflammatory cytokines and reactive oxygen and nitrogen intermediates. These macrophages are typically $IL\text{-}12^{high}$, $IL\text{-}23^{high}$, and $IL\text{-}10^{low}$, promote Th1 responses, are tumoricidal, and can elicit tissue destruction (Mantovani and Sica, 2010; Baay et al., 2011). M2 anti-inflammatory macrophages, on the other hand, are directly induced by interleukin (IL)-4 and IL-13 and indirectly by IL-5, IL-10, IL-21, IL-25, and IL-33 (Liu and Yang, 2013). The chemokines, CCL2, **CCL17**/thymus and activation-regulated chemokine/TARC, and **CCL22**/macrophage-derived chemokine/MDC have also been shown to promote M2 polarization of macrophages (Mantovani and Sica, 2010). These cells are usually $IL\text{-}12^{low}$, $IL\text{-}23^{low}$, and $IL\text{-}10^{high}$, and are involved in immunosuppression, tissue repair (including angiogenesis), and tumor promotion (Mantovani and Sica, 2010; Baay et al., 2011).

M1 and M2 macrophages also express different chemokines; M1 cells produce pro-inflammatory CXCL5/epithelial cell-derived neutrophil-activating factor-78/ENA-78, CXCL9/monokine induced by IFN-γ/MIG, and CXCL10/IFN-γ-inducible protein-10/IP-10, while M2 macrophages make CCL17, CCL22, and **CCL24**/eotaxin-2 (**Figure 1**) (Mantovani et al., 2004; Traves et al., 2012). Further classification of alternatively activated macrophages into M2a, M2b, and M2c has also been suggested (Gordon and Martinez, 2010). Typically, tumor associated macrophages (TAMs) are M2 macrophages and play important roles in angiogenesis, metastasis, and the generation of immunosuppressive regulatory T cells (Tregs) (Mantovani et al., 2009).

TUMOR-ASSOCIATED MACROPHAGES (TAMs)

The induction of angiogenesis is considered to be a "hallmark" of cancer, a distinctive capability that is necessary for tumor growth and dissemination (Hanahan and Weinberg, 2011). While angiogenesis was once thought occur after tumor cells acquired an invasive phenotype, it is now appreciated that this event occurs early in tumorigenesis during pre-malignant lesions of the breast, prostate, gastrointestinal tract, cervix, uterus, lung, and squamous cell carcinoma of the head and neck (Raica et al., 2009). Furthermore, it has been shown that TAMs play a key role in the induction of angiogenesis, and their infiltration precedes vascular remodeling in the PyMT (mammary epithelial cell restricted expression of the polyoma middle T oncoprotein) mouse model of breast cancer (Lin et al., 2006). Depletion of macrophages in this tumor model using mice carrying the homozygous null allele ($Csf1^{op}$) for the monocyte growth factor, colony stimulating factor (CSF)-1, caused a 50% reduction in vascular density and resulted in delayed tumor progression and metastasis (Lin et al., 2006; Murdoch et al., 2008).

Tumor-promoting inflammation is considered to be the seventh "hallmark" of cancer (Hanahan and Weinberg, 2011). The microenvironment of solid tumors comprises many other non-malignant cell types, including the cells of blood and lymphatic vessels, fibroblasts, adipocytes, and leukocytes such as macrophages, dendritic cells, lymphocytes, neutrophils, eosinophils, mast cells, and myeloid-derived immune suppressor cells (MDSCs), which are characterized by co-expression of the macrophage surface marker, CDllb, and the neutrophil surface marker, Gr1. TAMs are the most prominent component of the leukocyte infiltrate within tumors, and one meta-analysis found that intratumoral macrophage density correlated with a poor patient prognosis in over 80% of studies (Bingle et al., 2002; Halin et al., 2009). Macrophages are a heterogeneous population of leukocytes that play many important roles in immune regulation, angiogenesis, tumor progression, and metastasis. Accumulating data suggest that peripheral blood monocytes extravasate into tumors and differentiate into tissue macrophages, accumulating in distinct tumor microenvironments depending on chemokine expression pattern (Murdoch et al., 2008; Lee et al., 2013). This accumulation of macrophages occurs within hypoxic areas of

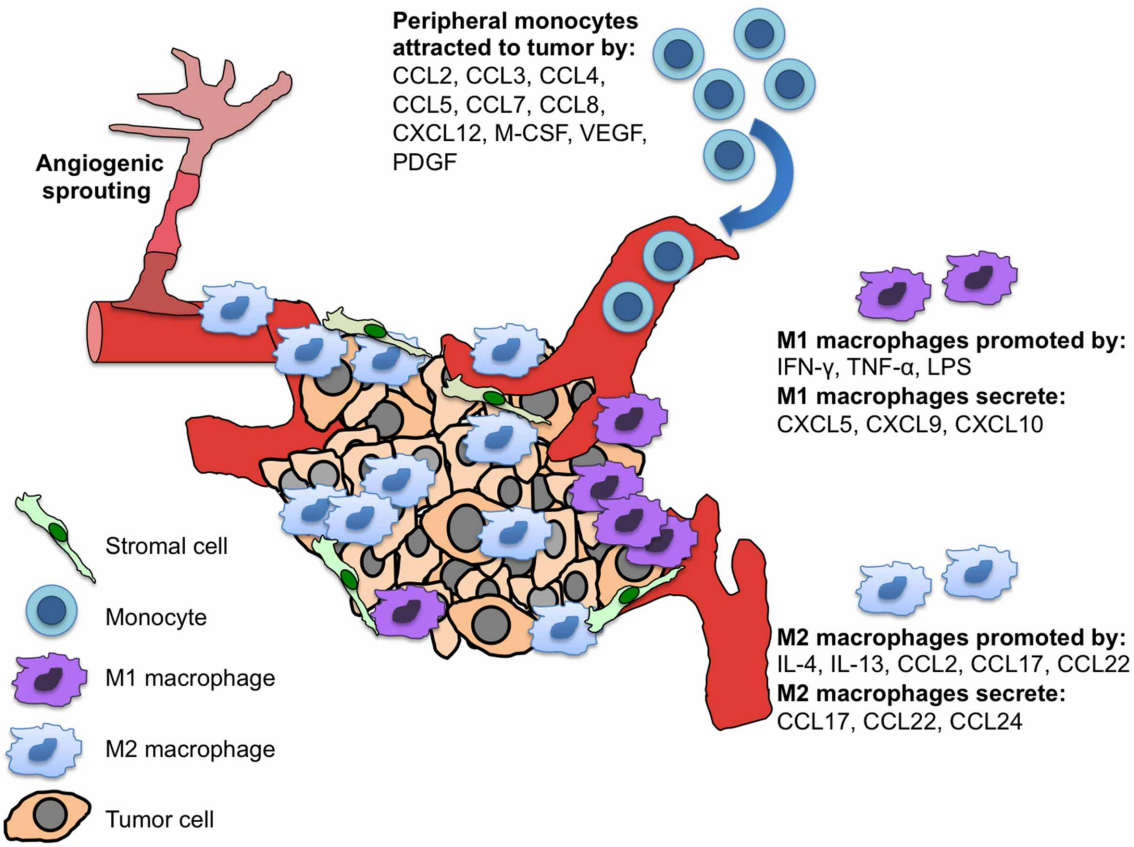

FIGURE 1 | M1 and M2 macrophages and their differential chemokine secretion in the tumor microenvironment. Tumor and tumor-associated stromal cells secrete chemokines and cytokines to recruit monocytes to the site of the tumor. These peripheral monocytes differentially develop into polarized tissue macrophages (M1 vs. M2), which have distinct chemokine secretion patterns and functions within the tumor.

the neoplasm that contain necrotic tissue and is mediated primarily by the CC chemokine, **CCL2**/monocyte chemoattractant protein-1/MCP-1 (Murdoch et al., 2008). CSF-1, VEGF, placental growth factor (PGF), CXCL12, CXCL8, and MMP-9 have also been reported to be involved in the mobilization and recruitment of hematopoietic cells from the bone marrow to the sites of tumors (De Palma et al., 2007).

CC CHEMOKINES

CC chemokines are chemotactic for monocytes, dendritic cells (DCs), eosinophils, basophils, lymphocytes, and NK cells (Zlotnik and Yoshie, 2012). Multiple studies have demonstrated a correlation between the levels of the inflammatory chemokines, CCL2 and CCL5, and the number of myeloid cells within tumors (Soria and Ben-Baruch, 2008; Allavena et al., 2011). The primary monocyte-recruiting chemokine, CCL2, regulates the trafficking of monocytes, macrophages, and other inflammatory cells by binding to its receptor, CCR2 (Zhang et al., 2010). CCL2 expression has been demonstrated in many types of cancer including, multiple myeloma, melanoma, esophageal, gastric, colorectal, lung, breast, ovary, and prostate cancer (Craig and Loberg, 2006; Zhang et al., 2010). CCL2 indirectly contributes to angiogenesis by attracting TAMS, which secrete pro-angiogenic cytokines such as VEGF, platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, and CXCL8/IL-8, and the proteolytic enzymes, MMP-2 and MMP-9 (Mantovani et al., 2010). CCL2 can also directly induce angiogenesis in endothelial cells, which express its receptor, CCR2 (Salcedo et al., 2000), and induce VEGF and hypoxic-inducible factor (HIF)-1 in tumor cells (Zhang et al., 2010).

Other tumor-derived chemotactic factors secreted by both malignant and stromal cells that attract peripheral monocytes to the site of tumors include **CCL3**/macrophage inflammatory protein-1α/MIP-1α, **CCL4**/macrophage inflammatory protein-1β/MIP-1β, **CCL5**/regulated on activation normal T cell expressed and secreted/RANTES, **CCL7**/MCP-3, **CCL8**/MCP-2, CXCL12, and the cytokines, macrophage colony stimulating factor (M-CSF), PGF, and VEGF (**Figure 1**) (Lewis and Pollard, 2006; Murdoch et al., 2008; Mukaida and Baba, 2012). After macrophages have successfully migrated into the hypoxic region of the tumor, their movements become restricted by decreased expression of CCR2 and CCR5 via hypoxia-mediated down-regulation of these receptors (Sica et al., 2000; Bosco et al., 2004; Lee et al., 2013). The authors' of these studies speculated that this may be a mechanism to retain recruited macrophages at hypoxic sites; and together with the observation that hypoxia

mediates up-regulation of CXCR4 within macrophages and tumor cells (Schioppa et al., 2003), is suggestive of plasticity in chemokine receptor expression within hypoxic tissues (Bosco et al., 2004).

Interestingly, investigators have revealed another connection in the angiogenesis nexus involving macrophages and CCL2. The transcription factor, Twist 1, has previously been shown to play multiple roles in tumor initiation and progression, including induction of the epithelial mesenchymal transition (EMT) and degradation of the extracellular matrix (Yang et al., 2004), with increased expression having a positive correlation with metastasis and poor survival in several aggressive human tumors, including breast and colorectal cancer (Gomez et al., 2011). Via, in part, to repression of E-cadherin transcription, the epithelial mesenchymal transition permits carcinoma cells to migrate away from the site of the primary tumor through the lymphatics and/or peripheral blood to form metastatic tumor foci (Qin et al., 2012). Low-Marchelli *et al.* recently demonstrated an additional and novel tumor-promoting role for this transcription factor in the induction of CCL2 production by human and murine mammary tumor cells, which serves to recruit infiltrating $CCR2^+$ macrophages and to induce angiogenesis (Low-Marchelli et al., 2013). These authors suggested that induction of CCL2 by Twist 1 in tumor cells recruits TAMs that then promote extravasation and metastatic seeding in other organs by virtue of the production of MMPs, which can degrade the extracellular matrix, release matrix-bound growth factors, and allow endothelial cells to invade the tumor during angiogenesis (Low-Marchelli et al., 2013).

A "CHEMOKINE-LIKE" FACTOR

A cytokine that is not a true chemokine but is considered to be "chemokine-like" is macrophage migration inhibitory factor (MIF), which a non-cognate ligand for both CXCR2 and CXCR4. The name of this cytokine may not be completely accurate, as studies have shown a role for MIF in the recruitment of monocytes in the pathogeneses of arthritis and glomerulonephritis (Bernhagen et al., 2007). Furthermore, it has been demonstrated that MIF is a critical molecule in vascular processes, and its expression is up-regulated in endothelial cells, smooth muscle cells, and macrophages during the development of atherosclerotic lesions in mice and humans (Bernhagen et al., 2007). Recently, investigators have also found that hypoxia-induced MIF recruits endothelial progenitor cells (EPCs) in a $CXCR4^+$dependent manner, suggesting a possible role for MIF in angiogenesis (Simons et al., 2011).

CONCLUDING REMARKS

Chemokines affect multiple signaling pathways of inflammatory diseases and tumor initiation and development including, cellular proliferation, survival and apoptosis, leukocyte recruitment, cellular migration/metastasis, and of course, angiogenesis. The newly appreciated complexity of the crosstalk between the chemokines, CXCL11 and CXCL12, and their receptors CXCR3, CXCR4, and CXCR7, illustrate the importance of additional studies in order to better understand the opposing and synergistic effects of pleiotropic chemokines and their promiscuous chemokine receptors. Like chemokines, macrophages also play multiple roles in inflammatory diseases and tumor progression. Thus, identifying the mechanisms by which macrophages are recruited to sites of inflammation or tumor, and exactly how these leukocytes influence angiogenesis may lead to better targeted therapeutic applications in patients with cancer and other inflammatory diseases involving vascular pathology.

ACKNOWLEDGMENTS

This work was supported in part by NIH Grant 1R01AI098833-01, DoD Grant CA111002, NIH/NCRR Clinical and Translational Science Award to the University of Florida (UL1 RR029890), and the Ocala Royal Dames Cancer Research project 00100297.

REFERENCES

Ahn, G. O., and Brown, J. M. (2009). Role of endothelial progenitors and other bone marrow-derived cells in the development of the tumor vasculature. *Angiogenesis* 12, 159–164. doi: 10.1007/s10456-009-9135-7

Airoldi, I., and Ribatti, D. (2011). Regulation of angiostatic chemokines driven by IL-12 and IL-27 in human tumors. *J. Leukoc. Biol.* 90, 875–882. doi: 10.1189/jlb.0511237

Allavena, P., Germano, G., Marchesi, F., and Mantovani, A. (2011). Chemokines in cancer related inflammation. *Exp. Cell Res.* 317, 664–673. doi: 10.1016/j.yexcr.2010.11.013

Asahara, T., Kawamoto, A., and Masuda, H. (2011). Concise review: circulating endothelial progenitor cells for vascular medicine. *Stem cells* 29, 1650–1655. doi: 10.1002/stem.745

Asahara, T., Murohara, T., Sullivan, A., Silver, M., Van Der Zee, R., Li, T., et al. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275, 964–967. doi: 10.1126/science.275.5302.964

Baay, M., Brouwer, A., Pauwels, P., Peeters, M., and Lardon, F. (2011). Tumor cells and tumor-associated macrophages: secreted proteins as potential targets for therapy. *Clin. Dev. Immunol.* 2011:565187. doi: 10.1155/2011/565187

Balkwill, F. (2004). The significance of cancer cell expression of the chemokine receptor CXCR4. *Semin. Cancer Biol.* 14, 171–179. doi: 10.1016/j.semcancer.2003.10.003

Ben-Baruch, A. (2012). The tumor-promoting flow of cells into, within and out of the tumor site: regulation by the inflammatory axis of TNFα and Chemokines. *Cancer Microenviron.* 5, 151–164. doi: 10.1007/s12307-011-0094-3

Bernhagen, J., Krohn, R., Lue, H., Gregory, J. L., Zernecke, A., Koenen, R. R., et al. (2007). MIF is a non-cognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. *Nat. Med.* 13, 587–596. doi: 10.1038/nm1567

Bingle, L., Brown, N. J., and Lewis, C. E. (2002). The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J. Pathol.* 196, 254–265. doi: 10.1002/path.1027

Bosco, M. C., Reffo, G., Puppo, M., and Varesio, L. (2004). Hypoxia inhibits the expression of the CCR5 chemokine receptor in macrophages. *Cell. Immunol.* 228, 1–7. doi: 10.1016/j.cellimm.2004.03.006

Bussolati, B., Grange, C., and Camussi, G. (2011). Tumor exploits alternative strategies to achieve vascularization. *FASEB J.* 25, 2874–2882. doi: 10.1096/fj.10-180323

Claesson-Welsh, L., and Welsh, M. (2013). VEGFA and tumour angiogenesis. *J. Int. Med.* 273, 114–127. doi: 10.1111/joim.12019

Collins, C. B., Mcnamee, E. N., Wermers, J. D., Lebsack, M. D., and Rivera-Nieves, J. (2010). Chemokine decoy receptor D6 in inflammatory bowel disease (IBD) and IBD-associated colon cancer. *Gut* 59, 151–152. doi: 10.1136/gut.2009.192708

Craig, M. J., and Loberg, R. D. (2006). CCL2 (Monocyte Chemoattractant Protein-1) in cancer bone metastases. *Cancer Metastasis Rev.* 25, 611–619. doi: 10.1007/s10555-006-9027-x

De Palma, M., Murdoch, C., Venneri, M. A., Naldini, L., and Lewis, C. E. (2007). Tie2-expressing monocytes: regulation of tumor angiogenesis and therapeutic implications. *Trends Immunol.* 28, 519–524. doi: 10.1016/j.it.2007.09.004

Domanska, U. M., Kruizinga, R. C., Nagengast, W. B., Timmer-Bosscha, H., Huls, G., De Vries, E. G., et al. (2013). A review on CXCR4/CXCL12 axis in oncology: no place to hide. *Eur. J. Cancer* 49, 219–230. doi: 10.1016/j.ejca.2012.05.005

Dubrac, A., Quemener, C., Lacazette, E., Lopez, F., Zanibellato, C., Wu, W. G., et al. (2010). Functional divergence between 2 chemokines is conferred by single amino acid change. *Blood* 116, 4703–4711. doi: 10.1182/blood-2010-03-274852

Egami, K., Murohara, T., Aoki, M., and Matsuishi, T. (2006). Ischemia-induced angiogenesis: role of inflammatory response mediated by P-selectin. *J. Leukoc. Biol.* 79, 971–976. doi: 10.1189/jlb.0805448

Fagiani, E., and Christofori, G. (2013). Angiopoietins in angiogenesis. *Cancer Lett.* 328, 18–26. doi: 10.1016/j.canlet.2012.08.018

Fokas, E., Mckenna, W. G., and Muschel, R. J. (2012). The impact of tumor microenvironment on cancer treatment and its modulation by direct and indirect antivascular strategies. *Cancer Metastasis Rev.* 31, 823–842. doi: 10.1007/s10555-012-9394-4

Gao, D., Nolan, D. J., Mellick, A. S., Bambino, K., Mcdonnell, K., and Mittal, V. (2008). Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis. *Science* 319, 195–198. doi: 10.1126/science.1150224

Gijsbers, K., Gouwy, M., Struyf, S., Wuyts, A., Proost, P., Opdenakker, G., et al. (2005). GCP-2/CXCL6 synergizes with other endothelial cell-derived chemokines in neutrophil mobilization and is associated with angiogenesis in gastrointestinal tumors. *Exp. Cell Res.* 303, 331–342. doi: 10.1016/j.yexcr.2004.09.027

Gomez, I., Pena, C., Herrera, M., Munoz, C., Larriba, M. J., Garcia, V., et al. (2011). TWIST1 is expressed in colorectal carcinomas and predicts patient survival. *PLoS ONE* 6:e18023. doi: 10.1371/journal.pone.0018023

Gordon, S., and Martinez, F. O. (2010). Alternative activation of macrophages: mechanism and functions. *Immunity* 32, 593–604. doi: 10.1016/j.immuni.2010.05.007

Halin, S., Rudolfsson, S. H., Van Rooijen, N., and Bergh, A. (2009). Extratumoral macrophages promote tumor and vascular growth in an orthotopic rat prostate tumor model. *Neoplasia* 11, 177–186.

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013

Hao, N. B., Lu, M. H., Fan, Y. H., Cao, Y. L., Zhang, Z. R., and Yang, S. M. (2012). Macrophages in tumor microenvironments and the progression of tumors. *Clin. Dev. Immunol.* 2012:948098. doi: 10.1155/2012/948098

Hernandez, L., Magalhaes, M. A., Coniglio, S. J., Condeelis, J. S., and Segall, J. E. (2011). Opposing roles of CXCR4 and CXCR7 in breast cancer metastasis. *Breast Cancer Res.* 13:R128. doi: 10.1186/bcr3074

Islam, S. A., Chang, D. S., Colvin, R. A., Byrne, M. H., Mccully, M. L., Moser, B., et al. (2011). Mouse CCL8, a CCR8 agonist, promotes atopic dermatitis by recruiting IL-5+ T(H)2 cells. *Nat. Immunol.* 12, 167–177. doi: 10.1038/ni.1984

Jodele, S., Chantrain, C. F., Blavier, L., Lutzko, C., Crooks, G. M., Shimada, H., et al. (2005). The contribution of bone marrow-derived cells to the tumor vasculature in neuroblastoma is matrix metalloproteinase-9 dependent. *Cancer Res.* 65, 3200–3208. doi: 10.1158/0008-5472.CAN-04-3770

Keeley, E. C., Mehrad, B., and Strieter, R. M. (2011). Chemokines as mediators of tumor angiogenesis and neovascularization. *Exp. Cell Res.* 317, 685–690. doi: 10.1016/j.yexcr.2010.10.020

Kiefer, F., and Siekmann, A. F. (2011). The role of chemokines and their receptors in angiogenesis. *Cell. Mol. Life Sci.* 68, 2811–2830. doi: 10.1007/s00018-011-0677-7

Kumar, A. H., Martin, K., Turner, E. C., Buneker, C. K., Dorgham, K., Deterre, P., et al. (2013). Role of CX3CR1 receptor in monocyte/macrophage driven neovascularization. *PLoS ONE* 8:e57230. doi: 10.1371/journal.pone.0057230

Kumar, A. H., Metharom, P., Schmeckpeper, J., Weiss, S., Martin, K., and Caplice, N. M. (2010). Bone marrow-derived CX3CR1 progenitors contribute to neointimal smooth muscle cells via fractalkine CX3CR1 interaction. *FASEB J.* 24, 81–92. doi: 10.1096/fj.09-132225

Lasagni, L., Francalanci, M., Annunziato, F., Lazzeri, E., Giannini, S., Cosmi, L., et al. (2003). An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. *J. Exp. Med.* 197, 1537–1549. doi: 10.1084/jem.20021897

Lee, H. W., Choi, H. J., Ha, S. J., Lee, K. T., and Kwon, Y. G. (2013). Recruitment of monocytes/macrophages in different tumor microenvironments. *Biochim. Biophys. Acta* 1835, 170–179.

Lewis, C. E., and Pollard, J. W. (2006). Distinct role of macrophages in different tumor microenvironments. *Cancer Res.* 66, 605–612. doi: 10.1158/0008-5472.CAN-05-4005

Liang, Z., Brooks, J., Willard, M., Liang, K., Yoon, Y., Kang, S., et al. (2007). CXCR4/CXCL12 axis promotes VEGF-mediated tumor angiogenesis through Akt signaling pathway. *Biochem. Biophys. Res. Commun.* 359, 716–722. doi: 10.1016/j.bbrc.2007.05.182

Lin, E. Y., Li, J. F., Gnatovskiy, L., Deng, Y., Zhu, L., Grzesik, D. A., et al. (2006). Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res.* 66, 11238–11246. doi: 10.1158/0008-5472.CAN-06-1278

Liu, G., and Yang, H. (2013). Modulation of macrophage activation and programming in immunity. *J. Cell. Physiol.* 228, 502–512. doi: 10.1002/jcp.24157

Low-Marchelli, J. M., Ardi, V. C., Vizcarra, E. A., Van Rooijen, N., Quigley, J. P., and Yang, J. (2013). Twist1 induces CCL2 and recruits macrophages to promote angiogenesis. *Cancer Res.* 73, 662–671. doi: 10.1158/0008-5472.CAN-12-0653

Lu, J., Pompili, V. J., and Das, H. (2011). Neovascularization and Hematopoietic Stem Cells. *Cell Biochem. Biophys.* doi: 10.1007/s12013-011-9298-x. [Epub ahead of print].

Lyden, D., Hattori, K., Dias, S., Costa, C., Blaikie, P., Butros, L., et al. (2001). Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat. Med.* 7, 1194–1201. doi: 10.1038/nm1101-1194

Maione, T. E., Gray, G. S., Petro, J., Hunt, A. J., Donner, A. L., Bauer, S. I., et al. (1990). Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. *Science* 247, 77–79. doi: 10.1126/science.1688470

Mantovani, A., Savino, B., Locati, M., Zammataro, L., Allavena, P., and Bonecchi, R. (2010). The chemokine system in cancer biology and therapy. *Cytokine Growth Factor Rev.* 21, 27–39. doi: 10.1016/j.cytogfr.2009.11.007

Mantovani, A., and Sica, A. (2010). Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr. Opin. Immunol.* 22, 231–237. doi: 10.1016/j.coi.2010.01.009

Mantovani, A., Sica, A., Allavena, P., Garlanda, C., and Locati, M. (2009). Tumor-associated macrophages and the related myeloid-derived suppressor cells as a paradigm of the diversity of macrophage activation. *Hum. Immunol.* 70, 325–330. doi: 10.1016/j.humimm.2009.02.008

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., and Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 25, 677–686. doi: 10.1016/j.it.2004.09.015

Moepps, B., Nuesseler, E., Braun, M., and Gierschik, P. (2006). A homolog of the human chemokine receptor CXCR1 is expressed in the mouse. *Mol. Immunol.* 43, 897–914. doi: 10.1016/j.molimm.2005.06.043

Motz, G. T., and Coukos, G. (2011). The parallel lives of angiogenesis and immunosuppression: cancer and other tales. *Nat. Rev. Immunol.* 11, 702–711. doi: 10.1038/nri3064

Mukaida, N., and Baba, T. (2012). Chemokines in tumor development and progression. *Exp. Cell Res.* 318, 95–102. doi: 10.1016/j.yexcr.2011.10.012

Murdoch, C., Muthana, M., Coffelt, S. B., and Lewis, C. E. (2008). The role of myeloid cells in the promotion of tumour angiogenesis. *Nat. Rev. Cancer* 8, 618–631. doi: 10.1038/nrc2444

Nagy, J. A., Chang, S. H., Shih, S. C., Dvorak, A. M., and Dvorak, H. F. (2010). Heterogeneity of the tumor vasculature. *Semin. Thromb. Hemost.* 36, 321–331. doi: 10.1055/s-0030-1253454

Nomiyama, H., Osada, N., and Yoshie, O. (2010). The evolution of mammalian chemokine genes. *Cytokine Growth Factor Rev.* 21, 253–262. doi: 10.1016/j.cytogfr.2010.03.004

Nozawa, H., Chiu, C., and Hanahan, D. (2006). Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12493–12498. doi: 10.1073/pnas.0601807103

Petit, I., Jin, D., and Rafii, S. (2007). The SDF-1-CXCR4 signaling pathway: a molecular hub modulating

neo-angiogenesis. *Trends Immunol.* 28, 299–307. doi: 10.1016/j.it.2007.05.007

Purhonen, S., Palm, J., Rossi, D., Kaskenpaa, N., Rajantie, I., Yla-Herttuala, S., et al. (2008). Bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumor growth. *Proc. Natl. Acad. Sci. U.S.A.* 105, 6620–6625. doi: 10.1073/pnas.0710516105

Qin, Q., Xu, Y., He, T., Qin, C., and Xu, J. (2012). Normal and disease-related biological functions of Twist1 and underlying molecular mechanisms. *Cell Res.* 22, 90–106. doi: 10.1038/cr.2011.144

Raica, M., Cimpean, A. M., and Ribatti, D. (2009). Angiogenesis in pre-malignant conditions. *Eur. J. Cancer* 45, 1924–1934. doi: 10.1016/j.ejca.2009.04.007

Raman, D., Sobolik-Delmaire, T., and Richmond, A. (2011). Chemokines in health and disease. *Exp. Cell Res.* 317, 575–589. doi: 10.1016/j.yexcr.2011.01.005

Reed, J. R., Stone, M. D., Beadnell, T. C., Ryu, Y., Griffin, T. J., and Schwertfeger, K. L. (2012). Fibroblast growth factor receptor 1 activation in mammary tumor cells promotes macrophage recruitment in a CX3CL1-dependent manner. *PLoS ONE* 7:e45877. doi: 10.1371/journal.pone.0045877

Risau, W. (1997). Mechanisms of angiogenesis. *Nature* 386, 671–674. doi: 10.1038/386671a0

Saederup, N., Chan, L., Lira, S. A., and Charo, I. F. (2008). Fractalkine deficiency markedly reduces macrophage accumulation and atherosclerotic lesion formation in CCR2-/- mice: evidence for independent chemokine functions in atherogenesis. *Circulation* 117, 1642–1648. doi: 10.1161/CIRCULATIONAHA.107.743872

Salcedo, R., Ponce, M. L., Young, H. A., Wasserman, K., Ward, J. M., Kleinman, H. K., et al. (2000). Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood* 96, 34–40.

Schioppa, T., Uranchimeg, B., Saccani, A., Biswas, S. K., Doni, A., Rapisarda, A., et al. (2003). Regulation of the chemokine receptor CXCR4 by hypoxia. *J. Exp. Med.* 198, 1391–1402. doi: 10.1084/jem.20030267

Sica, A., Saccani, A., Bottazzi, B., Bernasconi, S., Allavena, P., Gaetano, B., et al. (2000). Defective expression of the monocyte chemotactic protein-1 receptor CCR2 in macrophages associated with human ovarian carcinoma. *J. Immunol.* 164, 733–738.

Simons, D., Grieb, G., Hristov, M., Pallua, N., Weber, C., Bernhagen, J., et al. (2011). Hypoxia-induced endothelial secretion of macrophage migration inhibitory factor and role in endothelial progenitor cell recruitment. *J. Cell. Mol. Med.* 15, 668–678. doi: 10.1111/j.1582-4934.2010.01041.x

Singh, A. K., Arya, R. K., Trivedi, A. K., Sanyal, S., Baral, R., Dormond, O., et al. (2013). Chemokine receptor trio: CXCR3, CXCR4 and CXCR7 crosstalk via CXCL11 and CXCL12. *Cytokine Growth Factor Rev.* 24, 41–49. doi: 10.1016/j.cytogfr.2012.08.007

Soria, G., and Ben-Baruch, A. (2008). The inflammatory chemokines CCL2 and CCL5 in breast cancer. *Cancer Lett.* 267, 271–285. doi: 10.1016/j.canlet.2008.03.018

Struyf, S., Salogni, L., Burdick, M. D., Vandercappellen, J., Gouwy, M., Noppen, S., et al. (2011). Angiostatic and chemotactic activities of the CXC chemokine CXCL4L1 (platelet factor-4 variant) are mediated by CXCR3. *Blood* 117, 480–488. doi: 10.1182/blood-2009-11-253591

Tazzyman, S., Niaz, H., and Murdoch, C. (2013). Neutrophil-mediated tumour angiogenesis: subversion of immune responses to promote tumour growth. *Semin. Cancer Biol.* 23, 149–158. doi: 10.1016/j.semcancer.2013.02.003

Teicher, B. A., and Fricker, S. P. (2010). CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clin. Cancer Res.* 16, 2927–2931. doi: 10.1158/1078-0432.CCR-09-2329

Toulza, F., Eliaou, J. F., and Pinet, V. (2005). Breast tumor cell soluble factors induce monocytes to produce angiogenic but not angiostatic CXC chemokines. *Int. J. Cancer* 115, 429–436. doi: 10.1002/ijc.20705

Traves, P. G., Luque, A., and Hortelano, S. (2012). Macrophages, inflammation, and tumor suppressors: ARF, a new player in the game. *Mediators Inflamm.* 2012:568783. doi: 10.1155/2012/568783

Vandercappellen, J., Van Damme, J., and Struyf, S. (2011). The role of the CXC chemokines platelet factor-4 (CXCL4/PF-4) and its variant (CXCL4L1/PF-4var) in inflammation, angiogenesis and cancer. *Cytokine Growth Factor Rev.* 22, 1–18. doi: 10.1016/j.cytogfr.2010.10.011

Verbeke, H., Struyf, S., Laureys, G., and Van Damme, J. (2011). The expression and role of CXC chemokines in colorectal cancer. *Cytokine Growth Factor Rev.* 22, 345–358. doi: 10.1016/j.cytogfr.2011.09.002

White, G. E., and Greaves, D. R. (2009). Fractalkine: one chemokine, many functions. *Blood* 113, 767–768. doi: 10.1182/blood-2008-11-189860

Xu, L., Duda, D. G., Di Tomaso, E., Ancukiewicz, M., Chung, D. C., Lauwers, G. Y., et al. (2009). Direct evidence that bevacizumab, an anti-VEGF antibody, upregulates SDF1alpha, CXCR4, CXCL6, and neuropilin 1 in tumors from patients with rectal cancer. *Cancer Res.* 69, 7905–7910. doi: 10.1158/0008-5472.CAN-09-2099

Yamaguchi, K., Ogawa, K., Katsube, T., Shimao, K., Konno, S., Shimakawa, T., et al. (2005). Platelet factor 4 gene transfection into tumor cells inhibits angiogenesis, tumor growth and metastasis. *Anticancer Res.* 25, 847–851.

Yang, J., Mani, S. A., Donaher, J. L., Ramaswamy, S., Itzykson, R. A., Come, C., et al. (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* 117, 927–939. doi: 10.1016/j.cell.2004.06.006

Yoshimura, T., and Oppenheim, J. J. (2011). Chemokine-like receptor 1 (CMKLR1) and chemokine (C-C motif) receptor-like 2 (CCRL2); two multifunctional receptors with unusual properties. *Exp. Cell Res.* 317, 674–684. doi: 10.1016/j.yexcr.2010.10.023

Zhang, J., Patel, L., and Pienta, K. J. (2010). CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis. *Cytokine Growth Factor Rev.* 21, 41–48. doi: 10.1016/j.cytogfr.2009.11.009

Zlotnik, A., Burkhardt, A. M., and Homey, B. (2011). Homeostatic chemokine receptors and organ-specific metastasis. *Nat. Rev. Immunol.* 11, 597–606. doi: 10.1038/nri3049

Zlotnik, A., and Yoshie, O. (2000). Chemokines: a new classification system and their role in immunity. *Immunity* 12, 121–127. doi: 10.1016/S1074-7613(00)80165-X

Zlotnik, A., and Yoshie, O. (2012). The chemokine superfamily revisited. *Immunity* 36, 705–716. doi: 10.1016/j.immuni.2012.05.008

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 April 2013; paper pending published: 23 May 2013; accepted: 11 June 2013; published online: 05 July 2013.

*Citation: Owen JL and Mohamadzadeh M (2013) Macrophages and chemokines as mediators of angiogenesis. Front. Physiol. **4**:159. doi: 10.3389/fphys.2013.00159*

This article was submitted to Frontiers in Vascular Physiology, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 18 June 2013
doi: 10.3389/fphys.2013.00144

Macrophage-tumor cell interactions regulate the function of nitric oxide

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Tumor cell-macrophage interactions change as the tumor progresses, and the generation of nitric oxide (NO) by the inducible nitric oxide synthase (iNOS) plays a major role in this interplay. In early stages, macrophages employ their killing mechanisms, particularly the generation of high concentrations of NO and its derivative reactive nitrogen species (RNS) to initiate tumor cell apoptosis and destroy emerging transformed cells. If the tumor escapes the immune system and grows, macrophages that infiltrate it are reprogramed *in situ* by the tumor microenvironment. Low oxygen tensions (hypoxia) and immunosuppressive cytokines inhibit iNOS activity and lead to production of low amounts of NO/RNS, which are pro-angiogenic and support tumor growth and metastasis by inducing growth factors (e.g., VEGF) and matrix metalloproteinases (MMPs). We review here the different roles of NO/RNS in tumor progression and inhibition, and the mechanisms that regulate iNOS expression and NO production, highlighting the role of different subtypes of macrophages and the microenvironment. We finally claim that some tumor cells may become resistant to macrophage-induced death by increasing their expression of microRNA-146a (miR-146a), which leads to inhibition of iNOS translation. This implies that some cooperation between tumor cells and macrophages is required to induce tumor cell death, and that tumor cells may control their fate. Thus, in order to induce susceptibility of tumors cells to macrophage-induced death, we suggest a new therapeutic approach that couples manipulation of miR-146a levels in tumors with macrophage therapy, which relies on *ex vivo* stimulation of macrophages and their re-introduction to tumors.

Keywords: inducible nitric oxide synthase (iNOS), tumor cells, macrophage activation, apoptosis, angiogenesis, miR-146a, macrophage-induced death, macrophage therapy

IMMUNOEDITING: INTERPLAY BETWEEN MACROPHAGES AND TUMOR CELLS

Tumors arise when tissue cells accumulate genetic alterations/mutations that disrupt the tightly controlled cell growth and division systems (Hanahan and Weinberg, 2011), leading to uncontrolled proliferation of these cells and increased tumor mass. After overcoming intrinsic tumor-suppressor mechanisms (Vesely et al., 2011), the cells have to evade the immune system. In fact, most of the time the immune system succeeds in eliminating those aberrant cells, in a process once known as immunosurveilence (Dunn et al., 2004; Vesely et al., 2011). To better describe the complex interactions between tumor cells and the immune system, the term "immnoediting" has been coined (Dunn et al., 2004; Bui and Schreiber, 2007; Reiman et al., 2007; Schreiber et al., 2011) and consists of three stages; In the first stage (the elimination stage, previously known as immunosurveilence) immune cells destroy emerging transformed cells and prevent their development into a tumor. If this process is unsuccessful, there is a transition period to the second phase (equilibrium), where the immune system is able to contain but not eliminate the tumor. During equilibrium, the tumor cells are under constant immune pressure that eliminates many of the original variants but additional mutations may allow for new variants to be generated. Eventually, some variants may escape from the immune pressure triggering the third phase (escape), and becoming free to grow in an immunologically unrestricted manner. This sequence of events means that there is a constant and dynamic interplay between the tumor cells and the stroma immune cells, which continuously changes according to the shift in conditions. Among the immune cells, macrophages are the most prominent, as they infiltrate deep into the low oxygen tension (hypoxic) regions of the tumor and accumulate there, so that in some cases they can make up as much as 50% of the tumor mass (Murdoch et al., 2004; Mantovani et al., 2008).

Generally, macrophages are cells known to infiltrate tissues in order to combat and eradicate invading pathogens and tumor cells. Actually, they have additional tasks, including patrolling their surroundings and maintaining homeostasis, orchestrating tissue healing and repair and resolving inflammation. It is obvious that such opposing functions cannot be simultaneously performed by the same macrophage, and therefore, it was suggested that macrophages can be differentially activated depending on the signals they receive from their immediate microenvironment. Thus, macrophages display enormous plasticity (Stout and

Suttles, 2004; Stout et al., 2009), and can shift from one activation mode to another, unlike lymphocytes that remain committed to only one kind of activation. This concept has been extensively reviewed before (Mosser and Edwards, 2008; Murdoch et al., 2008; Martinez et al., 2009; Gordon and Martinez, 2010; Qian and Pollard, 2010) and will be only briefly mentioned here.

In recent years many studies have shown that during the escape phase macrophages become supportive and even critical to tumor progression, growth and metastasis, as they produce growth factors, cytokines and chemokines which are necessary for these processes. In order to escape immune killing, tumor cells activate several mechanisms to control the immune response, which include acquiring defects in the antigen processing and presentation pathways to facilitate evasion from adaptive immune recognition (Rabinovich et al., 2007), secretion of immunosuppressive mediators (e.g., TGFβ, IL-10, IL-13, PGE_2), and recruitment of regulatory immune cells (Bui and Schreiber, 2007; Rabinovich et al., 2007).

In this review we focus on the interplay between tumor cells and macrophages during different stages of tumor development, as manifested by the complex roles of a single molecule—inducible nitric oxide synthase (iNOS) and its product nitric oxide (NO). We review the conditions that regulate its expression and activity in different cell types and in changing microenvironments, and explore the significance of these differences. Finally, we describe possible future approaches that explore whether these interactions can be modulated in order to manipulate expression of iNOS or its activity, and to effectively enhance tumor eradication.

NO PRODUCTION IN DIFFERENT TYPES OF MACROPHAGE ACTIVATION

Generally, three types of macrophage activation can be described. Classically activated or M1 macrophages are activated by ligands of toll-like receptors (TLRs) and pro-inflammatory cytokines (e.g., interferon γ—IFNγ, tumor necrosis factor α—TNFα, interleukin 1β—IL-1β), they activate the Th1 immune response and secrete high amounts of pro-inflammatory mediators that kill the invading pathogens or tumor cells, such as the cytotoxic TNFα and NO. In fact, the high expression of the iNOS that produces NO is the hallmark of these macrophages (Mosser, 2003; Mosser and Zhang, 2008). Alternatively or M2 activated macrophages are activated by and secrete anti-inflammatory mediators (e.g., IL-10, IL-13, tumor growth factor β—TGFβ, and prostaglandin E_2—PGE_2), which together generate a microenvironment that suppresses the activity of M1 macrophages and Th1 lymphocytes. M2 macrophages are involved mainly in homeostasis, tissue remodeling and wound healing, as they remove cellular debris, support phagocytosis (by expressing scavenger receptors. the mannose receptor CD206), and deposit extracellular matrix (ECM) proteins (e.g., fibronectin). M2 macrophages express high levels of arginase-I, which produces ornithine, the precursor of collagen. Arginase-I also competes with iNOS for their common substrate L-arginine, and prevents NO production (Martinez et al., 2009; Gordon and Martinez, 2010). Regulatory macrophages, a third type of activation, can be activated by TLR and immune complexes, by anti-inflammatory cytokines or mediators (e.g., adenosine), or by phagocytosis of apoptotic cells (Mosser, 2003; Mosser and Edwards, 2008). While several subtypes of regulatory activations have been identified, all types inhibit pro-inflammatory reactions, partly by secreting anti-inflammatory cytokines (e.g., IL-10, TGFβ). As macrophages exhibit great plasticity, they may exhibit additional types of activation within the range defined by these three main types to yield many different sub-populations with different roles and functions (Mosser and Edwards, 2008).

Three main macrophage subsets have been identified within the tumor mass and can be localized in different niches of the tumor (Lewis and Pollard, 2006). Tumor-associated macrophages (TAMs) support tumor progression and metastasis, as they secrete pro-angiogenic growth factors (e.g., vascular endothelial cell growth factor—VEGF), and matrix metalloproteinases (MMPs). TAMs infiltrate deep into the tumor and are found in perinecrotic and hypoxic areas. In addition to the secretion of many pro-angiogenic factors (e.g., FGF2, IL-8, PDGF, VEGF, MMP-7, and MMP-12), TAMs also use several mechanisms to render M1 macrophages as well as $CD4^+$ and $CD8^+$ T cells non-responsive to tumor-specific antigens, including secretion of immunosuppressive mediators (e.g., IL-10) and depletion of L-arginine by the activity of arginase-I (Coffelt et al., 2009). Moreover, TAMs are necessary for metastasis, and their ability to secrete EGF together with the ability of tumor cells to secrete M-CSF/CSF-1 stimulate mutual migration in both cell types (Wyckoff et al., 2004; Condeelis and Pollard, 2006; Coffelt et al., 2009; Hernandez et al., 2009).

Tie2-expressing monocytes (TEMs), that unlike TAMs reside very close to blood vessels (Venneri et al., 2007), are similar to TAMs in their support for tumor progression and metastasis via pro-angiogenic growth factors such as VEGF and MMPs. In fact, TEMs are essential for tumor progression, as their depletion markedly inhibits tumor angiogenesis (De Palma et al., 2005; Venneri et al., 2007). In addition, TEMs are potent immunosuppressive cells, as they can secrete high levels of IL-10, suppress T cell proliferation and promote the expansion of regulatory T cells (Treg) (Coffelt et al., 2011).

In tumor-bearing mice and humans, expanded populations of myeloid-derived suppressor cells (MDSCs) are found within the tumors, spleen and bone marrow in proportion to the tumor size. MDSCs are a mixture of immature granulocytic and monocytic cells, and monocytic MDSCs belong to the regulatory macrophages. MDSCs are triggered by a combination of IFNγ and IL-13, and secrete IFNγ, IL-13, IL-10, and TGFβ, which help them suppress Th1 cell-mediated immune response, induce regulatory T cells and inhibit M1 macrophages (Bronte, 2009; Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg and Sinha, 2009). Production of NO and peroxynitrite help MDSCs to nitrate the TCR and CD8 molecules on cytotoxic T cells, inhibiting the ability of the latter to bind to MHC class I molecules and rendering them non-responsive (Nagaraj et al., 2007; Nagaraj and Gabrilovich, 2008).

TAMs, TEMs, and MDSCs express similar activation markers, which place them between regulatory macrophages and M2 macrophages (De Palma et al., 2007; Mosser and Edwards, 2008; Murdoch et al., 2008). More accurately, these macrophages

exhibit a mixed expression profile, of both M1 and M2 markers. In this respect, TEMs are considered more M2-skewed than TAMs, as they express more arginase-I but less iNOS (Pucci et al., 2009), whereas MDSCs express both arginase-I and iNOS (Corzo et al., 2010). It is possible that these subsets represent different linages that develop separately (Pucci et al., 2009), or they may gradually progress from one to the other, as they migrate from the blood vessels into the perinecrotic areas and continue to be polarized or reprogrammed by the local tumoral microenvironment that consists of a gradient of cytokines and hypoxia, and by the interactions with the tumor cells.

THE MULTIPLE BIOLOGICAL ROLES OF NO IN TUMORS

NO is a small, short-lived, lipophilic gas molecule, which can easily cross membranes, and rapidly reacts with oxygen or superoxide to generate the derivatives that exert its biological activity. NO has been shown to both promote and inhibit tumor growth and metastasis. Although first recognized as a cytotoxic molecule that serves as a major killing mechanism of macrophages during pathogen infection or tumor cell killing, it also functions as a regulator of wound healing, tissue repair and suppression of the immune response-properties required to promote tumor growth. In fact, the different levels of iNOS expression in TAMs, TEMs, and MDSCs suggest multiple roles.

NO is produced by three isoforms of nitric oxide synthase (NOS) that convert L-arginine to L-citrulline. The endothelial (eNOS/NOS3) and neuronal (nNOS/NOS1) isoforms produce low amounts of NO (in the pM-nM range), and produce only a small fraction of the total NO in tumors. The bulk of NO in tumors is produced by the high output inducible isoform (iNOS/NOS2), which is strongly induced in macrophages and in tumor cells, and produces high concentrations of NO (in the μM range) (Xie and Nathan, 1994). It is important to note that unlike other inflammatory mediators that need to be enzymatically modulated or degraded, NO can chemically and directly react with other molecules (e.g., oxygen, superoxide) to produce multiple derivatives. Some of these derivatives are relatively stable (e.g., nitrites, hydroxylamine), and some are reactive nitrogen species (RNS) (e.g., peroxynitrite, nitrogen dioxide, nitroxyl) that are also biologically active (Donzelli et al., 2006). More details on the complex NO chemistry can be found elsewhere (Lechner et al., 2005; Wink et al., 2011). Since it is very difficult to separate between the effects of NO and its active derivatives, we will refer to them collectively as NO/RNS.

Depending on their concentrations, NO/RNS react with DNA, proteins and lipids and can act either as a signaling molecule that initiates signaling pathways or as a molecule that causes damage. Depending on the balance with other ROS, especially superoxide, NO/RNS may deaminate the DNA bases guanine, cytosine and adenine, causing DNA breaks and mutations, or it can affect proteins in one of four ways: (1) oxidation of metal prosthetic groups (heme or non-heme); (2) nitration, the covalent attachment of a nitro group (Tyr–NO_2) to tyrosine and tryptophan residues; (3) S-nitrosylation of thiol and amine groups, which covalently attaches NO to form a weak and reversible S-nitrosothiol (S-NO) bond; (4) oxidation of thiol groups in cysteine and methionine residues, that yield intramolecular disulfide bonds (-S-S-), cysteine sulfenic acid (R-S-OH), sulfinic acid (R-SO_2H) or sulfonic acids (R-SO_3H) (Lala and Chakraborty, 2001; Leon et al., 2008). These post-translational modifications can potentially activate or inhibit target proteins, with different biological consequences. The final biological outcome depends on the NO concentrations produced, on the cellular redox state and bioavailability of other ROS, on the cellular location of production, on the distance of the impacted proteins from the generated NO, and on the cell type (Leon et al., 2008). Research conducted with NO donors revealed threshold concentrations of NO/RNS that are needed to activate specific pathways. For example, 50 nM of NO were sufficient to phosphorylate ERK, 100 nM stabilized HIF-1α and activated the Akt pathway, more than 300 nM were required to cause DNA damage and induce p53 and 1 μM was considered nitrosative stress (Wink et al., 2011). In tumors, NO was described to have both pro- and anti-tumoral effects, depending first and foremost on its concentrations (summarized schematically in **Figure 1**). As these aspects have been extensively reviewed elsewhere (Lala and Chakraborty, 2001; Lechner et al., 2005; Lancaster and Xie, 2006; Weigert and Brune, 2008), we will only briefly mention these here.

TUMOR INHIBITING ACTIVITIES

In general, high concentrations of NO/RNS can arrest cell cycle (cytostatic effect) or induce death, whereas low concentrations may protect cells from death. In fact, generation of high levels of NO/RNS is a very effective tool to induce death, and macrophages use it as a major weapon in their arsenal against invading pathogens and tumor cells (Weigert and Brune, 2008). High levels of NO/RNS post-transnationally modify death-related target proteins, and could mediate inhibition of cellular respiration in target cells, leading to their cell cycle arrest.

Modification of death receptors of the TNFα superfamily (e.g., Fas, TRAIL, and TNFRI, DR4, and DR5), or of mitochondrial targets that affect the mitochondrial respiratory chain and its outer membrane permeability leading to the release of cytochrome c and initiation of apoptosis, are the two main pathways leading to cell death [extensively reviewed in Lechner et al. (2005), Jeannin et al. (2008), Leon et al. (2008)]. Thus, S-nitrosylation of the YY1 transcription factor alleviates its suppression on the Fas promoter, resulting in increased apoptosis, and NO-donors enhance apoptosis by increasing the expression of TNFα receptors. NO/RNS can bind to the heme-copper center of the reduced form of cytochrome c oxidase, compete for the binding of oxygen, and cause inhibition of the mitochondrial respiratory chain and finally increased mitochondrial membrane permeability and release of cytochrome c (Brune, 2003; Jeannin et al., 2008).

Additional mechanisms may support initiation of apoptosis. For example, NO/RNS may enhance phosphorylation of serine residue 15 of the wild type p53, causing its activation and increased nuclear retention, thereby initiating apoptosis (Brune, 2003; Jeannin et al., 2008), as well as transiently and reversibly down-regulating mdm2, thus contributing to p53 activation (Brune, 2003). NO/RNS in amounts that favor generation of peroxynitrite and DNA damage, lead to accumulation of nitrated p53, improve its DNA binding and cause apoptosis (Leon et al., 2008). Another example is the S-nitrosylation of the p50 subunit

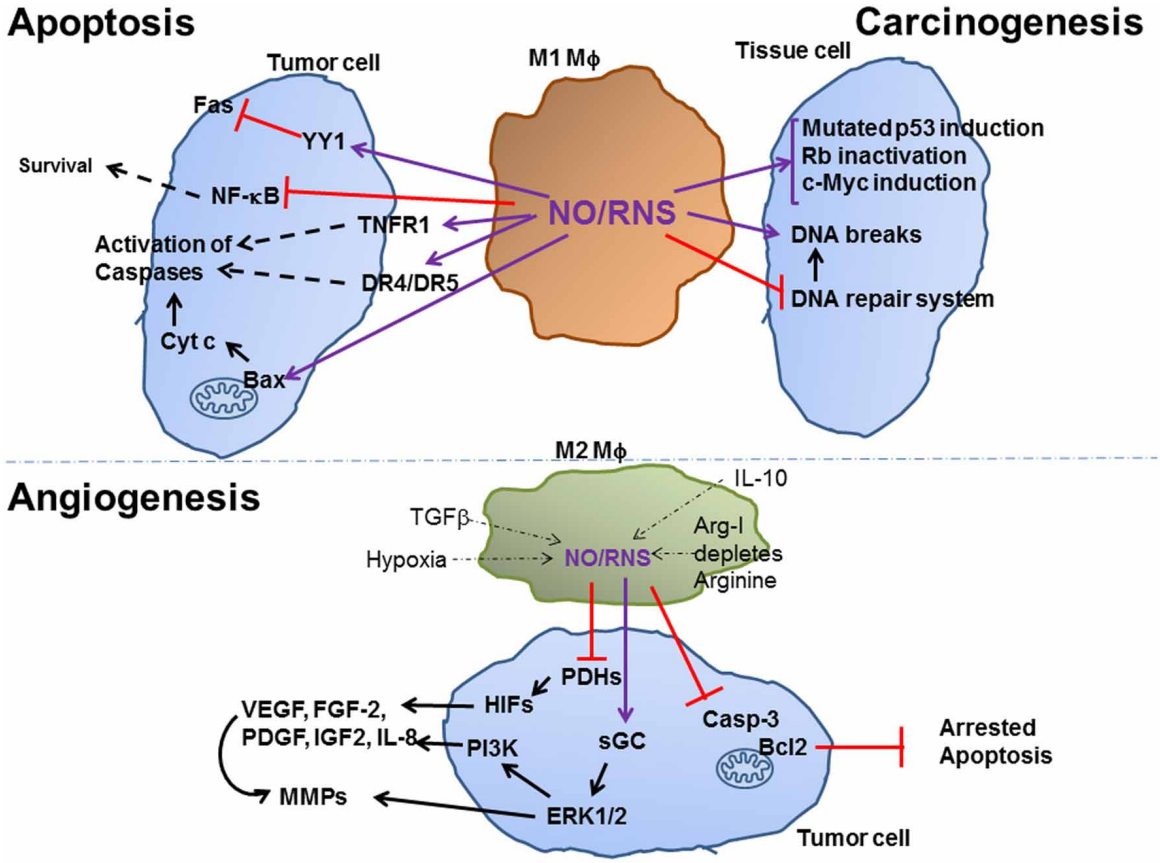

FIGURE 1 | Effect of Macrophage NO on tumor carcinogenesis, apoptosis and angiogenesis. M1-activated macrophages that produce high amounts of NO/RNS help carcinogenesis by increasing DNA breaks and mutations, arresting the DNA repair system and inducing oncogenes or inactivating tumor suppressor genes. High levels of NO/RNS may also drive apoptosis of established tumor cells, by modifying death receptors an increasing their expression, by inhibiting NF-kB and by enhancing cytochrome c release from the mitochondria. Low levels of NO/RNS produced in different subsets of M2-activated macrophages, and are reduced due to the effects of inhibitory microenvironmental factors, such as hypoxia. Hypoxia and NO/RNS stabilize the HIF family of transcription factors, and activate the MAPK ERK1/2 and the PI3K pathways, thus inducing the expression of pro-angiogenic factors, as described in the text. Purple arrows indicate post-translation modification (e.g., S-nitrosylation) of proteins, black arrows indicate activation of a protein/process, dashed arrows indicate a multi-step process, thin dashed arrows indicate the inhibitory effects of microenvironmental factors on NO/RNS production.

of NF-κB on cysteine residue 62 that inhibits its DNA binding activity and reduces NF-κB activity, which is generally considered an anti-apoptotic factor.

TUMOR PROMOTING ACTIVITIES

When discussing the tumor-promoting activities of NO/RNS, it is necessary to distinguish between carcinogenic activities that promote tumor generation, and activities that support a pre-exiting tumor at the stage of escape from the immune system.

Carcinogenesis

Chronic inflammation is linked to tumors and is recognized as a predisposing factor for malignant transformation of tissue cells (Kundu and Surh, 2008). In particular, the high amounts of ROS and RNS that are generated by recruited macrophages and neutrophils, as part of their killing mechanisms, play an important role. Since NO/RNS are lipophilic and can easily cross membranes, tissue cell DNA is exposed to the high concentrations, which may oxidize and/or deaminate the DNA bases, especially during transcription or replication where single strand DNA is more prevalently found. This may result in DNA breaks, DNA base modifications or DNA cross-links, which cause mutations, and may activate oncogenes or deactivate tumor suppressor genes (Lechner et al., 2005; Kundu and Surh, 2008). In addition, NO/RNS-driven protein modifications such as S-nitrosylation or nitration may inhibit proteins belonging to the DNA repair systems and hamper attempts to correct mutations. Thus, NO/RNS drive genomic instability.

In addition, there are many isolated examples for NO-driven protein modifications that further explain the carcinogenic effects of NO/RNS. For example, a negative feedback loop exists between iNOS and p53. NO activates wild-type p53, which is itself a negative regulator of iNOS that binds to its promoter and inhibits iNOS transcription. However, in tumors, p53 is often mutated and cannot inhibit iNOS expression (Lechner et al., 2005). Thus, in a model of chronic inflammation in p53 knockout mice,

increased NO production accelerated spontaneous tumor development, compared to the control mice (Hussain et al., 2008). NO-driven hyperphosphorylation of retinoblastoma (Rb) inactivated this tumor suppressor gene in a model of mouse colitis, (Ying et al., 2007). High amounts of NO induced the expression of c-Myc in a breast cancer cell line (Glynn et al., 2010), and activated EGFR and Src in estrogen receptor negative breast cancer patients through S-nitrosylation (Switzer et al., 2012). All of these examples provide a link between chronic inflammation, sustained production of NO/RNS and carcinogenesis.

In the escape phase

When tumor cells are already at the escape phase, they employ many pathways to maintain low levels of NO/RNS, as low amounts of NO/RNS are actually beneficial to the tumor cells. Anti-inflammatory mediators in the tumor microenvironment (e.g., TGFβ) reduce transcription of iNOS mRNA and effectively lower production of NO. Arginase-I that is highly expressed by the TAMs and MDSCs depletes L-arginine and leaves insufficient amounts of this common substrate for iNOS activity (Heller, 2008; Wink et al., 2011). In addition, if NO is secreted from TAMs, it is likely to be captured by red blood cells, where it S-nitrosylates their glutathione and hemoglobin, resulting in additional decrease in NO/RNS concentrations (Heller, 2008). Moreover, although hypoxia increases the expression of the iNOS mRNA and protein through the transcription factors HIF-1α and NF-κB, the hypoxic microenvironment actually inactivates the enzyme activity (Melillo et al., 1996), either because of the lack in the enzyme substrate or due to disruption of its protein–protein interactions with α-actinin-4 (Daniliuc et al., 2003). Collectively, these mechanisms ensure that only low amounts of NO/RNS are generated within the tumoral microenvironment.

Low amounts of NO/RNS are anti-apoptotic and beneficial for tumor cells. S-nitrosylation of caspases, especially caspase-3, inhibits the enzymes, and blocks apoptosis (Leon et al., 2008). S-nitrosylation of the FLIP adaptor protein prevents its Fas-induced ubiquitination and degradation and enables it to exert its anti-apoptotic activity (Iyer et al., 2008). Similarly, S-nitrosylation of Bcl2 also protected this protein from ubiquitination and degradation (Iyer et al., 2008). Thus, low amounts of NO/RNS which activate S-nitrosylation of proteins may be a general mechanism to prevent degradation of anti-apoptotic proteins and protect cells from death.

The role of NO in Angiogenesis. Angiogenesis, the process in which vascular endothelial cells proliferate and reorganize to form new vessels sprouting from pre-existing blood vessels, is essential for the growth of most primary tumors and their subsequent metastasis. Hypoxic core regions in tumors, which lack oxygen and nutrients, initiate the process of angiogenesis to generate growth of new blood vessels into the tumor. Many pro-angiogenic factors, including the most potent regulator and pivotal mediator VEGF, as well as FGF-2, PDGF, IGF2, TGFβ, and IL-8, are all induced by hypoxia inducible factor 1 or 2, which are transcription factors that bind to the hypoxia response element (HRE) located in the promoters of these genes (Black et al., 2008; Wink et al., 2011; Chowdhury et al., 2012).

Both hypoxia (<5% O_2) and NO/RNS can stabilize HIF-1α and HIF2α Both HIF-α subunits are constitutively transcribed and translated, but immediately directed for degradation in normoxia, through their hydroxylation of proline residues by the prolyl hydroxylases (PHDs) that rely on oxygen as their substrate. This hydroxylation recruits the von Hippel Lindau (VHL) protein, which has an E3 ubiquitin ligase activity that marks HIF-α subunits for degradation in the proteasome. Hypoxia inactivates PHDs due to the limited oxygen substrate, and therefore stabilizes the HIF-α subunits, allowing their heterodimerization with the HIF-1β subunit (Nizet and Johnson, 2009; Walmsley et al., 2009; Rahat et al., 2011). Low levels of NO/RNS can also stabilize HIF proteins by inactivating PHDs through oxidation of their non-heme Fe^{+2}-group, thereby causing reduced hydroxylation of HIF-1α and its accumulation even in normoxic regions of the tumor, close to the rims (Kimura et al., 2000, 2001).

Low amounts of NO further promote the induction of these aforementioned pro-angiogenic genes by activating guanylate cyclase and increasing cGMP levels, which help phosphorylate the MAP kinases ERK1/2 and activate PI3K/Akt, that activate additional transcription factors that are needed for the induction of the factors (Dulak and Jozkowicz, 2003; Ridnour et al., 2006). Such pro-angiogenic factors directly affect endothelial cells, as they are growth factors needed for their survival and proliferation, as well as for their spatial reorganization into tube-like formation (Ridnour et al., 2006).

While helping to induce pro-angiogenic factors, NO/RNS suppress the expression of thrombospondin-1 (Tsp1) (Ridnour et al., 2005), which limit angiogenesis by reducing the migration and proliferation of endothelial cells. This cross-talk between NO and Tsp1 is regulated by the concentrations of NO, as low NO levels down-regulate Tsp-1 expression, and increased levels of Tsp-1 inhibit the pro-angiogenic effects of NO (Ridnour et al., 2006).

Low levels of NO/RNS can directly and indirectly via VEGF enhance angiogenesis by activating MMP-1, MMP-9, and MMP-13 (Ridnour et al., 2007; Ziche and Morbidelli, 2009), MMPs are critical for angiogenesis, as they degrade components of the ECM and pave the way for migration of endothelial cells into the tumor, and of tumor cells out of the tumor to the nearest blood vessel. High levels of MMPs, particularly MMP-9, release and activate VEGF that is trapped by the ECM, and allow migration of endothelial cells, as well as leukocytes and metastatic tumor cells. In addition to its direct pro-angiogenic properties, VEGF is also a regulator of MMP-9, thus creating a positive feedback loop whereby MMP-9 and VEGF enhance each other (Hollborn et al., 2007). Low levels of NO/RNS control MMPs by activating JNK and NF-κB (Yang et al., 2011), and simultaneously down-regulate MMP's endogenous inhibitor TIMP-2 (Ziche and Morbidelli, 2009). Reduced levels of TIMP-2 not only allow the activity of MMPs, but are also pro-angiogenic, independently of their effect on MMPs (Lahat et al., 2011).

Thus, low NO/RNS levels enable multiple paths for angiogenesis, and shift the balance between pro- and anti-angiogenic factors to enhance angiogenesis.

Immune evasion. NO/RNS further contribute to the inhibition of anti-tumor immune responses and the ability of tumors

to evade the immune system by increasing T cell apoptosis, and by nitrating TCR on CD8+ T cells, thereby inhibiting their ability to kill antigen-specific tumor cells (Ostrand-Rosenberg and Sinha, 2009; Jia et al., 2010). A recent paper now describes an additional role for tumor-produced NO/RNS in attracting MDSCs and inducing their function (Jayaraman et al., 2012), thus enhancing immunosuppression and helping the tumors to evade immune recognition. This study further illustrates the importance of the cell type producing NO/RNS, and its critical role in mediating tumor cell-macrophage interactions.

Thus, tumor cells have a vested interest to lower NO concentrations in the tumor microenvironment. They employ different strategies, including the secretion of immunosuppressive cytokines (e.g., IL-10, TGFβ and PGE_2), the use of the hypoxic microenvironment that inactivate iNOS activity, and the depletion of L-arginine by arginase-I, to reduce NO production in the infiltrating macrophages. By doing so, tumor cells reprogram macrophages to ensure their pro-angiogenic activation, thus "enslaving" them to the tumor needs.

REGULATION OF iNOS EXPRESSION AND NO ACTIVITY

The regulation of iNOS expression and its activity have been extensively reviewed before (Alderton et al., 2001; Aktan, 2004; Pautz et al., 2010) and we will only briefly describe it here. The main regulatory checkpoint on iNOS expression is usually considered to be transcriptional. In mouse, stimulation by lipopolysaccharide (LPS) or by one of the pro-inflammatory cytokines (e.g., IL-1β, TNFα, IFNγ) is sufficient to induce high amounts of iNOS, whereas in human cells a mixture of several stimuli is needed to achieve iNOS induction (Xie and Nathan, 1994). These species-dependent differences were explained by the many differences found between the human and mouse iNOS promoters (Xie and Nathan, 1994; De Vera et al., 1996). The human promoter is longer and more complex than the mouse promoter, and consists of many binding sites for transcription factors that mediate both enhancement and inhibition of iNOS transcription, such as AP-1, C/EBPβ, EGFR-STAT3, HMGA1, p53, KLF6, five NF-κB sites, Oct-1, two binding sites for IRF-1/STAT-1α, HIF-1, Tcf-4, YY1 and many more (Taylor et al., 1998; Pautz et al., 2010). Only some of these sites can be found in the mouse promoter, which is shorter, and contains proximal and a distal regulatory regions that include mostly NF-κB and IRF-1 binding sites that mediate induction by LPS and IFNγ, respectively. Because of these differences, it was suggested that iNOS effects in mouse tumor models are different than in human tumors, as human cells tend to express lower levels of iNOS and generate less NO (Ambs and Glynn, 2011). However, high amounts of iNOS can be expressed in human cells, provided that a sufficiently strong stimulation is introduced consisting of a mixture of several cytokines *in vitro*, or during inflammation *in vivo* (Xie and Nathan, 1994; Albina, 1995). Furthermore, the hypoxic microenvironment in the tumor dictates a reduced production of NO, regardless of the high expression of the protein (Melillo et al., 1996; Daniliuc et al., 2003). Thus, we maintain that NO concentrations in the tumor are reduced in all species in correlation to the tumor size, indicating that NO production in large, hypoxic tumors is reduced while iNOS protein may be highly expressed in the tumor cells and infiltrating macrophages (Perske et al., 2010). Therefore, the role of iNOS protein expression as a prognostic indicator must be re-examined.

The cytokine network that regulates tumor cell-macrophage interactions is quite complex. In addition to the anti-inflammatory microenvironment (e.g., TGFβ, IL-10, and PGE_2) that invokes immunosuppression and reprograms macrophage toward M2 activation, pro-inflammatory cytokines (e.g. TNFα, IL-1β and IFNγ) are also present, albeit in relatively low concentrations. At such levels, these cytokines serve to induce adhesion molecules, MMPs, VEGF, and even COX-2 and PGE_2 production (Dinarello, 2006, 2010). Another microenvironmental factor is the presence of apoptotic cells that release many factors, including shingosine-1-phosphate (S1P) that is taken up by TAMs and repolarizes them toward M2 activation. M2 activated macrophages increase the expression of arginase-I, which changes their iNOS/arginase-I ratio and reduces their ability to produce NO/RNS (Weigert and Brune, 2008). A special role was highlighted for CSF-1, which is secreted from tumor cells and helps recruit macrophages and sustain them in the tumoral microenvironment, and to EGF, which is secreted from the infiltrating macrophages and serves to induce tumor cell migration and invasion (Hernandez et al., 2009). In respect to iNOS regulation, these central mediators also affect its expression, as macrophage EGF induces iNOS in tumor cells (Lo et al., 2005) and tumor cell CSF-1 induces iNOS in macrophages (Lin et al., 2010).

Another important checkpoint is the stability of the iNOS mRNA, which is mediated primarily by the AU-rich elements (ARE) found in the 3′-UTR regions of the transcript. Different RNA binding proteins compete for the binding to the 3′-UTR of iNOS mRNA, including HuR which usually stabilizes mRNAs and is increased upon cytokine induction, and KSRP and tristetraprolin (TTP), which usually mediate destabilization (Pautz et al., 2010). In murine cells, iNOS mRNA degradation was enhanced by TGFβ, and was mediated by the RNA binding proteins PTB (hnRNP I) and hnRNP L (Pautz et al., 2010). Thus, the balance between these proteins may mediate cell type-specific regulation of iNOS expression.

Translation of iNOS protein may be inhibited by small, non-coding RNA molecules known as microRNAs (miRNA). However, there is no direct evidence for the binding of specific miRNAs to iNOS mRNA. One report mentions the indirect translational inhibition of iNOS mRNA through the inhibition of the suppressor of cytokine signal (SOCS-1) mRNA by miR-155 (Wang et al., 2009), and we (Perske et al., 2010) and others (Dai et al., 2008) have shown the involvement of miR-146 in iNOS regulation.

Finally, the activity of the iNOS enzyme is also tightly regulated. Since the enzyme requires L-arginine as its substrate, arginine availability, transport or consumption may have profound implications on iNOS activity. Likewise, mechanisms regulating the availability of additional co-factors, like tetrahydrobiopterin (BH4), also affect iNOS activity (Pautz et al., 2010). The activity of iNOS demands that the protein is homodimerized to ensure

correct electron transfer. Protein–protein interactions with additional proteins, such as NAP110 (Ratovitski et al., 1999b) and kalirin (Ratovitski et al., 1999a) were shown to inhibit iNOS activity, whereas other proteins, such as rac2 (Kuncewicz et al., 2001) and α-acitinin-4 (Daniliuc et al., 2003) are required for its activity. The latter two proteins ensure that iNOS is properly localized at the cortical zone, just underneath the plasma membrane, and similar to the other NOS isoforms, enable its activity at this cellular compartment. Disruption of this interaction (e.g., by hypoxia) displaces the enzyme back to the cytoplasm and renders it inactive.

NO PRODUCTION BY TUMOR CELLS

Tumor cells, and not only macrophages, can induce iNOS expression and NO production. However, the potential biological relevance of iNOS expression in different malignant human tumors is still controversial, mostly because of technical reasons. Expression of iNOS is often determined by immunohistochemistry, western blot analysis or by real-time RT-PCR—all of which are basically semi-quantitative approaches. Most times, these techniques are applied on paraffin-embedded archival specimens, but these may produce unreliable results due to mRNA degradation in the paraffin-embedded blocks, or due to the recently emerging observations that iNOS protein expression does not necessarily correspond to NO production. Moreover, different ways to score iNOS immune reactivity (e.g., % of positive cells and/or intensity of staining) make comparison of these studies difficult. Measurement of the activity of the protein is thus restricted to fresh tissues, using primarily the indirect Griess reaction to measure accumulation of nitrates and nitrites (Cianchi et al., 2004), or the direct approach of measuring the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline (Koh et al., 1999; Franchi et al., 2006). Another indirect approach to indicate iNOS activity is the immunohistochemical detection of nitrotyrosinated proteins (Goto et al., 1999; Gochman et al., 2012) or 8-nitro-guanine DNA adducts (Chaiyarit et al., 2005; Ma et al., 2006) that can also be applied on paraffin embedded tumor specimens. However, these indirect approaches do not quantify the extent of iNOS activity, they may be influenced by high activity of other NOS isoforms or by the generation of other RNS (e.g., hypochlorous acid and nitrites that may also nitrotyrosinate proteins) (Radi, 2004), and they are very difficult to compare due to the use of different antibodies or different staining protocols.

Bearing in mind those difficulties, we have tried to critically review the literature, asking whether iNOS expression is correlated with tumoral grade and stage and with poor prognosis, and whether it is limited to macrophages or to tumor cells in specific types of cancer. **Table 1** present the conflicting results of this comparison, and emphasizes how poorly understood the role of NO in tumor biology remains.

In certain types of cancer (e.g., gastric cancer, melanoma) increased iNOS expression is found to be associated with tumor stage and grade or with tumor progression toward metastases, as well as with poor prognosis. In contrast, in other types of cancer (e.g., ovarian cancer), iNOS expression is reduced with tumor progression and with poor prognosis. Studies of some tumor types (e.g., colorectal, breast, brain, lung, and cervical cancers) are controversial, indicating either increased or reduced iNOS expression as tumor progresses, whereas in yet other types of cancer (e.g., bladder carcinoma, pancreatic, cervical cancers) positive and even strong iNOS expression was not correlated with either grade/stage or with prognosis. However, in all the studies we found (**Table 1**), moderate or strong expression of iNOS could be detected in the immunohistochemical images within stromal or inflammatory infiltrating cells, which in some studies were even identified as macrophages. Macrophage iNOS expression, however, was not correlated with prognosis, survival rates, invasiveness or tumor recurrence after therapy.

Evidently, these conflicting results reflect our lack of understanding of the many roles NO plays within the tumor, so that we can only speculate on what may be happening. These results might indicate a different role for iNOS expression in macrophages vs. tumor cells. Macrophages in the tumor stroma exhibit strong iNOS expression regardless of tumor grade and stage, and may produce high levels of NO/RNS that are gradually diminished as they infiltrate the hypoxic core of the tumor. The same is probably true for the tumor cells, and we can assume that tumor cells that are close to the hypoxic core produce less NO/RNS. Thus, the ability of the tumor microenvironment to uncouple iNOS expression and NO production (e.g., via hypoxia) may result in a gradient of NO/RNS concentrations and make it very difficult to assess their true levels within the tumor. The few studies (**Table 1**) that showed accumulation of nitrotyrosinylated proteins and interpreted these as a measure of increased NO/RNS production are not necessarily right, as protein nitrotyrosinylation is an irreversible reaction that may accumulate over time as the tumor progresses. It is possible that generation of high NO/RNS levels induce genetic instability, not only during the early stages of tumor development, but also as an on-going process, which helps tumor cells accumulate more mutations and further advance to the next malignant stage. It is equally possible that despite the high expression of iNOS protein, the enzyme is rendered inactive, and produces low amounts of NO/RNS that are pro-angiogenic and contribute to tumor aggressiveness. Thus, it is highly important to develop new techniques that will allow to precisely determine NO/RNS concentrations within tumors, preferably in paraffin-embedded archival specimens.

Finally, the fact that such conflicting data are observed in certain cancer types, whereas other cancer types reveal a more consistent behavior, may suggest that other, yet unidentified factors, are involved in the regulation of iNOS activity. Such factors may include components of the specific tissue (e.g., ECM proteins, interstitial cells), or the tumor cells themselves. The fact that macrophages express iNOS in all types of tumors may suggest that tumor cells differently regulate their iNOS expression and NO production.

TUMOR CELL PRODUCTION OF NO—FUTURE PERSPECTIVES

High levels of NO are strongly associated with initiation of apoptosis, and therefore, it seems reasonable to try and manipulate tumor cells to maintain high levels of NO/RNS concentrations as means of therapeutic intervention. In fact, early studies demonstrated that manipulating tumor cells to produce high NO/RNS

Table 1 | Patterns of iNOS expression and NO production in tumor cells, as influenced by tumor grade and stage.

Tumor type	Prognosis/survival	iNOS activity	Expression of iNOS in infiltrating immune cells	References
EXPRESSION OF INOS INVERSELY CORRELATES WITH TUMOR GRADE AND STAGE OR WITH METASTASES				
Ovarian cancer	No effect on prognosis; Low iNOS expression correlates with poor prognosis	N/A[a]; Low intra-cystic NO levels in advanced grade	N/M[b]; Strong iNOS staining in macrophages or mononuclear cells	Klimp et al., 2001; Ozel et al., 2006; Anttila et al., 2007; Nomelini et al., 2008
Colorectal cancer	N/M; Low iNOS expression correlates with low survival	N/A; Reduced in tumors relative to normal tissue	Strong staining in mononuclear cells	Moochhala et al., 1996; Ropponen et al., 2000; Hao et al., 2001; Ohta et al., 2006
Breast cancer	N/M	N/A	Strong iNOS staining of macrophages only in grade III tumors	Tschugguel et al., 1999
Brain cancer	No effect	N/A	Some stromal staining of iNOS	Giannopoulou et al., 2006
Lung cancer (NSCLC)	High iNOS expression predicts better survival	N/A	Strong iNOS staining in alveolar macrophages	Puhakka et al., 2003
Cervical cancer	N/M; High iNOS expression correlates with favorable prognosis, low risk for recurrence	N/A	N/M; Some expression in inflammatory infiltrate	Mazibrada et al., 2008; Eggen et al., 2011
EXPRESSION OF INOS DIRECTLY CORRELATES WITH TUMOR PROGRESSION, GRADE/STAGE, OR METASTASES				
Malignant melanoma	High iNOS expression is associated with invasiveness, metastases, and increased risk for death. No expression in melanocytic naevi	N/A	N/M, Intense staining of macrophages as tumor progresses	Massi et al., 2001; Ekmekcioglu et al., 2006
Colorectal cancer	N/M; High iNOS expression associated with poor survival	N/A; Increased nitrotyrosine staining	Expression of iNOS in few inflammatory mononuclear cells	Zafirellis et al., 2010; Gochman et al., 2012
Breast cancer	No prognostic effect; Strong iNOS associated with poor prognosis in ER-negative patients or with lower disease-free survival rates	N/A	Strong iNOS staining of stromal cells; No iNOS staining in stromal cells	Vakkala et al., 2000; Bulut et al., 2005; Glynn et al., 2010
Brain cancer	No prognostic effect	N/A	N/M	Hara and Okayasu, 2004
Lung cancer (NSCLC)	High expression relative to no-tumor tissues	Elevated in tumors (Griess)	Few stromal cells may be stained	Lee et al., 2003
Cervical cancer	High iNOS expression is associated with decreased survival and metastases	N/A	N/M	Chen et al., 2005
Gastric cancer	High iNOS expression, especially when accompanied by COX-2 staining, is associated with poor prognosis, invasiveness and/or metastasis	N/A; Increased nitrotyrosine staining	N/M; Weak to moderate positive staining in stromal mononuclear cells	Rajnakova et al., 2001; Feng et al., 2002; Li and Xu, 2005; Chen et al., 2006; Zhang et al., 2011
Head and neck (HNSCC)	N/M; High iNOS expression correlates with metastases and poor prognosis or increased 5-year recurrence rate	N/A; Elevated in carcinoma	Positive iNOS staining in inflammatory cells, probably macrophages	Chen et al., 2002; Franchi et al., 2002; Zhang et al., 2005; Ou Yang et al., 2011
Oral cancer	Expression of iNOS correlated with metastasis	N/A	Positive iNOS staining in stroma cells, probably macrophages	Chen et al., 2002
Pancreatic cancer	High iNOS expression is associated with lymph node metastases	N/A	N/A	Kasper et al., 2004
NO CORRELATION TO TUMOR GRADE AND STAGE/NOT CONCLUSIVE				
Cervical cancer	No effect	N/A	N/M; Some positive stromal cell	Oka et al., 2003

(Continued)

Table 1 | Continued

Tumor type	Prognosis/survival	iNOS activity	Expression of iNOS in infiltrating immune cells	References
Head and neck (HNSCC)	No prognostic effect; iNOS expression is not associated with tumor grade; iNOS activity is associated with lymph node metastasis	N/A; Elevated in tumor periphery	Occasional staining of mononuclear cells; positive staining of macrophages	Pukkila et al., 2002; Jayasurya et al., 2003; Franchi et al., 2006
Bladder cancer	Strong iNOS staining in all bladder tissue, regardless of stage and grade	N/A; No change or elevated nitrites in urine samples from TCC relative to controls	N/M; Strong staining in inflammatory cells (macrophages and neutrophils)	Swana et al., 1999; Eijan et al., 2002; Lin et al., 2003; Sandes et al., 2005
Pancreatic cancer	No prognostic effect	N/A	N/M; iNOS positive stroma cells	Vickers et al., 1999; Kong et al., 2002

[a] *N/A, not assayed.*

[b] *N/M, not mentioned.*

inhibited tumor growth. For example, orthotopically implanting pancreatic tumor cell lines that expressed different levels of iNOS showed that tumor cells with low iNOS expression developed pancreatic tumors with metastases to the liver and formed ascites, while tumor cells with high level of iNOS expression did not develop tumors (Wang et al., 2003). In other studies, transfection of tumor cells with the iNOS gene using adenoviral or retroviral vectors lead to their ability to produce NO and other pro-angiogenic proteins, but these cells did not form tumors in nude mice due to initiation of apoptosis (Le et al., 2005) or developed small tumors with no lung metastases in comparison to non-transfected cells (Juang et al., 1998). These studies highlighted the importance of tumor cell iNOS expression, but did not take into account the effects of the infiltrating macrophages or the changing microenvironment. Furthermore, since iNOS was continuously overexpressed in the tumor cells, it is likely that their apoptotic death occurred at an early stage of tumor development, before macrophages were recruited and "re-educated" to become pro-angiogenic and immunosuppressive. Moreover, such a manipulation of tumor cells that involves their transfection with an iNOS construct designed to cause high iNOS expression is clearly not easily feasible in the clinical real-life scenario, where tumors are often diagnosed after they have gained considerable mass and created an immunosuppressive microenvironment.

A different approach to treat tumors with NO/RNS was to use macrophages. Macrophages were isolated from a patient, activated *ex vivo* as M1 macrophages, and then re-introduced back to the same patient. Three qualities make this autologous macrophage adoptive transfer an appealing approach: (1) their tumoricidial abilities that is based on production of high concentration of cytotoxic molecules such as NO/RNS; (2) the ease to isolate them from patients in large numbers and to activate them *ex vivo* before their re-infusion; (3) their ability to home directly to the tumor, thereby specifically targeting the tumor cells (Murdoch et al., 2004; Allavena et al., 2008).

However, previous experiments performed on human subjects, where monocytes were collected, classically stimulated *ex vivo* with IFNγ and/or LPS, and autologously re-infused into the patient, proved that although the process was safe with only minor side effects, no significant beneficial clinical effects were observed (Andreesen et al., 1998; Hennemann et al., 1998). Another study showed that autologous IFNγ-activated macrophages that were intrapleurally injected into patients suffering from malignant mesothelioma showed only limited and insignificant (about 14%) anti-tumor response (Monnet et al., 2002), although these macrophages produced high levels of TNFα and NO/RNS and proved to be cytotoxic to tumor cells *in vitro*. In mice, such treatment resulted in inhibition of metastasis formation, with sometimes attenuated growth, but no regression of the primary tumor (Andreesen et al., 1998; Perske et al., 2010). Adoptive transfer of activated macrophages that were first transduced with macrophage colony stimulating factor (M-CSF) and IFNγ by recombinant adenovirus infection and were tumor-pulsed prior to their re-infusion, succeeded in reducing pulmonary metastases in a B16F10 melanoma model. These gene-modulated macrophages exhibited increased secretion of cytotoxic molecules, including NO, and increased antigen presentation when pulsed with tumor lysates, suggesting that on-going activation of macrophages *in vivo* is critical to their anti-tumor effects and to their ability to recruit specific cytotoxic T cells (Lei et al., 2000). However, such an approach, which demands isolation of macrophages in large amounts followed by their gene-modulation in combination with isolation of enough tumor tissue to produce lysates for macrophage pulsing, seems very elaborate and difficult to achieve in humans.

In retrospect, the macrophage therapy approach probably failed to take into account the ability of the hypoxic and immunosuppressive microenvironment to skew the *ex vivo* M1-activated macrophages back toward an M2 mode of activation, which resulted in failure of these trials. The tumor microenvironment, which is rich in anti-inflammatory mediators (e.g., TGFβ, IL-10, PGE_2) and with apoptotic cell debris, directly neutralizes such pre-treated M1-activated macrophages (Kees and Egeblad, 2011). Specifically, even if such macrophages expressed high levels of the iNOS protein, the hypoxic microenvironment would inhibit

their production of high amounts of NO/RNS. Additionally, macrophage therapy approach failed to provide on-going signals that would maintain the anti-tumoral phenotype of the infused macrophages.

How can we, then, manipulate tumor cell-macrophage interactions in order to eradicate the tumor? It is still advisable to use macrophages, but only as long as we can maintain their skew toward M1 activation. It is possible that after surgery, radio- or chemotherapy, when the tumor mass is reduced, regulatory cells (e.g., Treg or MDSCs) are diminished, and the microenvironment is less hypoxic and immunosuppressive, thus generating a small window of opportunity for a more successful macrophage therapy. Indeed, attempts to combine such therapies and activate the innate immune cells in a timely manner are now beginning to be explored (Kees and Egeblad, 2011).

We can also use NO as a radio- or chemo-sensitizer to enhance the beneficial effects or radio- and chemotherapy. It has been shown that well-oxygenated tumor cells that reside near blood vessels or at the tumor rim are radiosensitive, whereas those that are located in hypoxic areas may be 3-times more radio-resistant. Irradiation kills proliferating tumor cells through accumulation of DNA damage that is dependent on presence of oxygen and the free radicals it generates. Hypoxia is believed to increase radioresistance through the accumulation of HIF-1, which in turn, down-regulates pro-apoptotic genes, enhances multidrug resistant proteins and induces expression of genes like VEGF and enzymes of the glycolytic pathway, thus ensuring blood supply and energy required for tumor cell survival and proliferation (Fitzpatrick et al., 2008; Yasuda, 2008). The potential use of NO as a radio- and chemo-sensitizer for such resistant tumor cells is currently being explored, and several mechanisms could explain its effects. By binding to cytochrome c oxidase, NO can inhibit mitochondrial respiration and generate ROS that activate PHDs and HIF-1 hydroxylation, leading to increased degradation of HIF-1 in hypoxia (Fitzpatrick et al., 2008; Yasuda, 2008). Inhibition of the mitochondria also diverts oxygen from this organelle to the cytoplasm, thus protecting cells from death. Much like oxygen, NO can directly damage DNA, lipids and proteins (probably though generation of peroxynitrite), and systemically NO has a vasodilative effect that provides more blood supply to the tumor cells and maintain their oxygenation. Thus, NO or NO-donors have been explored as potential adjuvants for radiotherapy. However, results remain controversial, and studies show both beneficial and detrimental effects, depending on the tumor microenvironment, NO concentrations, the oxygenated state of the tumor, systemic responses and more (Oronsky et al., 2012). Use of NO-donors to radio-sensitize tumor cells may also cause serious systemic side effects, such as hypotension, which may result in further increasing tumor hypoxia and tumor cell radio-resistance, and the use of IFNγ administration to induce iNOS expression is limited because of its toxicity and vascular effects (Fitzpatrick et al., 2008). However, this highlights again the importance of endogenous NO production by the tumor cells.

NO/RNS production plays a key role in tumor cell-macrophage interactions, as both cell types can produce it. **Table 1** demonstrates that in some tumor types, high grade tumors or metastatic tumor cells tend to reduce their iNOS protein expression or lose it completely, as a means of escaping the immune system. We have previously shown in a murine renal cell carcinoma (RENCA) model injected subcutaneously, that even high concentrations of NO/RNS within the tumor *in vivo*, whether delivered by an NO donor (NOC-18) or by M1-activated macrophages, could only attenuate tumor growth, but did not regress the tumor (Perske et al., 2010). Furthermore, *in vitro* co-culture of RENCA tumor cells that did not express iNOS with RAW 264.7 macrophages, in the presence of IFNγ and LPS that strongly induced macrophage iNOS expression, did not result in tumor cell death. Only when these tumor cells were induced to express iNOS, even in low levels, by alleviating the translational inhibition on the protein through neutralization of microRNA-146a (miR-146a), macrophage-induced tumor cell death was initiated (Perske et al., 2010). Thus, high exogenous concentrations of NO/RNS in the tumor microenvironment are not sufficient to kill tumor cells, and the decision whether tumor cells will undergo apoptosis depends on their own ability to produce NO. Other studies that demonstrated the importance of endogenous tumor cell NO production to their susceptibility to apoptosis support our findings (Le et al., 2005). Different tumor cells were transfected with wild type or mutant iNOS constructs that resulted in different degrees of iNOS activity, and then implanted *s.c.* into nude mice. NO production in the wild type cells strongly suppressed tumor cell proliferation and tumor growth by inducing their apoptosis in a concentration-dependent way, whereas induction of the expression of pro-angiogenic factors, such as VEGF and IL-8 remained constant (Le et al., 2005). These findings and our own, highlight one strategy that tumor cells may take to evade macrophage-induced death by reducing or abrogating their iNOS expression. It is possible that miRNA-146a affects additional targets besides iNOS, and thus acts as a general stimulator, this time of the tumor cells rather than the macrophages. These findings also highlight the importance of the dialogue between tumor cells and macrophages, and underscore the degree of control that tumor cells exert over their environment and the functioning of infiltrating cells.

This current understanding of the important translational regulation of iNOS expression through miRNA-146a, that allows tumor cells to evade macrophage-induced death, may be expanded to envision new therapeutic approaches that are based on the ability to manipulate NO production in the tumor cells. To do this, we must first better understand the precise machinery that allows miR-146a to inhibit iNOS translation, and then find an efficient delivery system of anti-miR-146a specifically into tumor cells, so we can manipulate iNOS production in these cells. Such manipulation of iNOS expression in tumor cells, combined with infusion of *ex vivo* M1-activated macrophages could become an attractive therapeutic approach, which overrides both the immunosuppressive effects of the microenvironment and the evasion strategy of tumor cells.

In conclusion, it is the overall concentrations of NO/RNS, rather than the extent of iNOS expression, that ultimately determine their activities. Low levels of NO/RNS are pro-angiogenic and support immune evasion, whereas high amounts trigger apoptosis. Thus, our goal is to increase NO/RNS production in both tumor cells and macrophages, by overcoming

their respective inhibitory mechanisms, so that the pro-angiogenic effects of NO/RNS are inhibited, the immune system regains recognition of the tumor cells and its pro-apoptotic effects are enhanced to effectively eradicate the tumor.

ACKNOWLEDGMENT

This work was funded by a grant from the Ministry of Science and Culture in Lower Saxony, Germany and by a grant from the Israel Cancer Association.

REFERENCES

Aktan, F. (2004). iNOS-mediated nitric oxide production and its regulation. *Life Sci.* 75, 639–653. doi: 10.1016/j.lfs.2003.10.042

Albina, J. E. (1995). On the expression of nitric oxide synthase by human macrophages. Why no NO? *J. Leukoc. Biol.* 58, 643–649.

Alderton, W. K., Cooper, C. E., and Knowles, R. G. (2001). Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* 357, 593–615. doi: 10.1042/0264-6021:3570593

Allavena, P., Sica, A., Garlanda, C., and Mantovani, A. (2008). The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. *Immunol. Rev.* 222, 155–161. doi: 10.1111/j.1600-065X.2008.00607.x

Ambs, S., and Glynn, S. A. (2011). Candidate pathways linking inducible nitric oxide synthase to a basal-like transcription pattern and tumor progression in human breast cancer. *Cell Cycle* 10, 619–624. doi: 10.4161/cc.10.41.4864

Andreesen, R., Hennemann, B., and Krause, S. W. (1998). Adoptive immunotherapy of cancer using monocyte-derived macrophages: rationale, current status, and perspectives. *J. Leukoc. Biol.* 64, 419–426.

Anttila, M. A., Voutilainen, K., Merivalo, S., Saarikoski, S., and Kosma, V. M. (2007). Prognostic significance of iNOS in epithelial ovarian cancer. *Gynecol. Oncol.* 105, 97–103. doi: 10.1016/j.ygyno.2006.10.049

Black, S. M., Devol, J. M., and Wedgwood, S. (2008). Regulation of fibroblast growth factor-2 expression in pulmonary arterial smooth muscle cells involves increased reactive oxygen species generation. *Am. J. Physiol. Cell Physiol.* 294, C345–C354. doi: 10.1152/ajpcell.00216.2007

Bronte, V. (2009). Myeloid-derived suppressor cells in inflammation: uncovering cell subsets with enhanced immunosuppressive functions. *Eur. J. Immunol.* 39, 2670–2672. doi: 10.1002/eji.200939892

Brune, B. (2003). Nitric oxide: NO apoptosis or turning it ON? *Cell Death Differ.* 10, 864–869. doi: 10.1038/sj.cdd.4401261

Bui, J. D., and Schreiber, R. D. (2007). Cancer immunosurveillance, immunoediting and inflammation: independent or interdependent processes? *Curr. Opin. Immunol.* 19, 203–208. doi: 10.1016/j.coi.2007.02.001

Bulut, A. S., Erden, E., Sak, S. D., Doruk, H., Kursun, N., and Dincol, D. (2005). Significance of inducible nitric oxide synthase expression in benign and malignant breast epithelium: an immunohistochemical study of 151 cases. *Virchows Arch.* 447, 24–30. doi: 10.1007/s00428-005-1250-2

Chaiyarit, P., Ma, N., Hiraku, Y., Pinlaor, S., Yongvanit, P., Jintakanon, D., et al. (2005). Nitrative and oxidative DNA damage in oral lichen planus in relation to human oral carcinogenesis. *Cancer Sci.* 96, 553–559. doi: 10.1111/j.1349-7006.2005.00096.x

Chen, C. N., Hsieh, F. J., Cheng, Y. M., Chang, K. J., and Lee, P. H. (2006). Expression of inducible nitric oxide synthase and cyclooxygenase-2 in angiogenesis and clinical outcome of human gastric cancer. *J. Surg. Oncol.* 94, 226–233. doi: 10.1002/jso.20372

Chen, H. H., Su, W. C., Chou, C. Y., Guo, H. R., Ho, S. Y., Que, J., et al. (2005). Increased expression of nitric oxide synthase and cyclooxygenase-2 is associated with poor survival in cervical cancer treated with radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* 63, 1093–1100. doi: 10.1016/j.ijrobp.2005.03.062

Chen, Y. K., Hsue, S. S., and Lin, L. M. (2002). Increased expression of inducible nitric oxide synthase for human buccal squamous-cell carcinomas: immunohistochemical, reverse transcription-polymerase chain reaction (RT-PCR) and *in situ* RT-PCR studies. *Head Neck* 24, 925–932. doi: 10.1002/hed.10131

Chowdhury, R., Godoy, L. C., Thiantanawat, A., Trudel, L. J., Deen, W. M., and Wogan, G. N. (2012). Nitric oxide produced endogenously is responsible for hypoxia-induced HIF-1alpha stabilization in colon carcinoma cells. *Chem. Res. Toxicol.* 25, 2194–2202. doi: 10.1021/tx300274a

Cianchi, F., Cortesini, C., Fantappie, O., Messerini, L., Sardi, I., Lasagna, N., et al. (2004). Cyclooxygenase-2 activation mediates the proangiogenic effect of nitric oxide in colorectal cancer. *Clin. Cancer Res.* 10, 2694–2704. doi: 10.1158/1078-0432.CCR-03-0192

Coffelt, S. B., Chen, Y. Y., Muthana, M., Welford, A. F., Tal, A. O., Scholz, A., et al. (2011). Angiopoietin 2 stimulates TIE2-expressing monocytes to suppress T cell activation and to promote regulatory T cell expansion. *J. Immunol.* 186, 4183–4190. doi: 10.4049/jimmunol.1002802

Coffelt, S. B., Hughes, R., and Lewis, C. E. (2009). Tumor-associated macrophages: effectors of angiogenesis and tumor progression. *Biochim. Biophys. Acta* 1796, 11–18.

Condeelis, J., and Pollard, J. W. (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 124, 263–266. doi: 10.1016/j.cell.2006.01.007

Corzo, C. A., Condamine, T., Lu, L., Cotter, M. J., Youn, J. I., Cheng, P., et al. (2010). HIF-1alpha regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. *J. Exp. Med.* 207, 2439–2453. doi: 10.1084/jem.20100587

Dai, R., Phillips, R. A., Zhang, Y., Khan, D., Crasta, O., and Ahmed, S. A. (2008). Suppression of LPS-induced Interferon-gamma and nitric oxide in splenic lymphocytes by select estrogen-regulated microRNAs: a novel mechanism of immune modulation. *Blood* 112, 4591–4597. doi: 10.1182/blood-2008-04-152488

Daniliuc, S., Bitterman, H., Rahat, M. A., Kinarty, A., Rosenzweig, D., and Lahat, N. (2003). Hypoxia inactivates inducible nitric oxide synthase in mouse macrophages by disrupting its interaction with alpha-actinin 4. *J. Immunol.* 171, 3225–3232.

De Palma, M., Murdoch, C., Venneri, M. A., Naldini, L., and Lewis, C. E. (2007). Tie2-expressing monocytes: regulation of tumor angiogenesis and therapeutic implications. *Trends Immunol.* 28, 519–524. doi: 10.1016/j.it.2007.09.004

De Palma, M., Venneri, M. A., Galli, R., Sergi Sergi, L., Politi, L. S., Sampaolesi, M., et al. (2005). Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell* 8, 211–226. doi: 10.1016/j.ccr.2005.08.002

De Vera, M. E., Shapiro, R. A., Nussler, A. K., Mudgett, J. S., Simmons, R. L., Morris, S. M., et al. (1996). Transcriptional regulation of human inducible nitric oxide synthase (NOS2) gene by cytokines: initial analysis of the human NOS2 promoter. *Proc. Natl. Acad. Sci. U.S.A.* 93, 1054–1059. doi: 10.1073/pnas.93.3.1054

Dinarello, C. A. (2006). The paradox of pro-inflammatory cytokines in cancer. *Cancer Metastasis Rev.* 25, 307–313. doi: 10.1007/s10555-006-9000-8

Dinarello, C. A. (2010). Why not treat human cancer with interleukin-1 blockade? *Cancer Metastasis Rev.* 29, 317–329. doi: 10.1007/s10555-010-9229-0

Donzelli, S., Switzer, C. H., Thomas, D. D., Ridnour, L. A., Espey, M. G., Isenberg, J. S., et al. (2006). The activation of metabolites of nitric oxide synthase by metals is both redox and oxygen dependent: a new feature of nitrogen oxide signaling. *Antioxid. Redox Signal.* 8, 1363–1371. doi: 10.1089/ars.2006.8.1363

Dulak, J., and Jozkowicz, A. (2003). Regulation of vascular endothelial growth factor synthesis by nitric oxide: facts and controversies. *Antioxid. Redox Signal.* 5, 123–132. doi: 10.1089/152308603321223612

Dunn, G. P., Old, L. J., and Schreiber, R. D. (2004). The three Es of cancer immunoediting. *Annu. Rev. Immunol.* 22, 329–360. doi: 10.1146/annurev.immunol.22.012703.104803

Eggen, T., Sager, G., Arnes, M., Pettersen, I., and Orbo, A. (2011). Expression of iNOS–a

favourable prognostic marker for early-stage carcinoma of the uterine cervix. *Anticancer Res.* 31, 2319–2325.

Eijan, A. M., Piccardo, I., Riveros, M. D., Sandes, E. O., Porcella, H., Jasnis, M. A., et al. (2002). Nitric oxide in patients with transitional bladder cancer. *J. Surg. Oncol.* 81, 203–208. doi: 10.1002/jso.10170

Ekmekcioglu, S., Ellerhorst, J. A., Prieto, V. G., Johnson, M. M., Broemeling, L. D., and Grimm, E. A. (2006). Tumor iNOS predicts poor survival for stage III melanoma patients. *Int. J. Cancer* 119, 861–866. doi: 10.1002/ijc.21767

Feng, C. W., Wang, L. D., Jiao, L. H., Liu, B., Zheng, S., and Xie, X. J. (2002). Expression of p53, inducible nitric oxide synthase and vascular endothelial growth factor in gastric precancerous and cancerous lesions: correlation with clinical features. *BMC Cancer* 2:8. doi: 10.1186/1471-2407-2-8

Fitzpatrick, B., Mehibel, M., Cowen, R. L., and Stratford, I. J. (2008). iNOS as a therapeutic target for treatment of human tumors. *Nitric Oxide* 19, 217–224. doi: 10.1016/j.niox.2008.05.001

Franchi, A., Gallo, O., Paglierani, M., Sardi, I., Magnelli, L., Masini, E., et al. (2002). Inducible nitric oxide synthase expression in laryngeal neoplasia: correlation with angiogenesis. *Head Neck* 24, 16–23. doi: 10.1002/hed.10045

Franchi, A., Massi, D., Santucci, M., Masini, E., Rossi Degl'innocenti, D., Magnelli, L., et al. (2006). Inducible nitric oxide synthase activity correlates with lymphangiogenesis and vascular endothelial growth factor-C expression in head and neck squamous cell carcinoma. *J. Pathol.* 208, 439–445. doi: 10.1002/path.1892

Gabrilovich, D. I., and Nagaraj, S. (2009). Myeloid-derived suppressor cells as regulators of the immune system. *Nat. Rev. Immunol.* 9, 162–174. doi: 10.1038/nri2506

Giannopoulou, E., Ravazoula, P., Kalofonos, H., Makatsoris, T., and Kardamakis, D. (2006). Expression of HIF-1alpha and iNOS in astrocytic gliomas: a clinicopathological study. *In Vivo* 20, 421–425.

Glynn, S. A., Boersma, B. J., Dorsey, T. H., Yi, M., Yfantis, H. G., Ridnour, L. A., et al. (2010). Increased NOS2 predicts poor survival in estrogen receptor-negative breast cancer patients. *J. Clin. Invest.* 120, 3843–3854. doi: 10.1172/JCI42059

Gochman, E., Mahajna, J., Shenzer, P., Dahan, A., Blatt, A., Elyakim, R., et al. (2012). The expression of iNOS and nitrotyrosine in colitis and colon cancer in humans. *Acta Histochem.* 114, 827–835. doi: 10.1016/j.acthis.2012.02.004

Gordon, S., and Martinez, F. O. (2010). Alternative activation of macrophages: mechanism and functions. *Immunity* 32, 593–604. doi: 10.1016/j.immuni.2010.05.007

Goto, T., Haruma, K., Kitadai, Y., Ito, M., Yoshihara, M., Sumii, K., et al. (1999). Enhanced expression of inducible nitric oxide synthase and nitrotyrosine in gastric mucosa of gastric cancer patients. *Clin. Cancer Res.* 5, 1411–1415.

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013

Hao, X. P., Pretlow, T. G., Rao, J. S., and Pretlow, T. P. (2001). Inducible nitric oxide synthase (iNOS) is expressed similarly in multiple aberrant crypt foci and colorectal tumors from the same patients. *Cancer Res.* 61, 419–422.

Hara, A., and Okayasu, I. (2004). Cyclooxygenase-2 and inducible nitric oxide synthase expression in human astrocytic gliomas: correlation with angiogenesis and prognostic significance. *Acta Neuropathol.* 108, 43–48. doi: 10.1007/s00401-004-0860-0

Heller, A. (2008). Apoptosis-inducing high (.)NO concentrations are not sustained either in nascent or in developed cancers. *Chem. Med. Chem.* 3, 1493–1499. doi: 10.1002/cmdc.200800257

Hennemann, B., Beckmann, G., Eichelmann, A., Rehm, A., and Andreesen, R. (1998). Phase I trial of adoptive immunotherapy of cancer patients using monocyte-derived macrophages activated with interferon gamma and lipopolysaccharide. *Cancer Immunol. Immunother.* 45, 250–256. doi: 10.1007/PL00006671

Hernandez, L., Smirnova, T., Kedrin, D., Wyckoff, J., Zhu, L., Stanley, E. R., et al. (2009). The EGF/CSF-1 paracrine invasion loop can be triggered by heregulin beta1 and CXCL12. *Cancer Res.* 69, 3221–3227. doi: 10.1158/0008-5472.CAN-08-2871

Hollborn, M., Stathopoulos, C., Steffen, A., Wiedemann, P., Kohen, L., and Bringmann, A. (2007). Positive feedback regulation between MMP-9 and VEGF in human RPE cells. *Invest. Ophthalmol. Vis. Sci.* 48, 4360–4367. doi: 10.1167/iovs.06-1234

Hussain, S. P., He, P., Subleski, J., Hofseth, L. J., Trivers, G. E., Mechanic, L., et al. (2008). Nitric oxide is a key component in inflammation-accelerated tumorigenesis. *Cancer Res.* 68, 7130–7136. doi: 10.1158/0008-5472.CAN-08-0410

Iyer, A. K., Azad, N., Wang, L., and Rojanasakul, Y. (2008). Role of S-nitrosylation in apoptosis resistance and carcinogenesis. *Nitric Oxide* 19, 146–151. doi: 10.1016/j.niox.2008.04.019

Jayaraman, P., Parikh, F., Lopez-Rivera, E., Hailemichael, Y., Clark, A., Ma, G., et al. (2012). Tumor-expressed inducible nitric oxide synthase controls induction of functional myeloid-derived suppressor cells through modulation of vascular endothelial growth factor release. *J. Immunol.* 188, 5365–5376. doi: 10.4049/jimmunol.1103553

Jayasurya, A., Dheen, S. T., Yap, W. M., Tan, N. G., Ng, Y. K., and Bay, B. H. (2003). Inducible nitric oxide synthase and bcl-2 expression in nasopharyngeal cancer: correlation with outcome of patients after radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* 56, 837–845. doi: 10.1016/S0360-3016(03)00122-6

Jeannin, J. F., Leon, L., Cortier, M., Sassi, N., Paul, C., and Bettaieb, A. (2008). Nitric oxide-induced resistance or sensitization to death in tumor cells. *Nitric Oxide* 19, 158–163. doi: 10.1016/j.niox.2008.04.024

Jia, W., Jackson-Cook, C., and Graf, M. R. (2010). Tumor-infiltrating, myeloid-derived suppressor cells inhibit T cell activity by nitric oxide production in an intracranial rat glioma + vaccination model. *J. Neuroimmunol.* 223, 20–30. doi: 10.1016/j.jneuroim.2010.03.011

Juang, S. H., Xie, K., Xu, L., Shi, Q., Wang, Y., Yoneda, J., et al. (1998). Suppression of tumorigenicity and metastasis of human renal carcinoma cells by infection with retroviral vectors harboring the murine inducible nitric oxide synthase gene. *Hum. Gene Ther.* 9, 845–854. doi: 10.1089/hum.1998.9.6-845

Kasper, H. U., Wolf, H., Drebber, U., Wolf, H. K., and Kern, M. A. (2004). Expression of inducible nitric oxide synthase and cyclooxygenase-2 in pancreatic adenocarcinoma: correlation with microvessel density. *World J. Gastroenterol.* 10, 1918–1922.

Kees, T., and Egeblad, M. (2011). Innate immune cells in breast cancer–from villains to heroes? *J. Mammary Gland Biol. Neoplasia* 16, 189–203. doi: 10.1007/s10911-011-9224-2

Kimura, H., Weisz, A., Kurashima, Y., Hashimoto, K., Ogura, T., D'acquisto, F., et al. (2000). Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. *Blood* 95, 189–197.

Kimura, H., Weisz, A., Ogura, T., Hitomi, Y., Kurashima, Y., Hashimoto, K., et al. (2001). Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide. *J. Biol. Chem.* 276, 2292–2298.

Klimp, A. H., Hollema, H., Kempinga, C., Van Der Zee, A. G., De Vries, E. G., and Daemen, T. (2001). Expression of cyclooxygenase-2 and inducible nitric oxide synthase in human ovarian tumors and tumor-associated macrophages. *Cancer Res.* 61, 7305–7309.

Koh, E., Noh, S. H., Lee, Y. D., Lee, H. Y., Han, J. W., Lee, H. W., et al. (1999). Differential expression of nitric oxide synthase in human stomach cancer. *Cancer Lett.* 146, 173–180. doi: 10.1016/S0304-3835(99)00265-7

Kong, G., Kim, E. K., Kim, W. S., Lee, K. T., Lee, Y. W., Lee, J. K., et al. (2002). Role of cyclooxygenase-2 and inducible nitric oxide synthase in pancreatic cancer. *J. Gastroenterol. Hepatol.* 17, 914–921. doi: 10.1046/j.1440-1746.2002.02829.x

Kuncewicz, T., Balakrishnan, P., Snuggs, M. B., and Kone, B. C. (2001). Specific association of nitric oxide synthase-2 with Rac isoforms in activated murine macrophages. *Am. J. Physiol. Renal Physiol.* 281, F326–F336.

Kundu, J. K., and Surh, Y. J. (2008). Inflammation: gearing the journey to cancer. *Mutat. Res.* 659, 15–30. doi: 10.1016/j.mrrev.2008.03.002

Lahat, N., Bitterman, H., Engelmayer-Goren, M., Rosenzweig, D., Weiss-Cerem, L., and Rahat, M. A. (2011). Reduced TIMP-2 in hypoxia enhances angiogenesis. *Am. J. Physiol. Cell Physiol.* 300, C557–C566. doi: 10.1152/ajpcell.00177.2010

Lala, P. K., and Chakraborty, C. (2001). Role of nitric oxide in carcinogenesis and tumour progression. *Lancet Oncol.* 2, 149–156. doi: 10.1016/S1470-2045(00)00256-4

Lancaster, J. R. Jr., and Xie, K. (2006). Tumors face NO problems? *Cancer Res.* 66, 6459–6462. doi: 10.1158/0008-5472.CAN-05-2900

Le, X., Wei, D., Huang, S., Lancaster, J. R. Jr., and Xie, K. (2005). Nitric oxide synthase II suppresses the growth and metastasis of human cancer regardless of its up-regulation of protumor factors. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8758–8763. doi: 10.1073/pnas.0409581102

Lechner, M., Lirk, P., and Rieder, J. (2005). Inducible nitric oxide synthase (iNOS) in tumor biology: the two sides of the same coin. *Semin. Cancer Biol.* 15, 277–289. doi: 10.1016/j.semcancer.2005.04.004

Lee, T. W., Chen, G. G., Xu, H., Yip, J. H., Chak, E. C., Mok, T. S., et al. (2003). Differential expression of inducible nitric oxide synthase and peroxisome proliferator-activated receptor gamma in non-small cell lung carcinoma. *Eur. J. Cancer* 39, 1296–1301. doi: 10.1016/S0959-8049(02)00733-5

Lei, H., Ju, D. W., Yu, Y., Tao, Q., Chen, G., Gu, S., et al. (2000). Induction of potent antitumor response by vaccination with tumor lysate-pulsed macrophages engineered to secrete macrophage colony-stimulating factor and interferon-gamma. *Gene Ther.* 7, 707–713. doi: 10.1038/sj.gt.3301162

Leon, L., Jeannin, J. F., and Bettaieb, A. (2008). Post-translational modifications induced by nitric oxide (NO): implication in cancer cells apoptosis. *Nitric Oxide* 19, 77–83. doi: 10.1016/j.niox.2008.04.014

Lewis, C. E., and Pollard, J. W. (2006). Distinct role of macrophages in different tumor microenvironments. *Cancer Res.* 66, 605–612. doi: 10.1158/0008-5472.CAN-05-4005

Li, L. G., and Xu, H. M. (2005). Inducible nitric oxide synthase, nitrotyrosine and apoptosis in gastric adenocarcinomas and their correlation with a poor survival. *World J. Gastroenterol.* 11, 2539–2544.

Lin, C. W., Shen, S. C., Ko, C. H., Lin, H. Y., and Chen, Y. C. (2010). Reciprocal activation of macrophages and breast carcinoma cells by nitric oxide and colony-stimulating factor-1. *Carcinogenesis* 31, 2039–2048. doi: 10.1093/carcin/bgq172

Lin, Z., Chen, S., Ye, C., and Zhu, S. (2003). Nitric oxide synthase expression in human bladder cancer and its relation to angiogenesis. *Urol. Res.* 31, 232–235. doi: 10.1007/s00240-003-0302-9

Lo, H. W., Hsu, S. C., Ali-Seyed, M., Gunduz, M., Xia, W., Wei, Y., et al. (2005). Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway. *Cancer Cell* 7, 575–589. doi: 10.1016/j.ccr.2005.05.007

Ma, N., Tagawa, T., Hiraku, Y., Murata, M., Ding, X., and Kawanishi, S. (2006). 8-Nitroguanine formation in oral leukoplakia, a premalignant lesion. *Nitric Oxide* 14, 137–143. doi: 10.1016/j.niox.2005.09.012

Mantovani, A., Allavena, P., Sica, A., and Balkwill, F. (2008). Cancer-related inflammation. *Nature* 454, 436–444. doi: 10.1038/nature07205

Martinez, F. O., Helming, L., and Gordon, S. (2009). Alternative activation of macrophages: an immunologic functional perspective. *Annu. Rev. Immunol.* 27, 451–483. doi: 10.1146/annurev.immunol.021908.132532

Massi, D., Franchi, A., Sardi, I., Magnelli, L., Paglierani, M., Borgognoni, L., et al. (2001). Inducible nitric oxide synthase expression in benign and malignant cutaneous melanocytic lesions. *J. Pathol.* 194, 194–200. doi: 10.1002/1096-9896(200106)194:2<194::AID-PATH851>3.0.CO;2-S

Mazibrada, J., Ritta, M., Mondini, M., De Andrea, M., Azzimonti, B., Borgogna, C., et al. (2008). Interaction between inflammation and angiogenesis during different stages of cervical carcinogenesis. *Gynecol. Oncol.* 108, 112–120. doi: 10.1016/j.ygyno.2007.08.095

Melillo, G., Taylor, L. S., Brooks, A., Cox, G. W., and Varesio, L. (1996). Regulation of inducible nitric oxide synthase expression in IFN-gamma-treated murine macrophages cultured under hypoxic conditions. *J. Immunol.* 157, 2638–2644.

Monnet, I., Breau, J. L., Moro, D., Lena, H., Eymard, J. C., Menard, O., et al. (2002). Intrapleural infusion of activated macrophages and gamma-interferon in malignant pleural mesothelioma: a phase II study. *Chest* 121, 1921–1927. doi: 10.1378/chest.121.6.1921

Moochhala, S., Chhatwal, V. J., Chan, S. T., Ngoi, S. S., Chia, Y. W., and Rauff, A. (1996). Nitric oxide synthase activity and expression in human colorectal cancer. *Carcinogenesis* 17, 1171–1174. doi: 10.1093/carcin/17.5.1171

Mosser, D. M. (2003). The many faces of macrophage activation. *J. Leukoc. Biol.* 73, 209–212. doi: 10.1189/jlb.0602325

Mosser, D. M., and Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8, 958–969. doi: 10.1038/nri2448

Mosser, D. M., and Zhang, X. (2008). Activation of murine macrophages. *Curr. Protoc. Immunol.* Chapter 14:Unit 14.12. doi: 10.1002/0471142735.im1402s83

Murdoch, C., Giannoudis, A., and Lewis, C. E. (2004). Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood* 104, 2224–2234. doi: 10.1182/blood-2004-03-1109

Murdoch, C., Muthana, M., Coffelt, S. B., and Lewis, C. E. (2008). The role of myeloid cells in the promotion of tumour angiogenesis. *Nat. Rev. Cancer* 8, 618–631. doi: 10.1038/nrc2444

Nagaraj, S., and Gabrilovich, D. I. (2008). Tumor escape mechanism governed by myeloid-derived suppressor cells. *Cancer Res.* 68, 2561–2563. doi: 10.1158/0008-5472.CAN-07-6229

Nagaraj, S., Gupta, K., Pisarev, V., Kinarsky, L., Sherman, S., Kang, L., et al. (2007). Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat. Med.* 13, 828–835. doi: 10.1038/nm1609

Nizet, V., and Johnson, R. S. (2009). Interdependence of hypoxic and innate immune responses. *Nat. Rev. Immunol.* 9, 609–617. doi: 10.1038/nri2607

Nomelini, R. S., De Abreu Ribeiro, L. C., Tavares-Murta, B. M., Adad, S. J., and Murta, E. F. (2008). Production of nitric oxide and expression of inducible nitric oxide synthase in ovarian cystic tumors. *Mediators Inflamm.* 2008:186584. doi: 10.1155/2008/186584

Ohta, T., Takahashi, M., and Ochiai, A. (2006). Increased protein expression of both inducible nitric oxide synthase and cyclooxygenase-2 in human colon cancers. *Cancer Lett.* 239, 246–253. doi: 10.1016/j.canlet.2005.08.014

Oka, K., Suzuki, Y., Iida, H., and Nakano, T. (2003). Pd-ECGF positivity correlates with better survival, while iNOS has no predictive value for cervical carcinomas treated with radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* 57, 217–221. doi: 10.1016/S0360-3016(03)00436-X

Oronsky, B. T., Knox, S. J., and Scicinski, J. J. (2012). Is nitric oxide (NO) the last word in radiosensitization? a review. *Transl. Oncol.* 5, 66–71.

Ostrand-Rosenberg, S., and Sinha, P. (2009). Myeloid-derived suppressor cells: linking inflammation and cancer. *J. Immunol.* 182, 4499–4506. doi: 10.4049/jimmunol.0802740

Ou Yang, K. X., Liang, J., and Huang, Z. Q. (2011). Association of clinicopathologic parameters with the expression of inducible nitric oxide synthase and vascular endothelial growth factor in mucoepidermoid carcinoma. *Oral. Dis.* 17, 590–596. doi: 10.1111/j.1601-0825.2011.01813.x

Ozel, E., Pestereli, H. E., Simsek, T., Erdogan, G., and Karaveli, F. S. (2006). Expression of cyclooxygenase-2 and inducible nitric oxide synthase in ovarian surface epithelial carcinomas: is there any correlation with angiogenesis or clinicopathologic parameters? *Int. J. Gynecol. Cancer* 16, 549–555. doi: 10.1111/j.1525-1438.2006.00567.x

Pautz, A., Art, J., Hahn, S., Nowag, S., Voss, C., and Kleinert, H. (2010). Regulation of the expression of inducible nitric oxide synthase. *Nitric Oxide* 23, 75–93. doi: 10.1016/j.niox.2010.04.007

Perske, C., Lahat, N., Levin, S. S., Bitterman, H., Hemmerlein, B., and Rahat, M. A. (2010). Loss of inducible nitric oxide synthase expression in the mouse renal cell carcinoma cell line RENCA is mediated by microRNA miR-146a. *Am. J. Pathol.* 177, 2046–2054. doi: 10.2353/ajpath.2010.091111

Pucci, F., Venneri, M. A., Biziato, D., Nonis, A., Moi, D., Sica, A., et al. (2009). A distinguishing gene signature shared by tumor-infiltrating Tie2-expressing monocytes, blood "resident" monocytes, and embryonic macrophages suggests common functions and developmental relationships. *Blood* 114, 901–914. doi: 10.1182/blood-2009-01-200931

Puhakka, A., Kinnula, V., Napankangas, U., Saily, M., Koistinen, P., Paakko, P., et al. (2003). High expression of nitric oxide synthases is a favorable prognostic sign in non-small cell lung carcinoma. *APMIS* 111, 1137–1146. doi: 10.1111/j.1600-0463.2003.apm1111210.x

Pukkila, M. J., Kellokoski, J. K., Virtaniemi, J. A., Kumpulainen, E. J., Johansson, R. T., Halonen, P. M.,

et al. (2002). Inducible nitric oxide synthase expression in pharyngeal squamous cell carcinoma: relation to p53 expression, clinicopathological data, and survival. *Laryngoscope* 112, 1084–1088. doi: 10.1097/00005537-200206000-00027

Qian, B. Z., and Pollard, J. W. (2010). Macrophage diversity enhances tumor progression and metastasis. *Cell* 141, 39–51. doi: 10.1016/j.cell.2010.03.014

Rabinovich, G. A., Gabrilovich, D., and Sotomayor, E. M. (2007). Immunosuppressive strategies that are mediated by tumor cells. *Annu. Rev. Immunol.* 25, 267–296. doi: 10.1146/annurev.immunol.25.022106.141609

Radi, R. (2004). Nitric oxide, oxidants, and protein tyrosine nitration. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4003–4008. doi: 10.1073/pnas.0307446101

Rahat, M. A., Bitterman, H., and Lahat, N. (2011). Molecular mechanisms regulating macrophage response to hypoxia. *Front. Immunol.* 2:45. doi: 10.3389/fimmu.2011.00045

Rajnakova, A., Moochhala, S., Goh, P. M., and Ngoi, S. (2001). Expression of nitric oxide synthase, cyclooxygenase, and p53 in different stages of human gastric cancer. *Cancer Lett.* 172, 177–185. doi: 10.1016/S0304-3835(01)00645-0

Ratovitski, E. A., Alam, M. R., Quick, R. A., McMillan, A., Bao, C., Kozlovsky, C., et al. (1999a). Kalirin inhibition of inducible nitric-oxide synthase. *J. Biol. Chem.* 274, 993–999.

Ratovitski, E. A., Bao, C., Quick, R. A., Mcmillan, A., Kozlovsky, C., and Lowenstein, C. J. (1999b). An inducible nitric-oxide synthase (NOS)-associated protein inhibits NOS dimerization and activity. *J. Biol. Chem.* 274, 30250–30257.

Reiman, J. M., Kmieciak, M., Manjili, M. H., and Knutson, K. L. (2007). Tumor immunoediting and immunosculpting pathways to cancer progression. *Semin. Cancer Biol.* 17, 275–287. doi: 10.1016/j.semcancer.2007.06.009

Ridnour, L. A., Isenberg, J. S., Espey, M. G., Thomas, D. D., Roberts, D. D., and Wink, D. A. (2005). Nitric oxide regulates angiogenesis through a functional switch involving thrombospondin-1. *Proc. Natl. Acad. Sci. U.S.A.* 102, 13147–13152. doi: 10.1073/pnas.0502979102

Ridnour, L. A., Thomas, D. D., Donzelli, S., Espey, M. G., Roberts, D. D., Wink, D. A., et al. (2006). The biphasic nature of nitric oxide responses in tumor biology. *Antioxid. Redox Signal.* 8, 1329–1337. doi: 10.1089/ars.2006.8.1329

Ridnour, L. A., Windhausen, A. N., Isenberg, J. S., Yeung, N., Thomas, D. D., Vitek, M. P., et al. (2007). Nitric oxide regulates matrix metalloproteinase-9 activity by guanylyl-cyclase-dependent and -independent pathways. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16898–16903. doi: 10.1073/pnas.0702761104

Ropponen, K. M., Kellokoski, J. K., Lipponen, P. K., Eskelinen, M. J., Alanne, L., Alhava, E. M., et al. (2000). Expression of inducible nitric oxide synthase in colorectal cancer and its association with prognosis. *Scand. J. Gastroenterol.* 35, 1204–1211. doi: 10.1080/003655200750056709

Sandes, E. O., Faletti, A. G., Riveros, M. D., Vidal Mdel, C., Gimenez, L., Casabe, A. R., et al. (2005). Expression of inducible nitric oxide synthase in tumoral and non-tumoral epithelia from bladder cancer patients. *Nitric Oxide* 12, 39–45. doi: 10.1016/j.niox.2004.11.003

Schreiber, R. D., Old, L. J., and Smyth, M. J. (2011). Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 331, 1565–1570. doi: 10.1126/science.1203486

Stout, R. D., and Suttles, J. (2004). Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J. Leukoc. Biol.* 76, 509–513. doi: 10.1189/jlb.0504272

Stout, R. D., Watkins, S. K., and Suttles, J. (2009). Functional plasticity of macrophages: *in situ* reprogramming of tumor-associated macrophages. *J. Leukoc. Biol.* 86, 1105–1109. doi: 10.1189/jlb.0209073

Swana, H. S., Smith, S. D., Perrotta, P. L., Saito, N., Wheeler, M. A., and Weiss, R. M. (1999). Inducible nitric oxide synthase with transitional cell carcinoma of the bladder. *J. Urol.* 161, 630–634. doi: 10.1016/S0022-5347(01)61985-2

Switzer, C. H., Glynn, S. A., Cheng, R. Y., Ridnour, L. A., Green, J. E., Ambs, S., et al. (2012). S-Nitrosylation of EGFR and src activates an oncogenic signaling network in human basal-like breast cancer. *Mol. Cancer Res.* 10, 1203–1215. doi: 10.1158/1541-7786.MCR-12-0124

Taylor, B. S., De Vera, M. E., Ganster, R. W., Wang, Q., Shapiro, R. A., Morris, S. M. Jr., et al. (1998). Multiple NF-kappaB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. *J. Biol. Chem.* 273, 15148–15156. doi: 10.1074/jbc.273.24.15148

Tschugguel, W., Schneeberger, C., Unfried, G., Czerwenka, K., Weninger, W., Mildner, M., et al. (1999). Expression of inducible nitric oxide synthase in human breast cancer depends on tumor grade. *Breast Cancer Res. Treat.* 56, 145–151. doi: 10.1023/A:1006288526311

Vakkala, M., Kahlos, K., Lakari, E., Paakko, P., Kinnula, V., and Soini, Y. (2000). Inducible nitric oxide synthase expression, apoptosis, and angiogenesis in *in situ* and invasive breast carcinomas. *Clin. Cancer Res.* 6, 2408–2416.

Venneri, M. A., De Palma, M., Ponzoni, M., Pucci, F., Scielzo, C., Zonari, E., et al. (2007). Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. *Blood* 109, 5276–5285. doi: 10.1182/blood-2006-10-053504

Vesely, M. D., Kershaw, M. H., Schreiber, R. D., and Smyth, M. J. (2011). Natural innate and adaptive immunity to cancer. *Annu. Rev. Immunol.* 29, 235–271. doi: 10.1146/annurev-immunol-031210-101324

Vickers, S. M., Macmillan-Crow, L. A., Green, M., Ellis, C., and Thompson, J. A. (1999). Association of increased immunostaining for inducible nitric oxide synthase and nitrotyrosine with fibroblast growth factor transformation in pancreatic cancer. *Arch. Surg.* 134, 245–251. doi: 10.1001/archsurg.134.3.245

Walmsley, S. R., Chilvers, E. R., and Whyte, M. K. (2009). Hypoxia. Hypoxia, hypoxia inducible factor and myeloid cell function. *Arthritis Res. Ther.* 11, 219. doi: 10.1186/ar2632

Wang, B., Wei, D., Crum, V. E., Richardson, E. L., Xiong, H. H., Luo, Y., et al. (2003). A novel model system for studying the double-edged roles of nitric oxide production in pancreatic cancer growth and metastasis. *Oncogene* 22, 1771–1782. doi: 10.1038/sj.onc.1206386

Wang, X., Zhao, Q., Matta, R., Meng, X., Liu, X., Liu, C. G., et al. (2009). Inducible nitric-oxide synthase expression is regulated by mitogen-activated protein kinase phosphatase-1. *J. Biol. Chem.* 284, 27123–27134. doi: 10.1074/jbc.M109.051235

Weigert, A., and Brune, B. (2008). Nitric oxide, apoptosis and macrophage polarization during tumor progression. *Nitric Oxide* 19, 95–102. doi: 10.1016/j.niox.2008.04.021

Wink, D. A., Hines, H. B., Cheng, R. Y., Switzer, C. H., Flores-Santana, W., Vitek, M. P., et al. (2011). Nitric oxide and redox mechanisms in the immune response. *J. Leukoc. Biol.* 89, 873–891. doi: 10.1189/jlb.1010550

Wyckoff, J., Wang, W., Lin, E. Y., Wang, Y., Pixley, F., Stanley, E. R., et al. (2004). A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res.* 64, 7022–7029. doi: 10.1158/0008-5472.CAN-04-1449

Xie, Q., and Nathan, C. (1994). The high-output nitric oxide pathway: role and regulation. *J. Leukoc. Biol.* 56, 576–582.

Yang, L., Guo, A., and Gu, J. C. (2011). c-Jun N-terminal kinase and nuclear factor kappaB mediate nitric oxide-induced expression of matrix metalloproteinase-13. *Int. Orthop.* 35, 1261–1266. doi: 10.1007/s00264-010-1056-y

Yasuda, H. (2008). Solid tumor physiology and hypoxia-induced chemo/radio-resistance: novel strategy for cancer therapy: nitric oxide donor as a therapeutic enhancer. *Nitric Oxide* 19, 205–216. doi: 10.1016/j.niox.2008.04.026

Ying, L., Hofseth, A. B., Browning, D. D., Nagarkatti, M., Nagarkatti, P. S., and Hofseth, L. J. (2007). Nitric oxide inactivates the retinoblastoma pathway in chronic inflammation. *Cancer Res.* 67, 9286–9293. doi: 10.1158/0008-5472.CAN-07-2238

Zafirellis, K., Zachaki, A., Agrogiannis, G., and Gravani, K. (2010). Inducible nitric oxide synthase expression and its prognostic significance in colorectal cancer. *APMIS* 118, 115–124. doi: 10.1111/j.1600-0463.2009.02569.x

Zhang, J., Peng, B., and Chen, X. (2005). Expressions of nuclear factor kappaB, inducible nitric oxide synthase, and vascular endothelial growth factor in adenoid cystic carcinoma of salivary glands: correlations with the angiogenesis and clinical outcome. *Clin. Cancer Res.* 11, 7334–7343. doi: 10.1158/1078-0432.CCR-05-0241

Zhang, W., He, X. J., Ma, Y. Y., Wang, H. J., Xia, Y. J., Zhao, Z. S., et al. (2011). Inducible nitric oxide synthase expression

correlates with angiogenesis, lymphangiogenesis, and poor prognosis in gastric cancer patients. *Hum. Pathol.* 42, 1275–1282. doi: 10.1016/j.humpath.2010.09.020

Ziche, M., and Morbidelli, L. (2009). Molecular regulation of tumour angiogenesis by nitric oxide. *Eur. Cytokine Netw.* 20, 164–170.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 March 2013; paper pending published: 14 April 2013; accepted: 29 May 2013; published online: 18 June 2013.

*Citation: Rahat MA and Hemmerlein B (2013) Macrophage-tumor cell interactions regulate the function of nitric oxide. Front. Physiol. **4**:144. doi: 10.3389/fphys.2013.00144*

This article was submitted to Frontiers in Vascular Physiology, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 28 March 2014
doi: 10.3389/fphys.2014.00114

The role IL-1 in tumor-mediated angiogenesis

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Tumor angiogenesis is one of the hallmarks of tumor progression and is essential for invasiveness and metastasis. Myeloid inflammatory cells, such as immature myeloid precursor cells, also termed myeloid-derived suppressor cells (MDSCs), neutrophils, and monocytes/macrophages, are recruited to the tumor microenvironment by factors released by the malignant cells that are subsequently "educated" *in situ* to acquire a pro-invasive, pro-angiogenic, and immunosuppressive phenotype. The proximity of myeloid cells to endothelial cells (ECs) lining blood vessels suggests that they play an important role in the angiogenic response, possibly by secreting a network of cytokines/chemokines and inflammatory mediators, as well as via activation of ECs for proliferation and secretion of pro-angiogenic factors. Interleukin-1 (IL-1) is an "alarm," upstream, pro-inflammatory cytokine that is generated primarily by myeloid cells. IL-1 initiates and propagates inflammation, mainly by inducing a local cytokine network and enhancing inflammatory cell infiltration to affected sites and by augmenting adhesion molecule expression on ECs and leukocytes. Pro-inflammatory mediators were recently shown to play an important role in tumor-mediated angiogenesis and blocking their function may suppress tumor progression. In this review, we summarize the interactions between IL-1 and other pro-angiogenic factors during normal and pathological conditions. In addition, the feasibility of IL-1 neutralization approaches for anti-cancer therapy is discussed.

Keywords: IL-1α, IL-1β, VEGF, VEGFR1, VEGFR2, angiogenesis, myeloid cells, inflammation

INTRODUCTION

Postnatal angiogenesis, first described by Folkman in the 70's, referred to a process by which mature endothelial cells (ECs) proliferate and sprout to create new blood vessels. Usually, quiescent ECs are inactive for months and even years; they proliferate only upon angiogenic activation due to various pathological conditions (Carmeliet and Jain, 2000; Carmeliet, 2003, 2005; Ribatti, 2009). Initially, the angiogenic process was considered to represent the outcome of straightforward interactions between pro-angiogenic factors and specific signaling receptors on the membranes of ECs (Folkman, 1995, 2001, 2003; Folkman and D'Amore, 1996). Although many molecules are involved in angiogenesis, VEGF stands out because it has a direct mitogenic effect on ECs and functions as a key regulator of both physiological and pathological angiogenesis (Thomas, 1996; Alitalo and Carmeliet, 2002; Shibuya, 2006a,b). VEGFA belongs to the VEGF family and through activation of two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (FLK-1), plays a major role in the angiogenic response. The biological effects of VEGF are thought to be mediated mainly by VEGFR2, whereas VEGFR1 can transduce a weak, intracellular signal in ECs and was initially thought to be a decoy receptor, thus serving as a negative regulator of angiogenesis (Hiratsuka et al., 1998). At the same time, VEGFR1 is expressed on different myeloid cells, such as macrophages and is directly involved in the migration of these cells to inflammatory areas (Shibuya, 2006a,b). Recently, it was found that inflammation usually accompanies tumor development and the mechanism by which inflammation contributes to tumor-mediated angiogenesis has been the subject of intensive study.

Pro-inflammatory cytokines are potent factors in the induction and support of angiogenesis. Of special relevance to angiogenesis are the "alarm cytokines" IL-1 and TNFα that are generated by macrophages immediately after confrontation with inflammatory stimuli. These stimuli induce the expression of pro-inflammatory genes in diverse stromal/inflammatory cells, which ultimately results in a local cascade of cytokines and small effector molecules that initiate, propagate, and sustain inflammation (Dinarello, 1996; Apte and Voronov, 2002, 2008; Balkwill, 2002; Apte et al., 2006a,b; Balkwill and Mantovani, 2012). IL-1 and TNFα also increase the expression of adhesion molecules on both ECs and leukocytes, promoting leukocyte infiltration from the blood into inflamed tissues (Apte and Voronov, 2002, 2008; Apte et al., 2006a,b).

The direct relationship between inflammation and tumor-mediated angiogenesis has prompted the development of novel anti-tumor therapies based on the attenuation of inflammation.

In this review, the interaction of IL-1 molecules with a critical pro-angiogenic factor, VEGF, and the effects of IL-1 on ECs and myeloid cells during the angiogenic response are discussed.

Abbreviations: IL-1, Interleukin-1; IL-1Ra, Interleukin-1 receptor antagonist; VEGF, Vascular endothelial growth factor; VEGFR, Vascular endothelial growth factor receptor; TNF, Tumor necrosis factor; TGFβ, Tumor growth factor beta; PlGF, Placental growth factor; bFGF, Basic Fibroblast growth factor.

THE IL-1 MOLECULES

The IL-1 family consists of agonistic and antagonistic molecules, as well as receptors. The two major agonistic proteins are IL-1α and IL-1β. The third important molecule is the IL-1 receptor antagonist (IL-1Ra), which is a physiological inhibitor of preformed IL-1; it binds to IL-1 receptors without transmitting an activation signal (reviewed in Dinarello, 1996; Stylianou and Saklatvala, 1998; Sims et al., 2001; Apte and Voronov, 2002, 2008; Arend, 2002; Dinarello, 2002; O'Neill, 2002; Braddock and Quinn, 2004; Apte et al., 2006a,b; Mantovani et al., 2008; Mantovani and Sica, 2010).

Many cell types produce and secrete IL-1α, IL-1β, and IL-1Ra upon activation with microbes, microbial products, cytokines, and other environmental stimuli, as well as products of damaged tissue (reviewed in Dinarello, 1996, 2006; Apte and Voronov, 2008; Voronov et al., 2013). IL-1α and IL-1β are synthesized as precursors of 31 kD that are further processed by proteases to their mature secreted 17 kD forms. IL-1 differs from most other cytokines by the lack of a signal sequence, thus not passing through the endoplasmic reticulum-Golgi pathway. The mechanisms of IL-1 secretion are not yet completely understood. IL-1Ra, which has a signal peptide, is secreted in the ER-Golgi exocytic pathway.

IL-1β is not present in homeostatic conditions; it is induced and secreted only upon inflammatory signals. The IL-1β precursor is biologically inactive until it is enzymatically cleaved into the mature secreted form by the IL-1β-converting enzyme (caspase-1) that is activated in the cytosol on the inflammasome platform (reviewed in Dinarello, 2009, 2011a; Eisenbarth and Flavell, 2009; Franchi et al., 2009; Martinon et al., 2009; Latz, 2010; Schroder and Tschopp, 2010). IL-1β, due to the fact that it is extensively secreted, has been considered to be the major pro-inflammatory molecule. At low local doses, IL-1β induces limited inflammatory responses, followed by activation of specific immune mechanisms, while at high doses, IL-1β induces broad inflammation accompanied by tissue damage and immune suppression (reviewed in Voronov et al., 2013).

IL-1α, in contrast, is present in homeostatic conditions in many cells. IL-1α is only rarely secreted by living cells and in most cases is undetectable in body fluids. Its protein is translated as a precursor (ProIL-1α), which is further processed by the Ca^{2+}-dependent protease, calpain, into the mature 17 kD form and the 16 kD N-terminal cleavage product- the propiece of IL-1α, also known as the IL-1α N-terminal peptide (IL-1NTP). Intracellular ProIL-1α is present in many cells because they contain calpain inhibitors and are thus unable to process and secrete IL-1α (Afonina et al., 2011; Di Paolo and Shayakhmetov, 2013; Zheng et al., 2013). Recently, a novel mechanism to control IL-1α activity has been described by Zheng et al. (2013) and reviewed in Di Paolo and Shayakhmetov (2013). Thus, under normal conditions, IL-1α is synthesized as a p33 precursor that is sequestered in the cytosol by IL-1 receptor type 2 (IL-1R2), where it cannot be cleaved by proteases or activate IL-1 receptor type 1 (IL-1R1) signaling. However, under inflammatory signals, after inflammasome activation, IL-1R2 is cleaved by caspase-1 and ProIL-1α is further processed by calpain to the highly active p17 mature IL-1α form, which can be secreted from cells.

A biologically active membrane-associated form of IL-1α (23 kD) that is anchored to the membrane via a mannose-like receptor has been observed in activated cells that express the cytokine. However, it is not clear how IL-1α is inserted into the membrane.

IL-1α and IL-1β signal through the same IL-1Rs, which belong to the immunoglobulin (Ig) supergene family and are extensively expressed on many cell types. IL-1R1 (80 kD) is a signaling receptor, whereas IL-1R2 (68 kD) serves as a decoy target, acting to reduce excessive amounts of IL-1 (reviewed in Apte and Voronov, 2008; O'Neill, 2008; Dinarello, 2009, 2011b; Garlanda et al., 2009; Gabay et al., 2010; Sims and Smith, 2010; Voronov et al., 2013). Following the binding of IL-1 to IL-1R1, a second chain, i.e., the IL-1R acceptor protein (IL-1RAcP), is recruited. This heterodimeric complex triggers IL-1 signaling by activating the IL-1 receptor-associated kinase (IRAK) and ultimately leads to the activation of NF-κB and its target genes. On the contrary, IL-1R2 and the IL-1Ra do not form this heterodimeric complex with the IL-1RAcP and therefore do not recruit IRAK. While IL-1α and IL-1β signals through the same receptors, they differ dramatically in some biological functions (reviewed in Apte and Voronov, 2008; O'Neill, 2008; Dinarello, 2009, 2011b; Garlanda et al., 2009; Gabay et al., 2010; Sims and Smith, 2010; Voronov et al., 2013).

The differential effects of these molecules on angiogenesis are discussed in this review.

THE EFFECTS OF IL-1 ON ECs AND ITS CROSSTALK WITH PRO-ANGIOGENIC MOLECULES

ECs are the main cells that are involved in both the normal and pathological angiogenic response. Multiple studies have shown *in vitro* effects of recombinant IL-1 on parameters related to the physiology of ECs, including their activation, as evidenced by morphological changes, increased migration and proliferation and ultimately organization into tube-like structures (reviewed in Voronov et al., 2007, 2010a, 2013; Apte and Voronov, 2008). As IL-1 is a strong activator of ECs, it induces profound changes in gene expression and function that allow these cells to participate actively in inflammatory reactions, immunity, and blood vessel formation. For example, IL-1β induces morphological transformation in human dermal micro-vascular endothelial cells (HDMECs), accompanied by an increased growth rate, loss of contact inhibition, and an increase in the permeability of confluent EC monolayers (Bokhari et al., 2006). Nevertheless, it is not known whether the described effects of IL-1 on ECs are direct, due to its action, or indirect, due to IL-1-induced cytokines produced by ECs. It was found that *in vitro,* IL-1β increases expression of FGF2 in ECs through activation of NF-κB (Lee and Kay, 2012) and also induces expression of different chemokines, cytokines, direct angiogenic factors, and adhesion molecules on ECs (Breviario et al., 1990; Sica et al., 1990; Kang et al., 2006). In addition, IL-1β interaction with IL-1R1 on ECs induces migration of the cells and tube formation, mainly via activation of p38-mitogen-activated protein kinase (MAPK) and MAPK-activated protein kinase 2 (Jagielska et al., 2012). Moreover, IL-1β upregulates expression of VEGF and its receptors on ECs (Berse et al., 1999) or aortic smooth muscle cells (Stavri et al., 1995; Maruyama et al., 1999; Nasu et al., 2006) and VEGF secretion

from these cells was significantly inhibited by the addition of IL-1Ra. *In vivo*, IL-1 also plays a synergistic, pro-angiogenic role with some pro-angiogenic factors (Friesel and Maciag, 1999). For example, the synergistic effects of VEGF and IL-1 in the up-regulation of genes of growth factors and inflammatory cytokines in ECs have been described and a 60% overlapping of genes induced by each of these cytokines was observed (Schweighofer et al., 2009). VEGF/IL-1 induced genes comprise mainly a group of genes with NFAT, as well as NF-κB binding sites in their promoters; VEGF-A preferentially uses NFAT and IL-1 uses NF-κB to induce these genes. It was also observed that both VEGF and IL-1β increase the permeability of ECs via a Src-dependent pathway (Sheikpranbabu et al., 2009).

Using a Matrigel model, we found that both rIL-1β and rVEGF induce each other and are both essential to induce an angiogenic response. Thus, neutralization of either of these cytokines abrogated the angiogenic response. The importance of the interaction between IL-1β and VEGF was also demonstrated by the weak angiogenic response observed in Matrigel plugs loaded with rVEGF in mice deficient in IL-1β or its signaling receptor (Carmi et al., 2013). Inhibition of VEGFR1 signaling in Matrigel plugs supplemented with recombinant cytokines (IL-1β or VEGF) abrogated angiogenesis, probably by inhibiting recruitment of bone marrow-derived (BMD) myeloid cells. Thus, in multiple studies, synergy of IL-1β and VEGF has been described; however, our studies demonstrate the necessity of both factors in angiogenic responses. IL-1β is also an essential factor for endothelial precursor cells (EPCs) to mature into ECs and this IL-1β-induced effect was facilitated by the addition of VEGF. For example, when either EPCs or myeloid circulating angiogenic cells from lupus patients were co-cultured with IL-1β and VEGF, their capacity to proliferate and differentiate into mature ECs was synergistically increased (Thacker et al., 2010).

There are fewer studies on the role of IL-1α in angiogenesis. We found that IL-1α has less potent pro-angiogenic effects compared to IL-1β in its recombinant form (unpublished results). Nevertheless, *in vivo* studies on the effects of IL-1α and IL-1β on angiogenesis revealed that both agonistic molecules are important, but probably work through different pathways. Thus, IL-1α can stimulate a high angiogenic response by recruiting macrophages that are an abundant source of FGF (reviewed in Sano et al., 1990; Brogi et al., 1993; Dinarello, 1996; Rider et al., 2011, 2012), or other VEGF-expressing inflammatory cells (Salven et al., 2002). In addition, a dose-dependent effect of IL-1α in *de novo* synthesis of VEGF by human peripheral mononuclear cells (PBMCs) was shown. This effect was blocked by treatment with VEGFR2 antibodies, whereas neutralization of VEGFR1 induced only marginal effects. Both *in vitro* and *in vivo,* IL-1α can stimulate ECs to secrete IL-8 and during *in vivo* angiogenesis, the source of IL-1 can be from PBMCs or activated platelets (Kaplanski et al., 1994a,b). IL-1α is also released from ECs following stress signals, such as starvation or TNF activation (Berda-Haddad et al., 2011) and in addition, IL-1α activates ECs to express CXCL1, VCAM-1, and ICAM-1, thus promoting trans-endothelial-migration of inflammatory cells (Thornton et al., 2010).

IL-1 IN INFLAMMATION-INDUCED ANGIOGENESIS

In the last decade, the link between inflammation and angiogenesis has become recognized. Extensive angiogenesis was observed in chronic inflammation, for example in rheumatoid arthritis and inflammatory bowel disease (Folkman, 2001, 2003). In both acute and chronic inflammation, functional changes in the vasculature were found. In acute inflammation, these changes include an increase in permeability, extensive EC mitotic activity, and remodeling of capillaries. Upon chronic inflammation, vascular dilation and an increase in capillary density was observed. During inflammation, ECs actively recruit immune cells from the circulation into the underlying tissue, where they play a role in angiogenesis. An increase in growth factor and cytokine production, due to inflammation, also leads to proliferation of ECs.

The role of the IL-1 molecules in promoting inflammation-induced angiogenesis was studied by us, using the model of Matrigel plugs supplemented with supernatants of hypoxic macrophages, as such, or after activation with LPS as an inflammatory stimulus (Carmi et al., 2009). We showed that neutralization of IL-1 in supernatants of hypoxic macrophages, particularly IL-1β, completely abrogated cell infiltration and angiogenesis in Matrigel plugs, concomitant to dramatically reduced VEGF levels. Supernatants from macrophages of IL-1β knockout (KO) mice did not induce this inflammatory or angiogenic response. The importance of IL-1 signaling in the host was demonstrated by the dramatic reduction of inflammation-induced angiogenic responses in Matrigel plugs that contained supernatants derived from WT macrophages implanted in IL-1RI KO mice. Using the aortic sprouting assay, it was shown that IL-1 does not directly activate EC migration, proliferation, and organization into blood vessel-like structures, but rather activates infiltrating myeloid cells to produce a cascade of cytokines/chemokines, which further activate tissue resident ECs to produce direct pro-angiogenic factors, such as VEGF (Carmi et al., 2009). Similarly, Nakao et al., demonstrated that ectopic expression of IL-1β in the cornea induces increased angiogenesis, which is dependent on infiltration of COX-2-positive macrophages that activate the angiogenic process in a complex manner (Nakao et al., 2005). An example of an indirect effect of IL-1β on angiogenesis is the pro-angiogenic activity of osteopontin-treated monocytes on chick embryo chorioallantoic membranes that is completely abrogated by neutralization with anti-IL-1β antibodies (Naldini et al., 2006). In addition, systemic treatment with IL-1Ra prevented the formation of new blood vessels in corneas impregnated with VEGF or basic FGF (Coxon et al., 2002). In conclusion, IL-1, most probably the actively secreted form of IL-1β, functions directly or indirectly on myeloid infiltrating cells, as well as ECs, and thus regulates inflammation-induced angiogenic responses.

IL-1 MOLECULES PLAY A KEY ROLE IN THE TUMOR MICROENVIRONMENT

The induction of angiogenesis is considered to be a hallmark of cancer and is important for tumor growth and dissemination (Folkman, 1995, 2001, 2003; Folkman and D'Amore, 1996). An initially developing tumor is dormant until it undergoes the angiogenic switch, as initially described by Folkman. During this switch, the balance between the pro-angiogenic and

anti-angiogenic factors changes and angiogenic stimuli are predominant. One of the major mechanisms of the angiogenic switch is enhanced expression and secretion of angiogenic factors, mainly VEGF, by the malignant cells. However, the mechanisms of the angiogenic switch are not completely elucidated, as detectable tumors usually have undergone this switch and already have an established vascular network. As indicated, tumor vascularization occurs through "classical" angiogenesis, as well as via vasculogenesis, which involves cells recruited from the BM (Carmeliet, 2005; De Palma et al., 2005; Bertolini et al., 2006). Thus, circulating VEGFR2-positive cells were shown to be initiators of vasculogenesis; however, conflicting findings on their ability to incorporate into newly formed blood vessels at tumor sites have been reported (De Palma et al., 2003; Gothert et al., 2004; Peters et al., 2005; Purhonen et al., 2008; Carmi et al., 2013). EPCs were initially described by Asahara, who reported on their ability to repair angiogenesis in ischemic tissues (Asahara et al., 1999). It was found that IL-1β can mobilize EPCs in a VEGF-dependent manner by regulation of VEGF and VEGFR2 expression on ECs, thus supporting neovascularization (Amano et al., 2004).

In further studies, a population of hematopoietic cells expressing endothelial markers, such as CD31, VEGFR2, and Tie2, was described and termed myeloid angiogenic cells (MACs) (Medina et al., 2010). These cells express high levels of pro-angiogenic and macrophage type 2 markers (Medina et al., 2011) and their function at the tumor site is still disputed. In some models, MACs were shown to differentiate into mature ECs that incorporate into the vasculature (Kawamoto et al., 2001; Urbich et al., 2003); however, upon intravitreal injection, MACs enhanced vascular repair of ischemic retinopathy, but preserved their original phenotype and did not differentiate into mature ECs. In addition, BMD cells of myeloid origin were shown to be essential for tumor-mediated angiogenesis. The proximity of BMD cells, including MACs, to ECs lining blood vessels suggests their paracrine role, facilitating the EC response during angiogenesis. These cells probably do not incorporate into blood vessels but function as supporting cells (Ziegelhoeffer et al., 2004).

Various populations of BMD myeloid cells are found in the tumor microenvironment. These include circulating monocytes, Tie-2 expressing monocytes (TEMs), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs) and neutrophils (reviewed in Murdoch et al., 2008; Wels et al., 2008; Coffelt et al., 2010; Ferrara, 2010a; Ruffell et al., 2012; Sica et al., 2012; Chambers et al., 2013; De Palma and Lewis, 2013; Favre et al., 2013). The precise relationship among the myeloid cell types is uncertain and it is not known whether they act in the tumor microenvironment in redundant or specific manners. In various tumor experimental systems, diverse myeloid cell populations dominate. Mantovani et al. suggested a unifying hypothesis that myeloid cells at tumor sites actually represent a continuum of cell populations that all share properties of M2-macrophages, which promote tumor-mediated angiogenesis and progression (Mantovani and Sica, 2010; Allavena and Mantovani, 2012). Myeloid cells perform paracrine functions in the tumor-mediated environment, secreting angiogenic factors, and providing the inflammatory milieu for the angiogenic response. For example, MDSCs and TAMs secrete VEGF and MMP9, which increases the bioavailability of VEGF sequestered in the extracellular matrix by its proteolytic activity (reviewed in Murdoch et al., 2008; Mantovani et al., 2009; Gabrilovich et al., 2012).

IL-1, as a major hematopoietic and pro-inflammatory cytokine has dramatic effects on the generation of myeloid cells in the BM and on their recruitment to tumor sites, as well as on the *in situ* phenotype/function of these cells in the tumor microenvironment (reviewed in Apte and Voronov, 2008; Voronov et al., 2013). For example, *in vitro,* addition of IL-1 to BMCs increases secretion of VEGF and the total number of BMCs expressing CD34 or Flk-1 (Qin et al., 2006). Indeed, the absence of IL-1β in the tumor microenvironment limited the recruitment of FGF1-producing mononuclear cells to tumor sites (Prudovsky et al., 2003). Thus, it was suggested that IL-1 can affect BMD differentiation or induce them to secrete pro-inflammatory or angiogenic factors (reviewed in Apte and Voronov, 2008; Voronov et al., 2013), which are also involved in the recruitment of additional myeloid cells to tumor sites. Indeed, the importance of the VEGFR1/VEGF-A axis in recruiting myeloid cells that express VEGFR1 to tumor sites has been noted, and in mice deficient in VEGFR1, decreased recruitment of macrophages and other myeloid cells into tumors was observed (Duyndam et al., 2002). In addition, depletion of VEGFR1 in the renal cell carcinoma model leads to inhibition of macrophage recruitment to tumors (Li et al., 2011).

In our studies, the role of microenvironment-derived IL-1 on the recruitment of VEGFR1-positive BMD cells to tumor sites was confirmed. We characterized a new auto-induction circuit, IL-1β/VEGF, which acts via interactions between BMDs, mainly $VEGFR1^+/IL\text{-}1R1^+$immature myeloid cells (MDSCs) and to a lesser extent macrophages, and tissue-resident ECs (Carmi et al., 2013). Myeloid cells do not directly stimulate ECs for migration, proliferation, and subsequent blood vessel formation. However, they produce IL-1β and a network of other pro-inflammatory cytokines/molecules, such as Bv8, CCL2 and CCL3, etc., which subsequently activate resting tissue-resident ECs to produce VEGF, as well as other direct pro-angiogenic factors, such as PlGF and bFGF. Thus, IL-1β provides the inflammatory microenvironment for angiogenesis and tumor progression. We have shown that IL-1β inhibition stably reduces tumor growth, by limiting inflammation and inducing the maturation of MDSCs into M1 macrophages, which do not promote tumor invasiveness and can be cytotoxic/cytostatic for tumor cells. In addition, M1 macrophages also serve as antigen-presenting cells for inducing anti-tumor immunity. These results suggest that IL-1β, apart from its ability to affect recruitment of myeloid cells, controls their maturation or activation for a pro-tumorigenic phenotype (Song et al., 2005; Bunt et al., 2006; Tu et al., 2008; Carmi et al., 2013). For example, Hagenmann et al. showed that IL-1R signaling in macrophages is essential for M2 polarization induced by conditioned medium of tumor cells (Hagemann et al., 2008). Other studies also showed the involvement of IL-1β in myeloid cell differentiation. Thus, IL-1β inhibits the skewing of myeloid cells present in proangiogenic cultures from circulating angiogenic cells to mature dendritic cells (DCs) (Mohty et al., 2003; Denny et al., 2007). In addition, IL-1β can impair maturation in DCs treated with rapamycin (Turnquist et al., 2008).

Our experimental system of tumor cells encapsulated in Matrigel plugs allows the study of the early angiogenic response and even the angiogenic switch. In this case, VEGF produced by ECs possibly synergizes with direct angiogenic factors secreted by the malignant cells. Traditionally, it was thought that ECs respond to VEGF during the angiogenic response and produce very little or undetectable amounts of VEGF (Kerbel, 2008). In other studies, using large, established tumors that are densely infiltrated by "activated" myeloid cells, it was shown that VEGF mainly originates from such myeloid cells and it controls tumor angiogenesis (reviewed in Apte and Voronov, 2008; Murdoch et al., 2008; Coffelt et al., 2010; Ferrara, 2010a; Mantovani and Sica, 2010; Qian and Pollard, 2010; Ruffell et al., 2010; Allavena and Mantovani, 2012; Gabrilovich et al., 2012). In the early stages of tumor development, as assessed in our studies, myeloid cells are probably not yet sufficiently activated to produce VEGF. Thus, at different stages of tumor progression, VEGF is secreted by diverse microenvironmental cells and it acts together with angiogenic factors of tumor cell origin.

Results of our studies have shown that IL-1β is a major mediator in the tumor microenvironment, which plays a crucial role in the angiogenic response. IL-1β acts together with VEGF in mounting and maintaining tumor-mediated angiogenesis.

THE DIFFERENTIAL EFFECTS OF IL-1 MOLECULES IN TUMOR-INDUCED ANGIOGENESIS AND INVASIVENESS

High levels of IL-1 molecules are found in experimental tumor models and in human malignancies and IL-1 has been implicated as a key factor in tumor progression. IL-1 exerts its proliferative and angiogenic effects in the tumor microenvironment, mainly via interaction with stromal, inflammatory, as well as the malignant cells, stimulating tumor cell proliferation and invasion through autocrine or paracrine loops. For example, IL-1β induces secretion of chemokines by both tumor and tumor-microenvironmental cells and thus promotes cancer invasiveness (Portier et al., 1993; Suswam et al., 2005; Apte et al., 2006a; Naldini et al., 2010). A few studies have documented constitutive IL-1β protein production in human and animal cancer cell lines, including sarcomas and ovarian cell carcinomas (Dinarello, 1996; Lewis et al., 2006). In patients, overexpression of IL-1β has been described in solid tumors, including breast, colon, lung, head and neck cancers, and melanomas. In some tumors, IL-1, as well as other pro-inflammatory cytokines, is induced by oncogenes that transform the cell, thus providing the microenvironment for invasiveness of the malignant cells. In other types of tumors, IL-1 is induced only in the invasiveness and metastasis phases. The switch-on of IL-1 genes in malignant cells is induced by genetic alterations and possibly also by microenvironment-derived signals. Indeed, high IL-1 concentrations within the tumor microenvironment have been reported in numerous studies in cancer patients and experimental models and are associated with a more virulent tumor phenotype (reviewed in Elaraj et al., 2006; Lewis et al., 2006).

In the tumor milieu, IL-1 induces expression of various metastatic mediators, such as matrix metalloproteinases (MMP), VEGF, IL-8, IL-6, TNFα, and TGFβ (reviewed in Dinarello, 1996; Apte and Voronov, 2002, 2008; Apte et al., 2006b; Lewis et al., 2006; Voronov et al., 2013). Previously, in a transgenic model of Myc-dependent carcinogenesis, IL-1β was characterized as the principal effector molecule in the onset of angiogenesis, via MMP-mediated sequestration of extracellular matrix-associated VEGF and its further ligation to its cognate receptor on ECs (Shchors et al., 2006).

Our studies throughout the years have assessed the role of the IL-1 molecules in different phases of the malignant process, such as carcinogenesis, tumor angiogenesis, and invasiveness (Song et al., 2003, 2005; Krelin et al., 2007). Using a transplantable fibrosarcoma cell line transfected with cDNAs of the active forms of IL-1β, i.e., the mature form of IL-1β or the mature form of IL-1β ligated to a signal sequence (ssIL-1β), in which IL-1β is actively secreted through the endoplasmic reticulum-Golgi pathway, we observed that invasiveness of the different tumor cell lines was directly proportional to the amount of IL-1β secreted by the malignant cells (Song et al., 2003, 2005). In this system, invasiveness correlated with increased angiogenesis and accumulation of MDSCs in the spleen and tumor, which leads to general anergy. Similar observations were described in other experimental systems using IL-1β-transfected tumor cells (Saijo et al., 2002; Nakao et al., 2005; Bunt et al., 2006). We have hypothesized that initially small amounts of tumor cell-derived IL-1β induce a local inflammatory response, which subsequently recruits and activates BMDs that further secrete IL-1β, as well as an entire cytokine network that promotes tumor-mediated angiogenesis and tumor progression.

In various tumor models, we found that IL-1β induces increased tumor growth, invasiveness, and angiogenesis, when compared to IL-1α (Song et al., 2003; Voronov et al., 2003, 2010b). However, in some tumor cells, active secretion of IL-1α is observed (Apte and Voronov, 2008; Voronov et al., 2013).

In some tumors, such as breast carcinoma or pancreatic cancer, a pro-angiogenic signature correlated with IL-1α signaling (Matsuo et al., 2009; Guo and Gonzalez-Perez, 2011; Zhou et al., 2011). In these studies, IL-1α was shown to be involved in the induction of VEGF/VEGFR2 in the tumor microenvironment, through a cascade involving leptin/Notch. Secreted IL-1α may promote invasiveness in a similar manner to secreted IL-1β in these tumors.

However, in most malignancies, IL-1 is produced not only by tumor cells, but by other cells, such as neutrophils, macrophages, and MDSCs, recruited to the tumor microenvironment (reviewed in Apte and Voronov, 2008; Witz, 2008; Balkwill and Mantovani, 2010; Demaria et al., 2010; Grivennikov et al., 2010; Hanahan and Weinberg, 2011; Voronov et al., 2013).

These recruited cells thereby promote inflammation in the tumor microenvironment and thus increase the angiogenic response, tumor cell invasiveness and metastasis (Goldberg and Schwertfeger, 2010; Naldini et al., 2010; Schmid et al., 2011; Carmi et al., 2013).

Using IL-1 KO mice, we demonstrated that microenvironment-derived IL-1β, and to a much lesser extent IL-1α, is responsible for *in vivo* tumor angiogenesis and invasiveness of B16 melanoma cells. Significantly decreased angiogenesis was observed in IL-1β deficient mice injected with either melanoma cells or melanoma cell-containing Matrigel

plugs, when compared to wild-type mice. In IL-1α KO mice, tumor growth and angiogenesis in Matrigel plugs were observed to a lesser extent than in WT mice, but higher than those in IL-1β KO mice (Voronov et al., 2003). Addition of recombinant IL-1 into Matrigel plugs containing B16 cells in IL-1β KO mice partially restored the angiogenic response, while addition of IL-1Ra to B16-containing Matrigel plugs in wild-type mice inhibited the ingrowth of the blood vessel network into the plugs. In subsequent studies, the angiogenic potential of IL-1 was confirmed by Elaraj et al., who observed high expression of IL-1 mRNA in more than half of all tested metastatic human tumor specimens, including non-small-cell lung carcinoma, colorectal adenocarcinoma, and melanoma tumor samples. Supernatants from IL-1 expressing cell lines induced a significant increase in EC monolayer permeability, a hallmark event in early angiogenesis. Systemic treatment with recombinant IL-1Ra resulted in significant inhibition of xenograft growth and neovessel density of IL-1-producing tumor cell lines. Nevertheless, the addition of IL-1Ra did not have any effect in non-IL-1-producing tumor lines (Elaraj et al., 2006).

ANTI-IL-1 THERAPY IN ANTI-TUMOR APPROACHES

Since tumor development is related to and dependent on the angiogenic response, anti-angiogenic therapy targeting blood vessel formation was used to suppress existing tumors and to prevent metastasis. Various studies used different anti-angiogenic molecules or agents to limit tumor angiogenesis. The main molecule used in these experiments was VEGF, which is the most abundant pro-angiogenic molecule. A number of preclinical studies demonstrated a significant inhibition of tumor growth in various types of cancer by blocking VEGF (Ferrara and Kerbel, 2005). VEGF has been approved by the FDA to be used in cancer patients to inhibit tumor growth. Anti-VEGF monoclonal antibodies (bevacizumab, also called Avastin) were tested in various metastatic cancers in humans, usually in combination with chemotherapy (Ferrara et al., 2005; Carmeliet and Jain, 2011). However, some patients are refractory to this treatment and tumor recurrence or even metastases were observed after initial tumor shrinkage. It is beyond the scope of this review to summarize the effects of anti-VEGF therapy in cancer patients. In animal models, it was also shown that termination or interruption of VEGF neutralization induces rapid vascular regrowth in tumors and limits tumor-suppressive effects of VEGF treatment (reviewed in Kubota, 2012; Bellou et al., 2013). The dramatic response of the tumor microenvironment to VEGF inhibition may be due to the key role VEGF plays in normal cell viability, including ECs, myeloid cells, and possibly other cell types involved in tissue homeostasis (Chung and Ferrara, 2011; Lazarus and Keshet, 2011; Luo et al., 2011; Potente et al., 2011). For example, blocking VEGF induces EC apoptosis, resulting in the loss of integrity of blood vessels, and disturbance of tissue homeostasis. Due to these effects, VEGF blockage damages not only tumor blood vessels, but also healthy vessels, thus occasionally resulting in severe complications, such as hemorrhagic or thrombotic events (Verheul and Pinedo, 2007).

Different mechanisms have been proposed to explain rebound angiogenesis following VEGF neutralization (Casanovas et al., 2005; Ferrara, 2010b; Chung and Ferrara, 2011; Carmi et al., 2013). For example, intrinsic mechanisms, such as the adaptation of malignant cells to low VEGF levels by selecting tumor cell variants that are less dependent on VEGF for their survival/proliferation or variants with enhanced expression/secretion of other pro-angiogenic factors, such as bFGF or PlGF have been described (Casanovas et al., 2005; Fischer et al., 2007; Kubota, 2012). Following VEGF neutralization, extrinsic mechanisms also contribute to tumor recurrence by altering microenvironment cells to express different or additional angiogenic factors, thus facilitating tumor recurrence. These include induced secretion of Bv8 by MDSCs and a subsequent VEGF-independent angiogenic response (Shojaei et al., 2007; Carmi et al., 2013) or expression of PDGF-C in cancer-associated fibroblasts (Bergers and Hanahan, 2008; Crawford et al., 2009). In our studies, we described a novel mechanism of rebound angiogenesis after VEGF inhibition in Matrigel plugs containing tumor cells (Carmi et al., 2013). This resulted in significant and consistently elevated expression of direct angiogenic factors, such as bFGF, PlGF, and PDGF, as well as VEGF in VEGFR1$^+$ immature myeloid cells, mostly MDSCs. The re-programming of VEGFR1$^+$ immature myeloid cells into active pro-angiogenic cells also involves increased expression of hypoxia-inducible factor 1α, a key transcription factor regulating angiogenesis (Cramer and Johnson, 2003; Semenza, 2011). In contrast, in "uninterrupted angiogenesis" (without anti-VEGF treatment), as described above, ECs, rather than myeloid cells, are the major cells that produce VEGF and other direct angiogenic factors (Carmi et al., 2013).

In recent years, myeloid, cell-driven angiogenesis was shown to contribute to refractoriness or resistance to VEGF inhibition. For example, as discussed above, MDSCs can secrete Bv8, which further upregulates granulocyte colony-stimulating factor that promotes tumor growth and angiogenesis in a VEGF-independent manner. Therefore, intervention in myeloid cell recruitment to tumor sites in order to modulate differentiation or neutralization of specific pro-inflammatory or pro-angiogenic factors secreted by these cells, are now widely discussed. For example, blocking CSF-1 was shown to be effective in suppressing tumor angiogenesis (Kubota, 2012). In our studies, we found that blocking a major, alarm, pro-inflammatory molecule derived from myeloid cells, i.e., IL-1β can lead to a significant amelioration of tumor angiogenesis (Voronov et al., 2003; Carmi et al., 2013). The inhibition of IL-1β does not affect the healthy vascular system or tissue homeostasis and is more suitable for anti-angiogenic therapy than anti-VEGF approaches. IL-1β neutralization largely inhibited infiltration of myeloid cells, which are obligatory for tumor-mediated angiogenesis. Furthermore, IL-1β neutralization induces maturation of immature myeloid cells into anti-invasive M1 macrophages.

We recently found that IL-1β neutralization inhibited pro-inflammatory genes, such as Bv8, CCL2 and CCL3 in myeloid cells, and did not significantly alter the expression of angiogenic factors. IL-1β inhibition also reduced *in vivo* tumor development in mice injected subcutaneously with tumor cells. This inhibition was long-lasting and without recurrence a contrast to that observed with anti-VEGF (Carmi et al., 2013).

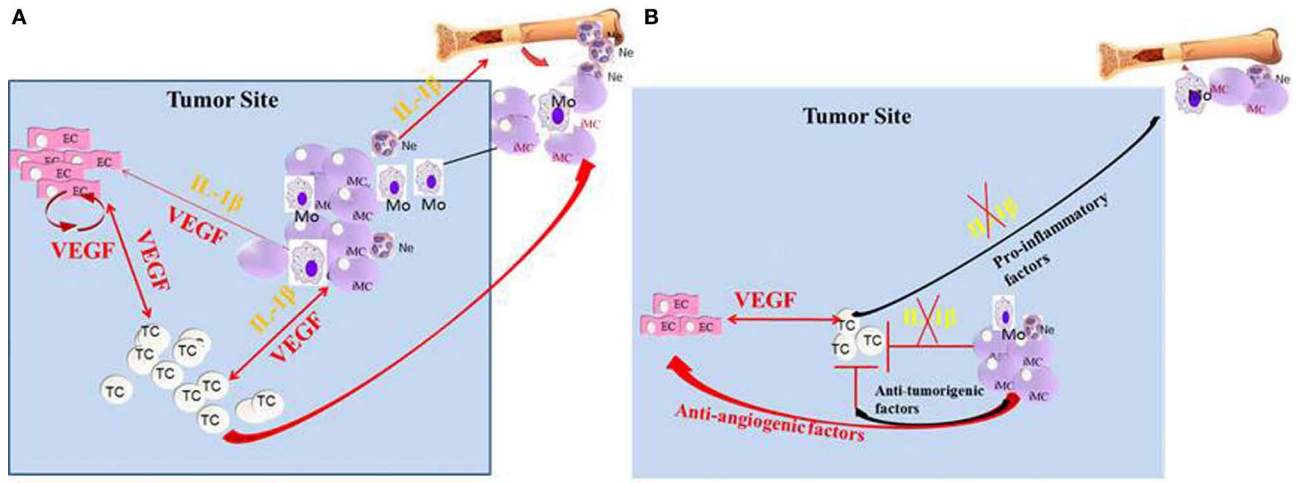

FIGURE 1 | Crosstalk between IL-1β and VEGF in the tumor microenvironment. (A) In the tumor microenvironment, IL-1β can be expressed initially by the malignant cells. In turn, IL-1β can recruit myeloid cells from the BM and further activate them to secrete pro-inflammatory and pro-angiogenic molecules, such as VEGF and IL-1β. Furthermore, IL-1β keeps myeloid cells in their immature stage, where they are pro-invasive and immunosuppressive. IL-1β can also activate tissue-resident ECs to produce VEGF and other pro-angiogenic factors. The malignant cells can also be activated by IL-1β for increased invasiveness. VEGF in the tumor microenvironment activates ECs and myeloid cells and also has homeostatic effects on these cells. **(B)** In the absence of IL-1β, inflammation, as well as VEGF production is diminished, which results in reduced invasiveness. Lack of IL-1β in the tumor microenvironment also induces the maturation of MDSCs into anti-tumor M1 macrophages.

These results have provided the rationale to use IL-1β neutralization as a treatment for tumor-mediated angiogenesis, and subsequent tumor progression.

Indeed, the positive role IL-1Ra plays in reducing the angiogenic response and in attenuating tumor progression has been demonstrated by us (Song et al., 2003; Voronov et al., 2003) and by others (Saijo et al., 2002; Nakao et al., 2005; Bunt et al., 2006; Schmid et al., 2011). IL-1 neutralization agents are available at the present time (Dinarello, 2010a,b; Dinarello and van der Meer, 2012). The IL-1Ra, also called Anakirna (Kineret; Amgen/Biovitrum) has been shown to be safe and effective in alleviating symptoms of rheumatoid arthritis and other autoinflammatory diseases. Continuous delivery systems of recombinant IL-1Ra or cells over-expressing IL-1Ra, encapsulated within alginate-poly (L-lysine)-alginate (APA) microspheres, reduced the tumor burden and inhibited tumor-mediated angiogenesis when implanted into tumor-bearing mice (Bar et al., 2004; Lavi et al., 2007). Similarly, over-expression of the IL-1Ra in human melanoma cell lines expressing endogenous IL-1, inhibits tumor growth and metastasis in human melanoma xenografts in nude mice through its effects on the microenvironment (Weinreich et al., 2003; Elaraj et al., 2006). Altogether, these results provide pre-clinical support for the use of IL-1Ra in cancer therapy.

Recently, some novel IL-1 neutralization agents, such as humanized anti-IL-1β, an IL-1 trap, as well as other agents that indirectly inhibit IL-1β production, have been developed (Dinarello, 2011b). These agents are now ready for testing in cancer patients. Optimally, IL-1 neutralization should be most effective in patients with minimal residual disease (MRD), to prevent tumor-induced angiogenesis, recurrence, and metastasis.

CONCLUSIONS

Finally, this review demonstrated the important role of the IL-1 family molecules, especially IL-1β, in the tumor microenvironment. IL-1β represents a major upstream cytokine that controls the local pro-inflammatory cascade and thereby affects the balance between protective immunity and destructive inflammation. IL-1 modulates diverse cells in the tumor microenvironment and acts together with VEGF in mounting and maintaining tumor-mediated angiogenesis (**Figure 1**). Thus, its neutralization should reduce tumor progression and invasiveness via its affect on pro-tumorigenic cells, and on the tumor-induced angiogenic switch. Further characterization of the optimal conditions for IL-1 neutralization could lead to the application of anti-IL-1 approaches in cancer therapy.

ACKNOWLEDGMENTS

The authors would like to thank their collaborators: Prof. Charles A. Dinarello, University of Colorado, Denver; Prof. Yochiro Iwakura, University of Tokyo, Tokyo, Japan; Prof. Hynda K. Kleinman, NIH, Bethesda, MD, USA, and Dr Monika Huszar, Kaplan Hospital, Rehovot, Israel. We would like to thank our technician, Mrs. Malka White for her devoted technical and editorial help, as well as to our students, whose work is discussed in this review. Ron N. Apte was supported by the following grants: FP7: Cancer and Inflammation (INFLA-CARE), Israel Ministry of Science (MOS) jointly with the Deutsches Krebsforschungscentrum (DKFZ), Heidelberg, Germany, the Israel Science Foundation funded by the Israel Academy of Sciences and Humanities, the United States-Israel Bi-national Foundation (BSF), the Israel Cancer Association and the Israel Ministry of Health Chief Scientist's Office. Prof. Ron N. Apte is an incumbent of the Irving Isaac Sklar Chair in Endocrinology

and Cancer, Ben Gurion University of the Negev. Elena Voronov was supported by the FP7: Cancer and Inflammation and the Israel Science Foundation, Israel Cancer Association, the Israel Ministry of Health Chief Scientist's Office, the United States-Israel Bi-national Foundation (BSF), and the Israel Science Foundation funded by the Israel Academy of Sciences and Humanities.

REFERENCES

Afonina, I. S., Tynan, G. A., Logue, S. E., Cullen, S. P., Bots, M., Luthi, A. U., et al. (2011). Granzyme B-dependent proteolysis acts as a switch to enhance the proinflammatory activity of IL-1alpha. *Mol. Cell* 44, 265–278. doi: 10.1016/j.molcel.2011.07.037

Alitalo, K., and Carmeliet, P. (2002). Molecular mechanisms of lymphangiogenesis in health and disease. *Cancer Cell* 1, 219–227. doi: 10.1016/S1535-6108(02)00051-X

Allavena, P., and Mantovani, A. (2012). Immunology in the clinic review series; focus on cancer: tumour-associated macrophages: undisputed stars of the inflammatory tumour microenvironment. *Clin. Exp. Immunol.* 167, 195–205. doi: 10.1111/j.1365-2249.2011.04515.x

Amano, K., Okigaki, M., Adachi, Y., Fujiyama, S., Mori, Y., Kosaki, A., et al. (2004). Mechanism for IL-1 beta-mediated neovascularization unmasked by IL-1 beta knock-out mice. *J. Mol. Cell. Cardiol.* 36, 469–480. doi: 10.1016/j.yjmcc.2004.01.006

Apte, R. N., Dotan, S., Elkabets, M., White, M. R., Reich, E., Carmi, Y., et al. (2006b). The involvement of IL-1 in tumorigenesis, tumor invasiveness, metastasis and tumor-host interactions. *Cancer Metastasis Rev.* 25, 387–408. doi: 10.1007/s10555-006-9004-4

Apte, R. N., Krelin, Y., Song, X., Dotan, S., Recih, E., Elkabets, M., et al. (2006a). Effects of micro-environment- and malignant cell-derived interleukin-1 in carcinogenesis, tumour invasiveness and tumour-host interactions. *Eur. J. Cancer* 42, 751–759. doi: 10.1016/j.ejca.2006.01.010

Apte, R. N., and Voronov, E. (2002). Interleukin-1-a major pleiotropic cytokine in tumor-host interactions. *Semin. Cancer Biol.* 12, 277–290. doi: 10.1016/S1044-579X(02)00014-7

Apte, R. N., and Voronov, E. (2008). Is interleukin-1 a good or bad "guy" in tumor immunobiology and immunotherapy? *Immunol. Rev.* 222, 222–241. doi: 10.1111/j.1600-065X.2008.00615.x

Arend, W. P. (2002). The balance between IL-1 and IL-1Ra in disease. *Cytokine Growth Factor Rev.* 13, 323–340. doi: 10.1016/S1359-6101(02)00020-5

Asahara, T., Masuda, H., Takahashi, T., Kalka, C., Pastore, C., Silver, M., et al. (1999). Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res.* 85, 221–228. doi: 10.1161/01.RES.85.3.221

Balkwill, F. (2002). Tumor necrosis factor or tumor promoting factor? *Cytokine Growth Factor Rev.* 13, 135–141. doi: 10.1016/S1359-6101(01)00020-X

Balkwill, F., and Mantovani, A. (2010). Cancer and inflammation: implications for pharmacology and therapeutics. *Clin. Pharmacol. Ther.* 87, 401–406. doi: 10.1038/clpt.2009.312

Balkwill, F. R., and Mantovani, A. (2012). Cancer-related inflammation: common themes and therapeutic opportunities. *Semin. Cancer Biol.* 22, 33–40. doi: 10.1016/j.semcancer.2011.12.005

Bar, D., Apte, R. N., Voronov, E., Dinarello, C. A., and Cohen, S. (2004). A continuous delivery system of IL-1 receptor antagonist reduces angiogenesis and inhibits tumor development. *FASEB. J.* 18, 161–163. doi: 10.1096/fj.03-0483fje

Bellou, S., Pentheroudakis, G., Murphy, C., and Fotsis, T. (2013). Anti-angiogenesis in cancer therapy: hercules and hydra. *Cancer Lett.* 338, 219–228. doi: 10.1016/j.canlet.2013.05.015

Berda-Haddad, Y., Robert, S., Salers, P., Zekraoui, L., Farnarier, C., Dinarello, C. A., et al. (2011). Sterile inflammation of endothelial cell-derived apoptotic bodies is mediated by interleukin-1alpha. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20684–20689. doi: 10.1073/pnas.1116848108

Bergers, G., and Hanahan, D. (2008). Modes of resistance to anti-angiogenic therapy. *Nat. Rev. Cancer* 8, 592–603. doi: 10.1038/nrc2442

Berse, B., Hunt, J. A., Diegel, R. J., Morganelli, P., Yeo, K., Brown, F., et al. (1999). Hypoxia augments cytokine (transforming growth factor-beta (TGF-beta) and IL-1)-induced vascular endothelial growth factor secretion by human synovial fibroblasts. *Clin. Exp. Immunol.* 115, 176–182. doi: 10.1046/j.1365-2249.1999.00775.x

Bertolini, F., Shaked, Y., Mancuso, P., and Kerbel, R. S. (2006). The multifaceted circulating endothelial cell in cancer: towards marker and target identification. *Nat. Rev. Cancer* 6, 835–845. doi: 10.1038/nrc1971

Bokhari, S. M., Zhou, L., Karasek, M. A., Paturi, S. G., and Chaudhuri, V. (2006). Regulation of skin microvasculature angiogenesis, cell migration, and permeability by a specific inhibitor of PKCalpha. *J. Invest. Dermatol.* 126, 460–467. doi: 10.1038/sj.jid.5700071

Braddock, M., and Quinn, A. (2004). Targeting IL-1 in inflammatory disease: new opportunities for therapeutic intervention. *Nat. Rev. Drug Discov.* 3, 330–339. doi: 10.1038/nrd1342

Breviario, F., Proserpio, P., Bertocchi, F., Lampugnani, M. G., Mantovani, A., and Dejana, E. (1990). Interleukin-1 stimulates prostacyclin production by cultured human endothelial cells by increasing arachidonic acid mobilization and conversion. *Arteriosclerosis* 10, 129–134. doi: 10.1161/01.ATV.10.1.129

Brogi, E., Winkles, J. A., Underwood, R., Clinton, S. K., Alberts, G. F., and Libby, P. (1993). Distinct patterns of expression of fibroblast growth factors and their receptors in human atheroma and nonatherosclerotic arteries. Association of acidic FGF with plaque microvessels and macrophages. *J. Clin. Invest.* 92, 2408–2418. doi: 10.1172/JCI116847

Bunt, S. K., Sinha, P., Clements, V. K., Leips, J., and Ostrand-Rosenberg, S. (2006). Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. *J. Immunol.* 176, 284–290.

Carmeliet, P. (2003). Angiogenesis in health and disease. *Nat. Med.* 9, 653–660. doi: 10.1038/nm0603-653

Carmeliet, P. (2005). Angiogenesis in life, disease and medicine. *Nature* 438, 932–936. doi: 10.1038/nature04478

Carmeliet, P., and Jain, R. K. (2000). Angiogenesis in cancer and other diseases. *Nature* 407, 249–257. doi: 10.1038/35025220

Carmeliet, P., and Jain, R. K. (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473, 298–307. doi: 10.1038/nature10144

Carmi, Y., Dotan, S., Rider, P., Kaplanov, I., White, M. R., Baron, R., et al. (2013). The role of IL-1beta in the early tumor cell-induced angiogenic response. *J. Immunol.* 190, 3500–3509. doi: 10.4049/jimmunol.1202769

Carmi, Y., Voronov, E., Dotan, S., Lahat, N., Rahat, M. A., Fogel, M., et al. (2009). The role of macrophage-derived IL-1 in induction and maintenance of angiogenesis. *J. Immunol.* 183, 4705–4714. doi: 10.4049/jimmunol.0901511

Casanovas, O., Hicklin, D. J., Bergers, G., and Hanahan, D. (2005). Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell* 8, 299–309. doi: 10.1016/j.ccr.2005.09.005

Chambers, S. E., O'Neill, C. L., O'Doherty, T. M., Medina, R. J., and Stitt, A. W. (2013). The role of immune-related myeloid cells in angiogenesis. *Immunobiology* 218, 1370–1375. doi: 10.1016/j.imbio.2013.06.010

Chung, A. S., and Ferrara, N. (2011). Developmental and pathological angiogenesis. *Annu. Rev. Cell Dev. Biol.* 27, 563–584. doi: 10.1146/annurev-cellbio-092910-154002

Coffelt, S. B., Lewis, C. E., Naldini, L., Brown, J. M., Ferrara, N., and De Palma, M. (2010). Elusive identities and overlapping phenotypes of proangiogenic myeloid cells in tumors. *Am. J. Pathol.* 176, 1564–1576. doi: 10.2353/ajpath.2010.090786

Coxon, A., Bolon, B., Estrada, J., Kaufman, S., Scully, S., Rattan, A., et al. (2002). Inhibition of interleukin-1 but not tumor necrosis factor suppresses neovascularization in rat models of corneal angiogenesis and adjuvant arthritis. *Arthritis Rheum.* 46, 2604–2612. doi: 10.1002/art.10546

Cramer, T., and Johnson, R. S. (2003). A novel role for the hypoxia inducible transcription factor HIF-1alpha: critical regulation of inflammatory cell function. *Cell Cycle* 2, 192–193. doi: 10.4161/cc.2.3.402

Crawford, Y., Kasman, I., Yu, L., Zhong, C., Wu, X., Modrusan, Z., et al. (2009). PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment. *Cancer Cell* 15, 21–34. doi: 10.1016/j.ccr.2008.12.004

Demaria, S., Pikarsky, E., Karin, M., Coussens, L. M., Chen, Y. C., El-Omar, E. M., et al. (2010). Cancer and inflammation: promise for biologic therapy. *J. Immunother.* 33, 335–351. doi: 10.1097/CJI.0b013e3181d32e74

Denny, M. F., Thacker, S., Mehta, H., Somers, E. C., Dodick, T., Barrat, F. J., et al. (2007). Interferon-alpha promotes abnormal vasculogenesis in lupus: a potential pathway for premature atherosclerosis. *Blood* 110, 2907–2915. doi: 10.1182/blood-2007-05-089086

De Palma, M., and Lewis, C. E. (2013). Macrophage regulation of tumor responses to anticancer therapies. *Cancer Cell* 23, 277–286. doi: 10.1016/j.ccr.2013.02.013

De Palma, M., Venneri, M. A., Galli, R., Sergi Sergi, L., and Politi, L. S., Sampaolesi, M, et al. (2005). Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell* 8, 211–226. doi: 10.1016/j.ccr.2005.08.002

De Palma, M., Venneri, M. A., and Naldini, L. (2003). *In vivo* targeting of tumor endothelial cells by systemic delivery of lentiviral vectors. *Hum. Gene Ther.* 14, 1193–1206. doi: 10.1089/104303403322168028

Dinarello, C. A. (1996). Biologic basis for interleukin-1 in disease. *Blood* 87, 2095–2147.

Dinarello, C. A. (2002). The IL-1 family and inflammatory diseases. *Clin. Exp. Rheumatol.* 20, S1–13.

Dinarello, C. A. (2006). Interleukin 1 and interleukin 18 as mediators of inflammation and the aging process. *Am. J. Clin. Nutr.* 83, 447S-455S.

Dinarello, C. A. (2009). Immunological and inflammatory functions of the interleukin-1 family. *Annu. Rev. Immunol.* 27, 519–550. doi: 10.1146/annurev.immunol.021908.132612

Dinarello, C. A. (2010a). Anti-inflammatory agents: present and future. *Cell* 140, 935–950. doi: 10.1016/j.cell.2010.02.043

Dinarello, C. A. (2010b). Why not treat human cancer with interleukin-1 blockade? *Cancer Metastasis Rev.* 29, 317–329. doi: 10.1007/s10555-010-9229-0

Dinarello, C. A. (2011a). Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood* 117, 3720–3732. doi: 10.1182/blood-2010-07-273417

Dinarello, C. A. (2011b). A clinical perspective of IL-1beta as the gatekeeper of inflammation. *Eur. J. Immunol.* 41, 1203–1217. doi: 10.1002/eji.201141550

Dinarello, C. A. S. A., and van der Meer, J. W. (2012). Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat. Rev. Drug Discov.* 11, 633–652. doi: 10.1038/nrd3800

Di Paolo, N. C., and Shayakhmetov, D. M. (2013). Interleukin-1 receptor 2 keeps the lid on interleukin-1alpha. *Immunity* 38, 203–205. doi: 10.1016/j.immuni.2013.02.001

Duyndam, M. C., Hilhorst, M. C., Schluper, H. M., Verheul, H. M., van Diest, P. J., Kraal, G., et al. (2002). Vascular endothelial growth factor-165 overexpression stimulates angiogenesis and induces cyst formation and macrophage infiltration in human ovarian cancer xenografts. *Am. J. Pathol.* 160, 537–548. doi: 10.1016/S0002-9440(10)64873-0

Eisenbarth, S. C., and Flavell, R. A. (2009). Innate instruction of adaptive immunity revisited: the inflammasome. *EMBO Mol. Med.* 1, 92–98. doi: 10.1002/emmm.200900014

Elaraj, D. M., Weinreich, D. M., Varghese, S., Puhlmann, M., Hewitt, S. M., Carroll, N. M., et al. (2006). The role of interleukin 1 in growth and metastasis of human cancer xenografts. *Clin. Cancer Res.* 12, 1088–1096. doi: 10.1158/1078-0432.CCR-05-1603

Favre, J., Terborg, N., and Horrevoets, A. J. (2013). The diverse identity of angiogenic monocytes. *Eur. J. Clin. Invest.* 43, 100–107. doi: 10.1111/eci.12009

Ferrara, N. (2010a). Role of myeloid cells in vascular endothelial growth factor-independent tumor angiogenesis. *Curr. Opin. Hematol.* 17, 219–224. doi: 10.1097/MOH.0b013e3283386660

Ferrara, N. (2010b). Pathways mediating VEGF-independent tumor angiogenesis. *Cytokine Growth Factor Rev.* 21, 21–26. doi: 10.1016/j.cytogfr.2009.11.003

Ferrara, N., Hillan, K. J., and Novotny, W. (2005). Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. *Biochem. Biophys. Res. Commun.* 333, 328–335. doi: 10.1016/j.bbrc.2005.05.132

Ferrara, N., and Kerbel, R. S. (2005). Angiogenesis as a therapeutic target. *Nature* 438, 967–974. doi: 10.1038/nature04483

Fischer, C., Jonckx, B., Mazzone, M., Zacchigna, S., Loges, S., Pattarini, L., et al. (2007). Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell* 131, 463–475. doi: 10.1016/j.cell.2007.08.038

Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* 1, 27–31. doi: 10.1038/nm0195-27

Folkman, J. (2001). Angiogenesis-dependent diseases. *Semin. Oncol.* 28, 536–542. doi: 10.1016/S0093-7754(01)90021-1

Folkman, J. (2003). Fundamental concepts of the angiogenic process. *Curr. Mol. Med.* 3, 643–651. doi: 10.2174/1566524033479465

Folkman, J., and D'Amore, P. A. (1996). Blood vessel formation: what is its molecular basis? *Cell* 87, 1153–1155. doi: 10.1016/S0092-8674(00)81810-3

Franchi, L., Eigenbrod, T., Munoz-Planillo, R., and Nunez, G. (2009). The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat. Immunol.* 10, 241–247. doi: 10.1038/ni.1703

Friesel, R., and Maciag, T. (1999). Fibroblast growth factor prototype release and fibroblast growth factor receptor signaling. *Thromb. Haemost.* 82, 748–754.

Gabay, C., Lamacchia, C., and Palmer, G. (2010). IL-1 pathways in inflammation and human diseases. *Nat. Rev. Rheumatol.* 6, 232–241. doi: 10.1038/nrrheum.2010.4

Gabrilovich, D. I., Ostrand-Rosenberg, S., and Bronte, V. (2012). Coordinated regulation of myeloid cells by tumours. *Nat. Rev. Immunol.* 12, 253–268. doi: 10.1038/nri3175

Garlanda, C., Anders, H. J., and Mantovani, A. (2009). TIR8/SIGIRR: an IL-1R/TLR family member with regulatory functions in inflammation and T cell polarization. *Trends Immunol.* 30, 439–446. doi: 10.1016/j.it.2009.06.001

Goldberg, J. E., and Schwertfeger, K. L. (2010). Proinflammatory cytokines in breast cancer: mechanisms of action and potential targets for therapeutics. *Curr. Drug Targets* 11, 1133–1146. doi: 10.2174/138945010792006799

Gothert, J. R., Gustin, S. E., van Eekelen, J. A., Schmidt, U., Hall, M. A., Jane, S. M., et al. (2004). Genetically tagging endothelial cells *in vivo*: bone marrow-derived cells do not contribute to tumor endothelium. *Blood* 104, 1769–1777. doi: 10.1182/blood-2003-11-3952

Grivennikov, S. I., Greten, F. R., and Karin, M. (2010). Immunity, inflammation, and cancer. *Cell* 140, 883–899. doi: 10.1016/j.cell.2010.01.025

Guo, S., and Gonzalez-Perez, R. R. (2011). Notch, IL-1 and leptin crosstalk outcome (NILCO) is critical for leptin-induced proliferation, migration and VEGF/VEGFR-2 expression in breast cancer. *PLoS ONE* 6:e21467. doi: 10.1371/journal.pone.0021467

Hagemann, T., Lawrence, T., McNeish, I., Charles, K. A., Kulbe, H., Thompson, R. G., et al. (2008). "Re-educating" tumor-associated macrophages by targeting NF-kappaB. *J. Exp. Med.* 205, 1261–1268. doi: 10.1084/jem.20080108

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013

Hiratsuka, S., Minowa, O., Kuno, J., Noda, T., and Shibuya, M. (1998). Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9349–9354. doi: 10.1073/pnas.95.16.9349

Jagielska, J., Kapopara, P. R., Salguero, G., Scherr, M., Schutt, H., Grote, K., et al. (2012). Interleukin-1 assembles a proangiogenic signaling module consisting of caveolin-1, tumor necrosis factor receptor-associated factor 6, p38-mitogen-activated protein kinase (MAPK), and MAPK-activated protein kinase 2 in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 32, 1280–1288. doi: 10.1161/ATVBAHA.111.243477

Kang, H. W., Torres, D., Wald, L., Weissleder, R., and Bogdanov, A. A. Jr. (2006). Targeted imaging of human endothelial-specific marker in a model of adoptive cell transfer. *Lab. Invest.* 86, 599–609. doi: 10.1038/labinvest.3700421

Kaplanski, G., Farnarier, C., Benoliel, A. M., Foa, C., Kaplanski, S., and Bongrand, P. (1994b). A novel role for E- and P-selectins: shape control of endothelial cell monolayers. *J. Cell Sci.* 107(Pt 9), 2449–2457.

Kaplanski, G., Farnarier, C., Kaplanski, S., Porat, R., Shapiro, L., Bongrand, P., et al. (1994a). Interleukin-1 induces interleukin-8 secretion from endothelial cells by a juxtacrine mechanism. *Blood* 84, 4242–4248.

Kawamoto, A., Gwon, H. C., Iwaguro, H., Yamaguchi, J. I., Uchida, S., Masuda, H., et al. (2001). Therapeutic potential of *ex vivo* expanded endothelial progenitor cells for myocardial ischemia. *Circulation* 103, 634–637. doi: 10.1161/01.CIR.103.5.634

Kerbel, R. S. (2008). Tumor angiogenesis. *N. Engl. J. Med.* 358, 2039–2049. doi: 10.1056/NEJMra0706596

Krelin, Y., Voronov, E., Dotan, S., Elkabets, M., Reich, E., Fogel, M., et al. (2007). Interleukin-1beta-driven inflammation promotes the development and invasiveness of chemical carcinogen-induced tumors. *Cancer Res.* 67, 1062–1071. doi: 10.1158/0008-5472.CAN-06-2956

Kubota, Y. (2012). Tumor angiogenesis and anti-angiogenic therapy. *Keio J. Med.* 61, 47–56. doi: 10.2302/kjm.61.47

Latz, E. (2010). The inflammasomes: mechanisms of activation and function. *Curr. Opin. Immunol.* 22, 28–33. doi: 10.1016/j.coi.2009.12.004

Lavi, G., Voronov, E., Dinarello, C. A., Apte, R. N., and Cohen, S. (2007). Sustained delivery of IL-1Ra from biodegradable microspheres reduces the number of murine B16 melanoma lung metastases. *J. Control Release* 123, 123–130. doi: 10.1016/j.jconrel.2007.07.015

Lazarus, A., and Keshet, E. (2011). Vascular endothelial growth factor and vascular homeostasis. *Proc. Am. Thorac. Soc.* 8, 508–511. doi: 10.1513/pats.201102-021MW

Lee, J. G., and Kay, E. P. (2012). NF-kappaB is the transcription factor for FGF-2 that causes endothelial mesenchymal transformation in cornea. *Invest. Ophthalmol. Vis. Sci.* 53, 1530–1538. doi: 10.1167/iovs.11-9102

Lewis, A. M., Varghese, S., Xu, H., and Alexander, H. R. (2006). Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. *J. Transl. Med.* 4, 48. doi: 10.1186/1479-5876-4-48

Li, C., Liu, B., Dai, Z., and Tao, Y. (2011). Knockdown of VEGF receptor-1 (VEGFR-1) impairs macrophage infiltration, angiogenesis and growth of clear cell renal cell carcinoma (CRCC). *Cancer Biol. Ther.* 12, 872–880. doi: 10.4161/cbt.12.10.17672

Luo, J., Xiong, Y., Han, X., and Lu, Y. (2011). VEGF non-angiogenic functions in adult organ homeostasis: therapeutic implications. *J. Mol. Med. (Berl.)* 89, 635–645. doi: 10.1007/s00109-011-0739-1

Mantovani, A., Romero, P., Palucka, A. K., and Marincola, F. M. (2008). Tumour immunity: effector response to tumour and role of the microenvironment. *Lancet* 371, 771–783. doi: 10.1016/S0140-6736(08)60241-X

Mantovani, A., and Sica, A. (2010). Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr. Opin. Immunol.* 22, 231–237. doi: 10.1016/j.coi.2010.01.009

Mantovani, A., Sica, A., Allavena, P., Garlanda, C., and Locati, M. (2009). Tumor-associated macrophages and the related myeloid-derived suppressor cells as a paradigm of the diversity of macrophage activation. *Hum. Immunol.* 70, 325–330. doi: 10.1016/j.humimm.2009.02.008

Martinon, F., Mayor, A., and Tschopp, J. (2009). The inflammasomes: guardians of the body. *Annu. Rev. Immunol.* 27, 229–265. doi: 10.1146/annurev.immunol.021908.132715

Maruyama, K., Mori, Y., Murasawa, S., Masaki, H., Takahashi, N., Tsutusmi, Y., et al. (1999). Interleukin-1 beta upregulates cardiac expression of vascular endothelial growth factor and its receptor KDR/flk-1 via activation of protein tyrosine kinases. *J. Mol. Cell. Cardiol.* 31, 607–617. doi: 10.1006/jmcc.1998.0895

Matsuo, Y., Sawai, H., Ochi, N., Yasuda, A., Takahashi, H., Funahashi, H., et al. (2009). Interleukin-1alpha secreted by pancreatic cancer cells promotes angiogenesis and its therapeutic implications. *J. Surg. Res.* 153, 274–281. doi: 10.1016/j.jss.2008.04.040

Medina, R. J., O'Neill, C. L., O'Doherty, T. M., Knott, H., Guduric-Fuchs, J., Gardiner, T. A., et al. (2011). Myeloid angiogenic cells act as alternative M2 macrophages and modulate angiogenesis through interleukin-8. *Mol. Med.* 17, 1045–1055. doi: 10.2119/molmed.2011.00129

Medina, R. J., O'Neill, C. L., Sweeney, M., Guduric-Fuchs, J., Gardiner, T. A., Simpson, D. A., et al. (2010). Molecular analysis of endothelial progenitor cell (EPC) subtypes reveals two distinct cell populations with different identities. *BMC Med. Genomics* 3:18. doi: 10.1186/1755-8794-3-18

Mohty, M., Vialle-Castellano, A., Nunes, J. A., Isnardon, D., Olive, D., and Gaugler, B. (2003). IFN-alpha skews monocyte differentiation into Toll-like receptor 7-expressing dendritic cells with potent functional activities. *J. Immunol.* 171, 3385–3393.

Murdoch, C., Muthana, M., Coffelt, S. B., and Lewis, C. E. (2008). The role of myeloid cells in the promotion of tumour angiogenesis. *Nat. Rev. Cancer* 8, 618–631. doi: 10.1038/nrc2444

Nakao, S., Kuwano, T., Tsutsumi-Miyahara, C., Ueda, S., Kimura, Y. N., Hamano, S., et al. (2005). Infiltration of COX-2-expressing macrophages is a prerequisite for IL-1 beta-induced neovascularization and tumor growth. *J. Clin. Invest.* 115, 2979–2991. doi: 10.1172/JCI23298

Naldini, A., Filippi, I., Miglietta, D., Moschetta, M., Giavazzi, R., and Carraro, F. (2010). Interleukin-1beta regulates the migratory potential of MDAMB231 breast cancer cells through the hypoxia-inducible factor-1alpha. *Eur. J. Cancer* 46, 3400–3408. doi: 10.1016/j.ejca.2010.07.044

Naldini, A., Leali, D., Pucci, A., Morena, E., Carraro, F., Nico, B., et al. (2006). Cutting edge: IL-1beta mediates the proangiogenic activity of osteopontin-activated human monocytes. *J. Immunol.* 177, 4267–4270.

Nasu, K., Itoh, H., Yuge, A., Kawano, Y., Yoshimatsu, J., and Narahara, H. (2006). Interleukin-1beta regulates vascular endothelial growth factor and soluble fms-like tyrosine kinase-1 secretion by human oviductal epithelial cells and stromal fibroblasts. *Gynecol. Endocrinol.* 22, 495–500. doi: 10.1080/08916930600929487

O'Neill, L. A. (2002). Signal transduction pathways activated by the IL-1 receptor/toll-like receptor superfamily. *Curr. Top. Microbiol. Immunol.* 270, 47–61. doi: 10.1007/978-3-642-59430-4_3

O'Neill, L. A. (2008). The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. *Immunol. Rev.* 226, 10–18. doi: 10.1111/j.1600-065X.2008.00701.x

Peters, B. A., Diaz, L. A., Polyak, K., Meszler, L., Romans, K., Guinan, E. C., et al. (2005). Contribution of bone marrow-derived endothelial cells to human tumor vasculature. *Nat. Med.* 11, 261–262. doi: 10.1038/nm1200

Portier, M., Zhang, X. G., Ursule, E., Lees, D., Jourdan, M., Bataille, R., et al. (1993). Cytokine gene expression in human multiple myeloma. *Br. J. Haematol.* 85, 514–520. doi: 10.1111/j.1365-2141.1993.tb03341.x

Potente, M., Gerhardt, H., and Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. *Cell* 146, 873–887. doi: 10.1016/j.cell.2011.08.039

Prudovsky, I., Mandinova, A., Soldi, R., Bagala, C., Graziani, I., Landriscina, M., et al. (2003). The non-classical export routes: FGF1 and IL-1alpha point the way. *J. Cell Sci.* 116, 4871–4881. doi: 10.1242/jcs.00872

Purhonen, S., Palm, J., Rossi, D., Kaskenpaa, N., Rajantie, I., Yla-Herttuala, S., et al. (2008). Bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumor growth. *Proc. Natl. Acad. Sci. U.S.A.* 105, 6620–6625. doi: 10.1073/pnas.0710516105

Qian, B. Z., and Pollard, J. W. (2010). Macrophage diversity enhances tumor progression and metastasis. *Cell* 141, 39–51. doi: 10.1016/j.cell.2010.03.014

Qin, S. L., Li, T. S., Takahashi, M., and Hamano, K. (2006). *In vitro* assessment of the effect of interleukin-1beta on angiogenic potential of bone marrow cells. *Circ. J.* 70, 1195–1199. doi: 10.1253/circj.70.1195

Ribatti, D. (2009). The paracrine role of Tie-2-expressing monocytes in tumor angiogenesis. *Stem Cells Dev.* 18, 703–706. doi: 10.1089/scd.2008.0385

Rider, P., Carmi, Y., Guttman, O., Braiman, A., Cohen, I., Voronov, E., et al. (2011). IL-1alpha and IL-1beta recruit different myeloid cells and promote different stages of sterile inflammation. *J. Immunol.* 187, 4835–4843. doi: 10.4049/jimmunol.1102048

Rider, P., Kaplanov, I., Romzova, M., Bernardis, L., Braiman, A., Voronov, E., et al. (2012). The transcription of the alarmin cytokine interleukin-1 alpha is controlled by hypoxia inducible factors 1 and 2 alpha in hypoxic cells. *Front. Immunol.* 3:290. doi: 10.3389/fimmu.2012.00290

Ruffell, B., Affara, N. I., and Coussens, L. M. (2012). Differential macrophage programming in the tumor microenvironment. *Trends Immunol.* 33, 119–126. doi: 10.1016/j.it.2011.12.001

Ruffell, B., DeNardo, D. G., Affara, N. I., and Coussens, L. M. (2010). Lymphocytes in cancer development: polarization towards pro-tumor immunity. *Cytokine Growth Factor Rev.* 21, 3–10. doi: 10.1016/j.cytogfr.2009.11.002

Saijo, Y., Tanaka, M., Miki, M., Usui, K., Suzuki, T., Maemondo, M., et al. (2002). Proinflammatory cytokine IL-1 beta promotes tumor growth of Lewis lung carcinoma by induction of angiogenic factors: *in vivo* analysis of tumor-stromal interaction. *J. Immunol.* 169, 469–475.

Salven, P., Hattori, K., Heissig, B., and Rafii, S. (2002). Interleukin-1alpha promotes angiogenesis *in vivo* via VEGFR-2 pathway by inducing inflammatory cell VEGF synthesis and secretion. *FASEB J.* 16, 1471–1473.

Sano, H., Forough, R., Maier, J. A., Case, J. P., Jackson, A., Engleka, K., et al. (1990). Detection of high levels of heparin binding growth factor-1 (acidic fibroblast growth factor) in inflammatory arthritic joints. *J. Cell Biol.* 110, 1417–1426. doi: 10.1083/jcb.110.4.1417

Schmid, M. C., Avraamides, C. J., Foubert, P., Shaked, Y., Kang, S. W., Kerbel, R. S., et al. (2011). Combined blockade of integrin-alpha4beta1 plus cytokines SDF-1alpha or IL-1beta potently inhibits tumor inflammation and growth. *Cancer Res.* 71, 6965–6975. doi: 10.1158/0008-5472.CAN-11-0588

Schroder, K., and Tschopp, J. (2010). The inflammasomes. *Cell* 140, 821–832. doi: 10.1016/j.cell.2010.01.040

Schweighofer, B., Testori, J., Sturtzel, C., Sattler, S., Mayer, H., Wagner, O., et al. (2009). The VEGF-induced transcriptional response comprises gene clusters at the crossroad of angiogenesis and inflammation. *Thromb. Haemost.* 102, 544–554. doi: 10.1160/TH08-12-0830

Semenza, G. L. (2011). Hypoxia. Cross talk between oxygen sensing and the cell cycle machinery. *Am. J. Physiol. Cell Physiol.* 301, C550–C552. doi: 10.1152/ajpcell.00176.2011

Shchors, K., Shchors, E., Rostker, F., Lawlor, E. R., Brown-Swigart, L., and Evan, G. I. (2006). The Myc-dependent angiogenic switch in tumors is mediated by interleukin 1beta. *Genes Dev.* 20, 2527–2538. doi: 10.1101/gad.1455706

Sheikpranbabu, S., Kalishwaralal, K., Venkataraman, D., Eom, S. H., Park, J., and Gurunathan, S. (2009). Silver nanoparticles inhibit VEGF-and IL-1beta-induced vascular permeability via Src dependent pathway in porcine retinal endothelial cells. *J. Nanobiotechnol.* 7, 8. doi: 10.1186/1477-3155-7-8

Shibuya, M. (2006a). Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis. *J. Biochem. Mol. Biol.* 39, 469–478. doi: 10.5483/BMBRep.2006.39.5.469

Shibuya, M. (2006b). Vascular endothelial growth factor (VEGF)-Receptor2: its biological functions, major signaling pathway, and specific ligand VEGF-E. *Endothelium* 13, 63–69. doi: 10.1080/10623320600697955

Shojaei, F., Wu, X., Zhong, C., Yu, L., Liang, X. H., Yao, J., et al. (2007). Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature* 450, 825–831. doi: 10.1038/nature06348

Sica, A., Matsushima, K., Van Damme, J., Wang, J. M., Polentarutti, N., Dejana, E., et al. (1990). IL-1 transcriptionally activates the neutrophil chemotactic factor/IL-8 gene in endothelial cells. *Immunology* 69, 548–553.

Sica, A., Porta, C., Morlacchi, S., Banfi, S., Strauss, L., Rimoldi, M., et al. (2012). Origin and functions of tumor-associated myeloid cells (TAMCs). *Cancer Microenviron*. 5, 133–149. doi: 10.1007/s12307-011-0091-6

Sims, J. E., Nicklin, M. J., Bazan, J. F., Barton, J. L., Busfield, S. J., Ford, J. E., et al. (2001). A new nomenclature for IL-1-family genes. *Trends Immunol.* 22, 536–537. doi: 10.1016/S1471-4906(01)02040-3

Sims, J. E., and Smith, D. E. (2010). The IL-1 family: regulators of immunity. *Nat. Rev. Immunol.* 10, 89–102. doi: 10.1038/nri2691

Song, X., Krelin, Y., Dvorkin, T., Bjorkdahl, O., Segal, S., Dinarello, C. A., et al. (2005). CD11b+/Gr-1+ immature myeloid cells mediate suppression of T Cells in mice bearing tumors of IL-1{beta}-secreting cells. *J. Immunol.* 175, 8200–8208.

Song, X., Voronov, E., Dvorkin, T., Fima, E., Cagnano, E., Benharroch, D., et al. (2003). Differential effects of IL-1 alpha and IL-1 beta on tumorigenicity patterns and invasiveness. *J. Immunol.* 171, 6448–6456.

Stavri, G. T., Zachary, I. C., Baskerville, P. A., Martin, J. F., and Erusalimsky, J. D. (1995). Basic fibroblast growth factor upregulates the expression of vascular endothelial growth factor in vascular smooth muscle cells. Synergistic interaction with hypoxia.*Circulation* 92, 11–14. doi: 10.1161/01.CIR.92.1.11

Stylianou, E., and Saklatvala, J. (1998). Interleukin-1. *Int. J. Biochem. Cell Biol.* 30, 1075–1079. doi: 10.1016/S1357-2725(98)00081-8

Suswam, E. A., Nabors, L. B., Huang, Y., Yang, X., and King, P. H. (2005). IL-1beta induces stabilization of IL-8 mRNA in malignant breast cancer cells via the 3' untranslated region: involvement of divergent RNA-binding factors HuR, KSRP and TIAR. *Int. J. Cancer* 113, 911–919. doi: 10.1002/ijc.20675

Thacker, S. G., Berthier, C. C., Mattinzoli, D., Rastaldi, M. P., Kretzler, M., and Kaplan, M. J. (2010). The detrimental effects of IFN-alpha on vasculogenesis in lupus are mediated by repression of IL-1 pathways: potential role in atherogenesis and renal vascular rarefaction. *J. Immunol.* 185, 4457–4469. doi: 10.4049/jimmunol.1001782

Thomas, K. A. (1996). Vascular endothelial growth factor, a potent and selective angiogenic agent. *J. Biol. Chem.* 271, 603–606. doi: 10.1074/jbc.271.2.603

Thornton, P., McColl, B. W., Greenhalgh, A., Denes, A., Allan, S. M., and Rothwell, N. J. (2010). Platelet interleukin-1alpha drives cerebrovascular inflammation. *Blood* 115, 3632–3639. doi: 10.1182/blood-2009-11-252643

Tu, S., Bhagat, G., Cui, G., Takaishi, S., Kurt-Jones, E. A., Rickman, B., et al. (2008). Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer Cell* 14, 408–419. doi: 10.1016/j.ccr.2008.10.011

Turnquist, H. R., Sumpter, T. L., Tsung, A., Zahorchak, A. F., Nakao, A., Nau, G. J., et al. (2008). IL-1beta-driven ST2L expression promotes maturation resistance in rapamycin-conditioned dendritic cells. *J. Immunol.* 181, 62–72.

Urbich, C., Heeschen, C., Aicher, A., Dernbach, E., Zeiher, A. M., and Dimmeler, S. (2003). Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. *Circulation* 108, 2511–2516. doi: 10.1161/01.CIR.0000096483.29777.50

Verheul, H. M., and Pinedo, H. M. (2007). Possible molecular mechanisms involved in the toxicity of angiogenesis inhibition. *Nat. Rev. Cancer* 7, 475–485. doi: 10.1038/nrc2152

Voronov, E., Carmi, Y., and Apte, R. N. (2007). Role of IL-1-mediated inflammation in tumor angiogenesis. *Adv. Exp. Med. Biol.* 601, 265–270. doi: 10.1007/978-0-387-72005-0_28

Voronov, E., Dotan, S., Gayvoronsky, L., White, R. M., Cohen, I., Krelin, Y., et al. (2010a). IL-1-induced inflammation promotes development of leishmaniasis in susceptible BALB/c mice. *Int. Immunol.* 22, 245–257. doi: 10.1093/intimm/dxq006

Voronov, E., Dotan, S., Krelin, Y., Song, X., Elkabets, M., Carmi, Y., et al. (2013). Unique Versus Redundant Functions of IL-1alpha and IL-1beta in the Tumor Microenvironment. *Front. Immunol.* 4:177. doi: 10.3389/fimmu.2013.00177

Voronov, E., Reich, E., Dotan, S., Dransh, P., Cohen, I., Huszar, M., et al. (2010b). Effects of IL-1 molecules on growth patterns of 3-MCA-induced cell lines: an interplay between immunogenicity and invasive potential. *J. Immunotoxicol.* 7, 27–38. doi: 10.3109/15476910903405528

Voronov, E., Shouval, D. S., Krelin, Y., Cagnano, E., Benharroch, D., Iwakura, Y., et al. (2003). IL-1 is required for tumor invasiveness and angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 100, 2645–2650. doi: 10.1073/pnas.0437939100

Weinreich, D. M., Elaraj, D. M., Puhlmann, M., Hewitt, S. M., Carroll, N. M., Feldman, E. D., et al. (2003). Effect of interleukin 1 receptor antagonist gene transduction on human melanoma xenografts in nude mice. *Cancer Res.* 63, 5957–5961.

Wels, J., Kaplan, R. N., Rafii, S., and Lyden, D. (2008). Migratory neighbors and distant invaders: tumor-associated niche cells. *Genes Dev.* 22, 559–574. doi: 10.1101/gad.1636908

Witz, I. P. (2008). Tumor-microenvironment interactions: dangerous liaisons. *Adv. Cancer Res.* 100, 203–229. doi: 10.1016/S0065-230X(08)00007-9

Zheng, Y., Humphry, M., Maguire, J. J., Bennett, M. R., and Clarke, M. C. (2013). Intracellular interleukin-1 receptor 2 binding prevents cleavage and activity of interleukin-1alpha, controlling necrosis-induced sterile inflammation. *Immunity* 38, 285–295. doi: 10.1016/j.immuni.2013.01.008

Zhou, W., Guo, S., and Gonzalez-Perez, R. R. (2011). Leptin pro-angiogenic signature in breast cancer is linked to IL-1 signalling. *Br. J. Cancer* 104, 128–137. doi: 10.1038/sj.bjc.6606013

Ziegelhoeffer, T., Fernandez, B., Kostin, S., Heil, M., Voswinckel, R., Helisch, A., et al. (2004). Bone marrow-derived cells do not incorporate into the adult growing vasculature. *Circ. Res.* 94, 230–238. doi: 10.1161/01.RES.0000110419.50982.1C

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 December 2013; accepted: 07 March 2014; published online: 28 March 2014.

*Citation: Voronov E, Carmi Y and Apte RN (2014) The role IL-1 in tumor-mediated angiogenesis. Front. Physiol. **5**:114. doi: 10.3389/fphys.2014.00114*

This article was submitted to Vascular Physiology, a section of the journal Frontiers in Physiology.

frontiers in PHYSIOLOGY

REVIEW ARTICLE
published: 05 March 2014
doi: 10.3389/fphys.2014.00075

Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis

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Tumor angiogenesis is an essential process for supplying rapidly growing malignant tissues with essential nutrients and oxygen. An angiogenic switch allows tumor cells to survive and grow, and provides them access to vasculature resulting in metastatic disease. Monocyte-derived macrophages recruited and reprogrammed by tumor cells serve as a major source of angiogenic factors boosting the angiogenic switch. Tumor endothelium releases angiopoietin-2 and further facilitates recruitment of TIE2 receptor expressing monocytes (TEM) into tumor sites. Tumor-associated macrophages (TAM) sense hypoxia in avascular areas of tumors, and react by production of angiogenic factors such as VEGFA. VEGFA stimulates chemotaxis of endothelial cells (EC) and macrophages. In some tumors, TAM appeared to be a major source of MMP9. Elevated expression of MMP9 by TAM mediates extracellular matrix (ECM) degradation and the release of bioactive VEGFA. Other angiogenic factors released by TAM include basic fibroblast growth factor (bFGF), thymidine phosphorylase (TP), urokinase-type plasminogen activator (uPA), and adrenomedullin (ADM). The same factors used by macrophages for the induction of angiogenesis [like vascular endothelial growth factor A (VEGF-A) and MMP9] support lymphangiogenesis. TAM can express LYVE-1, one of the established markers of lymphatic endothelium. TAM support tumor lymphangiogenesis not only by secretion of pro-lymphangiogenic factors but also by trans-differentiation into lymphatic EC. New pro-angiogenic factor YKL-40 belongs to a family of mammalian chitinase-like proteins (CLP) that act as cytokines or growth factors. Human CLP family comprises YKL-40, YKL-39, and SI-CLP. Production of all three CLP in macrophages is antagonistically regulated by cytokines. It was recently established that YKL-40 induces angiogenesis *in vitro* and in animal tumor models. YKL-40-neutralizing monoclonal antibody blocks tumor angiogenesis and progression. The role of YKL-39 and SI-CLP in tumor angiogenesis and lymphangiogenesis remains to be investigated.

Keywords: tumor-associated macrophages, TIE2 receptor, VEGF, LYVE-1, stabilin-1, chitinase-like protein

INTRODUCTION

Tumor-associated macrophages (TAM) are key cells controlling tumor angiogenesis. TAM originate from circulating monocytes which are recruited to the tumor site and programmed by tumor-derived factors such as colony-stimulating factor-1 (CSF-1), vascular endothelial growth factor A (VEGF-A) and CC chemokine ligand 2 (CCL2) (Mantovani et al., 1992; Qian and Pollard, 2010). These and other factors in the tumor microenvironment shape the TAM phenotype and skew them toward tumor-supportive M2-polarized macrophages, although M1-polarized TAM with anti-tumor activity were also reported in several types of cancer (Forssell et al., 2007; Galarneau et al., 2007; Ong et al., 2012; Sica and Mantovani, 2012). Macrophage density correlates with poor prognosis in many types of human cancer. Tumor supporting functions of TAM including stimulation of tumor cell growth and the creation of favorable conditions for tumor cell intravasation into vessels and metastatic spread are well-described in animal models of breast cancer (Lin et al., 2006; Qian and Pollard, 2010). Numerous recent studies have demonstrated that TAM function as major producers of pro-angiogenic factors in malignant tumors. The angiogenic switch is an important step in cancer progression. The formation of new blood vessels is essential for fast growing tumor cells to be supplied with nutrients and oxygen. The angiogenic switch allows tumor cells to survive and provides them access to vasculature, which may result in the escape of malignant cells into circulation and onset of metastatic disease. Macrophages recruited and reprogrammed by tumor cells produce factors mediating the angiogenic switch (Huang et al., 2002; Lin et al., 2006; Du et al., 2008). The contribution of TAM to tumor angiogenesis was described in animal models of breast cancer, melanoma, prostate cancer, cervical cancer, and ovarian cancer (Huang et al., 2002; Egami et al., 2003; Giraudo et al., 2004;

Lin et al., 2006; Halin et al., 2009). A positive correlation between TAM infiltration and angiogenesis was found in many human cancers including breast cancer, melanoma, pulmonary adenocarcinoma, glioma, gastric cancer, B-cell non-Hodgkin's lymphoma, mucoepidermoid carcinoma of salivary glands, and leiomyosarcoma (Leek et al., 1996; Nishie et al., 1999; Takanami et al., 1999; Vacca et al., 1999; Torisu et al., 2000; Shieh et al., 2009; Espinosa et al., 2011; Wu et al., 2012).

Clear evidence for the role of TAM in tumor angiogenesis was reported by Lin and colleagues using the polyoma virus middle T oncogene (MMTV-PyMT) spontaneous mouse model of mammary adenocarcinoma (Lin et al., 2006). Increased infiltration of the primary tumor with macrophages was associated with an angiogenic switch. In CSF-1-null mice, macrophage infiltration of the tumor site was significantly lower and accompanied by impaired development of the vasculature network. This was a direct effect of the absence of macrophages in the tumors since the restoration of macrophage numbers in the tumors of CSF-1-null mice by the transgenic expression of CSF-1, specifically in the mammary epithelium, resulted in the increase of vessel density. VEGF was depleted in the stromal cells of tumors of CSF-1 null mice suggesting that this is a significant reason of impaired angiogenesis. Another in vivo study demonstrated that human breast cancer spheroids implanted into nude mice induced more pronounced vascularization if they were infiltrated with macrophages before implantation (Huang et al., 2002). In this model, macrophages contributed significantly to VEGF release by spheroids and increased the angiogenic response. Up to date, VEGF-A is the best characterized TAM-derived cytokine involved in tumor angiogenesis.

VEGF PRODUCTION AND PROCESSING BY TAM

The ability of TAM to accelerate vessel growth is mediated through the up-regulation and release of several pro-angiogenic factors. The tumor microenvironment polarizes macrophages toward M2 or a mixed M1/M2 phenotype, which is characterized by elevated expression of potent pro-angiogenic factors (Ly et al., 2010; Rolny et al., 2011). Re-polarization of the TAM phenotype toward M1 manifests in inhibition of pro-angiogenic activity and elevated expression of anti-angiogenic factors such as CXC-chemokine ligand 9 (CXCL9) and IFN-β (Rolny et al., 2011). VEGF-A is known as a major pro-angiogenic cytokine released by TAM. Its levels correlate with TAM density in several types of human cancer (Valkovic et al., 2002; Shieh et al., 2009). In breast cancer, TAM produce VEGF-A in hypoxic avascular areas of tumors (Lewis et al., 2000). Recently, the $MHCII^{low}$ subset of TAM which resides in hypoxic areas of tumors was shown to be associated with a pro-angiogenic gene signature and increased VEGF-A expression (Laoui et al., 2013). The accumulation and retention of TAM in hypoxic areas of tumors seems to be specifically regulated by hypoxia-induced factor semaphorin 3A which triggers macrophage recruitment through VEGFR1 (Casazza et al., 2013). In macrophages, hypoxia induces expression of hypoxia inducible factor (HIF-1α and HIF-2α) transcription factors, the major master regulators of VEGF-A expression (Bingle et al., 2002; Burke et al., 2002; Imtiyaz et al., 2010; Staples et al., 2011). However, recent studies reported that HIF-1α and HIF-2α may play opposing roles in tumor angiogenesis (Eubank et al., 2011; Roda et al., 2012). In a mouse melanoma model, chemical stabilization of HIF-2α in TAM stimulated production of a soluble form of the VEGF receptor (sVEGFR-1) which neutralized biological activity of VEGF-A. This resulted in reduced angiogenesis and tumor growth (Roda et al., 2012). In contrast, HIF-1α expression in TAM was responsible for VEGF-A production. In agreement with this study, co-culture of breast cancer spheroids with wild type or HIF-1α knocked out macrophages revealed an indispensable role of macrophage-expressed HIF-1α in tumor angiogenesis (Werno et al., 2010). Besides hypoxia, HIF-1-controlled VEGF-A expression can be induced by several cytokines. The production of IL1β by TAM is able to induce HIF-1α expression and VEGF-A release in tumors. It was shown that IL1β stimulates HIF-1α production in several cancer cell lines even under normoxic conditions (Jung et al., 2003). Another common TAM-produced cytokine, transforming growth factor β1 (TGFβ1), also contributes to VEGF-A expression in mouse macrophages through HIF-1α/β- and Smad3/4-dependent mechanisms (Jeon et al., 2007). Alternatively, VEGF-A expression can be induced by tumor-released CSF-1 (M-CSF), which acts through NF-κB activation and, in combination with CCL2, promotes pro-angiogenic functions of macrophages (Eubank et al., 2003; Wyckoff et al., 2004). Moreover, irradiation stimulates tumor cells to produce higher levels of CSF-1 resulting in the enhanced infiltration of pro-angiogenic myeloid cells into the tumor site (Rego et al., 2013; Xu et al., 2013). Upon secretion from cells, VEGF-A associates with extracellular matrix (ECM) and its soluble form can be released by enzymatic cleavage of ECM by matrix metalloproteinases (MMPs) (Lee et al., 2005). Several studies revealed that TAM can significantly contribute to this process. In a mouse model of human ovarian cancer, TAM were found to be a major source of MMP9. Furthermore, the presence of MMP9-expressing TAM positively correlated with tumor angiogenesis, tumor growth and VEGF-A levels (Huang et al., 2002). Elevated expression of MMP9 by tumor-infiltrating inflammatory cells in a mouse pancreatic cancer model mediated the release of bioactive VEGF-A from its extracellular reservoir (Bergers et al., 2000). In a similar study using a mouse glioblastoma tumor model, MMP9-producing macrophages and TIE2+ monocytes (TEM) contributed to the release of bioactive VEGF-A from its ECM-bound form. These bone marrow-derived cells were recruited into tumors by the HIF-1 target molecule CXC-chemokine ligand CXCL12 (SDF-1α) released by tumor cells (Du et al., 2008). It should be noted that targeting CXCL12 and its receptor CXCR4 in mouse glioblastoma prevented HIF-1-mediated recruitment of pro-angiogenic TAM and TEM, reduced vasculogenesis and abrogated tumor growth after irradiation (Kioi et al., 2010). Interestingly, a recent study demonstrated that in contrast to high-dose tumor irradiation, local low-dose gamma irradiation of pancreatic carcinomas induced TAM with an anti-tumor immunostimulatory phenotype. These TAM downregulated HIF-1 expression and suppressed intratumoral VEGF-A production resulting in the normalization of tumor vasculature. This study emphasizes the plasticity of the TAM phenotype and the possibility to revert their pro-angiogenic properties through dampening of VEGF-A-dependent angiogenesis (De Palma et al.,

2013; Klug et al., 2013). Altogether, most of the studies demonstrate the indispensable role of TAM in the induction of tumor angiogenesis either through the direct production of VEGF-A or modulation of its accessibility in the tumor microenvironment.

Despite the undeniable role of VEGF in tumor angiogenesis, several studies revealed that other molecular factors (presumably HIF-1-induced factors) can significantly contribute to this process (Kioi et al., 2010; Chen et al., 2012).

OTHER ANGIOGENIC FACTORS RELEASED OR INTERNALIZED BY TAM

Besides VEGF, TAM release a panel of pro-angiogenic factors which include tumor necrosis factor α (TNFα), basic fibroblast growth factor (bFGF), thymidine phosphorylase (TP), urokinase-type plasminogen activator (uPA), adrenomedullin (ADM), and semaphorin 4D (Sema4D) (Hildenbrand et al., 1995; Leek et al., 1998; Mantovani et al., 2007; Sierra et al., 2008; Chen et al., 2011). TP stimulates the migration of endothelial cells (EC), whereas uPA mediates ECM degradation and increases vascular invasion (Hotchkiss et al., 2003; Piao et al., 2005; Basire et al., 2006; Bijnsdorp et al., 2011). Macrophage-derived TP was associated with angiogenesis and reduced survival in human glioma and intestinal type gastric cancer (Yao et al., 2001; Kawahara et al., 2010). Elevated expression of TP is correlated with a poor prognosis in breast cancer and pancreatic cancer, and uPA expression is correlated with a poor prognosis in breast cancer (Takao et al., 1998; Toi et al., 1999; Harbeck et al., 2002). Macrophage-derived IL1α directly stimulated endothelial tube formation and neovascularization, as well as growth of mouse prostate tumors (Kwon et al., 2013). TAM-derived ADM was shown to induce angiogenesis and tumor growth in a mouse model of melanoma, and can potentially be implicated in human melanoma angiogenesis (Chen et al., 2011). In mouse models of breast cancer, TAM-produced Sema4D was found to be critical for tumor angiogenesis, vessel maturation, and tumor growth (Sierra et al., 2008). Sema4D induced motility of EC via the engagement of receptor plexin B1. In Sema4D KO mice, interactions between EC and pericytes (an essential process in vessel formation) were disrupted. Another macrophage-derived pro-angiogenic factor, prostaglandin E2 (PGE_2), can be potentially involved in pathological angiogenesis induced in tumors by therapeutic interventions which stimulate apoptosis. It was shown that apoptotic cells activated an angiogenic program in human macrophages resulting in PGE_2-mediated endothelial cell migration (Brecht et al., 2011).

Macrophages communicate with other cell types and control tissue turnover not only by the release of various factors, but also by their internalization and degradation. This clearance function is especially effective in alternatively activated macrophages (M2) (Kzhyshkowska and Krusell, 2009). Thus, the clearance of secreted protein acidic and rich in cystein (SPARC), a soluble component of ECM that inhibits angiogenesis by the modulation of expression of VEGF and MMPs (Zhang et al., 2012a), has been demonstrated by us in human alternatively activated macrophages (Kzhyshkowska et al., 2006a). Multifunctional scavenger receptor stabilin-1 is responsible for SPARC uptake and targeting for degradation in lysosomes. We and others also found in several tumor models that stabilin-1 is expressed by TAM (Schledzewski et al., 2006; Werno et al., 2010; Algars et al., 2012; David et al., 2012), leading to the hypothesis that stabilin-1-mediated clearance of SPARC can affect tumor angiogenesis. The clearance function of TAM as a mechanism of the regulation of angiogenesis is underestimated at the moment and has to be addressed experimentally.

TAM INDUCE ANGIOGENIC ACTIVITY IN TUMOR CELLS

The involvement of TAM in tumor angiogenesis is not limited to the self-production of angiogenic factors. TAM are able to release cytokines that indirectly contribute to tumor angiogenesis by the induction of a pro-angiogenic program in tumor cells. It was demonstrated that human monocytes and macrophages induced the production of the pro-angiogenic factors IL-8 and VEGF from melanoma and glioma cells during co-culture (Torisu et al., 2000; Hong et al., 2009). Elevated expression of IL-8 during co-culture with macrophages was also found in several lung cancer, osteogenic sarcoma and hepatoma cell lines (Chen et al., 2003). Up-regulation of IL-8 expression was presumably mediated by TAM-derived TNFα and IL1α, which stimulated NF-κB activity in tumor cells (Yao et al., 2005). Macrophage-derived TNFα and IL-1α were necessary for the release of IL-8 and VEGF from melanoma cells (Torisu et al., 2000). *In vitro* M2-polarized monocyte-derived macrophages enhanced the angiogenic potential of human basal cell carcinoma cells through the induction of cyclooxygenase-2 (COX-2) expression resulting in the elevated release of VEGF and bFGF from tumor cells (Tjiu et al., 2009). Thus, tumor cells and recruited TAM cooperate in the tumor microenvironment to amplify the production of pro-angiogenic factors resulting in an angiogenic switch (**Figure 1**).

TIE2 EXPRESSING MONOCYTES AS MAJOR INDUCERS OF TUMOR ANGIOGENESIS

There is a growing set of evidence that specific subpopulations of TIE2 receptor expressing monocytes (TEM) in mice and humans significantly contribute to tumor angiogenesis (Lewis et al., 2007; Matsubara et al., 2013). These monocytes/macrophages are attracted into the tumors by endothelial cell (EC)-derived cytokine angiopoietin-2 (ANG-2), which interacts with its receptor TIE2 (Huang et al., 2011). In addition, TEM express chemokine receptor CXCR4 and can be attracted into tumors by CXCL12 (Welford et al., 2011). This subpopulation of macrophages is associated with vessels and is highly angiogenic acting in a paracrine manner (De Palma et al., 2005). It is not known whether TIE2 expressing monocytes are recruited into specific types of solid tumors and whether stimulation of TIE2 expression in the conventional TAM associated with blood vessels can result into development of TEM phenotype. Blood circulating TIE2 expressing monocytes are already pre-programmed to exert pro-angiogenic activity and express elevated levels of MMP9, VEGF-A, COX-2, and Wnt5a (Coffelt et al., 2010). When stimulated by EC-derived ANG-2, TEM additionally upregulate several pro-angiogenic factors including TP and cathepsin B. Moreover, ANG-2 induces the expression of IL-10 and CCL17 by TEM, factors which suppress T-cell proliferation and promote the expansion of regulatory T-cells providing tumor cells with a way to escape from immune responses (Coffelt et al.,

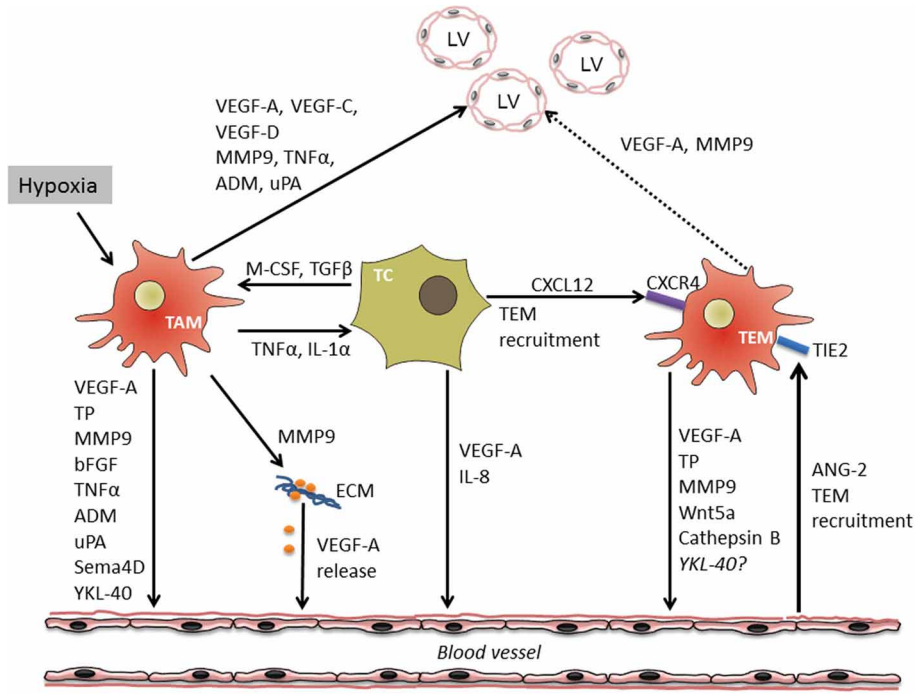

FIGURE 1 | Cross-talk between macrophages, tumor cells and endothelium during tumor angiogenesis and lymphangiogenesis. Tumor hypoxia and cytokine crosstalk between tumor cells (TC) and TAM results in the induction of a pro-angiogenic program in both cell types followed by the release of multiple soluble factors stimulating migration and proliferation of vascular endothelial cells (VEC) either directly or through the mobilization of extracellular matrix (ECM)-bound VEGF-A. In addition, TC and VEC are able to attract specific population of TIE-2 expressing monocytes (TEM) with high angiogenic potential. TAM and TEM-derived factors as well-induce growth of tumor lymphatic vessels (LV). Through the production of factors such as VEGF-A and MMP9 influencing growth of both lymphatic and vascular EC, TAM link processes of tumor angio- and lymphangiogenesis.

2011). TEM were described to cause re-growth of subcutaneous breast and lung carcinomas after local irradiation (Kozin et al., 2010). In addition, they limited the efficacy of vascular-disrupting compounds in murine mammary tumors, presumably initiating vascular repair (Welford et al., 2011). Several studies reported an important role of EC in the differentiation and functional activity of TEM. The interaction of TEM with activated EC was important for TIE2 upregulation and the establishment of a pro-angiogenic program in TEM (Mazzieri et al., 2011). A blockade of EC-derived ANG-2 in mouse MMTV-PyMT mammary cancer downregulated TIE2 expression in TEM and disrupted their association with angiogenic blood vessels. The inhibition of TIE2 expression by TEM resulted in reduced tumor angiogenesis. In another study, co-culture of primary EC with bone marrow-derived hematopoietic progenitor cells drove the differentiation of pro-angiogenic TIE2 expressing macrophages, which established tight associations with EC and supported tumor growth. The expansion of macrophage colonies was induced by EC-derived CSF-1 (He et al., 2012). Interestingly, expression profiles of TEM, resident monocytes and TIE2 expressing embryonic macrophages are related, suggesting that these myeloid populations represent developmental stages of TEM (Pucci et al., 2009). Recently, Medina and colleagues characterized a subpopulation of pro-angiogenic monocytes from human peripheral blood which were also referred to as myeloid angiogenic cells (MACs) (Medina et al., 2011). These cells had a signature of M2-polarized macrophages and expressed a panel of markers including CD163, IL-10 and macrophage scavenger receptor-1 (MSR-1). Moreover, they expressed and released a spectrum of pro-angiogenic factors such as IL-8, MMP9, and VEGF. According to the gene expression profile, MACs resembled TEM and induced endothelial tubule formation mediated mainly through IL-8 release. The role of this monocytic subpopulation in tumor angiogenesis was not described. However, their clear pro-angiogenic properties and predisposition to M2 polarization suggest a potential contribution to tumor angiogenesis once these cells are recruited to the tumor site. A summary of the complex regulation of angiogenesis by TAM is schematically presented on **Figure 1**.

EFFECT OF TAM ON LYMPHANGIOGENESIS

In recent years, evidence has accumulated that macrophages are not only critical regulators of angiogenesis, but also crucial participants in lymphangiogenesis, both in inflammatory settings and in tumors (Ran and Montgomery, 2012). Importantly, macrophages may simultaneously induce both angiogenesis and lymphangiogenesis by the production of VEGF-A and MMP9. These cytokines which are abundantly produced by subpopulations of TAM were shown to induce the development of both blood and lymphatic vessels. Thus, TAM-derived factors can link tumor angiogenesis and lymphangiogenesis (Scavelli et al., 2004; Coffelt et al., 2009; Gomes et al., 2013) (see **Figure 1**).

Macrophages can utilize two main pathways to stimulate lymphangiogenesis: either by the direct secretion of prolymphangiogenic factors or by trans-differentiation into lymphatic EC, actively taking part in the formation of lymphatic vessels (Kerjaschki, 2005). TAM can express a major marker of lymphatic vessels, LYVE-1 (lymphatic vessel endothelial hyaluronan receptor (1), both in murine and human tumors (Schledzewski et al., 2006; Zumsteg et al., 2009). It was reported that in mouse models of pancreatic insulinoma and prostate cancer F4/80$^+$Lyve-1$^+$ TAM directly integrated into peritumoral lymphatic vessels and presumably lost their macrophage features upon this integration (Zumsteg et al., 2009). Even though not all researchers agree with the trans-differentiation hypothesis (Gordon et al., 2010), the fact that certain macrophages secrete pro-lymphangiogenic factors in certain circumstances, such as in inflammation or tumors, is undisputed. Below we review the pro-lymphangiogenic factors secreted by tumor associated macrophages (TAM), as well as the role of TAM density in evaluating tumor lymphangiogenesis.

FACTORS PRODUCED BY TAM THAT REGULATE LYMPHANGIOGENESIS

There are a few factors produced by TAM that are responsible for the induction of lymphangiogenesis, with VEGFR-3 and its ligands, VEGF-C and VEFG-D, thought to have a key role in it. Studies have shown that VEGF-C producing tumor cells significantly increase intratumoral lymphangiogenesis together with regional metastasis (Skobe et al., 2001a). Additionally, the inhibition of VEGFR-3 with receptor-specific antagonist antibodies was shown to suppress tumor lymphangiogenesis as well as regional and distant metastasis (Roberts et al., 2006). Schoppmann et al. found that TAM expressing VEGF-C, VEGF-D, and VEGFR-3 substantially increased tumor lymphatic microvessel density (LVD) in cervical cancer (Schoppmann et al., 2002). In another study, they showed that TAM expressing VEGF-C increased tumor lymphangiogenesis and lymphovascular invasion in breast cancer. Moreover, a positive correlation was found between VEGF-C$^+$ stromal cells and VEGF-C$^+$ tumor cells (Schoppmann et al., 2006). Similar findings were obtained in a study of Lewis lung carcinoma cells in which M2 macrophages displayed the ability to induce VEGF-C expression in tumor cells (Zhang et al., 2012b). By depletion of VEGFR-3$^+$ TAM with clodronate liposomes, Yang et al. were able to obtain a considerable reduction (>80%) in the secretion of VEGF-C and VEGF-D in the tumor mass and also a significant reduction in LVD (Yang et al., 2011). Recently, the critical role of VEGF-C producing TAM in lymphangiogenesis and tumor dissemination was found in mantle cell lymphoma (Song et al., 2013). The description of VEGF-C expressing TAM and their prolymphangiogenic influence can also be found in a variety of other studies (Jeon et al., 2008; Moussai et al., 2011; Ding et al., 2012; Werchau et al., 2012; Wu et al., 2012).

Although VEGF-C, VEGF-D, and VEGFR-3 are mainly regarded as prolymphangiogenic factors, there are studies suggesting their role in angiogenesis. The mature forms of VEGF-C and VEFG-D possess the ability to bind to VEGFR-2, a receptor associated with angiogenesis (Joukov et al., 1997; Stacker et al., 1999; Lohela et al., 2009). Moreover, VEGF-C was shown to promote angiogenesis in vivo and to promote angiogenesis, in addition to lymphangiogenesis, in melanoma models (Cao et al., 1998; Skobe et al., 2001b). VEGFR-3 is also associated with the promotion of angiogenesis. In a study on mouse angiogenesis models, the blockage of VEGFR-3 with monoclonal antibodies was found to decrease sprouting, vascular density and endothelial cell proliferation. Additionally a blockage of VEGFR-3 in combination with the blockage of VEGFR-2 resulted in additive inhibition of angiogenesis and tumor growth (Tammela et al., 2008).

VEGF-A, a classical pro-angiogenic factor expressed by TAM, was found to be involved also in lymphangiogenesis. Apart from indirectly stimulating lymphangiogenesis by recruiting macrophages (Cursiefen et al., 2004), VEGF-A has been shown to induce proliferation and migration of VEGFR-2 expressing lymphatic endothelial cells (LEC) *in vitro* (Hong et al., 2004) and to induce sentinel lymph node lymphangiogenesis in a skin cancer model (Hirakawa et al., 2005). In a fibrosarcoma model, VEGF-A displayed the ability to induce peritumoral lymphangiogenesis as well as contribute to lymphatic metastasis (Bjorndahl et al., 2005a). Anti-VEGF-A therapy proved to be an effective way to reduce both blood and lymphatic vascular densities in a breast cancer model. Moreover, it decreased the VEGFR-3 expression levels in LEC and reduced the incidence of regional and distant metastasis (Bjorndahl et al., 2005b; Whitehurst et al., 2007).

In addition to the expression of direct inducers of lymphangiogenesis, TAM regulate lymphangioegensis, also indirectly, by the production of enzymes, such as MMP, plasmin and urokinase plasminogen activator (uPA), that regulate matrix remodeling and growth factor activation (Allavena et al., 2008). Matrix remodeling and growth factor activation are very important processes, both in angiogenesis and lymphangiogenesis. MMP-2 and MMP-9 have been shown to have a role in governing the formation of lymphatic vessels. MMP-2 facilitates LEC migration through collagen fibers, which is otherwise affected by physical matrix constraints. Inhibition or downregulation of MMP-2 and MMP-9 reduces lymphangiogenesis, the invasive ability and tube-forming properties of LEC (Nakamura et al., 2004; Detry et al., 2012) that supports the idea that MMP-2 and MMP-9-mediated lymphangiogenesis contribute their ability to promote several types of tumors. Along with MMP-1 and MMP-2, uPA was shown to have a role in lymphatic progression of oral tongue squamous cell carcinoma (Zhang et al., 2011a). Plasmin has been reported to activate the lymphangiogenic growth factors VEGF-C and VEGF-D. Proteolytic processing of these growth factors by plasmin greatly enhanced their affinity to VEGFR-3 and binding to both VEGFR-2 and VEGFR-3 (McColl et al., 2003).

Numerous other pro-lymphangiogenic factors have been identified in recent years. Among them are ADM, angiopoietin 1 and 2 (Ang-1, Ang-2), COX-2, endothelin-1, fibroblast growth factor-2 (FGF-2), growth hormone, heparanase, hepatocyte growth factor (HGF), insulin-like growth factors 1 and 2 (IGF-1, IFG-1), platelet-derived growth factor-BB (PDGF-BB) and tumor necrosis factor alpha (TNF-α) (Duong et al., 2012; Ran and Montgomery, 2012). Although these factors have been linked to lymphangiogenesis, the evidence that TAM are expressing them during carcinogenesis as part of their lymphangiogenic activity

is still insufficient. Future studies will shed light on the role of TAM in the complex control of tumor lymphangiogenesis by the production of cocktails of regulators of lymphangiogenesis.

THE ROLE OF TAM DENSITY IN TUMOR TISSUE AND ITS ASSOCIATION WITH LYMPHANGIOGENESIS

The idea that TAM regulate tumor lymphangiogenesis is strongly supported by a significant correlation between the density of TAM and LVD in tumor tissues. In human cervical cancer, VEGF-C expressing TAM were found to correlate with increased LVD in peritumoral stroma. All TAM producing VEGF-C were also expressing VEGF-D and VEGFR-3 and were distinguished from other cells by CD68, CD14, CD23, HLA-DR, and CD45 (Schoppmann et al., 2002). In a mouse model of breast cancer, Ito et al. demonstrated a clear correlation between the density of LYVE-1^+ lymphatic vessels, $CD68^+$ macrophage infiltration, and VEGF-C expression. By comparison of breast cancer cell lines BJMC338 and BJMC3879 having low and high metastatic potential, respectively, more aggressive tumors were found to have an increased infiltration of $CD68^+$ macrophages, higher expression of VEGF-C and a higher LVD (Ito et al., 2011).

In human breast cancer, higher numbers of TAM expressing VEGF-C were associated with a higher LVD and lymph node metastasis or lymph vessel invasion (LVI) (Ding et al., 2012). Similar results were found in other types of tumors as well. A study on ciliary body melanoma showed a significant correlation between LYVE-1^+ and D2-40^+ intraocular lymphatic vessels, higher $CD68^+$ macrophage infiltration rate and an increased mortality rate (Heindl et al., 2010). Another study on melanoma revealed a correlation between $CD68^+$ macrophages and D2-40^+ lymphatic vessel invasion, but showed no association with clinical outcomes (Storr et al., 2012). In human skin squamous cell carcinoma, increased LYVE-1^+ LVD was found to be associated with increased VEGF-C secretion by $CD68^+$ and/or $CD163^+$ macrophages (Moussai et al., 2011). Similar results were obtained by Werchau et al. in a study of Merkel cell carcinoma. Here, VEGF-C secreting $CD68^+$ and/or $CD163^+$ macrophages were also found to increase LYVE-1^+ or D2-40^+ LVD (Werchau et al., 2012). Unfortunately, both of these studies failed to provide a prognostic significance for these correlations. In a study of lymphangiogenesis in gastric cancer, $CD68^+$ TAM were shown to be associated with a higher D2-40^+ LVD and were closely related to serosa invasion and lymph node metastasis (Wu et al., 2012). In pancreatic cancer, VEGF-C expressing M2-polarized macrophages had an association with increased LVD density and incidence of tumor cells in regional lymph nodes (Kurahara et al., 2013).

Positive correlations between the number of TAM, LVD and tumor progression was also demonstrated for lung and esophageal cancers (Ohta et al., 2002; Kurahara et al., 2011; Zhang et al., 2011b). Not all studies were able to show a correlation between TAM, LVD and tumor progression, and some explanation to this phenomenon can be found in a recent review (Ran and Montgomery, 2012). Moreover, our examination of the TAM phenotype in pancreatic insulinoma and melanoma mouse models demonstrated that LYVE-1 can be expressed not only by lymphatic vessels, but also by TAM themselves (Schledzewski et al., 2006). Subtraction of CD68+LYVE+ macrophages has to be done for precise quantification of lymphatic vessels in tumors without counting LYVE-1+ macrophages. LYVE-1 is not the only marker expressed by both TAM and the microvascular cells in tumors. Stabilin-1 is also abundantly expressed on TAM and non-continuous endothelium and probably on lymphatic vessels (Kzhyshkowska et al., 2006b; Martens et al., 2006; Karikoski et al., 2009; Kzhyshkowska, 2010). The available information about the role of TAM in tumor lymphangiogenesis is summarized in **Table 1**, and a solid body of data indicate that TAM are able to support tumor lymphangiogenesis by direct and indirect effects on EC using a broad spectrum of growth factors, cytokines and enzymes overlapping with pro-angiogenic factors.

CHITINASE-LIKE PROTEINS AS NEW REGULATORS OF TUMOR GROWTH AND VASCULARIZATION

Recently, a new potent inducer of angiogenesis, YKL40, has been reported for several types of cancer (Shao et al., 2009; Faibish et al., 2011; Francescone et al., 2011, 2013). YKL-40 belongs to the family of Glyco_18 containing proteins that comprises chitinases and chitinase-like proteins (CLPs) (Kzhyshkowska et al., 2006c, 2007). Mammalian CLPs include YKL-39, YKL-40, stabilin-1 interacting chitinase-like protein (SI-CLP) and YM1/YM2 (only in rodents) that contain a Glyco_18 domain only; they lack critical amino acids within the catalytic site (**Figure 2**) and therefore do not exhibit enzymatic activity (Kzhyshkowska et al., 2006c, 2007). Their biological activity is defined by specific interactions mediated by the enzymatically silent Glyco_18 domain. The Glyco_18 domain is characteristic for the evolutionary conserved chitinases, which belong to the family of 18 glycosyl hydrolases. Enzymatically active chitinases catalyze the hydrolysis of chitin, while their evolutionary conserved function in lower life forms is to provide host defense against chitin-containing organisms (Arakane and Muthukrishnan, 2010). However, at the moment we have only limited information about the biological functions of enzymatically silent CLPs that are induced during inflammation and cancer. Major cell types that express CLPs are macrophages and tumor cells. CLPs are secreted in the extracellular space and therefore can mediate cellular crass-talk. CLPs can also be found in circulation and can act both locally and systemically.

YKL-40, also called human cartilage glycoprotein-39 (HCgp-39), gp38k and Chitinase-3-like-1 (CHI3L1), is the best investigated human chitinase-like protein regarding its association with various disorders. Increased concentrations of YKL-40 in circulation are associated with disorders characterized by different types of inflammation and active tissue remodeling, including asthma, arthritis, atherosclerosis and other cardiovascular disorders (Wang et al., 2008; Kastrup, 2011; Harutyunyan et al., 2012; Jensen et al., 2013; Konradsen et al., 2013). Circulating YKL-40 can be detected in human serum or plasma using RIA based on polyclonal antibodies. Most intensively, circulating YKL-40 was investigated in tumor patients (Johansen et al., 2009; Allin et al., 2012). Elevated levels of YKL-40 were found in the circulation of patients with various solid tumors including glioma, breast cancer, colorectal cancer, ovarian cancer, metastatic renal and prostate cancer, and malignant melanoma. In breast cancer

Table 1 | Role of TAM in tumor lymphangiogenesis and tumor progression.

Model	TAM phenotype	Action/correlation	Method	References
Cervical cancer	CD68, CD14, CD23, HLA-DR, and CD45	Express VEGF-C,VEGF-D, VEGFR-3 Increased LVD in peritumoral stroma	IHC, IF, Confocal microscopy	Schoppmann et al., 2002
Lung adenocarcinoma	CD68, CD206	Increased LVD, increased lymph node metastasis rate, poor prognosis	IHC, IF, Confocal microscopy	Zhang et al., 2011b
Esophageal carcinoma	CD68	Increased microvessel density	IHC	Ohta et al., 2002
Pancreatic cancer	CD68, CD163, CD204	Increased LVD in cases with high number of CD163/CD204 TAM Increased lymph node metastasis rate in cases with high number of CD163/CD204 TAM	IHC	Kurahara et al., 2011, 2013
Gastric cancer	CD68	Higher LVD Increased lymph node metastasis rate	IHC	Wu et al., 2012
Merkel cell carcinoma	CD68 and/or CD163	VEGF-C expression Increased LVD	IHC, IF	Werchau et al., 2012
Skin squamous cell carcinoma	CD68 and/or CD163	VEGF-C expression Increased LVD	IHC, IF	Moussai et al., 2011
Melanoma	CD68	Increased LVD	IHC	Storr et al., 2012
Ciliary body melanoma	CD68	Increased LVD Increased mortality rate	IHC	Heindl et al., 2010
Mouse model of breast cancer	CD68	Increased LVD and VEGF-C expression	IHC	Ito et al., 2011

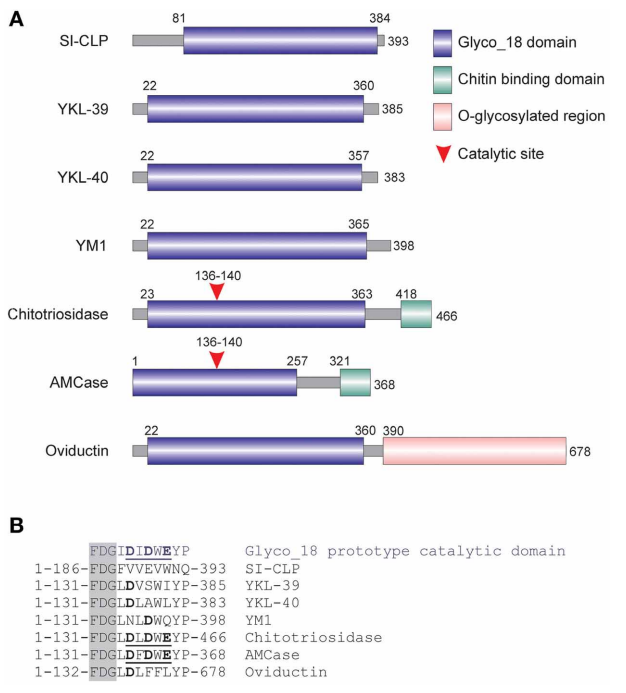

FIGURE 2 | Mammalian chitinase-like proteins (CLP) belong to the family of Glyc_18-containig proteins. (A) Schematic presentation of mammalian Glyco_18 domain containing proteins. **(B)** Critical amino acid in catalytic sites. The characteristic FDG sequence preceding the catalytic motif is shown in the shadowed box. Catalytic amino acids are shown in bold. Complete active catalytic motifs are underlined. This research was originally published in *Blood*. Kzhyshkowska et al. (2006c) © the American Society of Hematology.

patients, high levels of serum YKL-40 are associated with a poor prognosis (Jensen et al., 2003; Johansen et al., 2003; Kim et al., 2007; Yamac et al., 2008; Shao et al., 2011). In primary breast tumors, YKL-40 protein expression was found in tumor cells and in infiltrating inflammatory cells (Roslind et al., 2008). High expression was associated with positive estrogen and progesterone receptor status and high tumor differentiation. Serum levels of YKL-40 are indicative of a poor prognosis and rapid metastatic process. For example, increased plasma concentration of YKL-40 is related to poor prognosis and shorter survival of patients with ovarian cancer, colorectal carcinoma, metastatic prostate carcinoma and melanoma. A recent study demonstrated that YKL-40 expression in anal carcinoma is correlated with a poor outcome and can predict lymph node metastases (Mistrangelo et al., 2013). Differential levels of YKL-40 may reflect differences in the biology of cancer cells themselves, as well as the activation of innate immune responses in primary tumors, in particular, the activity and functional polarization of TAM.

YKL-40 is also the best functionally investigated protein out of all CLPs. Its stimulatory effect on tumor angiogenesis was demonstrated in several studies. Porcine homolog of YKL-40, gp38k (CHI3L1), specifically induces the migration of vascular smooth muscle cells (VSMC), but not fibroblasts. Moreover, gp38k promotes the attachment and spreading of VSMC (Nishikawa and Millis, 2003). Elevated serum levels of YKL-40 are associated with a worse prognosis among various advanced human cancers. Recently YKL-40 was found to act as a strong pro-angiogenic factor in cancer. It has been found that ectopic expression of YKL-40 in MDA-MB-231 breast cancer cells and in HCT-116 colon cancer cells led to larger tumor formation with an extensive angiogenic phenotype than did control cancer cells in

mice. Affinity-purified recombinant YKL-40 protein promoted vascular endothelial cell angiogenesis *in vitro*, the effects of which are similar to the activities observed using MDA-MB-231 and HCT-116 cell-conditioned medium after transfection with YKL-40. Blockade of YKL-40 using small interfering RNA (siRNA) suppressed tumor angiogenesis *in vitro* and *in vivo*. Immunohistochemical analysis of human breast cancer showed a correlation between YKL-40 expression and blood vessel density (Shao et al., 2009). Furthermore, a potential receptor for chitinase-like protein was identified for the first time in this study. YKL-40 is a heparin-binding protein and syndecans are a major source of cell surface heparin sulfate (HS). HS functions as a key mediator connecting membrane receptors with extracellular heparin-binding proteins, such as ECM protein vitronectin and angiogenic factors (FGF, VEGF) (Lambaerts et al., 2009). Shao et al. showed that YKL-40 induces a coordination of membrane-bound receptor syndecan-1 and integrin alpha$_v$beta$_3$ and activates an intracellular signaling cascade, including focal adhesion kinase and the MAP/Erk pathway (Shao et al., 2009). YKL-40 also stimulates VEGF expression in U87 glioblastoma cell line cells and synergistically with VEGF promote angiogenesis (Francescone et al., 2011). YKL-40 is also able to enhance contact of tumor and EC, to restrict vascular leakage and stabilize vascular networks (Francescone et al., 2013).

Blocking of YKL-40 activity with monoclonal antibodies demonstrated that this can be a promising therapeutic strategy for advanced tumors (Faibish et al., 2011). A mouse monoclonal anti-YKL-40 antibody (mAY) abolished YKL-40-induced activation of VEGF receptor 2 (Flk-1/KDR) and MAP-mediated intracellular signaling, and abrogated angiogenesis induced by YKL-40 conditioned medium of the glioblastoma cell line U87 with elevated levels of YKL-40 induced by γ-irradiation. Consequently, treatment of xenografted tumor mice with mAY suppressed tumor growth and angiogenesis (Faibish et al., 2011). More information about the mode of action of YKL-40 in tumors and its prognostic and therapeutic value can be found in the recent review of Shao (2013).

YKL-39 was identified as an abundantly secreted protein in primary culture of human articular chondrocytes (Halin et al., 2009). YKL-39 is currently recognized as a biomarker for the activation of chondrocytes and osteoarthritis (OA) progression in humans. YKL-39 might be an inducer of autoimmune processes related to arthritis, while antibodies against YKL-39 were found in patients with rheumatoid arthritis (RA) and OA (reviewed in Kzhyshkowska et al., 2007). For a long time, it was believed that macrophages do not secrete YKL-39. However, recently we demonstrated that the key regulatory factor of tumor progression, TGFß, strongly stimulates YKL-39 expression in macrophages *in vitro* (Gratchev et al., 2008) suggesting that YKL39 might be a biomarker for subpopulations of macrophages that underwent programming by TGFß in the tumor microenvironment. However expression of YKL39 on macrophages in vivo remains to be examined experimentally.

SI-CLP was identified by us as stabilin-1- interacting chitinase-like protein using yeast two-hybrid screening technology (Kzhyshkowska et al., 2006c). In parallel with its sorting receptor stabilin-1, expression of SI-CLP mRNA was strongly upregulated in macrophages stimulated by Th2 cytokine IL-4 and by dexamethasone. We developed a rat monoclonal antibody, 1C11, recognizing the N-terminal epitope of SI-CLP. This epitope is located upstream of the conservative Glyco_18 domain and has no similarity with sequences of other human Glyco_18 containing proteins. Using the 1C11 antibody, we demonstrated that the combination of IL-4 and dexamethasone increases SI-CLP expression in macrophages. 1C11 mAb recognized SI-CLP in the cellular fraction of bronchoalveolar lavage specimens obtained from patients with chronic inflammatory disorders of the respiratory tract and in peripheral blood leukocytes (PBLs) from these patients. SI-CLP is the only chitinase-like protein which is upregulated by glucocorticoids. However, the expression of SI-CLP in tumor cells and TAM, and role of SI-CLP in cancer remain to be investigated.

In summary, the identification of YKL-40 as a pro-angiogenic factor in animal models and *in vitro* studies opens a new field of investigation of the specific role of CLPs in regulation of tumor growth and angiogenesis. The role of YKL-39 and SI-CLP in tumor angiogenesis has not been reported up to date, however their homology with YKL-40 makes these proteins attractive candidates for the analysis of their effects on tumor angiogenesis and such studies are in progress in our laboratory. The ability of multiple macrophage-derived pro-angiogenic factors to induce growth of both lymphatic and blood vessels raises the possibility for the involvement of YKL-40 and other CLPs proteins in the tumor lymphangiogenesis. Further experimental efforts are required in order to address the role of CLPs in lymphangiogenesis.

CONCLUSIONS AND PERSPECTIVES

There are no doubts today that TAM are critical controllers of both tumor angiogenesis and lymphangiogenesis. They produce soluble factors which either directly induce vessel formation or enhance production of angiogenic factors by tumor cells. TAM-mediated support of vessel growth is associated with increased tumor growth and metastasis. Thus, targeting of TAM appears to be a promising approach for tumor therapy and can be achieved on both cellular and molecular levels. First, complete systemic depletion of macrophages can be performed. However, this approach raises significant concerns since prolonged absence of these cells in organs and circulation may result in sensitization to bacterial infections, and affect functionality of the whole immune system.

Another possibility is to block the recruitment of specific pro-angiogenic TAM populations into tumor site. Analysis of published data suggests that the disruption of CXCL12-CXCR4, ANG2-TIE2, and VEGF-VEGFR axes can prevent infiltration of tumors by angiogenic TAM populations. This approach appears to be promising to inhibit the repopulation of hypoxic tumor areas by TAM after therapeutic interventions. Finally, pro-angiogenic activity of TAM is based on, but not limited by, the release of common angiogenic factors such as VEGF-A and MMP9. Recent reports reveal novel TAM-derived angiogenic factors including ADM, Sema4D, and YKL-40 in specific tumor types. Thus, it is possible that a tumor-specific microenvironment induces the expression of distinct pro-angiogenic programs in TAM depending on tumor type and affected organ. Therefore,

the identification of novel TAM-derived angiogenic factors and the validation of their targeting in specific types of cancer is urgently needed. The limitation of this method is related to the fact that TAM release a cocktail of pro-angiogenic factors, and targeting of single factor can be insufficient for suppression of tumor vascularization. Macrophages are not terminally differentiated and show high level of plasticity that makes TAM attractive targets for therapeutic immunomodulation (Stout and Suttles, 2004; Gratchev et al., 2006; Stout et al., 2009). Manipulating TAM phenotype and their re-polarization from pro-angiogenic M2 into anti-angiogenic M1 cells is an advanced strategy to solve this problem. HIF-1, COX-2, and NF-kB can be considered as promising target molecules for macrophages re-polarization. Moreover, correction of macrophage polarization in combination with targeting of single soluble factors, despite being sophisticated and requiring solid experimental investment, opens new horizons for an efficient and personalized cancer therapy.

ACKNOWLEDGMENTS

This work was supported by the ERA.Net RUS grant DTEST-CLP (for Julia Kzhyshkowska and Alexander Orekhov), RFFI 11-04-01666-à (for Julia Kzhyshkowska) and the Chinese Research Scholarship (for Nan Wang).

REFERENCES

Algars, A., Irjala, H., Vaittinen, S., Huhtinen, H., Sundstrom, J., Salmi, M., et al. (2012). Type and location of tumor-infiltrating macrophages and lymphatic vessels predict survival of colorectal cancer patients. *Int. J. Cancer* 131, 864–873. doi: 10.1002/ijc.26457

Allavena, P., Sica, A., Solinas, G., Porta, C., and Mantovani, A. (2008). The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. *Crit. Rev. Oncol. Hematol.* 66, 1–9. doi: 10.1016/j.critrevonc.2007.07.004

Allin, K. H., Bojesen, S. E., Johansen, J. S., and Nordestgaard, B. G. (2012). Cancer risk by combined levels of YKL-40 and C-reactive protein in the general population. *Br. J. Cancer* 106, 199–205. doi: 10.1038/bjc.2011.501

Arakane, Y., and Muthukrishnan, S. (2010). Insect chitinase and chitinase-like proteins. *Cell. Mol. Life Sci.* 67, 201–216. doi: 10.1007/s00018-009-0161-9

Basire, A., Sabatier, F., Ravet, S., Lamy, E., Mialhe, A., Zabouo, G., et al. (2006). High urokinase expression contributes to the angiogenic properties of endothelial cells derived from circulating progenitors. *Thromb. Haemost.* 95, 678–688. doi: 10.1160/TH05-07-0511

Bergers, G., Brekken, R., McMahon, G., Vu, T. H., Itoh, T., Tamaki, K., et al. (2000). Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat. Cell Biol.* 2, 737–744. doi: 10.1038/35036374

Bijnsdorp, I. V., Capriotti, F., Kruyt, F. A., Losekoot, N., Fukushima, M., Griffioen AW., et al. (2011). Thymidine phosphorylase in cancer cells stimulates human endothelial cell migration and invasion by the secretion of angiogenic factors. *Br. J. Cancer* 104, 1185–1192. doi: 10.1038/bjc.2011.74

Bingle, L., Brown, N. J., and Lewis, C. E. (2002). The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J. Pathol.* 196, 254–265. doi: 10.1002/path.1027

Bjorndahl, M. A., Cao, R., Burton, J. B., Brakenhielm, E., Religa, P., Galter, D., et al. (2005a). Vascular endothelial growth factor-a promotes peritumoral lymphangiogenesis and lymphatic metastasis. *Cancer Res.* 65, 9261–9268. doi: 10.1158/0008-5472.CAN-04-2345

Bjorndahl, M., Cao, R., Nissen, L. J., Clasper, S., Johnson, L. A., Xue, Y., et al. (2005b). Insulin-like growth factors 1 and 2 induce lymphangiogenesis *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 102, 15593–15598. doi: 10.1073/pnas.0507865102

Brecht, K., Weigert, A., Hu, J., Popp, R., Fisslthaler, B., Korff, T., et al. (2011). Macrophages programmed by apoptotic cells promote angiogenesis via prostaglandin E2. *FASEB J.* 25, 2408–2417. doi: 10.1096/fj.10-179473

Burke, B., Tang, N., Corke, K. P., Tazzyman, D., Ameri, K., Wells, M., et al. (2002). Expression of HIF-1alpha by human macrophages: implications for the use of macrophages in hypoxia-regulated cancer gene therapy. *J. Pathol.* 196, 204–212. doi: 10.1002/path.1029

Cao, Y., Linden, P., Farnebo, J., Cao, R., Eriksson, A., Kumar, V., et al. (1998). Vascular endothelial growth factor C induces angiogenesis *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 95, 14389–14394. doi: 10.1073/pnas.95.24.14389

Casazza, A., Laoui, D., Wenes, M., Rizzolio, S., Bassani, N., Mambretti, M., et al. (2013). Impeding macrophage entry into hypoxic tumor areas by sema3a/Nrp1 Signaling blockade inhibits angiogenesis and restores antitumor immunity. *Cancer Cell* 24, 695–709. doi: 10.1016/j.ccr.2013.11.007

Chen, J. J., Yao, P. L., Yuan, A., Hong, T. M., Shun, C. T., Kuo, M. L., et al. (2003). Up-regulation of tumor interleukin-8 expression by infiltrating macrophages: its correlation with tumor angiogenesis and patient survival in non-small cell lung cancer. *Clin. Cancer Res.* 9, 729–737.

Chen, P., Huang, Y., Bong, R., Ding, Y., Song, N., Wang, X., et al. (2011). Tumor-associated macrophages promote angiogenesis and melanoma growth via adrenomedullin in a paracrine and autocrine manner. *Clin. Cancer Res.* 17, 7230–7239. doi: 10.1158/1078-0432.CCR-11-1354

Chen, W., Ma, T., Shen, X. N., Xia, X. F., Xu, G. D., Bai X. L., et al. (2012). Macrophage-induced tumor angiogenesis is regulated by the TSC2-mTOR pathway. *Cancer Res.* 72, 1363–1372. doi: 10.1158/0008-5472.CAN-11-2684

Coffelt, S. B., Chen, Y. Y., Muthana, M., Welford, A. F., Tal, A. O., Scholz, A., et al. (2011). Angiopoietin 2 stimulates TIE2-expressing monocytes to suppress T cell activation and to promote regulatory T cell expansion. *J. Immunol.* 186, 4183–4190. doi: 10.4049/jimmunol.1002802

Coffelt, S. B., Hughes, R., and Lewis, C. E. (2009). Tumor-associated macrophages: effectors of angiogenesis and tumor progression. *Biochim. Biophys. Acta* 1796, 11–18. doi: 10.1016/j.bbcan.2009.02.004

Coffelt, S. B., Tal, A. O., Scholz, A., De Palma, M., Patel, S., Urbich, C., et al. (2010). Angiopoietin-2 regulates gene expression in TIE2-expressing monocytes and augments their inherent proangiogenic functions. *Cancer Res.* 70, 5270–5280. doi: 10.1158/0008-5472.CAN-10-0012

Cursiefen, C., Chen, L., Borges, L. P., Jackson, D., Cao, J., Radziejewski, C., et al. (2004). VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J. Clin. Invest.* 113, 1040–1050. doi: 10.1172/JCI20465

David, C., Nance, J. P., Hubbard, J., Hsu, M., Binder, D., and Wilson, E. H. (2012). Stabilin-1 expression in tumor associated macrophages. *Brain Res.* 1481, 71–78. doi: 10.1016/j.brainres.2012.08.048

De Palma, M., Coukos, G., and Hanahan, D. (2013). A new twist on radiation oncology: low-dose irradiation elicits immunostimulatory macrophages that unlock barriers to tumor immunotherapy. *Cancer Cell* 24, 559–561. doi: 10.1016/j.ccr.2013.10.019

De Palma, M., Venneri, M. A., Galli, R., Sergi, S. L., Politi, L. S., Sampaolesi, M., et al. (2005). Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell* 8, 211–226. doi: 10.1016/j.ccr.2005.08.002

Detry, B., Erpicum, C., Paupert, J., Blacher, S., Maillard, C., Bruyere, F., et al. (2012). Matrix metalloproteinase-2 governs lymphatic vessel formation as an interstitial collagenase. *Blood* 119, 5048–5056. doi: 10.1182/blood-2011-12-400267

Ding, M., Fu, X., Tan, H., Wang, R., Chen, Z., and Ding, S. (2012). The effect of vascular endothelial growth factor C expression in tumor-associated macrophages on lymphangiogenesis and lymphatic metastasis in breast cancer. *Mol. Med. Rep.* 6, 1023–1029. doi: 10.3892/mmr.2012.1043

Du, R., Lu, K. V., Petritsch, C., Liu, P., Ganss, R., Passegue, E., et al. (2008). HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer Cell* 13, 206–220. doi: 10.1016/j.ccr.2008.01.034

Duong, T., Koopman, P., and Francois, M. (2012). Tumor lymphangiogenesis as a potential therapeutic target. *J. Oncol.* 2012:204946. doi: 10.1155/2012/204946

Egami, K., Murohara, T., Shimada, T., Sasaki, K., Shintani, S., Sugaya, T., et al. (2003). Role of host angiotensin II type 1 receptor in tumor angiogenesis and growth. *J. Clin. Invest.* 112, 67–75. doi: 10.1172/JCI16645

Espinosa, I., Edris, B., Lee, C. H., Cheng, H. W., Gilks, C. B., Wang, Y., et al. (2011). CSF1 expression in nongynecological leiomyosarcoma is associated with increased tumor angiogenesis. *Am. J. Pathol.* 179, 2100–2107. doi: 10.1016/j.ajpath.2011.06.021

Eubank, T. D., Galloway, M., Montague, C. M., Waldman, W. J., and Marsh, C. B. (2003). M-CSF induces vascular endothelial growth factor production and angiogenic activity from human monocytes. *J. Immunol.* 171, 2637–2643.

Eubank, T. D., Roda, J. M., Liu, H., O'Neil, T., and Marsh, C. B. (2011). Opposing roles for HIF-1alpha and HIF-2alpha in the regulation of angiogenesis by mononuclear phagocytes. *Blood* 117, 323–332. doi: 10.1182/blood-2010-01-261792

Faibish, M., Francescone, R., Bentley, B., Yan, W., and Shao, R. (2011). A YKL-40-neutralizing antibody blocks tumor angiogenesis and progression: a potential therapeutic agent in cancers. *Mol. Cancer Ther.* 10, 742–751. doi: 10.1158/1535-7163.MCT-10-0868

Forssell, J., Oberg, A., Henriksson, M. L., Stenling, R., Jung, A., and Palmqvist, R. (2007). High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. *Clin. Cancer Res.* 13, 1472–1479. doi: 10.1158/1078-0432.CCR-06-2073

Francescone, R. A., Scully, S., Faibish, M., Taylor, S. L., Oh, D., Moral, L., et al. (2011). Role of YKL-40 in the angiogenesis, radioresistance, and progression of glioblastoma. *J. Biol. Chem.* 286, 15332–15343. doi: 10.1074/jbc.M110.212514

Francescone, R., Ngernyuang, N., Yan, W., Bentley, B., and Shao, R. (2013). Tumor-derived mural-like cells coordinate with endothelial cells: role of YKL-40 in mural cell-mediated angiogenesis. *Oncogene*. doi: 10.1038/onc.2013.160. [Epub ahead of print].

Galarneau, H., Villeneuve, J., Gowing, G., Julien, J. P., and Vallieres, L. (2007). Increased glioma growth in mice depleted of macrophages. *Cancer Res.* 67, 8874–8881. doi: 10.1158/0008-5472.CAN-07-0177

Giraudo, E., Inoue, M., and Hanahan, D. (2004). An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. *J. Clin. Invest.* 114, 623–633. doi: 10.1172/JCI200422087

Gomes, F. G., Nedel, F., Alves, A. M., Nor, J. E., and Tarquinio, S. B. (2013). Tumor angiogenesis and lymphangiogenesis: tumor/endothelial crosstalk and cellular/microenvironmental signaling mechanisms. *Life Sci.* 92, 101–107. doi: 10.1016/j.lfs.2012.10.008

Gordon, E. J., Rao, S., Pollard, J. W., Nutt, S. L., Lang, R. A., and Harvey, N. L. (2010). Macrophages define dermal lymphatic vessel calibre during development by regulating lymphatic endothelial cell proliferation. *Development* 137, 3899–3910. doi: 10.1242/dev.050021

Gratchev, A., Kzhyshkowska, J., Kothe, K., Muller-Molinet, I., Kannookadan, S., Utikal, J., et al. (2006). Mphi1 and Mphi2 can be re-polarized by Th2 or Th1 cytokines, respectively, and respond to exogenous danger signals. *Immunobiology* 211, 473–486. doi: 10.1016/j.imbio.2006.05.017

Gratchev, A., Schmuttermaier, C., Mamidi, S., Gooi, L., Goerdt, S., and Kzhyshkowska, J. (2008). Expression of osteoarthritis marker YKL-39 is stimulated by transforming growth factor beta (TGF-beta) and IL-4 in differentiating macrophages. *Biomark. Insights* 3, 39–44.

Halin, S., Rudolfsson, S. H., van Rooijen, N., and Bergh, A. (2009). Extratumoral macrophages promote tumor and vascular growth in an orthotopic rat prostate tumor model. *Neoplasia* 11, 177–186.

Harbeck, N., Schmitt, M., Kates, R. E., Kiechle, M., Zemzoum, I., Janicke, F., et al. (2002). Clinical utility of urokinase-type plasminogen activator and plasminogen activator inhibitor-1 determination in primary breast cancer tissue for individualized therapy concepts. *Clin. Breast Cancer* 3, 196–200. doi: 10.3816/CBC.2002.n.023

Harutyunyan, M., Christiansen, M., Johansen, J. S., Kober, L., Torp-Petersen, C., and Kastrup, J. (2012). The inflammatory biomarker YKL-40 as a new prognostic marker for all-cause mortality in patients with heart failure. *Immunobiology* 217, 652–656. doi: 10.1016/j.imbio.2011.11.003

Harutyunyan, M., Gotze, J. P., Winkel, P., Johansen, J. S., Hansen, J. F., Jensen G. B., et al. (2013). Serum YKL-40 predicts long-term mortality in patients with stable coronary disease: a prognostic study within the CLARICOR trial. *Immunobiology* 218, 945–951. doi: 10.1016/j.imbio.2012.10.015

He, H., Xu, J., Warren, C. M., Duan, D., Li, X., Wu, L., et al. (2012). Endothelial cells provide an instructive niche for the differentiation and functional polarization of M2-like macrophages. *Blood* 120, 3152–3162. doi: 10.1182/blood-2012-04-422758

Heindl, L. M., Hofmann, T. N., Adler, W., Knorr, H. L., Holbach, L. M., Naumann G. O., et al. (2010). Intraocular tumor-associated lymphangiogenesis a novel prognostic factor for ciliary body melanomas with extraocular extension? *Ophthalmology* 117, 334–342. doi: 10.1016/j.ophtha.2009.06.057

Hildenbrand, R., Dilger, I., Horlin, A., and Stutte, H. J. (1995). Urokinase and macrophages in tumour angiogenesis. *Br. J. Cancer* 72, 818–823. doi: 10.1038/bjc.1995.419

Hirakawa, S., Kodama, S., Kunstfeld, R., Kajiya, K., Brown, L. F., and Detmar, M. (2005). VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. *J. Exp. Med.* 201, 1089–1099. doi: 10.1084/jem.20041896

Hong, T. M., Teng, L. J., Shun, C. T., Peng, M. C., and Tsai, J. C. (2009). Induced interleukin-8 expression in gliomas by tumor-associated macrophages. *J. Neurooncol.* 93, 289–301. doi: 10.1007/s11060-008-9786-z

Hong, Y. K., Lange-Asschenfeldt, B., Velasco, P., Hirakawa, S., Kunstfeld, R., Brown L. F., et al. (2004). VEGF-A promotes tissue repair-associated lymphatic vessel formation via VEGFR-2 and the alpha1beta1 and alpha2beta1 integrins. *FASEB J.* 18, 1111–1113.

Hotchkiss, K. A., Ashton, A. W., and Schwartz, E. L. (2003). Thymidine phosphorylase and 2-deoxyribose stimulate human endothelial cell migration by specific activation of the integrins alpha 5 beta 1 and alpha V beta 3. *J. Biol. Chem.* 278, 19272–19279. doi: 10.1074/jbc.M212670200

Huang, H., Lai, J. Y., Do, J., Liu, D., Li, L., Del Rosario, J., et al. (2011). Specifically targeting angiopoietin-2 inhibits angiogenesis, Tie2-expressing monocyte infiltration, and tumor growth. *Clin. Cancer Res.* 17, 1001–1011. doi: 10.1158/1078-0432.CCR-10-2317

Huang, S., Van Arsdall, M., Tedjarati, S., McCarty, M., Wu, W., Langley, R., et al. (2002). Contributions of stromal metalloproteinase-9 to angiogenesis and growth of human ovarian carcinoma in mice. *J. Natl. Cancer Inst.* 94, 1134–1142. doi: 10.1093/jnci/94.15.1134

Imtiyaz, H. Z., Williams, E. P., Hickey, M. M., Patel, S. A., Durham, A. C., Yuan LJ., et al. (2010). Hypoxia-inducible factor 2alpha regulates macrophage function in mouse models of acute and tumor inflammation. *J. Clin. Invest.* 120, 2699–2714. doi: 10.1172/JCI39506

Ito, Y., Shibata, M. A., Eid, N., Morimoto, J., and Otsuki, Y. (2011). Lymphangiogenesis and axillary lymph node metastases correlated with VEGF-C expression in two immunocompetent mouse mammary carcinoma models. *Int. J. Breast Cancer* 2011:867152. doi: 10.4061/2011/867152

Jensen, B. V., Johansen, J. S., and Price, P. A. (2003). High levels of serum HER-2/neu and YKL-40 independently reflect aggressiveness of metastatic breast cancer. *Clin. Cancer Res.* 9, 4423–4434.

Jensen, P., Wiell, C., Milting, K., Poggenborg, R. P., Ostergaard, M., Johansen JS., et al. (2013). Plasma YKL-40: a potential biomarker for psoriatic arthritis? *J. Eur. Acad. Dermatol. Venereol.* 27, 815–819. doi: 10.1111/j.1468-3083.2012.04570.x

Jeon, B. H., Jang, C., Han, J., Kataru, R. P., Piao, L., Jung, K., et al. (2008). Profound but dysfunctional lymphangiogenesis via vascular endothelial growth factor ligands from CD11b+ macrophages in advanced ovarian cancer. *Cancer Res.* 68, 1100–1109. doi: 10.1158/0008-5472.CAN-07-2572

Jeon, S. H., Chae, B. C., Kim, H. A., Seo, G. Y., Seo, D. W., Chun GT., et al. (2007). Mechanisms underlying TGF-beta1-induced expression of VEGF and Flk-1 in mouse macrophages and their implications for angiogenesis. *J. Leukoc. Biol.* 81, 557–566. doi: 10.1189/jlb.0806517

Johansen, J. S., Christensen, I. J., Riisbro, R., Greenall, M., Han, C., Price, P. A., et al. (2003). High serum YKL-40 levels in patients with primary breast cancer is related to short recurrence free survival. *Breast Cancer Res. Treat.* 80, 15–21. doi: 10.1023/A:1024431000710

Johansen, J. S., Schultz, N. A., and Jensen, B. V. (2009). Plasma YKL-40: a potential new cancer biomarker? *Future Oncol.* 5, 1065–1082. doi: 10.2217/fon.09.66

Joukov, V., Sorsa, T., Kumar, V., Jeltsch, M., Claesson-Welsh, L., Cao, Y., et al. (1997). Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J.* 16, 3898–3911. doi: 10.1093/emboj/16.13.3898

Jung, Y. J., Isaacs, J. S., Lee, S., Trepel, J., and Neckers, L. (2003). IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. *FASEB J.* 17, 2115–2117.

Karikoski, M., Irjala, H., Maksimow, M., Miiluniemi, M., Granfors, K., Hernesniemi, S., et al. (2009). Clever-1/Stabilin-1 regulates lymphocyte migration within lymphatics and leukocyte entrance to sites of inflammation. *Eur. J. Immunol.* 39, 3477–3487. doi: 10.1002/eji.200939896

Kastrup, J. (2011). Can YKL-40 be a new inflammatory biomarker in cardiovascular disease? *Immunobiology* 217, 483–491. doi: 10.1016/j.imbio.2011.04.007

Kawahara, A., Hattori, S., Akiba, J., Nakashima, K., Taira, T., Watari, K., et al. (2010). Infiltration of thymidine phosphorylase-positive macrophages is closely associated with tumor angiogenesis and survival in intestinal type gastric cancer. *Oncol. Rep.* 24, 405–415. doi: 10.3892/or_00000873

Kerjaschki, D. (2005). The crucial role of macrophages in lymphangiogenesis. *J. Clin. Invest.* 115, 2316–2319. doi: 10.1172/JCI26354

Kim, S. H., Das, K., Noreen, S., Coffman, F., and Hameed, M. (2007). Prognostic implications of immunohistochemically detected YKL-40 expression in breast cancer. *World J. Surg. Oncol.* 5:17. doi: 10.1186/1477-7819-5-17

Kioi, M., Vogel, H., Schultz, G., Hoffman, R. M., Harsh, G. R., and Brown, J. M. (2010). Inhibition of vasculogenesis, but not angiogenesis, prevents the recurrence of glioblastoma after irradiation in mice. *J. Clin. Invest.* 120, 694–705. doi: 10.1172/JCI40283

Klug, F., Prakash, H., Huber, P. E., Seibel, T., Bender, N., Halama, N., et al. (2013). Low-dose irradiation programs macrophage differentiation to an iNOS(+)/M1 phenotype that orchestrates effective T Cell immunotherapy. *Cancer Cell* 24, 589–602. doi: 10.1016/j.ccr.2013.09.014

Konradsen, J. R., James, A., Nordlund, B., Reinius, L. E., Soderhall, C., Melen, E., et al. (2013). The chitinase-like protein YKL-40: A possible biomarker of inflammation and airway remodeling in severe pediatric asthma. *J. Allergy. Clin. Immunol.* 132, 328–335. doi: 10.1016/j.jaci.2013.03.003

Kozin, S. V., Kamoun, W. S., Huang, Y., Dawson, M. R., Jain, R. K., and Duda, D. G. (2010). Recruitment of myeloid but not endothelial precursor cells facilitates tumor regrowth after local irradiation. *Cancer Res.* 70, 5679–5685. doi: 10.1158/0008-5472.CAN-09-4446

Kurahara, H., Shinchi, H., Mataki, Y., Maemura, K., Noma, H., Kubo, F., et al. (2011). Significance of M2-polarized tumor-associated macrophage in pancreatic cancer. *J. Surg. Res.* 167, e211–e219. doi: 10.1016/j.jss.2009.05.026

Kurahara, H., Takao, S., Maemura, K., Mataki, Y., Kuwahata, T., Maeda, K., et al. (2013). M2-polarized tumor-associated macrophage infiltration of regional lymph nodes is associated with nodal lymphangiogenesis and occult nodal involvement in pN0 pancreatic cancer. *Pancreas* 42, 155–159. doi: 10.1097/MPA.0b013e318254f2d1

Kwon, S. J., Lee, G. T., Lee, J. H., Iwakura, Y., Kim, W. J., and Kim, I. Y. (2013). Mechanism of pro-tumorigenic effect of BMP-6: neovascularization involving tumor-associated macrophages and IL-1alpha. *Prostate* 74, 121–133. doi: 10.1002/pros.22734

Kzhyshkowska, J. (2010). Multifunctional receptor stabilin-1 in homeostasis and disease. *ScientificWorldJournal* 10, 2039–2053. doi: 10.1100/tsw.2010.189

Kzhyshkowska, J., Gratchev, A., and Goerdt, S. (2006b). Stabilin-1, a homeostatic scavenger receptor with multiple functions. *J. Cell. Mol. Med.* 10, 635–649. doi: 10.1111/j.1582-4934.2006.tb00425.x

Kzhyshkowska, J., and Krusell, L. (2009). Cross-talk between endocytic clearance and secretion in macrophages. *Immunobiology* 214, 576–593. doi: 10.1016/j.imbio.2009.03.007

Kzhyshkowska, J., Gratchev, A., and Goerdt, S. (2007). Human chitinases and chitinase-like proteins as indicators for inflammation and cancer. *Biomark. Insights* 2, 128–146.

Kzhyshkowska, J., Mamidi, S., Gratchev, A., Kremmer, E., Schmuttermaier, C., Krusell, L., et al. (2006c). Novel stabilin-1 interacting chitinase-like protein (SI-CLP) is up-regulated in alternatively activated macrophages and secreted via lysosomal pathway. *Blood* 107, 3221–3228. doi: 10.1182/blood-2005-07-2843

Kzhyshkowska, J., Workman, G., Cardo-Vila, M., Arap, W., Pasqualini, R., Gratchev, A., et al. (2006a). Novel function of alternatively activated macrophages: stabilin-1-mediated clearance of, SPARC. *J. Immunol.* 176, 5825–5832.

Lambaerts, K., Wilcox-Adelman, S. A., and Zimmermann, P. (2009). The signaling mechanisms of syndecan heparan sulfate proteoglycans. *Curr. Opin. Cell Biol.* 21, 662–669. doi: 10.1016/j.ceb.2009.05.002

Laoui, D., Van Overmeire, E., Di Conza, G., Aldeni, C., Keirsse, J., Morias, Y., et al. (2013). Tumor hypoxia does not drive differentiation of tumor-associated macrophages but rather fine-tunes the M2-like macrophage population. *Cancer Res.* 74, 24–30. doi: 10.1158/0008-5472.CAN-13-1196

Lee, S., Jilani, S. M., Nikolova, G. V., Carpizo, D., and Iruela-Arispe, M. L. (2005). Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. *J. Cell Biol.* 169, 681–691. doi: 10.1083/jcb.200409115

Leek, R. D., Landers, R., Fox, S. B., Ng, F., Harris, A. L., and Lewis, C. E. (1998). Association of tumour necrosis factor alpha and its receptors with thymidine phosphorylase expression in invasive breast carcinoma. *Br. J. Cancer* 77, 2246–2251. doi: 10.1038/bjc.1998.373

Leek, R. D., Lewis, C. E., Whitehouse, R., Greenall, M., Clarke, J., and Harris, A. L. (1996). Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res.* 56, 4625–4629.

Lewis, C. E., De Palma, M., and Naldini, L. (2007). Tie2-expressing monocytes and tumor angiogenesis: regulation by hypoxia and angiopoietin-2. *Cancer Res.* 67, 8429–8432. doi: 10.1158/0008-5472.CAN-07-1684

Lewis, J. S., Landers, R. J., Underwood, J. C., Harris, A. L., and Lewis, C. E. (2000). Expression of vascular endothelial growth factor by macrophages is up- regulated in poorly vascularized areas of breast carcinomas. *J. Pathol.* 192, 150–158. doi: 10.1002/1096-9896(2000)9999:9999%3C::AID-PATH687%3E3.0.CO;2-G

Lin, E. Y., Li, J. F., Gnatovskiy, L., Deng, Y., Zhu, L., Grzesik D. A., et al. (2006). Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res.* 66, 11238–11246. doi: 10.1158/0008-5472.CAN-06-1278

Lohela, M., Bry, M., Tammela, T., and Alitalo, K. (2009). VEGFs and receptors involved in angiogenesis versus lymphangiogenesis. *Curr. Opin. Cell Biol.* 21, 154–165. doi: 10.1016/j.ceb.2008.12.012

Ly, L. V., Baghat, A., Versluis, M., Jordanova, E. S., Luyten, G. P., van Rooijen, N., et al. (2010). In aged mice, outgrowth of intraocular melanoma depends on proangiogenic M2-type macrophages. *J. Immunol.* 185, 3481–3488. doi: 10.4049/jimmunol.0903479

Mantovani, A., Bottazzi, B., Colotta, F., Sozzani, S., and Ruco, L. (1992). The origin and function of tumor-associated macrophages. *Immunol. Today* 13, 265–270. doi: 10.1016/0167-5699(92)90008-U

Mantovani, A., Marchesi, F., Porta, C., Sica, A., and Allavena, P. (2007). Inflammation and cancer: breast cancer as a prototype. *Breast* 16(Suppl. 2), S27–S33. doi: 10.1016/j.breast.2007.07.013

Martens, J. H., Kzhyshkowska, J., Falkowski-Hansen, M., Schledzewski, K., Gratchev, A., Mansmann, U., et al. (2006). Differential expression of a gene signature for scavenger/lectin receptors by endothelial cells and macrophages in human lymph node sinuses, the primary sites of regional metastasis. *J. Pathol.* 208, 574–589. doi: 10.1002/path.1921

Matsubara, T., Kanto, T., Kuroda, S., Yoshio, S., Higashitani, K., Kakita, N., et al. (2013). TIE2-expressing monocytes as a diagnostic marker for hepatocellular carcinoma correlates with angiogenesis. *Hepatology* 57, 1416–1425. doi: 10.1002/hep.25965

Mazzieri, R., Pucci, F., Moi, D., Zonari, E., Ranghetti, A., Berti, A., et al. (2011). Targeting the ANG2/TIE2 axis inhibits tumor growth and metastasis by impairing angiogenesis and disabling rebounds of proangiogenic myeloid cells. *Cancer Cell* 19, 512–526. doi: 10.1016/j.ccr.2011.02.005

McColl, B. K., Baldwin, M. E., Roufail, S., Freeman, C., Moritz, R. L., Simpson R. J., et al. (2003). Plasmin activates the lymphangiogenic growth factors VEGF-C and VEGF-D. *J. Exp. Med.* 198, 863–868. doi: 10.1084/jem.20030361

Medina, R. J., O'Neill, C. L., O'Doherty, T. M., Knott, H., Guduric-Fuchs, J., Gardiner TA., et al. (2011). Myeloid angiogenic cells act as alternative M2 macrophages and modulate angiogenesis through interleukin-8. *Mol. Med.* 17, 1045–1055. doi: 10.2119/molmed.2011.00129

Mistrangelo, M., Senetta, R., Racca, P., Castellano, I., Chiusa, L., Bello, M., et al. (2013). A novel biomarker-based analysis reliably predicts nodal metastases in anal carcinoma: preliminary evidence of therapeutic impact. *Colorectal Dis.* 15, 1382–1391. doi: 10.1111/codi.12289

Moussai, D., Mitsui, H., Pettersen, J. S., Pierson, K. C., Shah, K. R., Suarez-Farinas, M., et al. (2011). The human cutaneous squamous cell carcinoma microenvironment is characterized by increased lymphatic density and enhanced expression of macrophage-derived VEGF-C. *J. Invest. Dermatol.* 131, 229–236. doi: 10.1038/jid.2010.266

Nakamura, E. S., Koizumi, K., Kobayashi, M., and Saiki, I. (2004). Inhibition of lymphangiogenesis-related properties of murine lymphatic endothelial cells and lymph node metastasis of lung cancer by the matrix metalloproteinase inhibitor MMI270. *Cancer Sci.* 95, 25–31. doi: 10.1111/j.1349-7006.2004.tb03166.x

Nishie, A., Ono, M., Shono, T., Fukushi, J., Otsubo, M., Onoue, H., et al. (1999). Macrophage infiltration and heme oxygenase-1 expression correlate with angiogenesis in human gliomas. *Clin. Cancer Res.* 5, 1107–1113.

Nishikawa, K. C., and Millis, A. J. (2003). gp38k (CHI3L1) is a novel adhesion and migration factor for vascular cells. *Exp. Cell Res.* 287, 79–87. doi: 10.1016/S0014-4827(03)00069-7

Ohta, M., Kitadai, Y., Tanaka, S., Yoshihara, M., Yasui, W., Mukaida, N., et al. (2002). Monocyte chemoattractant protein-1 expression correlates with

macrophage infiltration and tumor vascularity in human esophageal squamous cell carcinomas. *Int. J. Cancer* 102, 220–224. doi: 10.1002/ijc.10705

Ong, S. M., Tan, Y. C., Beretta, O., Jiang, D., Yeap, W. H., Tai J. J., et al. (2012). Macrophages in human colorectal cancer are pro-inflammatory and prime T cells towards an anti-tumour type-1 inflammatory response. *Eur. J. Immunol.* 42, 89–100. doi: 10.1002/eji.201141825

Piao, Y. J., Lee, C. H., Zhu, M. J., Kye, K. C., Kim, J. M., Seo YJ., et al. (2005). Involvement of urokinase-type plasminogen activator in sphingosylphosphorylcholine-induced angiogenesis. *Exp. Dermatol.* 14, 356–362. doi: 10.1111/j.0906-6705.2005.00272.x

Pucci, F., Venneri, M. A., Biziato, D., Nonis, A., Moi, D., Sica, A., et al. (2009). A distinguishing gene signature shared by tumor-infiltrating Tie2-expressing monocytes, blood "resident" monocytes, and embryonic macrophages suggests common functions and developmental relationships. *Blood* 114, 901–914. doi: 10.1182/blood-2009-01-200931

Qian, B. Z., and Pollard, J. W. (2010). Macrophage diversity enhances tumor progression and metastasis. *Cell* 141, 39–51. doi: 10.1016/j.cell.2010.03.014

Ran, S., and Montgomery, K. E. (2012). Macrophage-Mediated Lymphangiogenesis: the emerging role of macrophages as lymphatic endothelial progenitors. *Cancers (Basel)*. 4, 618–657. doi: 10.3390/cancers4030618

Rego, S. L., Helms, R. S., and Dreau, D. (2013). Breast tumor cell TACE-shed MCSF promotes pro-angiogenic macrophages through NF-kappaB signaling. *Angiogenesis*. doi: 10.1007/s10456-013-9405-2. [Epub ahead of print].

Roberts, N., Kloos, B., Cassella, M., Podgrabinska, S., Persaud, K., Wu, Y., et al. (2006). Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. *Cancer Res.* 66, 2650–2657. doi: 10.1158/0008-5472.CAN-05-1843

Roda, J. M., Wang, Y., Sumner, L. A., Phillips, G. S., Marsh, C. B., and Eubank, T. D. (2012). Stabilization of HIF-2alpha induces sVEGFR-1 production from tumor-associated macrophages and decreases tumor growth in a murine melanoma model. *J. Immunol.* 189, 3168–3177. doi: 10.4049/jimmunol.1103817

Rolny, C., Mazzone, M., Tugues, S., Laoui, D., Johansson, I., Coulon, C., et al. (2011). HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. *Cancer Cell* 19, 31–44. doi: 10.1016/j.ccr.2010.11.009

Roslind, A., Knoop, A. S., Jensen, M. B., Johansen, J. S., Nielsen, D. L., Price, P. A., et al. (2008). YKL-40 protein expression is not a prognostic marker in patients with primary breast cancer. *Breast Cancer Res. Treat.* 112, 275–285. doi: 10.1007/s10549-007-9870-7

Scavelli, C., Vacca, A., Di Pietro, G., Dammacco, F., and Ribatti, D. (2004). Crosstalk between angiogenesis and lymphangiogenesis in tumor progression. *Leukemia* 18, 1054–1058. doi: 10.1038/sj.leu.2403355

Schledzewski, K., Falkowski, M., Moldenhauer, G., Metharom, P., Kzhyshkowska, J., Ganss, R., et al. (2006). Lymphatic endothelium-specific hyaluronan receptor LYVE-1 is expressed by stabilin-1(+), F4/80(+), CD11b(+) macrophages in malignant tumours and wound healing tissue *in vivo* and in bone marrow cultures *in vitro*: implications for the assessment of lymphangiogenesis. *J. Pathol.* 209, 67–77. doi: 10.1002/path.1942

Schoppmann, S. F., Birner, P., Stockl, J., Kalt, R., Ullrich, R., Caucig, C., et al. (2002). Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. *Am. J. Pathol.* 161, 947–956. doi: 10.1016/S0002-9440(10)64255-1

Schoppmann, S. F., Fenzl, A., Nagy, K., Unger, S., Bayer, G., Geleff, S., et al. (2006). VEGF-C expressing tumor-associated macrophages in lymph node positive breast cancer: impact on lymphangiogenesis and survival. *Surgery* 139, 839–846. doi: 10.1016/j.surg.2005.12.008

Shao, R. (2013). YKL-40 acts as an angiogenic factor to promote tumor angiogenesis. *Front. Physiol.* 4:122. doi: 10.3389/fphys.2013.00122

Shao, R., Cao, Q. J., Arenas, R. B., Bigelow, C., Bentley, B., and Yan, W. (2011). Breast cancer expression of YKL-40 correlates with tumour grade, poor differentiation, and other cancer markers. *Br. J. Cancer* 105, 1203–1209. doi: 10.1038/bjc.2011.347

Shao, R., Hamel, K., Petersen, L., Cao, Q. J., Arenas, R. B., Bigelow, C., et al. (2009). YKL-40, a secreted glycoprotein, promotes tumor angiogenesis. *Oncogene* 28, 4456–4468. doi: 10.1038/onc.2009.292

Shieh, Y. S., Hung, Y. J., Hsieh, C. B., Chen, J. S., Chou, K. C., and Liu, S. Y. (2009). Tumor-associated macrophage correlated with angiogenesis and progression of mucoepidermoid carcinoma of salivary glands. *Ann. Surg. Oncol.* 16, 751–760. doi: 10.1245/s10434-008-0259-6

Sica, A., and Mantovani, A. (2012). Macrophage plasticity and polarization: *in vivo* veritas. *J. Clin. Invest.* 122, 787–795. doi: 10.1172/JCI59643

Sierra, J. R., Corso, S., Caione, L., Cepero, V., Conrotto, P., Cignetti, A., et al. (2008). Tumor angiogenesis and progression are enhanced by Sema4D produced by tumor-associated macrophages. *J. Exp. Med.* 205, 1673–1685. doi: 10.1084/jem.20072602

Skobe, M., Hamberg, L. M., Hawighorst, T., Schirner, M., Wolf, G. L., Alitalo, K., et al. (2001b). Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. *Am. J. Pathol.* 159, 893–903. doi: 10.1016/S0002-9440(10)61765-8

Skobe, M., Hawighorst, T., Jackson, D. G., Prevo, R., Janes, L., Velasco, P., et al. (2001a). Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat. Med.* 7, 192–198. doi: 10.1038/84643

Song, K., Herzog, B. H., Sheng, M., Fu, J., McDaniel, J. M., Ruan, J., et al. (2013). Lenalidomide inhibits lymphangiogenesis in preclinical models of mantle cell lymphoma. *Cancer Res.* 73, 7254–7264. doi: 10.1158/0008-5472.CAN-13-0750

Stacker, S. A., Stenvers, K., Caesar, C., Vitali, A., Domagala, T., Nice, E., et al. (1999). Biosynthesis of vascular endothelial growth factor-D involves proteolytic processing which generates non-covalent homodimers. *J. Biol. Chem.* 274, 32127–32136. doi: 10.1074/jbc.274.45.32127

Staples, K. J., Sotoodehnejadnematalahi, F., Pearson, H., Frankenberger, M., Francescut, L., Ziegler-Heitbrock, L., et al. (2011). Monocyte-derived macrophages matured under prolonged hypoxia transcriptionally up-regulate HIF-1alpha mRNA. *Immunobiology* 216, 832–839. doi: 10.1016/j.imbio.2010.12.005

Storr, S. J., Safuan, S., Mitra, A., Elliott, F., Walker, C., Vasko MJ., et al. (2012). Objective assessment of blood and lymphatic vessel invasion and association with macrophage infiltration in cutaneous melanoma. *Mod. Pathol.* 25, 493–504. doi: 10.1038/modpathol.2011.182

Stout, R. D., and Suttles, J. (2004). Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J. Leukoc. Biol.* 76, 509–513. doi: 10.1189/jlb.0504272

Stout, R. D., Watkins, S. K., and Suttles, J. (2009). Functional plasticity of macrophages: in situ reprogramming of tumor-associated macrophages. *J. Leukoc. Biol.* 86, 1105–1109. doi: 10.1189/jlb.0209073

Takanami, I., Takeuchi, K., and Kodaira, S. (1999). Tumor-associated macrophage infiltration in pulmonary adenocarcinoma: association with angiogenesis and poor prognosis. *Oncology* 57, 138–142. doi: 10.1159/000012021

Takao, S., Takebayashi, Y., Che, X., Shinchi, H., Natsugoe, S., Miyadera, K., et al. (1998). Expression of thymidine phosphorylase is associated with a poor prognosis in patients with ductal adenocarcinoma of the pancreas. *Clin. Cancer Res.* 4, 1619–1624.

Tammela, T., Zarkada, G., Wallgard, E., Murtomaki, A., Suchting, S., Wirzenius, M., et al. (2008). Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. *Nature* 454, 656–660. doi: 10.1038/nature07083

Tjiu, J. W., Chen, J. S., Shun, C. T., Lin, S. J., Liao, Y. H., Chu C. Y., et al. (2009). Tumor-associated macrophage-induced invasion and angiogenesis of human basal cell carcinoma cells by cyclooxygenase-2 induction. *J. Invest. Dermatol.* 129, 1016–1025. doi: 10.1038/jid.2008.310

Toi, M., Ueno, T., Matsumoto, H., Saji, H., Funata, N., Koike, M., et al. (1999). Significance of thymidine phosphorylase as a marker of protumor monocytes in breast cancer. *Clin. Cancer Res.* 5, 1131–1137.

Torisu, H., Ono, M., Kiryu, H., Furue, M., Ohmoto, Y., Nakayama, J., et al. (2000). Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma: possible involvement of TNFalpha and IL-1alpha. *Int. J. Cancer* 85, 182–188. doi: 10.1002/(SICI)1097-0215(20000115)85:2%3C182::AID-IJC6%3E3.3.CO;2-D

Vacca, A., Ribatti, D., Ruco, L., Giacchetta, F., Nico, B., Quondamatteo, F., et al. (1999). Angiogenesis extent and macrophage density increase simultaneously with pathological progression in B-cell non-Hodgkin's lymphomas. *Br. J. Cancer* 79, 965–970. doi: 10.1038/sj.bjc.6690154

Valkovic, T., Dobrila, F., Melato, M., Sasso, F., Rizzardi, C., and Jonjic, N. (2002). Correlation between vascular endothelial growth factor, angiogenesis, and tumor-associated macrophages in invasive ductal breast carcinoma. *Virchows Arch.* 440, 583–588. doi: 10.1007/s004280100458

Wang, Y., Ripa, R. S., Johansen, J. S., Gabrielsen, A., Steinbruchel, D. A., Friis, T., et al. (2008). YKL-40 a new biomarker in patients with acute coronary syndrome or stable coronary artery disease. *Scand. Cardiovasc. J.* 42, 295–302. doi: 10.1080/14017430802220567

Welford, A. F., Biziato, D., Coffelt, S. B., Nucera, S., Fisher, M., Pucci, F., et al. (2011). TIE2-expressing macrophages limit the therapeutic efficacy of the vascular-disrupting agent combretastatin A4 phosphate in mice. *J. Clin. Invest.* 121, 1969–1973. doi: 10.1172/JCI44562

Werchau, S., Toberer, F., Enk, A., Dammann, R., and Helmbold, P. (2012). Merkel cell carcinoma induces lymphatic microvessel formation. *J. Am. Acad. Dermatol.* 67, 215–225. doi: 10.1016/j.jaad.2011.09.002

Werno, C., Menrad, H., Weigert, A., Dehne, N., Goerdt, S., Schledzewski, K., et al. (2010). Knockout of HIF-1alpha in tumor-associated macrophages enhances M2 polarization and attenuates their pro-angiogenic responses. *Carcinogenesis* 31, 1863–1872. doi: 10.1093/carcin/bgq088

Whitehurst, B., Flister, M. J., Bagaitkar, J., Volk, L., Bivens, C. M., Pickett, B., et al. (2007). Anti-VEGF-A therapy reduces lymphatic vessel density and expression of VEGFR-3 in an orthotopic breast tumor model. *Int. J. Cancer* 121, 2181–2191. doi: 10.1002/ijc.22937

Wu, H., Xu, J. B., He, Y. L., Peng, J. J., Zhang, X. H., Chen C. Q., et al. (2012). Tumor-associated macrophages promote angiogenesis and lymphangiogenesis of gastric cancer. *J. Surg. Oncol.* 106, 462–468. doi: 10.1002/jso.23110

Wyckoff, J., Wang, W., Lin, E. Y., Wang, Y., Pixley, F., Stanley E. R., et al. (2004). A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res.* 64, 7022–7029. doi: 10.1158/0008-5472.CAN-04-1449

Xu, J., Escamilla, J., Mok, S., David, J., Priceman, S., West, B., et al. (2013). CSF1R signaling blockade stanches tumor-infiltrating myeloid cells and improves the efficacy of radiotherapy in prostate cancer. *Cancer Res.* 73, 2782–2794. doi: 10.1158/0008-5472.CAN-12-3981

Yamac, D., Ozturk, B., Coskun, U., Tekin, E., Sancak, B., Yildiz, R., et al. (2008). Serum YKL-40 levels as a prognostic factor in patients with locally advanced breast cancer. *Adv. Ther.* 25, 801–809. doi: 10.1007/s12325-008-0082-2

Yang, H., Kim, C., Kim, M. J., Schwendener, R. A., Alitalo, K., Heston, W., et al. (2011). Soluble vascular endothelial growth factor receptor-3 suppresses lymphangiogenesis and lymphatic metastasis in bladder cancer. *Mol. Cancer* 10:36. doi: 10.1186/1476-4598-10-36

Yao, P. L., Lin, Y. C., Wang, C. H., Huang, Y. C., Liao, W. Y., Wang, S. S., et al. (2005). Autocrine and paracrine regulation of interleukin-8 expression in lung cancer cells. *Am. J. Respir. Cell Mol. Biol.* 32, 540–547. doi: 10.1165/rcmb.2004-0223OC

Yao, Y., Kubota, T., Sato, K., and Kitai, R. (2001). Macrophage infiltration-associated thymidine phosphorylase expression correlates with increased microvessel density and poor prognosis in astrocytic tumors. *Clin. Cancer Res.* 7, 4021–4026.

Zhang, B., Yao, G., Zhang, Y., Gao, J., Yang, B., Rao, Z., et al. (2011b). M2-polarized tumor-associated macrophages are associated with poor prognoses resulting from accelerated lymphangiogenesis in lung adenocarcinoma. *Clinics (Sao. Paulo)*. 66, 1879–1886. doi: 10.1590/S1807-59322011001100006

Zhang, B., Zhang, Y., Yao, G., Gao, J., Yang, B., Zhao, Y., et al. (2012b). M2-polarized macrophages promote metastatic behavior of Lewis lung carcinoma cells by inducing vascular endothelial growth factor-C expression. *Clinics (Sao. Paulo)*. 67, 901–906. doi: 10.6061/clinics/2012(08)08

Zhang, J. L., Chen, G. W., Liu, Y. C., Wang, P. Y., Wang, X., Wan YL., et al. (2012a). Secreted protein acidic and rich in cysteine (SPARC) suppresses angiogenesis by down-regulating the expression of VEGF and MMP-7 in gastric cancer. *PLoS ONE* 7:e44618. doi: 10.1371/journal.pone.0044618

Zhang, Z., Pan, J., Li, L., Wang, Z., Xiao, W., and Li, N. (2011a). Survey of risk factors contributed to lymphatic metastasis in patients with oral tongue cancer by immunohistochemistry. *J. Oral Pathol. Med.* 40, 127–134. doi: 10.1111/j.1600-0714.2010.00953.x

Zumsteg, A., Baeriswyl, V., Imaizumi, N., Schwendener, R., Ruegg, C., and Christofori, G. (2009). Myeloid cells contribute to tumor lymphangiogenesis. *PLoS ONE* 4:e7067. doi: 10.1371/journal.pone.0007067

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 June 2013; accepted: 06 February 2014; published online: 05 March 2014.
Citation: Riabov V, Gudima A, Wang N, Mickley A, Orekhov A and Kzhyshkowska J (2014) Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis. Front. Physiol. ***5****:75. doi: 10.3389/fphys.2014.00075*
This article was submitted to Vascular Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 05 February 2014
doi: 10.3389/fphys.2014.00017

Semaphorin7A promotes tumor growth and exerts a pro-angiogenic effect in macrophages of mammary tumor-bearing mice

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Semaphorins are a large family of molecules involved in axonal guidance during the development of the nervous system and have been recently shown to have both angiogenic and anti-angiogenic properties. Specifically, semaphorin 7A (SEMA7A) has been reported to have a chemotactic activity in neurogenesis and to be an immune modulator through α1β1integrins. SEMA7A has been shown to promote monocyte chemotaxis and induce them to produce proinflammatory mediators. In this study we explored the role of SEMA7A in a murine model of breast cancer. We show that SEMA7A is highly expressed by DA-3 murine mammary tumor cells in comparison to normal mammary cells (EpH4), and that peritoneal elicited macrophages from mammary tumor-bearing mice also express SEMA7A at higher levels compared to those derived from normal mice. We also show that murine macrophages treated with recombinant murine SEMA7A significantly increased their expression of proangiogenic molecule CXCL2/MIP-2. Gene silencing of SEMA7A in peritoneal elicited macrophages from DA-3 tumor-bearing mice resulted in decreased CXCL2/MIP-2 expression. Mice implanted with SEMA7A silenced tumor cells showed decreased angiogenesis in the tumors compared to the wild type tumors. Furthermore, peritoneal elicited macrophages from mice bearing SEMA7A-silenced tumors produce significantly ($p < 0.01$) lower levels of angiogenic proteins, such as CXCL2/MIP-2, CXCL1, and MMP-9, compared to those from control DA-3 mammary tumors. We postulate that SEMA7A in mammary carcinomas may skew monocytes into a pro-tumorigenic phenotype to support tumor growth. SEMA7A could prove to be valuable in establishing new research avenues toward unraveling important tumor-host immune interactions in breast cancer patients.

Keywords: SEMA7A, angiogenesis, macrophages, breast cancer, CXCL2/MIP-2, MMP-9

INTRODUCTION

Semaphorins (SEMAs) comprise a large family of transmembrane and secreted proteins that have been described as axon guidance molecules during neuronal development (Koppel et al., 1997; Pasterkamp and Kolodkin, 2003; Kikutani et al., 2007). Semaphorins, grouped into eight classes, are characterized by the presence of a conserved large SEMA domain (∼500 amino acids) at the N-terminal domain and differentiated by their C-terminus (Koppel et al., 1997). Of the 8 classes of semaphorins, classes 1 and 2 are mostly found in invertebrates while classes 3–7 are found in vertebrates and the viral (V) class encoded by viruses. Emerging evidence is revealing additional roles for semaphorins in the immune system where they seem to exert diverse effects on leukocyte migration, adhesion, and inflammatory responses (Kikutani et al., 2007; Sakurai et al., 2010).

A growing body of evidence demonstrates the participation of classical neuronal developmental molecules in either tumor growth or inhibition by their effects on angiogenesis (Banu et al., 2006; Basile et al., 2006; Guttmann-Raviv et al., 2007; Acevedo et al., 2008; Sierra et al., 2008; Casazza et al., 2011). Semaphorins have been found to affect tumor progression by either modulating tumor angiogenesis, recruiting bone marrow cells that could then influence tumor progression, or by directly affecting the behavior of tumor cells. While some semaphorins were found to inhibit angiogenesis, others enhanced new blood vessel growth. Proangiogenic semaphorins include semaphorin 4A (SEMA4A), semaphorin 4D (SEMA4D), and semaphorin 5A (SEMA5A) (Capparuccia and Tamagnone, 2009). However, some members in semaphorin 3 (SEMA3) class have antiangiogenic effects (Basile et al., 2004; Varshavsky et al., 2008; Sadanandam et al., 2010; Sakurai et al., 2010; Meda et al., 2012). Although many classes of semaphorins have been studied in different cancers, the role of sempahorin7A (SEMA7A) in cancer progression is largely unknown. SEMA7A is a novel transmembrane GPI-anchored

protein that has been described to function through plexin C1 and beta-integrins in multiple systems (Zhou et al., 2008). Recently, SEMA7A has been reported to be one of the proteins secreted by glioblastoma tumor cells that contribute to the highly invasive phenotype (Formolo et al., 2011). In this study we explore the role of SEMA7A in breast cancer progression using the DA-3 mammary tumor model. Specifically, we are investigating how SEMA7A can affect macrophage production of angiogenic molecules.

There is scarce information in literature on how SEMA7A affects macrophage induced angiogenesis. An angiogenic role for SEMA7A has been recently described to mediate vascular growth by bFGF stimulated fibroblasts in an experimental model of corneal neovascularization (Ghanem et al., 2011). In this manuscript using peritoneal elicited macrophages, a rich source of peripheral macrophages, we describe that SEMA7A induces macrophages to produce angiogenic molecules such as CXCL2/MIP-2 and that silencing the SEMA7A gene results in decreased production of these growth promoting molecules.

MATERIALS AND METHODS

MICE AND CELL LINES

Female BALB/c mice were used in all studies (Charles River Laboratories, 8–12 week-olds), and were housed and used according to the National Institutes of Health guidelines, under protocols approved by Florida Atlantic University Institutional Animal Care and Use Committee. In these studies, we used the DA-3 cell line which was derived from the D1-DMBA-3 mammary tumor syngeneic to BALB/c mice and were provided by Dr. Diana M. Lopez, University of Miami School of Medicine, Miami, FL (Sotomayor et al., 1991). EpH4 mammary cells, a normal mammary cell line, were provided by Dr. Jenifer Prosperi, Indiana University School of Medicine-South Bend, IN. Both DA-3 and EpH4 cells were grown in complete DMEM media (DMEM with 10% FBS). RAW 264.7 cells (American Type Culture Collection, Manassas, VA, USA) were grown and maintained in RPMI 1640 containing 5% FBS as described previously (Nishiyama et al., 2006, 2008). Female BALB/c mice were inoculated in the lower right ventral quadrant with 7.5×10^5 mammary tumor cells of the following types: (1) DA-3 cells silenced for the SEMA7A gene, (2) DA-3 cells with scramble shRNA, or (3) wild-type DA-3 cells. Imaging studies and caliper measurements of the primary tumors were performed up to 3 weeks post-tumor cell implantation and discontinued after this time point since the tumors become necrotic and fall off after 3 weeks. Tissues from 5-week tumor bearers were used in most of the studies, unless specified, based on our previous studies that production of tumor-derived factors peak at this time point (Lopez et al., 1991). At 5 weeks, tumors are not observed in the lung, liver, and bone. The establishment of metastastic colonies at distant sites occur at 10–12 weeks if $500–750 \times 10^3$ cells are inoculated. For determination of angiogenesis by AngioSense (PerkinElmer, Waltham, MA), mice were implanted with SEMA7A shRNA silenced mammary tumor cells or scramble shRNA control mammary tumor cells and imaged at 21 days post-tumor implantation while tissues were collected at 5 weeks post-tumor cell implantation.

CELL CULTURES

To obtain peritoneal elicited macrophages (PEMs), mice were injected intraperitoneally with 1.5 mL of 3% thioglycollate and 4 days post-thioglycollate injection and the peritoneal exudate cells were collected by peritoneal lavage with ice-cold RPMI 1640 with 10% fetal bovine serum. It is well-established that the optimal time point for harvesting PEMs is 4 days post-thioglycollate injection (Zhang et al., 2008). As our previous studies have shown increased chemokine and MMP-9 expression at 4–5 weeks post tumor cell inoculation, we chose 5 week time point to assess the role of SEMA7A in inducing proangiogenic factors by macrophages (Owen et al., 2003, 2011). PEMs from normal (N-PEM) and DA-3 tumor-bearing (DA-3 PEM) mice were then purified using CD11b magnetic beads (Miltenyi Biotec Inc., Auburn, CA). 2×10^6 cells/mL were preconditioned by culturing with rmSEMA7A (5 μg/mL) (R&D Systems, Minneapolis, MN) and incubated for 24 h followed by stimulation with LPS (500 ng/mL) (Sigma Aldrich, St.Louis, MO) for an additional 12 h for RNA and 18 h for protein collection. RAW 264.7 macrophages were also conditioned as described above. For cell signaling inhibition studies, RAW 264.7 cells were pretreated with 1 μM MAPK inhibitor, U0126 (Calbiochem, inhibitors, EMD Millipore, Billerica, MA) for 1 h, conditioned with rmSEMA7A for 12 h and then stimulated with LPS (500 ng/mL) for an additional 12 h.

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To determine the expression of SEMA7A, DA-3 mammary tumor cells were plated onto a confocal cover slide, post-fixed in 4% paraformaldehyde, blocked in 4% BSA and labeled with 0.1 μg/mL rat anti-SEMA7A (R&D Systems) followed by incubation in secondary antibody using donkey anti-rat IgG conjugated to AlexaFluor 488 (Molecular Probes, Eugene, OR). To visualize nuclei, DAPI (Vector Laboratories, Burlingame, CA) was added, cover-slipped with Vectashield and examined by confocal microscopy (Carl Zeiss Microimaging, Inc., Thornwood, NY).

RNA ISOLATION AND REAL-TIME REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Total RNA was extracted from murine tumor cells, RAW 264.7 macrophages or peritoneal elicited macrophages using the RNeasy Protect Mini Kit (QIAGEN) according to manufacturer's instructions. Briefly, cDNA was synthesized using Quantitech Reverse Transcription Kit (Qiagen, Valencia, CA) and gene expression was detected by SYBR Green real-time PCR analysis using SYBR RT²qPCR primers (Qiagen, proprietary primers, sequence not disclosed). The mRNA levels of gene of interest were normalized to β-actin mRNA levels. PCR cycles followed the sequence: 10 min at 95°C of initial denaturation; 15 s at 95°C; and 40 cycles of 1 min each at 60°C for annealing. The samples were amplified using the Strategene MX3005P cycler.

FLOW CYTOMETRY STUDIES

The expression of CD11b and CD29 on macrophages was assessed by flow cytometry (FACS-Calibur, BD Biosciences, San Jose, CA). N-PEMs and DA-3 PEMs were stained by incubating with

FITC-CD29 (0.125 μg/10^6 cells) and APC-CD11b (0.1 μg /10^6 cells) (both from BioLegend, San Diego, CA) for 20 min at 4°C. Surface expression was assessed by counting 10,000 cells and analyzed by FloJo software (Tree Star, Inc., Ashland, OR).

SILENCING OF SEMA7A IN MACROPHAGES

SEMA7A gene silencing in DA-3 PEMs was achieved by RNA interference via short hairpin RNA (Origene, Rockville, MD) as described above. Briefly, PT-67 packaging cells were transfected with one of the following plasmids: (1) plasmid encoding for shRNA sequence specifically for the SEMA7A gene and (2) scramble shRNA plasmid not specific for the SEMA7A gene, using Lipofectamine 2000 according to manufacturer's protocol. 0.45 μm filtered PT-67 transfected supernatants containing the retrovirus were used to silence SEMA7A gene in DA-3 PEMs for 36 h. Macrophages were then stimulated with LPS (100 ng/ml) for 12 h and q-PCR was performed to confirm SEMA7A gene silencing.

SILENCING OF SEMA7A IN DA-3 MURINE MAMMARY TUMOR CELLS

Semaphorin 7A gene silencing in DA-3 mammary tumor cells was achieved using RNA interference via short hairpin RNA (Origene). A retrovirus shRNA plasmid system was used for stable SEMA7A gene knockdown. To generate the retrovirus infecting particles, PT-67 packaging cells were transfected with one of the following plasmids: (1) plasmid encoding for shRNA sequence specifically for the SEMA7A gene and (2) scramble shRNA plasmid not specific to the SEMA7A gene. Transfection was performed using standard Lipofectamine 2000 according to manufacturer's protocol. The different variants of transfected PT-67 cells were selected for 2 weeks with puromycin (2 μg/mL) and the cell-free/retrovirus-rich supernatants from the different PT-67 variants and controls were used to infect DA-3 cells for 24–48 h. The different DA-3 variants were then selected with puromycin (1 μg/mL) for 4 weeks. To confirm gene knockdown, real time quantitative polymerase chain reaction (q-PCR) (Qiagen) was performed using the SEMA7A specific primers according to manufacturer's protocol. Cells were passaged and selected until at least a 5-fold decrease in the SEMA7A gene expression was achieved when compared to the scramble control. The results of gene expression were then confirmed by western blotting for the SEMA7A protein.

MONOCYTE MIGRATION ASSAY

To test migration, RAW 264.7 murine monocytes were labeled with Calcein-AM (10 μM) and used in a modified Boyden Chamber assay. Briefly, 10^5 RAW264.7 were placed in the transwell insert (8 μM pores) (BD Biosciences) of the upper chamber with lower chamber containing supernatants from: (1) DA-3 cells silenced for the SEMA7A gene, (2) DA-3 cells with scramble shRNA, and (3) wild-type DA-3 cells and incubated at 37°C in a CO_2 incubator for 12 h. RAW 264.7 macrophage migration was measured using a plate reader set at an excitation wavelength of ∼485 nm and an emission wavelength of ∼520 nm. Absorbance values among the various groups were measured at least 2 times in triplicate and fitted to a 7-point standard curve.

PROTEIN DETERMINATION

DA-3 murine mammary tumor cells were cultured under optimal conditions using DMEM culture media with 10% FBS until ∼80% confluency was achieved. DA-3 tumor cells and DA-3 SEMA7A-silenced cells or intraperitoneal macrophages from 5-week DA-3 mammary tumor-bearing mice were lysed with sample buffer (20 mM dithiothreitol, 6% SDS, 0.25 M Tris, pH 6.8, 10% glycerol, 10 mM NaF and bromophenyl blue) and used to extract total protein. 20 μg of total protein from DA-3 cells and PEMs were resolved on 4–20% Mini-Protean SDS-PAGE gradient gels (BioRad Life Sciences, Hercules, CA) and transferred to PVDF membrane (Pierce, Rockford, IL) using a semi-dry transfer transblotter (BioRad) at 20 Volts for 40 min. The membrane was blocked overnight at 4°C in SeaBlock (Calbiochem), and subsequently incubated at room temperature with anti-mouse SEMA7A monoclonal antibody (1 μg/ml) (R&D Systems) and anti-mouse beta actin polyclonal antibody (0.25 μg/ml) (Li-Cor Biosciences, Lincoln, NE). Western blots were washed for 10 min three times with 0.5% Tween-PBS followed by 1 h incubation at room temperature with corresponding fluorescent antibodies (Li-Cor Biosciences). Blots were washed again for 10 min three times with 0.5% Tween-PBS and then dried at 37°C for 20 min. The membranes were then were visualized with Li-Cor imager. Protein concentration was normalized to beta-actin as loading control.

ELISAs of CXCL1, CXCL2/MIP-2 and MMP-9 (R&D Systems) were performed following manufacturer suggested protocol from DA-3 tumor control mice.

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Formalin-fixed tissue from controls, SEMA7A scramble controls and SEMA7A silenced tumors was paraffin embedded and sectioned at 4-micron thickness. Pre-treatment of formalin-fixed, paraffin-embedded tissue sections with heat-induced epitope retrieval (HIER) was done using diluted EnVision™ FLEX Target Retrieval Solution, High pH (50×) (Dako Omnis, Carpinteria, CA) following manufacturer protocol. The sections were deparaffinized and stained with hematoxylin and eosin (H&E) with automated Tissue Tek® 2000 processor (Sakura-Finetek, Torrance, CA). Adjacent tumor sections were assessed for vascularity using CD31 antibody. Dako FLEX monoclonal mouse anti-human CD31 antibody (diluted 1:30, DAKO) was used to highlight the vasculature of the tumors. CD31, expressed almost exclusively on endothelial cells, is a brown antibody stain against a hematoxylin counter stain. Photographs were taken at 50× magnification with mineral oil immersion using Olympus MDOB3 microscope and photographed with OlympusDP21 digital camera (Center Valley, PA).

TUMOR MEASUREMENTS AND *IN VIVO* IMAGING FOR ANGIOGENESIS

Tumor size determination was performed by measuring the two longest perpendicular axes in the x/y plane of the tumor nearest to 0.1 mm by caliper measurement. The depth was assumed to be equivalent to the shortest of the perpendicular axes, defined as y and tumor volume = $x(y)^2/2$. To account for vascularization in mice injected with either wild type DA-3 tumor cells or those silenced for SEMA7A, near infrared blood pool agent

AngioSense 680 probe (2 nmol/mouse in 150 μL volume) (Perkin Elmer, Waltham, MA) was injected via tail vein 24 h before imaging. Mice were imaged using a bioluminescence optical imager (IVIS Lumina LTE, Perkin Elmer). Maximal near infrared signals were quantified using Living Image 2.5 (Xenogen, Perkin Elmer) image analysis software. Infrared signals are reported as photons/s.

Statistical analysis

Results are expressed as means ± standard deviation. Statistical analyses were performed using GraphPad Prism 3 software (LaJolla, CA). Statistical comparisons of paired groups were determined by Student's *t*-tests. Values of $p < 0.05$ were considered statistically significant.

RESULTS

SEMA7A IS EXPRESSED IN DA-3 MAMMARY TUMOR CELLS AND EXPRESSION IS INCREASED IN PERITONEAL ELICITED MACROPHAGES OF DA-3 MAMMARY TUMOR-BEARING MICE

Semaphorins have been described to be expressed by various cell types. Although it is known that SEMA7A is expressed by monocytes, activated T cells, and keratinocytes, it is not known if tumor cells express SEMA7A. We therefore cultured DA-3 mammary tumor cells and assessed for SEMA7A expression. Confocal image shows that SEMA7A is expressed by the DA-3 mammary tumor cell line (**Figure 1A**). We then asked if SEMA7A is expressed by EpH4 mammary cells, a normal mammary cell line, and how do these levels compare with those in DA-3 tumor cells? qPCR revealed very low levels of SEMA7A expression in EpH4 cells compared to DA-3 mammary tumor cells (**Figure 1B**).

Members of the semaphorin family have been reported to be cleaved to generate soluble forms that have effects on immune function (Kumanogoh and Kikutani, 2003). It was not known if SEMA7A is solubilized in our tumor model. Since there are no reliable ELISAs available to quantify secreted SEMA7A protein, dot blot analysis was used to determine if SEMA7A is solubilized. Analysis of total protein from supernatants of 3 day DA-3 mammary tumor cell cultures confirmed the soluble protein expression of SEMA7A, with increased levels reflected by increased cell numbers (**Figure 1C**). It is possible that circulating levels of cleaved SEMA7A could have effects on other cells.

In the immune system, SEMA7A has been reported to be expressed in the myeloid and the lymphoid lineage cells (Delorme et al., 2005). There are no studies to date describing the expression of SEMA7A in macrophages of mammary tumor bearers. Thus, thioglycollate peritoneal elicited macrophages from normal (N-PEMs) and DA-3 mammary tumor-bearing mice (DA-3 PEMs) were therefore tested to determine SEMA7A expression. It is well-established that the optimal time point for peritoneal elicited macrophages is 4 days post-thioglycollate injection (Zhang et al., 2008). At earlier time points (e.g., 4–24 h post-thioglycollate) the majority of cells in the peritoneal cavity consist of neutrophils (Melnicoff et al., 1989; Lam et al., 2013). SEMA7A expression was determined at 3, 4, and 5 days post-thioglycollate injection in normal and DA-3 mammary tumor-bearing mice. There were no significant differences in SEMA7A expression at these days in peritoneal elicited cells from either normal or tumor-bearing mice. We therefore opted for 4 days as our set time point for these studies. A 3-fold increase in SEMA7A expression at the mRNA level was found in peritoneal elicited macrophages from DA-3 PEMs (**Figure 1D**) compared to the expression in N-PEMs. Similarly, increased protein expression of SEMA7A was found in DA-3 PEMs compared to normal PEMs (**Figure 1E**). Quantification of the bands from western blot analysis confirmed increased SEMA7A protein expression in DA-3 PEMs.

EXPRESSION OF SEMA7A RECEPTOR, β1 INTEGRIN (CD29) IS INCREASED IN DA-3 MAMMARY TUMOR CELLS AND MACROPHAGES FROM MAMMARY TUMOR-BEARING MICE

The principal signaling function of SEMA7A in the nervous and immune systems is mediated through α1β1 integrin (Pasterkamp et al., 2003; Suzuki et al., 2007; Gan et al., 2011). Increased β1 signaling has previously been shown to be associated with decreased survival in invasive breast cancer (Yao et al., 2007). We first determined if there is a differential β1 integrin expression in EpH4 and DA-3 mammary tumor cells. Flow cytometric analysis showed that even though the percentage of β1 integrin (CD29) positive cells remained unchanged between the normal EpH4 cells and the DA-3 mammary tumor cells, the mean fluorescence intensity was almost doubled in the tumor cells (**Figure 2A**). The expression of SEMA7A's receptor, β1 integrin, in peripheral macrophages between normal and tumor bearers has not yet been well characterized. We determined if there are altered levels of β1 integrin expression in peritoneal elicited macrophages (PEMs) from normal and DA-3 mammary tumor-bearing mice. PEMs were gated based on the fluorescent intensity of CD11b expression (**Figure 2B**). Flow cytometric analysis of $CD11b^{low}$ PEMs from normal and DA-3 tumor bearing mice revealed no significant differences in the frequency of $CD29^{+}$ cells (**Figure 2C**). In contrast, expression of $CD11b^{hi}CD29^{+}$ in DA-3 PEMs was higher ($p < 0.05$) compared to the expression in normal PEMs (**Figure 2C**).

TREATMENT OF MACROPHAGES WITH rmSEMA7A INDUCES PRODUCTION OF ANGIOGENIC CXCL2/MIP-2

Macrophages from tumor-bearing mice are known to produce angiogenic molecules (Mantovani et al., 1992). Previous studies have shown that tumor-derived factors induce macrophages to produce angiogenic and proinflammatory molecules (Pollard, 2004). Holmes et al. have shown that SEMA7A induces the production of proinflammatory molecules including the IL-8 homolog of chemokine CXCL2/MIP-2, which also has angiogenic properties (Holmes et al., 2002). As shown in the previous section, DA-3 mammary tumor cells express and shed SEMA7A. We therefore determined whether soluble SEMA7A has an effect on macrophage function. Toward these studies we used the macrophage cell line RAW 264.7 in which SEMA7A mRNA was undetectable (CT value > 37). RAW264.7 macrophages, as a model of tissue macrophages isolated from normal mice, have been used frequently for *in vitro* studies of macrophage function. qPCR analysis of RAW 264.7 macrophages

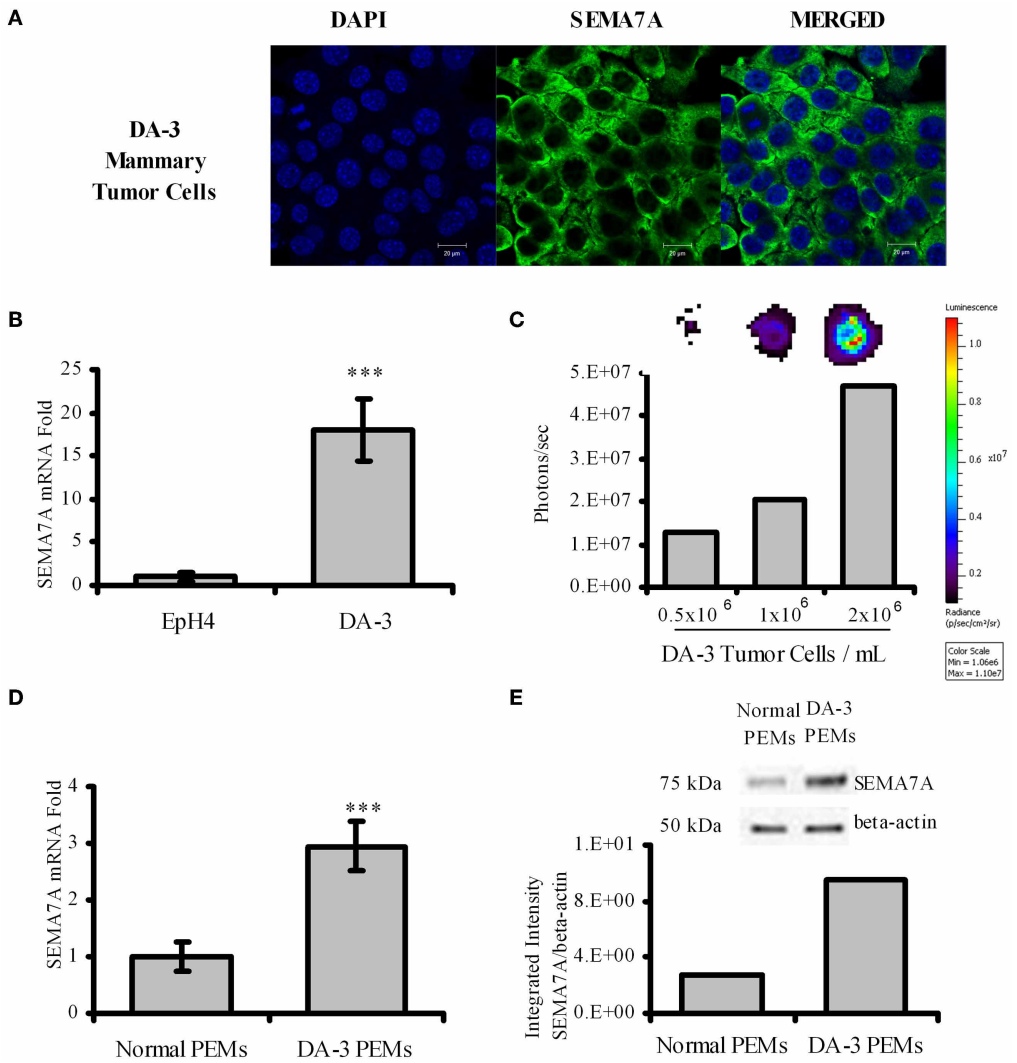

FIGURE 1 | DA-3 mammary tumor cells express SEMA7A. (A) Shown is the confocal images of DA-3 mammary tumor cells for SEMA7A expression; **(B)** SEMA7A expression in EpH4 and DA-3 mammary tumor cells as determined by qPCR; **(C)** DOT blot analysis of DA-3 mammary tumor cell culture showing that SEMA7A is solubilized; **(D)** mRNA expression of SEMA7A in peritoneal elicited macrophages (PEMs) from normal and DA-3 mammary tumor-bearing mice; **(E)** Western blot analysis of total protein from PEMs from control and DA-3 mammary tumor bearers with 7 mice/group; also shown is integrated intensity graph of western blot indicating higher levels of SEMA7A expression in tumor bearers' PEMs. *In-vitro* experiments are representative of three independent experiments, $^{***}p \leq 0.001$.

preconditioned with rmSEMA7A revealed that expression of proangiogenic molecules CXCL2/MIP-2 was increased by 5-fold ($p < 0.001$) (**Figure 3A**) after LPS stimulation. We found a significant ($p < 0.01$) increase in CXCL2/MIP-2 protein in RAW 264.7 macrophages treated with rmSEMA7A and LPS (**Figure 3B**). These studies also included culturing of RAW 264.7 cells with rmSEMA7A alone, which also showed an increase in CXCL2/MIP-2 (data not shown). SEMA7A has previously been reported to function through β1 integrin activation of MAPK signaling pathway to promote monocyte inflammatory response (Suzuki et al., 2007). To get insight if SEMA7A induces CXCL2/MIP-2 via MAPK pathway, RAW 264.7 macrophages were pretreated with a MAPK inhibitor (U0126). We found that U0126 conditioned and rmSEMA7A treated cells exhibited decreased ($p < 0.01$) production of CXCL2/MIP-2 compared to those cultured with rmSEMA7A alone (**Figure 3C**).

To determine if freshly isolated macrophages from normal and DA-3 mammary tumor bearers express CXCL2/MIP-2, peritoneal elicited macrophages from normal and DA-3 mammary tumor bearers were obtained and assessed for CXCL2/MIP-2 expression by qPCR. A greater than 5-fold increase ($p < 0.001$) in CXCL2/MIP-2 expression was observed in DA-3 PEMs compared to normal PEMs (**Figure 3D**). We have previously shown that tumor-derived factors have an effect on profile of PEMs (Lopez et al., 1996; DiNapoli et al., 1997; Handel-Fernandez et al., 1997; Torroella-Kouri et al., 2003). Therefore, peritoneal

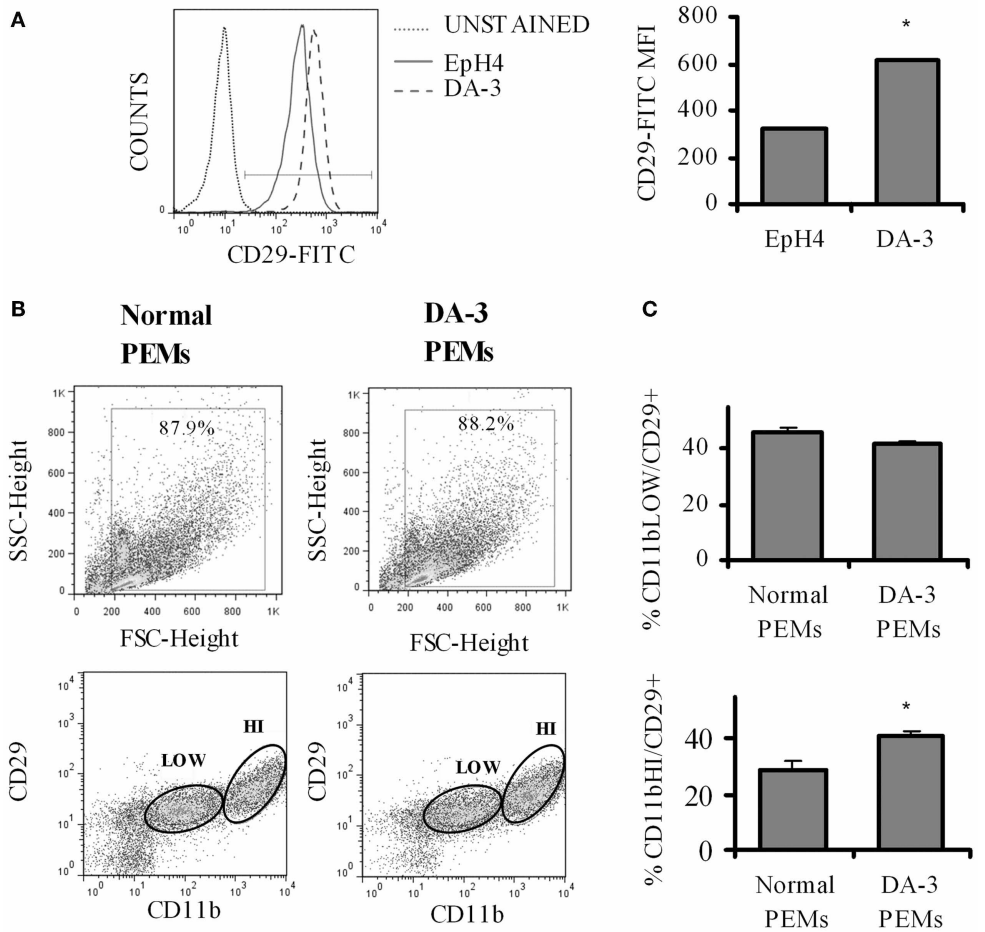

FIGURE 2 | β1 integrin expression is increased in DA-3 cells and macrophages of mammary tumor-bearing mice. (A) Flow cytometric analysis of DA-3 mammary tumor cells and EpH4 mammary epithelial cells for the expression of β1 integrin (CD29) (a representative plot of 2 independent experiments); **(B)** scatter plot of $CD11b^{+}$ and CD29 expression in PEMs from normal and DA-3 mammary tumor-bearing mice; **(C)** flow cytometric analysis of PEMs from control and DA-3 mammary tumor bearers gated on either $CD11b^{low}$ or $CD11b^{high}$ and assessed for the expression of CD29, $N = 8$, $^{*}p \leq 0.05$.

elicited macrophages were used as we wanted to determine the effect of SEMA7A in circulation on macrophages. Since RAW 264.7 macrophages treated with rmSEMA7A had increased expression of CXCL2/MIP-2, we determined if treatment of N-PEMs with rmSEMA7A had an effect on production of angiogenic molecule, CXCL2/MIP-2. A considerably ($p < 0.05$) enhanced expression of CXCL2/MIP-2 was observed in N-PEMs pretreated with rmSEMA7A and then stimulated with LPS (**Figure 3E**). Given that SEMA7A is known to induce CXCL2/MIP-2, and PEMs from DA-3 mammary tumor bearers have increased CXCL2/MIP-2 and SEMA7A, we silenced the SEMA7A gene in DA-3 PEMs using shRNA. Effectiveness of SEMA7A gene silencing as indicated in the 1st set of bars shows that SEMA7A gene was significantly ($p < 0.001$) silenced compared to the scramble control (**Figure 3F**). SEMA7A gene silenced DA-3 PEMs expressed significantly less CXCL2/MIP-2 compared to scramble control as determined by q-PCR (**Figure 3F**). It is important to note that our previous studies show that DA-3 cells express CXCL2/MIP-2. It is possible that SEMA7A could function in an autocrine manner to upregulate the expression of CXCL2/MIP-2.

DECREASED TUMOR-DERIVED SEMA7A RESULTS IN REDUCED *IN VITRO* MACROPHAGE MIGRATION AND CXCL2/MIP-2 PRODUCTION

Holmes et al. demonstrated that SEMA7A is a potent monocyte chemoattractant with 1000-times greater chemotactic activity than monocyte chemotactic protein, MCP-1. (Holmes et al., 2002). We hypothesized that silencing SEMA7A gene in DA-3 mammary tumor cells would result in decreased secretion of SEMA7A in tumor cell cultures and treatment of macrophages with this conditioned media would therefore have a negative influence on their migration. Thus, SEMA7A gene was silenced in DA-3 mammary tumor cells by shRNA. Western blotting was performed to test the effectiveness of SEMA7A gene silencing. Lane 1 indicates DA-3 wild type, lane 2 shows DA-3 scramble shRNA and lane 3 consists of DA-3 SEMA7A

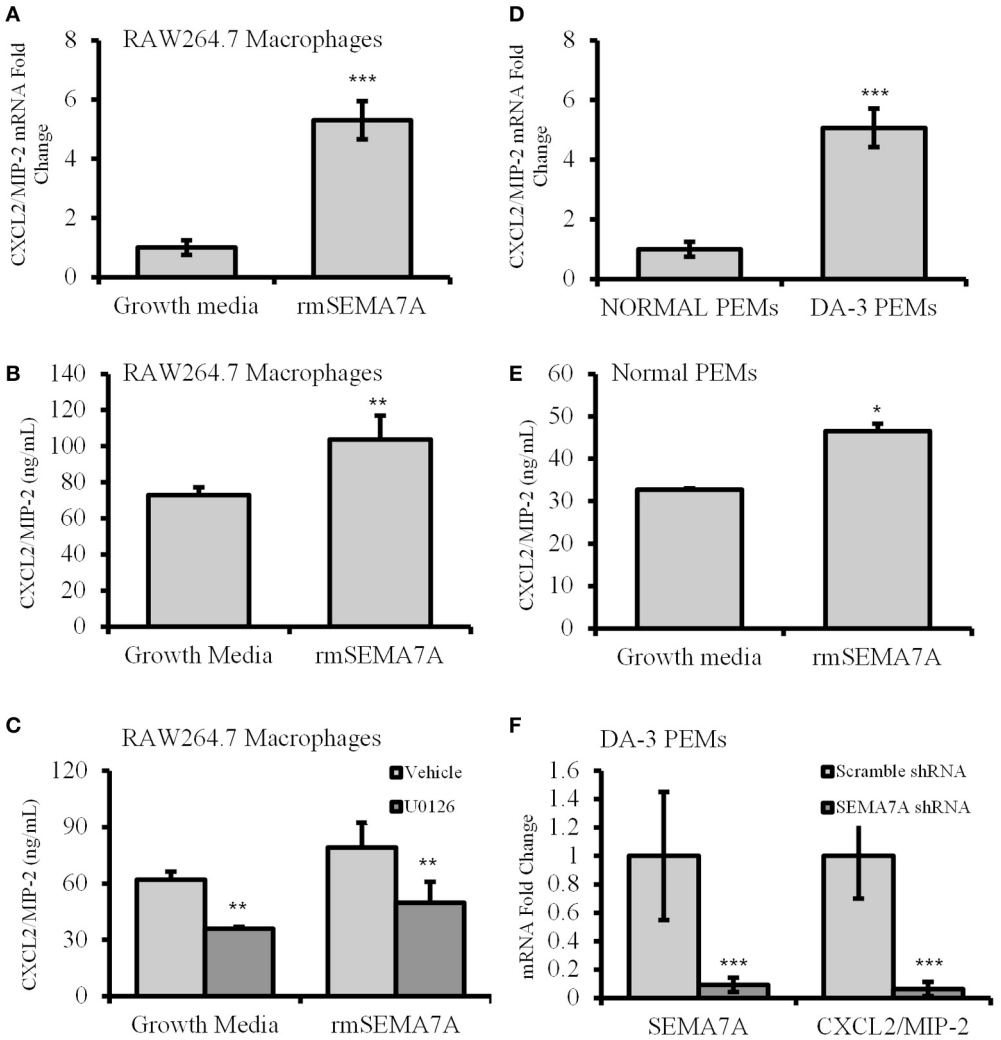

FIGURE 3 | Increased expression of angiogenic chemokine, CXCL2/MIP-2 in rmSEMA7A treated RAW264.7 macrophages and peritoneal elicited macrophages from mammary tumor-bearing mice. The effect of treatment with rmSEMA7A in RAW264.7 macrophages is shown in panels **(A–C)**. Expression of CXCL2/MIP-2 at mRNA level **(A)** and at protein level determined by ELISA **(B)** is increased in RAW 264.7 macrophages treated with rmSEMA7A; and **(C)** effect of treatment with U0126, a MAPK inhibitor (1 μM) on CXCL2/MIP-2 expression by RAW 264.7 macrophages treated with rmSEMA7A. CXCL2/MIP-2 expression is increased in peritoneal elicited macrophages (PEMs) from DA-3 mammary tumor bearers compared to N-PEMs: **(D)** increased mRNA expression of CXCL2/MIP-2 in DA-3 PEMs compared to PEMs from normal as determined by qPCR, $N = 8$; **(E)** CXCL2/MIP-2 ELISA of normal PEMs treated with rmSEMA7A and stimulated with LPS; and **(F)** the effect of SEMA7A gene knockdown in DA-3 PEMs on mRNA expression of SEMA7A and CXCL2/MIP-2 as determined by qPCR. *In-vitro* experiments are representative of three independent experiments. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

shRNA knockdown (**Figure 4A**, top panel). Integrated intensity graphs show a 6-fold decrease in SEMA7A expression in DA-3 SEMA7A shRNA knockdown cells compared to either DA-3 wild type or DA-3 scramble shRNA cells (**Figure 4A**, bottom panel). Although DA-3 cells express lower levels of CXCL2/MIP-2 compared to macrophages, silencing the SEMA7A gene also lead to a decrease in tumor-derived CXCL2/MIP-2. To determine if SEMA7A plays a role in monocyte migration, a modified Boyden chamber assay was performed using RAW 264.7 murine macrophages and conditioned media from wild type DA-3 tumor, DA-3 scramble shRNA, or DA-3 SEMA7A shRNA knockdown cells as possible chemoattractants. Fewer number of RAW 264.7 monocytes migrated towards the conditioned media from SEMA7A silenced DA-3 cells compared to media from either wild type DA-3 tumor cells or DA-3 cells with scramble shRNA (**Figure 4B**). Since we demonstrated that DA-3 mammary tumor cells produce SEMA7A, and that treatment of macrophages with rmSEMA7A induced the production of proangiogenic CXCL2/MIP-2, we hypothesized that silencing SEMA7A gene in DA-3 mammary tumor cells would have an inhibitory effect on production of CXCL2/MIP-2 by macrophages treated with tumor cell supernatants silenced for the SEMA7A gene. We therefore tested to see if SEMA7A gene silencing in tumor cells has an effect on CXCL2/MIP-2 chemokine expression. In

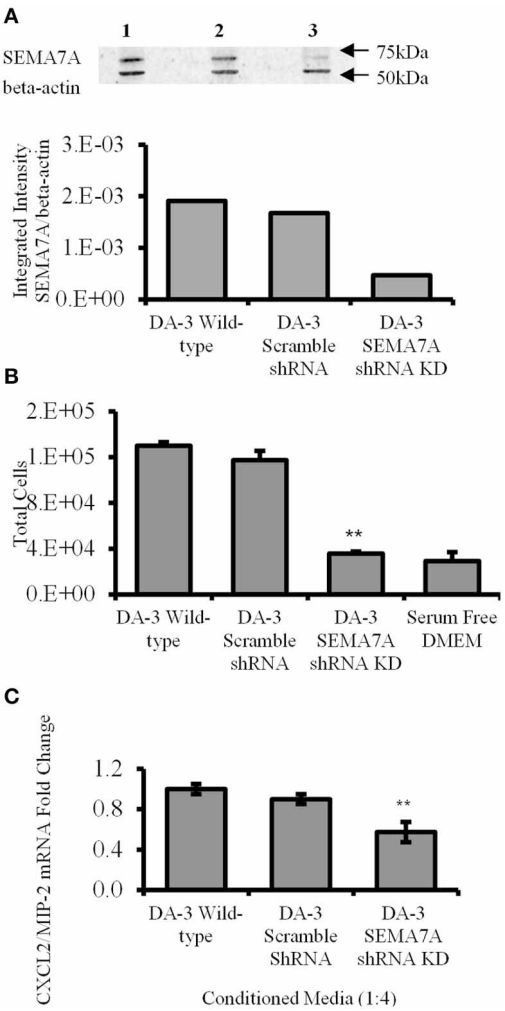

FIGURE 4 | Decreased tumor-derived SEMA7A results in reduced *in vitro* macrophage migration and CXCL2/MIP-2 production. (A) Effect of SEMA7A gene silencing on SEMA7A expression in DA-3 mammary tumor cells as determined by western blot analysis; **(B)** migration of Calcein AM labeled RAW 264.7 macrophages toward cell-free supernatants from either wild type DA-3 tumor cells, SEMA7A scramble shRNA DA-3 tumor cells, SEMA7A shRNA knockdown DA-3 cells or serum free media alone. **(C)** mRNA expression of CXCL2/MIP-2 on macrophages from normal mice *in vitro* treated with conditioned media from either wild type, SEMA7A scramble or SEMA7A knockdown DA-3 mammary tumor cells. *In-vitro* experiments are representative of three independent experiments. $^{**}p \leq 0.01$.

macrophage cultures with conditioned media from SEMA7A shRNA knockdown DA-3 cells, there was a significant ($p < 0.01$) reduction in CXCL2/MIP-2 expression compared to the cultures with SEMA7A (**Figure 4C**).

DECREASED TUMOR GROWTH IN MICE BEARING SEMA7A SILENCED MAMMARY TUMORS

Culturing of RAW 264.7 or thioglycollate elicited macrophages with rmSEMA7A induced the expression of CXCL2/MIP-2, a pro-angiogenic chemokine. We have previously shown that mice bearing either the parental D1-DMBA-3 or DA-3 mammary tumors exhibit higher levels of pro-angiogenic molecules (Owen et al., 2011). It is well-established that angiogenesis is required for invasive tumor growth and that tumors do not grow more than 1 mm^3 in the absence of angiogenesis (Folkman, 1971). We have shown in the previous section that SEMA7A induces production of angiogenic molecules by macrophages. We therefore determined if implantation of BALB/c mice with SEMA7A knockdown DA-3 mammary tumors has an inhibitory effect on tumor growth. To determine the *in vivo* role of SEMA7A, mice were implanted with either wild-type DA-3, scramble shRNA DA-3, or SEMA7A gene knockdown DA-3 (SEMA7A KD) mammary tumor cells. Mice implanted with SEMA7A KD tumors had significantly ($p < 0.01$) decreased primary tumor volume compared to the wild type or SEMA7A scramble control DA-3 mammary tumors (**Figure 5A**). Since SEMA7A KD tumors had lower tumor volume, we tested to see if there is decreased angiogenesis in these mice by use of AngioSense fluorescent probe and CD31 staining by immunohistochemistry. Thus, an AngioSense fluorescent probe was used to determine the extent of angiogenesis in the tumors by an *in vivo* imaging system. Shown in the upper panel are mice bearing wild type DA-3 tumors; the middle panel, scramble control for shRNA; while the bottom panel shows mice bearing SEMA7A KD tumors. Significantly ($p < 0.01$) decreased angiogenesis was observed in mice bearing the SEMA7A KD tumors compared to the scramble controls or wild type DA-3 mammary tumors (**Figure 5B**). We also show the quantification results of *in vivo* imaging indicating a similar trend in tumor growth. Decrease in angiogenesis in SEMA7A KD tumor sections was also observed by immunohistochemistry. H&E and immunohistochemical staining for CD31 highlighted angiogenesis in control tumors but minimally in SEMA7A KD tumors (**Figure 5C**).

PERITONEAL ELICITED MACROPHAGES FROM MICE BEARING SEMA7A KD TUMORS PRODUCE DECREASED LEVELS OF ANGIOGENIC MOLECULES

4–5 weeks post-tumor cell implantation, thioglycollate elicited macrophages from DA-3 scramble shRNA control or DA-3 SEMA7A shRNA mammary tumor-bearing mice were analyzed for the production of pro-angiogenic chemokines CXCL2/MIP-2, CXCL1 and matrix metalloprotease MMP-9. LPS-stimulated macrophages from mice implanted with SEMA7A gene silenced DA-3 mammary tumors produce significantly ($p < 0.01$) lower amounts of pro-angiogenic molecules compared to those implanted with SEMA7A scramble control DA-3 tumor cells. While there were no major differences in secretion of CXCL2/MIP-2 and CXCL1 in unstimulated macrophages from either SEMA7A scramble control or SEMA7A silenced mammary tumor-bearing mice, there were significant ($p < 0.01$) differences in the production of both these chemokines from LPS-stimulated (100 ng/ml) cultures (**Figures 6A,B**). Thus, LPS stimulated macrophages from scramble control DA-3 mammary tumors produced ∼25 ng/mL of CXCL2/MIP-2 while those from SEMA7A silenced tumor bearers produced ∼18 ng/mL (**Figure 6A**). Similarly, LPS stimulated macrophages from scramble controls produced ∼24 ng/mL and those from SEMA7A silenced DA-3 tumor-bearers' macrophages produced 16.8 ng/mL

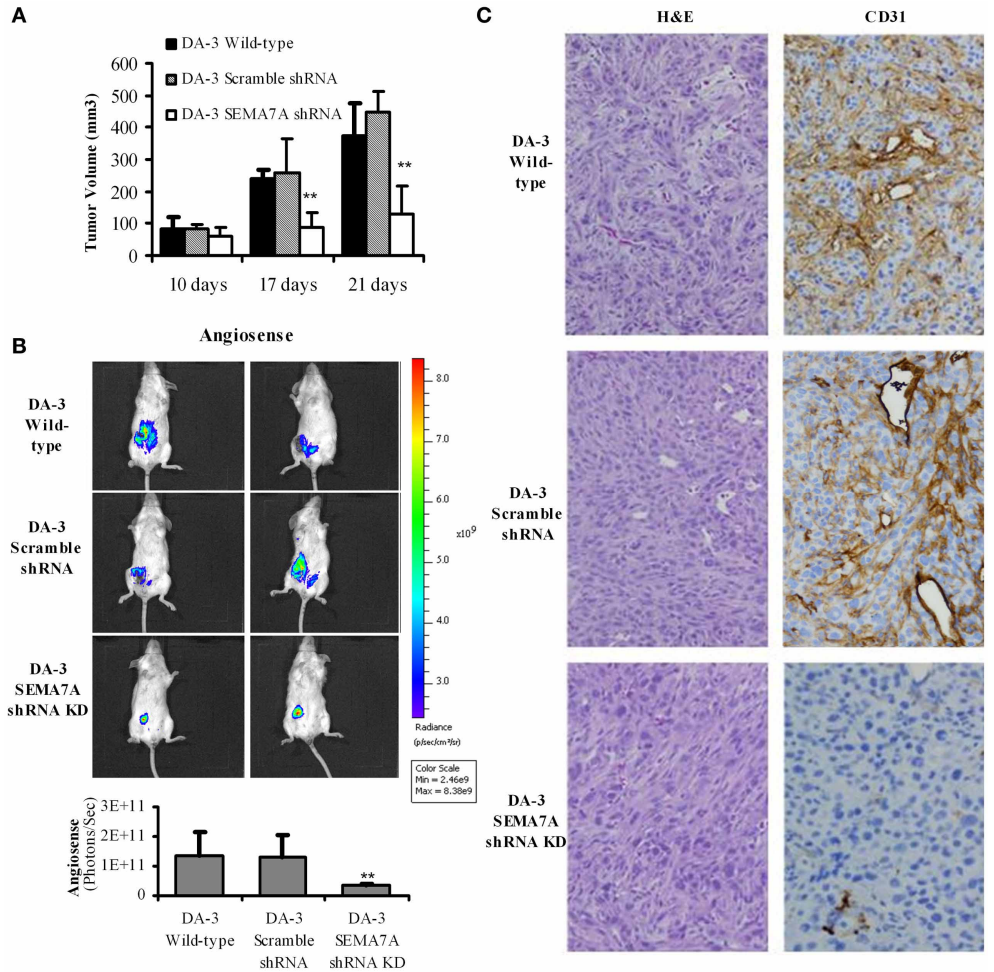

FIGURE 5 | Decreased tumor growth and angiogenesis in mice bearing SEMA7A silenced mammary tumors. (A) Tumor volume is decreased in mice bearing SEMA7A shRNA knockdown DA-3 mammary tumor compared with either wild type or SEMA7A scramble shRNA DA-3 mammary tumors; **(B)** ventral image of DA-wild type, SEMA7A scramble shRNA DA-3 or SEMA7A shRNA knockdown DA-3 mammary tumor bearing mice showing 1 out 20 mice/group; quantification (photons/sec) of AngioSense specific fluorescent signal indicating decreased angiogenesis at 21 days post-tumor implantation in the SEMA7A knockdown group, $N = 10$ per group, repeated twice $^{**}p \leq 0.01$. **(C)** H&E and CD31 staining in tumor sections from DA-3 wild type, scramble shRNA and SEMA7A shRNA knockdown tumor sections. Significance is indicated $^{*}p \geq 0.01$.

of CXCL1 (**Figure 6B**). Interestingly, implantation of SEMA7A knockdown tumor cells decreased the production of MMP-9 by intraperitoneal macrophages in both unstimulated and LPS-stimulated cultures (**Figure 6C**). Furthermore, we assayed a series of tumorigenesis-related genes by qPCR on peritoneal macrophages from SEMA7A shRNA KD or shRNA scramble control DA-3 mammary tumor-bearing mice. PEMs from SEMA7A KD tumor bearing mice showed a significant reduction in VEGF-A expression but not VEGF-B expression (**Figure 6D**). In contrast, expression of both epidermal growth factor (EGF) and platelet growth factor (PGF) was significantly reduced in PEMs from SEMA7A KD tumor bearing mice (**Figure 6E**). Interestingly, the levels of serpinf1, a secreted protein that has both anti-angiogenic and anti-tumorigenic functions, was significantly increased in PEMs from SEMA7A KD tumor bearers (**Figure 6F**).

DISCUSSION

The biological role of SEMA7A in breast cancer progression was explored in this study. First, we find that SEMA7A is expressed by mammary tumor cells. Second, we show that SEMA7A expression is upregulated in macrophages of mammary tumor-bearing mice. Third, we demonstrate that SEMA7A induces the expression of proangiogenic molecule CXCL2/MIP-2 in macrophages. Fourth, we find decreased tumor growth in mice implanted with SEMA7A shRNA DA-3 mammary tumor cells. Lastly, we find that there is decreased angiogenesis in mice implanted with SEMA7A knockdown mammary tumors. These findings suggest that SEMA7A could have a direct effect on tumor cell growth and macrophage function. We are the first to show that SEMA7A plays a role in breast cancer progression.

SEMA7A was first identified in the immune system, as myeloid and lymphoid lineage cells have been reported to express this

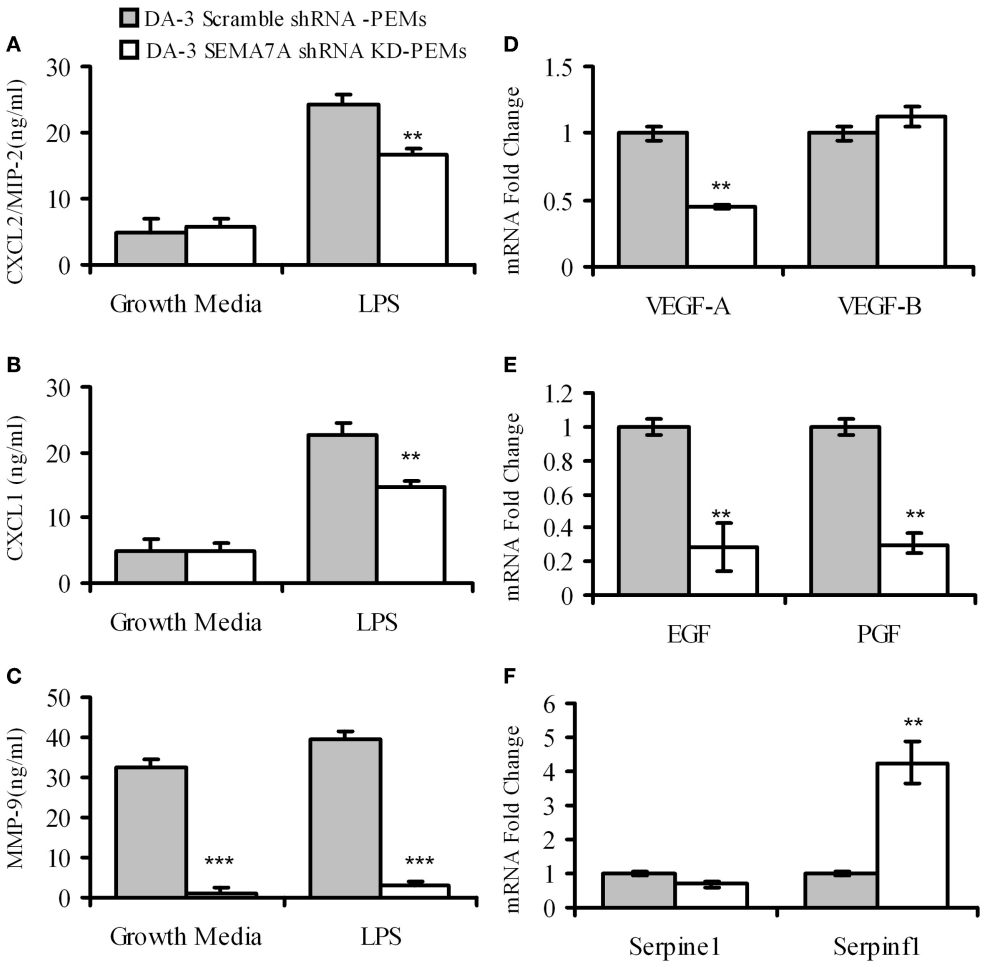

FIGURE 6 | Macrophages from mice bearing SEMA7A shRNA knockdown tumors produce decreased levels of angiogenic molecules. ELISA of peritoneal macrophages from scramble shRNA DA-3 and SEMA7A shRNA knockdown DA-3 tumor bearers cultured with and without LPS for protein levels of: **(A)** CXCL2/MIP-2; **(B)** CXCL1; and **(C)** MMP-9, $N = 16$. qPCR of peritoneal macrophages from scramble shRNA DA-3 and SEMA7A shRNA knockdown DA-3 tumor bearers for mRNA expression of: **(D)** VEGFA and VEGFB; **(E)** EGF and PGF; and **(F)** Serpine 1 and Serpinfl, $N = 6$, repeated twice. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

molecule (Comeau et al., 1998; Lange et al., 1998; Xu et al., 1998). There are very few reports on SEMA7A expression as it relates to cancer. We are the first to clearly demonstrate that SEMA7A is expressed by mammary tumor cells. Formolo et al. identified SEMA7A as one of the proteins in highly invasive astrocytoma cell line U87 while the less aggressive cells do not express this protein (Formolo et al., 2011). Our results parallel with these results as DA-3 mammary tumor cells had greater intensity in expression of this SEMA7A compared to the nontumorigenic mammary EpH4 cells. This raises the possibility that metastatic tumors express higher levels of SEMA7A. We are actively pursuing this in our laboratory by assessing different breast tumor cell lines with varying levels of metastatic potential for SEMA7A expression and correlating with aggressive behavior. Interestingly, while SEMA7A is known to affect monocyte activation *in vitro* via β1 integrin–mediated effects (Holmes et al., 2002), the role of SEMA7A in the activation of tumor cells has not yet been studied. We found that while PEMs from normal mice express low levels of SEMA7A, the expression of this protein is increased in PEMs from tumor bearers. So what induces the expression of this molecule in macrophages? In a murine fibrosis model, TGF-β has been reported to induce the expression of SEMA7A in the murine lung (Kang et al., 2007). We are testing tumor- and/or host-derived factors in inducing SEMA7A expression in PEMs.

Although the identification of SEMA7A receptors remains controversial, two potential receptors have been identified, i.e., plexin C1 and the β1 subunit of integrin receptor. The biological activities of SEMA7A in the immune system have only recently been elucidated. SEMA7A induces the production of inflammatory cytokines such as IL-6, TNF-α and IL-8 (Suzuki et al., 2007), an effect that could be mediated through direct interaction of GPI-anchored SEMA7A protein with α1β1 integrins on target cells. Alternatively, SEMA7A could be cleaved by ADAM-17 and have paracrine effects on other cells. Cell surface bound semaphorins have been found to be proteolytically cleaved in order to exert their biological function. For example, in order

to exert proangiogenic effect, SEMA4D is proteolytically cleaved by membrane type 1-matrix metalloproteinase, and the resulting soluble form acts on endothelial cells to enhance angiogenesis (Henningsen et al., 2010). SEMA7A is a GPI-anchored protein that has been found to be cleaved in platelets by ADAM-17 (Fong et al., 2011). We have previously reported increased expression of ADAM-17 in mammary tumor-bearing mice (Owen et al., 2003). It is possible that ADAM-17 in the tumor bearers could affect cleavage of SEMA7A. Biological effects of SEMA7A have been reported to function through both the soluble and membrane forms. Soluble SEMA7A has been shown to be an extremely potent monocyte chemoattaractant (Holmes et al., 2002) while membrane bound SEMA7A has been reported to stimulate monocytes and macrophages through α1β1 integrin and increase production of proinflammatory cytokines including IL-6 and TNF-α (Suzuki et al., 2007). SEMA7A has been shown to promote spreading and dendricity in human melanocytes through its receptor, β1-integrin. In this study, we report that peritoneal elicited macrophages from mammary tumor-bearing mice express higher levels of β1 integrins as well as its ligand SEMA7A compared to the control mice in tumor bearers' macrophages, suggesting that SEMA7A could function in a paracrine manner. In a cancerous system, it is probable that SEMA7A could mediate its functions through both membrane and soluble forms.

We have previously shown that macrophages from mammary tumor-bearing mice produce angiogenic molecules in response to tumor-derived factors (Libreros et al., 2012). Angiogenesis plays a crucial role in growth of tumors since solid tumors cannot grow beyond 1–2 mm^3 without establishing an adequate blood supply (Folkman, 1971). Using immunohistochemistry and an AngioSense probe, an *in vivo* blood pool vascular fluorescent imaging agent, we determined the *in vivo* role of SEMA7A by comparing angiogenesis in mice bearing scramble shRNA DA-3 mammary tumors with those bearing SEMA7A shRNA knockdown DA-3 mammary tumors. Since these studies showed a significant reduction in tumor volume in SEMA7A shRNA knockdown DA-3 mammary tumors, we hypothesized that these mice would produce decreased levels of angiogenic molecules. It is also possible that although we have knocked down the gene in the tumor cells, host derived SEMA7A may contribute toward angiogenesis. Using SEMA7A knockout mice, we are determining the effects of tumor-derived *vs.* host-derived SEMA7A.

Axonal guidance molecule expression is dysregulated in many types of cancer, including breast cancer, suggesting that they may be excellent targets for effective therapeutic strategies (Harburg and Hinck, 2011). In this report we provide novel data showing that macrophages from SEMA7A shRNA knockdown mammary tumor bearers have decreased production of angiogenic chemokines CXCL2/MIP-2 and CXCL1 as well as matrix degrading enzyme, MMP-9. Although it is known that cytokines such as TNF-α induce MMP-9 through MAPK pathway (Holvoet et al., 2003; Moon et al., 2004), there are no studies in literature describing induction of MMPs by SEMA7A. (Guo and Giancotti, 2004). We are the first to show a relationship between MMP-9 and SEMA7A. We speculate that SEMA7A-β1 integrin ligation may activate MAPK pathway. Activation of MAPK pathway has been shown to play an important role in tumor invasion and metastasis via interaction of integrins with specific receptors (Guo and Giancotti, 2004). Further, integrins have been reported to associate with receptor tyrosine kinases (RTKs) to activate signaling pathways, including MAPK pathways that are necessary for tumor invasion and metastasis. We have also shown that macrophages from SEMA7A shRNA knockdown mammary tumor bearers have increased levels of serpinf1, a secreted protein known to have anti-angiogenic and anti-tumorigenic functions (Filleur et al., 2009). It is possible that SEMA7A could act in an autocrine manner to upregulate the expression of not only angiogenic molecules, but also the integrins to enhance metastatic growth. We are now characterizing the effect of SEMA7A on different mammary tumor cells and their ability to migrate and metastasize. These findings could lead to further studies in the role of Semaphorin 7A in tumor progression in breast and many other cancers.

ACKNOWLEDGMENTS

The authors thank Mr. Keith Faber and Ms. Camila Castro for their critical review of the manuscript. This work was supported by NIH/NCI grant to Vijaya Iragavarapu-Charyulu: 2R15CA135513-02A1and R15 CA135513-01-OS1 and contributions from Mr. Jack Laub.

REFERENCES

Acevedo, L. M., Barillas, S., Weis, S. M., Gothert, J. R., and Cheresh, D. A. (2008). Semaphorin 3A suppresses VEGF-mediated angiogenesis yet acts as a vascular permeability factor. *Blood* 111, 2674–2680. doi: 10.1182/blood-2007-08-110205

Banu, N., Teichman, J., Dunlap-Brown, M., Villegas, G., and Tufro, A. (2006). Semaphorin 3C regulates endothelial cell function by increasing integrin activity. *FASEB J.* 20, 2150–2152. doi: 10.1096/fj.05-5698fje

Basile, J. R., Barac, A., Zhu, T., Guan, K. L., and Gutkind, J. S. (2004). Class IV semaphorins promote angiogenesis by stimulating Rho-initiated pathways through plexin-B. *Cancer Res.* 64, 5212–5224. doi: 10.1158/0008-5472.CAN-04-0126

Basile, J. R., Castilho, R. M., Williams, V. P., and Gutkind, J. S. (2006). Semaphorin 4D provides a link between axon guidance processes and tumor-induced angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 103, 9017–9022. doi: 10.1073/pnas.0508825103

Casazza, A., Fu, X., Johansson, I., Capparuccia, L., Andersson, F., Giustacchini, A., et al. (2011). Systemic and targeted delivery of semaphorin 3A inhibits tumor angiogenesis and progression in mouse tumor models. *Arterioscler. Thromb. Vasc. Biol.* 31, 741–749. doi: 10.1161/ATVBAHA.110.211920

Capparuccia, L., and Tamagnone, L. (2009). Semaphorin signaling in cancer cells and in cells of the tumor microenvironment–two sides of a coin. *J. Cell Sci.* 122, 1723–1736. doi: 10.1242/jcs.030197

Comeau, M. R., Johnson, R., DuBose, R. F., Petersen, M., Gearing, P., VandenBos, T., et al. (1998). A poxvirus-encoded semaphorin induces cytokine production from monocytes and binds to a novel cellular semaphorin receptor, VESPR. *Immunity* 8, 473–482. doi: 10.1016/S1074-7613(00)80552-X

Delorme, G., Saltel, F., Bonnelye, E., Jurdic, P., and Machuca-Gayet, I. (2005). Expression and function of semaphorin 7A in bone cells. *Biol. Cell* 97, 589–597. doi: 10.1042/BC20040103

DiNapoli, M. R., Calderon, C. L., and Lopez, D. M. (1997). Phosphatidyl serine is involved in the reduced rate of transcription of the inducible nitric oxide synthase gene in macrophages from tumor-bearing mice. *J. Immunol.* 158, 1810–1817.

Filleur, S., Nelius, T., de Riese, W., and Kennedy, R. C. (2009). Characterization of PEDF: a multi-functional serpin family protein. *J. Cell Biochem.* 106, 769–775. doi: 10.1002/jcb.22072

Folkman, J. (1971). Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.* 285, 1182–1186. doi: 10.1056/NEJM197111182852108

Fong, K. P., Barry, C., Tran, A. N., Traxler, E. A., Wannemacher, K. M., Tang, H. Y., et al. (2011). Deciphering the human platelet sheddome. *Blood* 117, e15–e26. doi: 10.1182/blood-2010-05-283838

Formolo, C. A., Williams, R., Gordish-Dressman, H., MacDonald, T. J., Lee, N. H., and Hathout, Y. (2011). Secretome signature of invasive glioblastoma multiforme. *J. Proteome Res.* 10, 3149–3159. doi: 10.1021/pr200210w

Gan, Y., Reilkoff, R., Peng, X., Russell, T., Chen, Q., Mathai, S. K., et al. (2011). Role of semaphorin 7a signaling in transforming growth factor beta1-induced lung fibrosis and scleroderma-related interstitial lung disease. *Arthritis Rheum.* 63, 2484–2494. doi: 10.1002/art.30386

Ghanem, R. C., Han, K. Y., Rojas, J., Ozturk, O., Kim, D. J., Jain, S., et al. (2011). Semaphorin 7A promotes angiogenesis in an experimental corneal neovascularization model. *Curr. Eye Res.* 36, 989–996. doi: 10.3109/02713683.2011.593730

Guo, W., and Giancotti, F. G. (2004). Integrin signalling during tumour progression. *Nat. Rev. Mol. Cell Biol.* 5, 816–826. doi: 10.1038/nrm1490

Guttmann-Raviv, N., Shraga-Heled, N., Varshavsky, A., Guimaraes-Sternberg, C., Kessler, O., and Neufeld, G. (2007). Semaphorin-3A and semaphorin-3F work together to repel endothelial cells and to inhibit their survival by induction of apoptosis. *J. Biol. Chem.* 282, 26294–26305. doi: 10.1074/jbc.M609711200

Handel-Fernandez, M. E., Cheng, X., Herbert, L. M., and Lopez, D. M. (1997). Down-regulation of IL-12, not a shift from a T helper-1 to a T helper-2 phenotype, is responsible for impaired IFN-gamma production in mammary tumor-bearing mice. *J. Immunol.* 158, 280–286.

Harburg, G. C., and Hinck, L. (2011). Navigating breast cancer: axon guidance molecules as breast cancer tumor suppressors and oncogenes. *J. Mamm. Gland Biol. Neoplasia* 16, 257–270. doi: 10.1007/s10911-011-9225-1

Henningsen, J., Rigbolt, K. T., Blagoev, B., Pedersen, B. K., and Kratchmarova, I. (2010). Dynamics of the skeletal muscle secretome during myoblast differentiation. *Mol. Cell Proteomics* 9, 2482–2496. doi: 10.1074/mcp.M110.002113

Holmes, S., Downs, A. M., Fosberry, A., Hayes, P. D., Michalovich, D., Murdoch, P., et al. (2002). Sema7A is a potent monocyte stimulator. *Scand. J. Immunol.* 56, 270–275. doi: 10.1046/j.1365-3083.2002.01129.x

Holvoet, S., Vincent, C., Schmitt, D., and Serres, M. (2003). The inhibition of MAPK pathway is correlated with down-regulation of MMP-9 secretion induced by TNF-alpha in human keratinocytes. *Exp. Cell Res.* 290, 108–119. doi: 10.1016/S0014-4827(03)00293-3

Kang, H. R., Lee, C. G., Homer, R. J., and Elias, J. A. (2007). Semaphorin 7A plays a critical role in TGF-beta1-induced pulmonary fibrosis. *J. Exp. Med.* 204, 1083–1093. doi: 10.1084/jem.20061273

Kikutani, H., Suzuki, K., and Kumanogoh, A. (2007). Immune semaphorins: increasing members and their diverse roles. *Adv. Immunol.* 93, 121–143. doi: 10.1016/S0065-2776(06)93003-X

Koppel, A. M., Feiner, L., Kobayashi, H., and Raper, J. A. (1997). A 70 amino acid region within the semaphorin domain activates specific cellular response of semaphorin family members. *Neuron* 19, 531–537. doi: 10.1016/S0896-6273(00)80369-4

Kumanogoh, A., and Kikutani, H. (2003). Immune semaphorins: a new area of semaphorin research. *J. Cell Sci.* 116, 3463–3470. doi: 10.1242/jcs.00674

Lam, D., Harris, D., and Qin, Z. (2013). Inflammatory mediator profiling reveals immune properties of chemotactic gradients and macrophage mediator production inhibition during thioglycollate elicited peritoneal inflammation. *Mediat. Inflamm.* 2013, 931562. doi: 10.1155/2013/931562

Lange, C., Liehr, T., Goen, M., Gebhart, E., Fleckenstein, B., and Ensser, A. (1998). New eukaryotic semaphorins with close homology to semaphorins of DNA viruses. *Genomics* 51, 340–350. doi: 10.1006/geno.1998.5256

Libreros, S., Garcia-Areas, R., Shibata, Y., Carrio, R., Torroella-Kouri, M., and Iragavarapu-Charyulu, V. (2012). Induction of proinflammatory mediators by CHI3L1 is reduced by chitin treatment: decreased tumor metastasis in a breast cancer model. *Int. J. Cancer.* 131, 377–386. doi: 10.1002/ijc.26379

Lopez, D. M., Handel-Fernandez, M. E., Cheng, X., Charyulu, V., Herbert, L. M., DiNapoli, M. R., et al. (1996). Cytokine production by lymphoreticular cells from mammary tumor bearing mice: the role of tumor-derived factors. *Anticancer Res.* 16, 3923–3929.

Lopez, D. M., Lopez-Cepero, M., Watson, G. A., Ganju, A., Sotomayor, E., and Fu, Y. X. (1991). Modulation of the immune system by mammary tumor-derived factors. *Cancer Invest.* 9, 643–653. doi: 10.3109/07357909109039876

Mantovani, A., Bottazzi, B., Colotta, F., Sozzani, S., and Ruco, L. (1992). The origin and function of tumor-associated macrophages. *Immunol. Today* 13, 265–270. doi: 10.1016/0167-5699(92)90008-U

Meda, C., Molla, F., De Pizzol, M., Regano, D., Maione, F., Capano, S., et al. (2012). Semaphorin 4A exerts a proangiogenic effect by enhancing vascular endothelial growth factor-A expression in macrophages. *J. Immunol.* 188, 4081–4092. doi: 10.4049/jimmunol.1101435

Melnicoff, M. J., Horan, P. K., and Morahan, P. S. (1989). Kinetics of changes in peritoneal cell populations following acute inflammation. *Cell Immunol.* 118, 178–191. doi: 10.1016/0008-8749(89)90367-5

Moon, S. K., Cha, B. Y., and Kim, C. H. (2004). ERK1/2 mediates TNF-alpha-induced matrix metalloproteinase-9 expression in human vascular smooth muscle cells via the regulation of NF-kappaB and AP-1: involvement of the ras dependent pathway. *J. Cell Physiol.* 198, 417–427. doi: 10.1002/jcp.10435

Nishiyama, A., Shinohara, T., Pantuso, T., Tsuji, S., Yamashita, M., Shinohara, S., et al. (2008). Depletion of cellular cholesterol enhances macrophage MAPK activation by chitin microparticles but not by heat-killed Mycobacterium bovis BCG. *Am. J. Physiol. Cell Physiol.* 295, C341–C349. doi: 10.1152/ajpcell.00446.2007

Nishiyama, A., Tsuji, S., Yamashita, M., Henriksen, R. A., Myrvik, Q. N., and Shibata, Y. (2006). Phagocytosis of N-acetyl-D-glucosamine particles, a Th1 adjuvant, by RAW 264.7 cells results in MAPK activation and TNF-alpha, but not IL-10, production. *Cell Immunol.* 239, 103–112. doi: 10.1016/j.cellimm.2006.04.003

Owen, J. L., Criscitiello, M. F., Libreros, S., Garcia-Areas, R., Guthrie, K., Torroella-Kouri, M., et al. (2011). Expression of the inflammatory chemokines CCL2, CCL5 and CXCL2 and the receptors CCR1-3 and CXCR2 in T lymphocytes from mammary tumor-bearing mice. *Cell Immunol.* 270, 172–182. doi: 10.1016/j.cellimm.2011.05.004

Owen, J. L., Iragavarapu-Charyulu, V., Gunja-Smith, Z., Herbert, L. M., Grosso, J. F., and Lopez, D. M. (2003). Up-regulation of matrix metalloproteinase-9 in T lymphocytes of mammary tumor bearers: role of vascular endothelial growth factor. *J. Immunol.* 171, 4340–4351.

Pasterkamp, R. J., and Kolodkin, A. L. (2003). Semaphorin junction: making tracks toward neural connectivity. *Curr. Opin. Neurobiol.* 13, 79–89. doi: 10.1016/S0959-4388(03)00003-5

Pasterkamp, R. J., Peschon, J. J., Spriggs, M. K., and Kolodkin, A. L. (2003). Semaphorin 7A promotes axon outgrowth through integrins and MAPKs. *Nature* 424, 398–405. doi: 10.1038/nature01790

Pollard, J. W. (2004). Tumour-educated macrophages promote tumour progression and metastasis. *Nat. Rev. Cancer* 4, 71–78. doi: 10.1038/nrc1256

Sadanandam, A., Rosenbaugh, E. G., Singh, S., Varney, M., and Singh, R. K. (2010). Semaphorin 5A promotes angiogenesis by increasing endothelial cell proliferation, migration, and decreasing apoptosis. *Microvasc. Res.* 79, 1–9. doi: 10.1016/j.mvr.2009.10.005

Sakurai, A., Gavard, J., Annas-Linhares, Y., Basile, J. R., Amornphimoltham, P., Palmby, T. R., et al. (2010). Semaphorin 3E initiates antiangiogenic signaling through plexin D1 by regulating Arf6 and R-Ras. *Mol. Cell Biol.* 30, 3086–3098. doi: 10.1128/MCB.01652-09

Sierra, J. R., Corso, S., Caione, L., Cepero, V., Conrotto, P., Cignetti, A., et al. (2008). Tumor angiogenesis and progression are enhanced by Sema4D produced by tumor-associated macrophages. *J. Exp. Med.* 205, 1673–1685. doi: 10.1084/jem.20072602

Sotomayor, E. M., Fu, Y. X., Lopez-Cepero, M., Herbert, L., Jimenez, J. J., Albarracin, C., et al. (1991). Role of tumor-derived cytokines on the immune system of mice bearing a mammary adenocarcinoma. II. Down-regulation of macrophage-mediated cytotoxicity by tumor-derived granulocyte-macrophage colony-stimulating factor. *J. Immunol.* 147, 2816–2823.

Suzuki, K., Okuno, T., Yamamoto, M., Pasterkamp, R. J., Takegahara, N., Takamatsu, H., et al. (2007). Semaphorin 7A initiates T-cell-mediated inflammatory responses through alpha1beta1 integrin. *Nature* 446, 680–684. doi: 10.1038/nature05652

Torroella-Kouri, M., Keith, J. C., Ivanova, M., and Lopez, D. M. (2003). IL-11-induced reduction of C/EBP transcription factor binding may contribute to the IL-12 downregulation in tumor-bearing mice. *Int. J. Oncol.* 22, 439–448.

Varshavsky, A., Kessler, O., Abramovitch, S., Kigel, B., Zaffryar, S., Akiri, G., et al. (2008). Semaphorin-3B is an angiogenesis inhibitor that is inactivated by furin-like pro-protein convertases. *Cancer Res.* 68, 6922–6931. doi: 10.1158/0008-5472.CAN-07-5408

Xu, X., Ng, S., Wu, Z. L., Nguyen, D., Homburger, S., Seidel-Dugan, C., et al. (1998). Human semaphorin K1 is glycosylphosphatidylinositol-linked and defines a new subfamily of viral-related semaphorins. *J. Biol. Chem.* 273, 22428–22434. doi: 10.1074/jbc.273.35.22428

Yao, E. S., Zhang, H., Chen, Y. Y., Lee, B., Chew, K., Moore, D., et al. (2007). Increased beta1 integrin is associated with decreased survival in invasive breast cancer. *Cancer Res.* 67**,** 659–664. doi: 10.1158/0008-5472.CAN-06-2768

Zhang, X., Goncalves, R., and Mosser, D. M. (2008). The isolation and characterization of murine macrophages. *Curr. Protoc. Immunol.* Chapter 14, Unit 14.1. doi: 10.1002/0471142735.im1401s83

Zhou, Y., Gunput, R. A. and Pasterkamp, R. J. (2008). Semaphorin signaling: progress made and promises ahead. *Trends Biochem Sci*. 33, 161–170. doi: 10.1016/j.tibs.2008.01.006

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 July 2013; accepted: 09 January 2014; published online: 05 February 2014.
*Citation: Garcia-Areas R, Libreros S, Amat S, Keating P, Carrio R, Robinson P, Blieden C and Iragavarapu-Charyulu V (2014) Semaphorin7A promotes tumor growth and exerts a pro-angiogenic effect in macrophages of mammary tumor-bearing mice. Front. Physiol. **5**:17. doi: 10.3389/fphys.2014.00017*
This article was submitted to Vascular Physiology, a section of the journal Frontiers in Physiology.

frontiers in PHYSIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 12 July 2013
doi: 10.3389/fphys.2013.00178

Tumor cell-macrophage interactions increase angiogenesis through secretion of EMMPRIN

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Tumor macrophages are generally considered to be alternatively/M2 activated to induce secretion of pro-angiogenic factors such as VEGF and MMPs. EMMPRIN (CD147, basigin) is overexpressed in many tumor types, and has been shown to induce fibroblasts and endothelial cell expression of MMPs and VEGF. We first show that tumor cell interactions with macrophages resulted in increased expression of EMMPRIN and induction of MMP-9 and VEGF. Human A498 renal carcinoma or MCF-7 breast carcinoma cell lines were co-cultured with the U937 monocytic-like cell line in the presence of TNFα (1 ng/ml). Membranal EMMPRIN expression was increased in the co-cultures (by 3–4-folds, $p < 0.01$), as was the secretion of MMP-9 and VEGF (by 2–5-folds for both MMP-9 and VEGF, $p < 0.01$), relative to the single cultures with TNFα. Investigating the regulatory mechanisms, we show that EMMPRIN was post-translationally regulated by miR-146a, as no change was observed in the tumoral expression of EMMPRIN mRNA during co-culture, expression of miR-146a was increased and its neutralization by its antagomir inhibited EMMPRIN expression. The secretion of EMMPRIN was also enhanced (by 2–3-folds, $p < 0.05$, only in the A498 co-culture) via shedding off of the membranal protein by a serine protease that is yet to be identified, as demonstrated by the use of wide range protease inhibitors. Finally, soluble EMMPRIN enhanced monocytic secretion of MMP-9 and VEGF, as inhibition of its expression levels by neutralizing anti-EMMPRIN or siRNA in the tumor cells lead to subsequent decreased induction of these two pro-angiogenic proteins. These results reveal a mechanism whereby tumor cell-macrophage interactions promote angiogenesis via an EMMPRIN-mediated pathway.

Keywords: membranal and secreted EMMPRIN, VEGF, MMP-9, tumor cells, macrophages, angiogenesis, mir-146a

INTRODUCTION

Solid tumors include tumor and stroma cells, particularly infiltrating macrophages, which may consist of up to half of the tumor mass (Mantovani et al., 2002; Murdoch et al., 2004). Macrophages can be activated to become effector killer cells (classically or M1 activated) or to be involved in wound healing and angiogenesis (alternatively or M2 activated) (Mosser and Edwards, 2008; Gordon and Martinez, 2010). However, since we now realize that macrophages are very plastic and can be activated in many additional modes according to the microenvironment, this dichotomy is really a simplified way to describe their activation and function.

The three main macrophage subsets that are recognized within the tumoral context are tumor-associated macrophages (TAMs) (Lewis and Murdoch, 2005), Tie2 expressing monocytes (TEMs) (De Palma et al., 2007), and myeloid-derived suppressor cells (MDSCs) (Serafini et al., 2006; Murdoch et al., 2008), all of which secrete varying amounts of pro-angiogenic factors (e.g., vascular endothelial growth factor-VEGF), anti-inflammatory mediators (e.g., TGF-β, IL-10, IL-13, PGE_2) that inhibit the tumoricidal activity of immune cells (Mantovani et al., 2004; Murdoch et al., 2005), and enzymes that degrade the extracellular matrix (ECM) and make room for the growing tumor mass (e.g., matrix metalloproteinases-MMPs). MMPs and VEGF are both crucial for tumor progression, invasiveness, metastasis and angiogenesis (Egeblad and Werb, 2002). High amounts of MMPs, particularly MMP-9, degrade the ECM, release and activate VEGF, and allow migration of cells (including infiltration of leukocytes, spreading of metastatic tumor cells, and integration of pericytes and endothelial cells into the tumor vasculature) (Murdoch et al., 2008). VEGF is an extremely potent pro-angiogenic factor, a chemoattractant for macrophages, and a regulator of MMP-9 (Owen et al., 2003). Thus, a regulatory positive loop exists, where MMP-9 regulates VEGF bioavailability, and VEGF regulates MMP-9 expression (Hollborn et al., 2007).

Thus, the tumoral microenvironment is rich in anti-inflammatory mediators (e.g., IL-10, IL-13, TGFβ, PGE_2) that skew macrophages toward alternative/M2 patterns of activation, while hypoxia immobilizes them at the site (Murdoch et al., 2004). However, the microenvironment also consists of low levels of pro-inflammatory cytokines (e.g., IL-1β, TNFα) which paradoxically enhance tumor angiogenesis and proliferation (Balkwill, 2009). TNFα in particular can be produced by the tumor cells not only to enhance their proliferation, but also to promote invasiveness

through its ability to induce macrophage MMP-9 (Hagemann et al., 2004)

In many solid tumors, expression of VEGF and MMPs, including MMP-9, is up-regulated by the extracellular matrix metalloproteinase inducer (EMMPRIN, also called basigin or CD147). This is a multifunctional protein that is expressed on the surface of both tumor and stroma cells, including macrophages (Yan et al., 2005; Nabeshima et al., 2006), and through homophilic EMMPRIN:EMMPRIN interactions between these two cell types may induce the expression of both MMPs and VEGF and increase angiogenesis (Tang et al., 2004, 2005; Yurchenko et al., 2010). Other protein partners for EMMPRIN, such as cyclophilin A and B, may also be responsible for its pro-angiogenic activity, as well as for inducing chemotaxis and recruitment of leukocytes to the tumor (Yurchenko et al., 2010). Overexpression of EMMPRIN was found in many types of tumors, and was correlated to VEGF and MMP-9 induction and increased tumor invasiveness (Zhou et al., 2005). Conversely, EMMPRIN neutralizing antibody reduced VEGF and MMP-9 expression (Tang et al., 2006) leading to reduced invasiveness. EMMPRIN may be found as a transmembranal protein or in its secreted form, and both forms can mediate its homophilic interactions (Belton et al., 2008). In this study we show that EMMPRIN is up-regulated by tumor cell-macrophage interactions and its proteolytic cleavage is enhanced in co-culture, resulting in increased amounts of the soluble form. Moreover, we show that the secreted form is sufficient to induce both VEGF and MMP-9, and is pro-angiogenic by itself.

METHODS AND MATERIALS

CELLS

The human renal carcinoma A498 (ATCC HTB-44), breast carcinoma MCF-7 (ATCC HTB-22) and U937 monocyte-like cells (ATCC CRL-1593) were cultured in RPMI-1640 medium with 10% fetal calf serum (FCS), 1% L-glutamine and antibiotics. The three cell lines were regularly tested for morphological changes and presence of mycoplasma. In some experiments cells were subjected to stimulation with TNFα (1 ng/ml, R&D systems, Minneapolis, MN), incubated with anti-EMMPRIN (LEAF™ Purified anti-human CD147 Antibody, BioLegend, San Diego, CA) or with recombinant EMMPRIN (R&D systems). To avoid possible masking of signals by exogenous stimuli, 10^6 cells were plated in 24-well plates in RPMI-1640 without FCS and TNFα was added for 48 h. In all co-cultures, tumor cells and U937 cells were plated at a 2:1 ratio. Cell viability was determined using the XTT kit (Biological industries, Beit-Haemek, Israel). The human endothelial cell line EaHy926 (gift of Dr. C. J. Edgell, University of North Carolina, Chapel Hill, NC) was cultured in DMEM with 2% glutamine, 10% FCS, 2% hypoxanthine-aminopterin-thymidine (HAT), and 1% antibiotics.

ELISA

The human EMMPRIN, MMP-9 and VEGF ELISA kits were performed according to the manufacturer's instructions (R&D systems). Samples were diluted 1:200 for determination of EMMPRIN and MMP-9, and 1:100 for determination of VEGF, according to previous calibration experiments.

FLOW CYTOMETRY

10^6 Cells were centrifuged and re-suspended in RPMI-1640 with 1% FCS, and then incubated with 1 μg of Alexa 647-conjugated anti-human CD147 or with its isotype control (BioLegend) for 30 min at 4°C. After washing, the cells were fixed in 0.1% formaldehyde and analyzed by flow cytometer, (LSRII, BD Biosciences, San Jose, CA). To distinguish between EMMPRIN expression on U937 cells and on tumor cells, A498 and MCF-7 cells were first labeled with 1 μM of Cell Tracker™ Green CMFDA (Life Technologies-InVitrogen, Darmstadt, Germany) according to the manufacturer's instructions, and only then were incubated in the experimental conditions. Dead cells were excluded from the analysis by their forward and sideway light-scattering properties.

QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from 10^6 U937, A498, or MCF-7 cells using the RNA extraction kit (Norgen biotek, Ontario, Canada), and 500 ng were transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). EMMPRIN mRNA expression and its reference gene GusB, or miR-146a and its reference gene U6 were quantified by real-time PCR using the TaqMan assay on demand kit with the StepOne system (Applied Biosystems). The comparative $\Delta\Delta C_T$ method was used for relative quantification, and non-stimulated cells served as a calibrator in each experiment, to allow comparison of relative quantity (RQ) between the samples.

REVERSE TRANSFECTION AND INHIBITION OF MIR-146a OR EMMPRIN EXPRESSION

The siPORT *NeoFX* transfection agent (Applied Biosystems/Ambion, Austin, TX) was diluted 1:25 with OPTI-MEM1 medium (Gibco, Invitrogen), combined with 30 nM of the anti-miR-146a inhibitor™ or its Cy3-labeled negative control (anti-miR-NC), or with 5 nM of EMMPRIN siRNA or its negative control (all reagents from Ambion). Solutions were incubated 10 min to allow transfection complexes to form and then dispensed into 24-well plates. 6×10^4 A498 or MCF-7 cells/well were overlaid in suspension over the transfection complexes and gently tilted to evenly distribute the complexes. Cells were incubated at 37°C overnight, followed by replacement with fresh medium and stimulation with TNFα for 48 h. These conditions were calibrated according to the manufacturer's instructions, reaching transfection efficiency of >92%.

ISOLATION OF EXOSOMES

10^6 A498 or MCF-7 cells were incubated in single- or co-cultures with 0.5×10^6 U937 cells in the presence of TNFα (1 ng/ml), supernatants were collected and centrifuged at 800 g for 10 min and then at 12,000 g for 30 min to sediment suspended cells. The resulting supernatants were ultra-centrifuged at 110,000 g (Micro-Ultracentrifuge RCM150, rotor S120AT2-0200; Thermo Scientific, Sorvall, Suwanee, GA, USA) for 1.5 h at 4°C to pellet the exosomes. Both pellets and supernatants were evaluated for the presence of EMMPRIN protein by ELISA.

In vitro "WOUND ASSAY"

EaHy926 monolayers (1×10^6 cells) in 24-well dishes were wounded with a wooden toothpick after overnight incubation, and the line of injury was marked. Detached cells were washed away with medium, and cells were incubated with or without human recombinant EMMPRIN (200 ng/ml) or with 100 μl of supernatants (diluted 1:4 with medium) derived from the siRNA experiments. Images of the field of injury were acquired at the beginning of the experiment and after 48 h. In each experiment, average distances between the two sides of the wound were measured in different locations along the wound (at least 10 locations per field), in day 0 and in day 2, and analyzed with ImagePro plus 4.5 software. The percent change was then calculated relative to day 0.

In vivo PLUG ASSAY

Liquid Matrigel (0.4 ml) was mixed with 200 ng/ml of human recombinant EMMPRIN and injected subcutaneously into the flank of BALB/c mice. As a control, Matrigel was mixed with serum-free DMEM and injected as above. Matrigel plugs were surgically removed after 7 days and photographed to give visual assessment of angiogenesis. All animal studies were approved by the Animal Care Committee of the Technion.

STATISTICAL ANALYSES

All values are presented as means ± SE. Significance between two groups was determined using two-tailed unpaired *t*-test. Differences between three or more experimental groups were analyzed using analysis of variance (ANOVA) and the Bonferroni's multiple comparison tests. *P*-values exceeding 0.05 were not considered significant.

RESULTS

CO-CULTURE INCREASES THE EXPRESSION OF EMMPRIN, MMP-9 AND VEGF

Preliminary studies were performed to calibrate the *in vitro* system. TNFα was added to each of the single cell cultures at a concentration of 1 ng/ml, which is similar to the concentration found in the tumor microenvironment (Elamin et al., 2008; Charles et al., 2009; Ali et al., 2012). At this concentration TNFα was sufficient to induce MMP-9, but did not induce cell death, as was estimated by the XTT assay (1.03 ± 0.04, 0.96 ± 0.02, and 0.99 ± 0.05 folds for A498, MCF-7, and U937 cells, respectively, relative to each of the non-stimulated cells). Furthermore, incubation time of 48 h was optimal to observe accumulation of VEGF and MMP-9 in the supernatants. As macrophages may make up as much as 50% of the tumor mass, tumor cells and monocytes were incubated at a ratio of 2:1, as was demonstrated before (Blot et al., 2003; Perske et al., 2010).

In all three cell lines examined separately or in co-culture, all of the cells (99.6 ± 0.3%) expressed surface EMMPRIN, however, with different intensities. The U937 cells expressed low levels of membranal EMMPRIN (**Figures 1A,B**), which increased by 4.5-folds during co-culture with both A498 and MCF-7 cells. Likewise, expression of membranal EMMPRIN on the tumor cells that was 2–3-fold higher in the single cultures than in the U937 cells, was increased during co-culture (by 4-folds and 3-folds in the A498 and MCF-7 cells, respectively, $p < 0.05$ and $p < 0.001$ relative to the singe cultures). TNFα had no effect on the membranal expression of EMMPRIN in all cell types, during separate incubation or in co-cultures. In contrast, TNFα elevated the amounts of secreted EMMPRIN from A498 cells cultured alone (by 2-folds, $p < 0.05$), but not from MCF-7 cells, which already secreted high levels of EMMPRIN (**Figure 1B**). Co-culture increased the accumulation of secreted EMMPRIN in the A498 experiments (by 3-folds and 1.5-folds relative to the U937 and A498 single cultures, respectively, $p < 0.001$, **Figure 1C**), although this increase was only additive. In contrast, using the MCF-7 cells, the amounts of the secreted EMMPRIN were not significantly different in the co-cultured cells relative to the single MCF-7 culture, suggesting that this cell line already secreted maximal amounts of EMMPRIN without further stimulation.

In both tumor cell lines and in the monocytic cell line, presence of TNFα was necessary to induce MMP-9 secretion in each of the single cultures, however, the co-cultures significantly elevated this level, synergistically for the A498 cells (5-folds, $p < 0.001$) and additively for the MCF-7 cells (1.6-folds, $p < 0.001$, **Figure 1D**). Similarly, co-cultures increased the accumulation of secreted VEGF (**Figure 1E**). In the A498 cells, co-cultures synergistically increased VEGF secretion (by 3-folds, $p < 0.001$ relative to each of the single cultures), and addition of TNFα, which had no effect in the single cultures, further stimulated secretion by 2-folds. In the MCF-7 cells, co-cultures increased the accumulation of VEGF additively only in the presence of TNFα (by 2-folds, $p < 0.01$ relative to each of the single cultures).

EMMPRIN EXPRESSION IS POST-TRANSCRIPTIONALLY REGULATED BY MIR-146a

We next evaluated the expression of EMMPRIN at the RNA levels. EMMPRIN has two known isoforms. The short isoform has only two Ig-like domains, whereas the long isoform has 3 such domains. Preliminary experiments indicated that both isoforms are expressed in the three cell lines, however, the short isoform was the prevalent one (average Ct values of 20 cycles), whereas the long isoform was scarce (average Ct values of 31). We, therefore, evaluated the effect of TNFα and co-culture on the accumulation of EMMPRIN mRNA levels of the short isoform only (**Figure 2A**). In order to estimate the level of EMMPRIN mRNA in each cell type, co-cultured cells were incubated in separate inserts (membrane of 0.3 μm pore size), so as to preclude cell migration between the two compartments. No significant change was observed in the EMMPRIN mRNA levels in either A498 or MCF-7 cells relative to each of the single cultures, whereas in the U937 cells co-culturing with either A498 or MCF-7 cells reduced these levels by 40 and 45% relative to the single culture ($p < 0.05$ and $p < 0.01$, respectively).

The discrepancy between the elevated protein levels observed before and the unchanged or decreased levels of the mRNA suggested a post-transcriptional regulation of EMMPRIN, which could be mediated through microRNA. We chose to examine the involvement of miR-146a that is a known inflammatory microRNA and has been implicated in tumor-macrophage interactions before (Perske et al., 2010). We could show (**Figure 2B**)

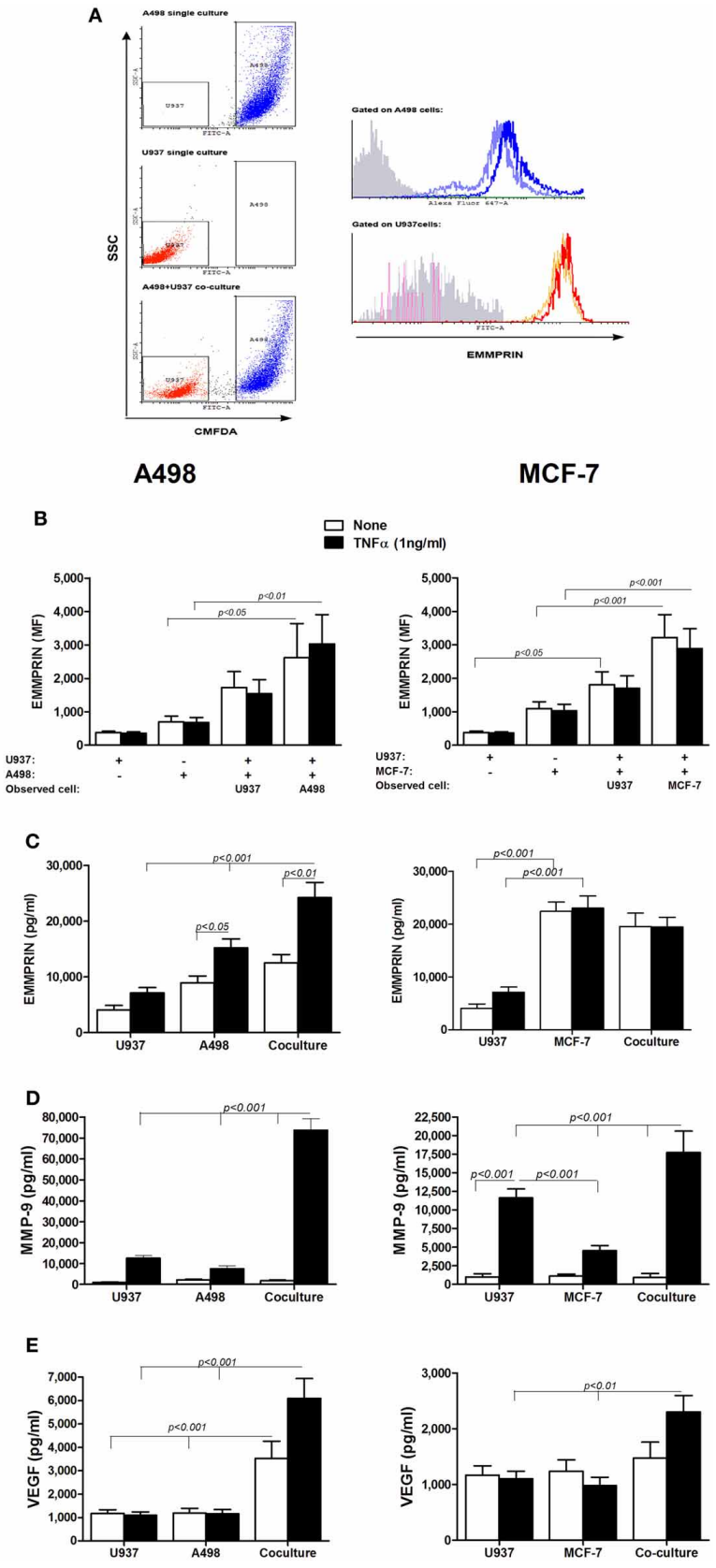

FIGURE 1 | Continued

FIGURE 1 | The effect of co-culture on the secretion of EMMPRIN, MMP-9 and VEGF. 10^6 A498 or MCF-7 cells were incubated in a serum-free medium either separately or with 0.5×10^6 U937 cells in co-culture for 48 h, with or without the addition of TNFα (1 ng/ml), and 0.5×10^6 U937 cells were incubated in a serum-free medium with or without the addition of TNFα (1 ng/ml). **(A)** Representative dot plot for the A498 and U937 co-cultures. Light blue and orange histograms—EMMPRIN expression in single cultures of A498 cells and U937 cells, respectively; Blue and red histograms—EMMPRIN expression measured separately on A498 or U937 cells, respectively, incubated in their co-cultures. Gray histogram—isotype control. **(B)** Mean fluorescence (MF) of the membranal expression of EMMPRIN that was evaluated by flow cytometry ($n = 8$). Concentrations of secreted proteins were determined in the supernatants by ELISA for **(C)** EMMPRIN ($n = 6$), **(D)** MMP-9 ($n = 8$) and **(E)** VEGF ($n = 8$).

that the expression of miR-146a was elevated by the co-culture relative to each of the non-stimulated single cultures (by 146-folds, ∼200-fold, and ∼300-folds for A498, MCF-7 and U937, $p < 0.05$). As miR-146a is an inflammatory miRNA, TNFα increased its levels, although not significantly (by 50-folds and 2.5-folds for A498 and U937 cells, respectively), and the co-culture further increased it in the stimulated A498 and U937 cells (by 3-folds and 160-folds, $p < 0.05$), whereas in the MCF-7 cells TNFα did not increase miR-146a expression relative to the non-stimulated cells.

To show that this microRNA regulates the expression of EMMPRIN, we next neutralized its activity by transfecting the tumor cells with its antagomir anti-miR-146a and then co-culturing them with the U937 monocytes in the presence of TNFα (**Figure 2C**). This neutralization resulted in decreased levels of secreted EMMPRIN protein, which did not reach significance in the A498 cells (by 1.5-folds), but was significantly different (3-folds, $p < 0.01$) for MCF-7 cells.

SOLUBLE EMMPRIN IS GENERATED BY A SERINE PROTEASE

The way that the secreted form of EMMPRIN is generated remains controversial, where some evidences indicate secretion by exosomes (Keller et al., 2009), and some demonstrate proteolytic cleavage by MMPs (Tang et al., 2004). We investigated several different possibilities for the generation of soluble EMMPRIN. First, we explored whether the co-culture of the tumor cell lines with the U937 monocytes induce alternative splicing of the EMMPRIN mRNA, so that the transmembranal portion of the molecule is deleted and the product cannot be anchored to the membrane. We, therefore, amplified the extracellular and transmembranal regions of the short EMMPRIN mRNA (indicated in **Figure 3A**) and quantified the transcript by both sets of primers. No change was observed between the extracellular (short isoform) and transmembranal regions of the single- and co-cultures of the A498 and MCF-7 cells (**Figure 3B**). Likewise, no such change was observed for the U937 cells, despite the reduction in the overall EMMPRIN transcript that was observed before (**Figure 2A**).

Next, we evaluated the presence of EMMPRIN protein in exosomes, by precipitating the exosomes using ultracentrifugation, an accepted method for exosome isolation (Lahat et al., 2011). However, EMMPRIN was exclusively found in the supernatants and was not sedimented at all after ultracentrifugation, indicating that it is not found in exosomes (**Figure 3C**).

Finally, we explored the possibility of proteolytic cleavage of the membranal EMMPRIN. To this end, we incubated the tumor cell lines in co-cultures with U937 monocytes in the presence of TNFα, to give rise to maximal secretion of EMMRPIN, and with a wide range of protease inhibitors (Aprotinin, the serine protease inhibitor; Leupeptin, the serine, cysteine and threonine protease inhibitor; Pepstatin A, the aspartyl protease inhibitor; Phenanthroline, the MMPs inhibitor), and with bafilomycin A1, the lysosomal inhibitor which prevents lysosomal acidification. These inhibitors collectively inhibit families of proteases with potential access to membranal EMMPRIN either at the plasma membrane or at the endosomal pathway. Different inhibitors in optimal concentrations, which were determined to be incapable of causing cell death in preliminary experiments (data not shown), were added to the co-cultures and their effect on the secretion of EMMPRIN was evaluated by ELISA. Each value is presented as fold from control (the co-cultured cells in the presence of TNFα) to allow better visualization of the differences exerted by the inhibitors. In both A498 and MCF-7 co-cultures with the monocytic U937 cell, only aprotinin (**Figure 3D**) caused a significant decrease in secreted EMMPRIN (a 14 and 46% reduction in A498 and MCF-7 co-cultures, respectively, $p < 0.001$).

EMMPRIN IS REQUIRED FOR FULL INDUCTION OF VEGF AND MMP-9

VEGF and MMP-9 are essential for tumor angiogenesis, and can be induced by a myriad of mediators found in tumor microenvironment, including hypoxia, TNFα or EMMPRIN. We used three approaches to demonstrate that EMMPRIN is required for maximal induction of both VEGF and MMP-9.

First, we incubated each of the cell lines, with or without TNFα, with increasing amounts of recombinant EMMPRIN. EMMPRIN alone had no significant effect on the secretion of MMP-9 in all three cell lines (**Figure 4**), but in the presence of TNFα the high amounts of the recombinant protein increased the secretion of MMP-9 by 2.5-folds from the U937 cells ($p < 0.001$), by 4-folds from the A498 cells ($p < 0.01$) and by 3-folds from the MCF-7 cells ($p < 0.05$), demonstrating a dose response. The secretion of VEGF was not affected by the presence of TNFα, and was significantly increased by the high amounts of the recombinant protein only in the case of the U937 cells (by 2-folds, $p < 0.01$). Since the recombinant EMMPRIN we used is a chimeric product where the IgG Fc fragment is fused to the carboxy-terminus of EMMPRIN, we made sure that this fragment alone does not induce MMP-9 or VEGF secretion from the cells (**Figure 4**) and used it as a control in all the experiments.

Secondly, we incubated the TNFα-stimulated cells in co-culture, and neutralized the effects of EMMPRIN by adding its specific antibody (at 2 ng/ml), a concentration that was determined after preliminary calibration experiments. In A498 cells co-cultured with U937 cells, anti-EMMPRIN reduced MMP-9 secretion by 32% ($p < 0.001$) and VEGF secretion by 48% ($p < 0.05$) (**Figure 5**). Likewise, in MCF-7 cells co-cultured with

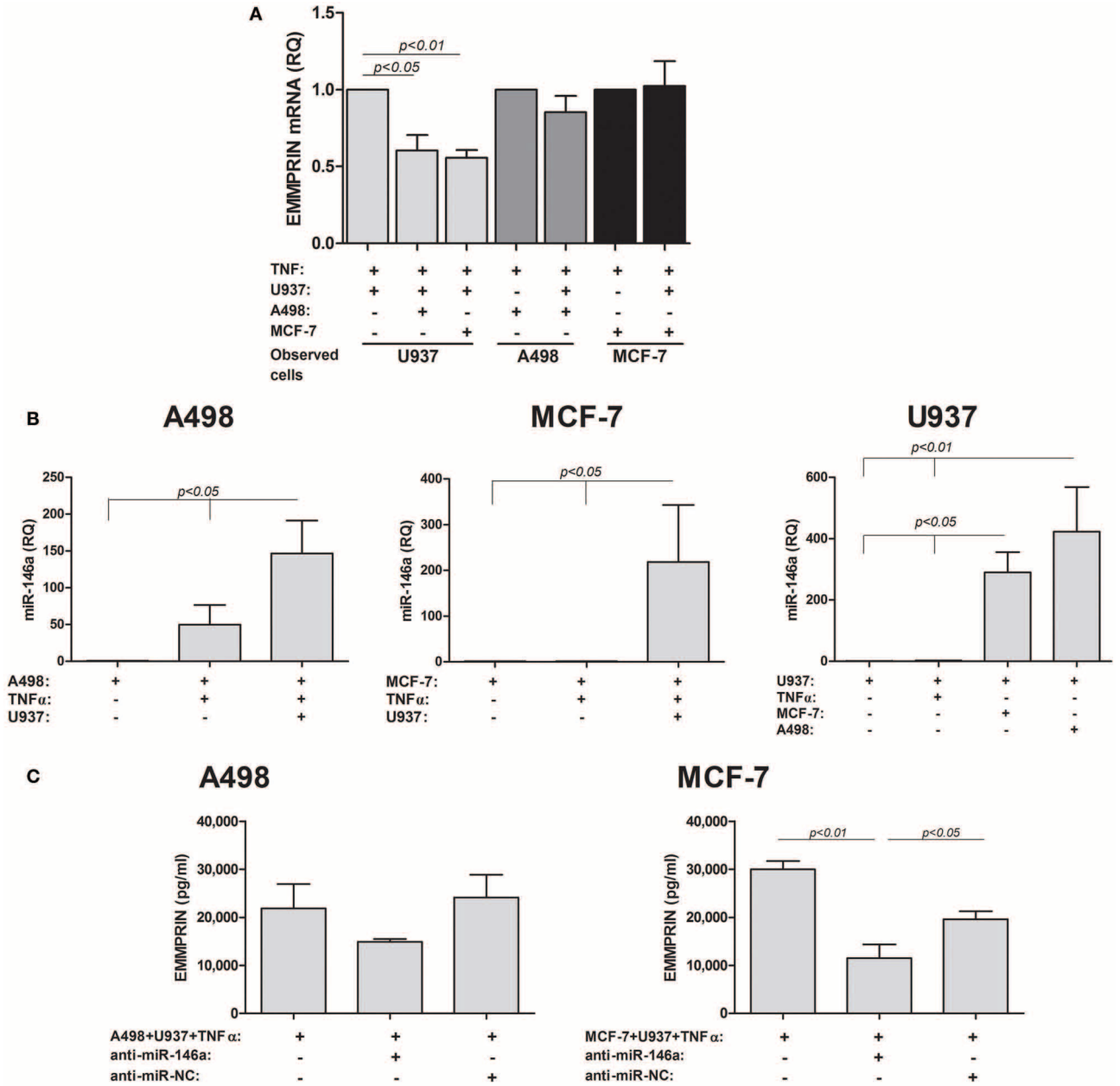

FIGURE 2 | EMMPRIN is regulated at the post-transcriptional level. 0.6×10^5 A498 or MCF-7 cells were incubated in single- or co-cultures with 0.3×10^5 U937 cells in the presence of TNFα (1 ng/ml) for 48 h. To allow estimation of mRNA or miRNA levels in each cell type, co-cultured cells were incubated in separate inserts (0.3 μm pore size), thus precluding cell migration between the two compartments. In some of the wells cells were transfected with anti-miR-146a or anti-miR-NC (scrambled negative control). Total RNA was extracted and transcribed to cDNA. **(A)** Expression of the short EMMPRIN mRNA isoform was determined by quantitative real-time PCR amplification ($n = 4$). **(B)** The expression of miR-146a was determined in each of the single and co-cultured cells cell lines ($n = 3$) **(C)** The effect of neutralization of miR-146a in the tumor cell lines on the expression of EMMPRIN protein in co-cultures was determined ($n = 3$).

U937 cells the antibody reduced MMP-9 secretion by 63% ($p < 0.05$) and VEGF secretion by 61% ($p < 0.01$). In the case of MCF-7, the antibody reduced the expression of both MMP-9 and VEGF to levels comparable to those induced by TNFα in the single cultures, whereas in the A498 cells the antibody reduced MMP-9 and VEGF levels only partially, and MMP-9 and VEGF levels were still higher than those found in the TNFα-stimulated single cultures (**Figure 5**).

Thirdly, we specifically reduced the expression of EMMPRIN in the tumoral cells using EMMPRIN siRNA. To make sure that EMMPRIN was maximally knocked down, we used two different siRNA sequences as well as their combination, and all

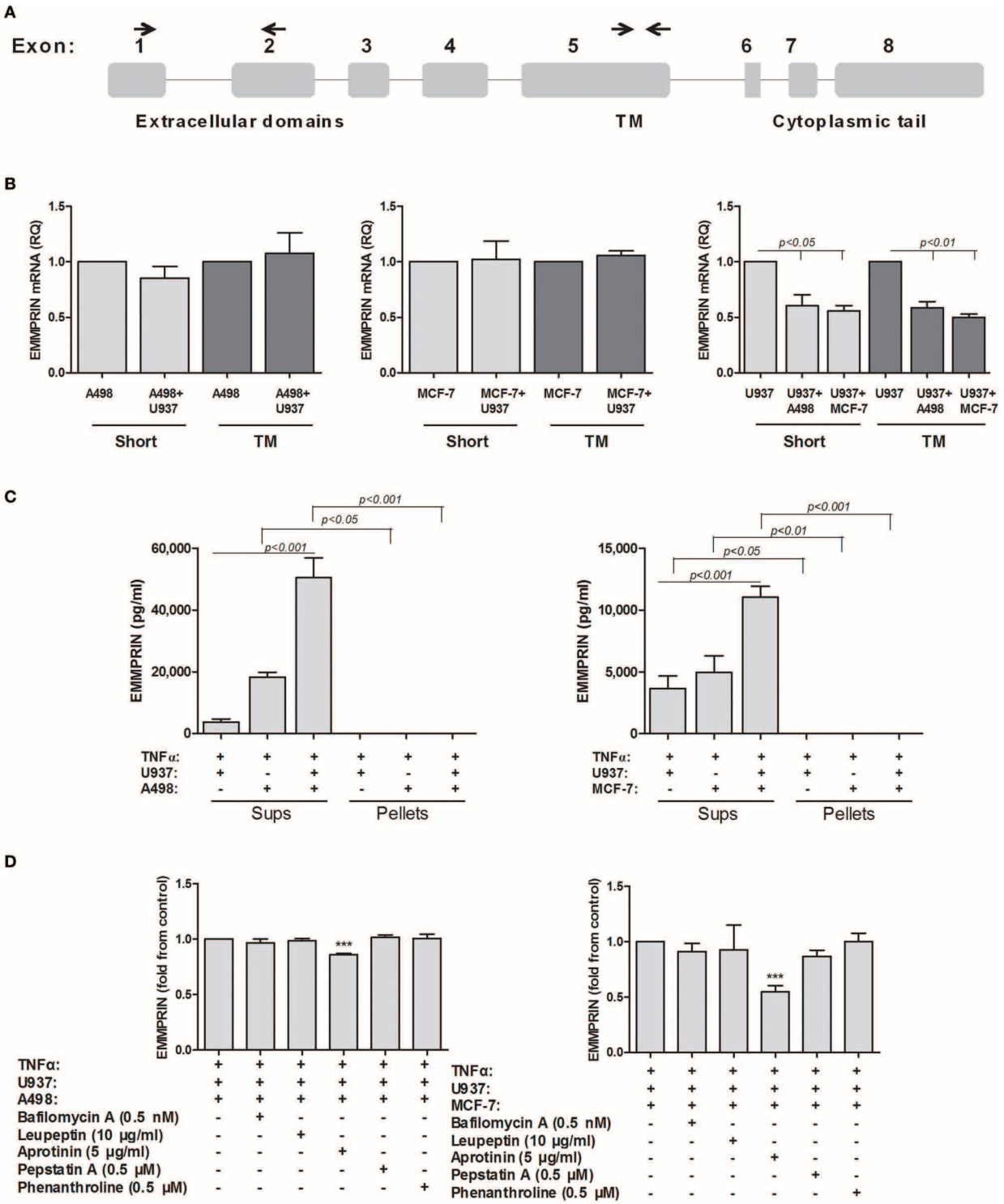

FIGURE 3 | Possible mechanisms for generating secreted EMMPRIN protein. 10^6 A498 or MCF-7 cells were incubated in single- or co-cultures with 0.5×10^6 U937 cells in the presence of TNFα (1 ng/ml) for 48 h. **(A)** A schematic representation of the different exons of the EMMPRIN mRNA. The primers used for the amplification of the transmembranal (TM) or extracellular regions of the short isoform are indicated by small arrows. **(B)** Total RNA was extracted and transcribed into cDNA. Amplification of both the extracellular and transmembranal regions by quantitative real-time PCR was used to assess alternative splicing ($n = 3$). **(C)** Supernatants were collected and ultra-centrifuged as described in the methods. Presence of the EMMRIN protein in exosomes was determined by evaluating its concentrations in both pellets and supernatants by ELISA ($n = 4$). **(D)** Ability of a wide range of protease inhibitors (optimal concentrations indicated) to inhibit EMMPRIN secretion was evaluated by ELISA ($n = 5$).

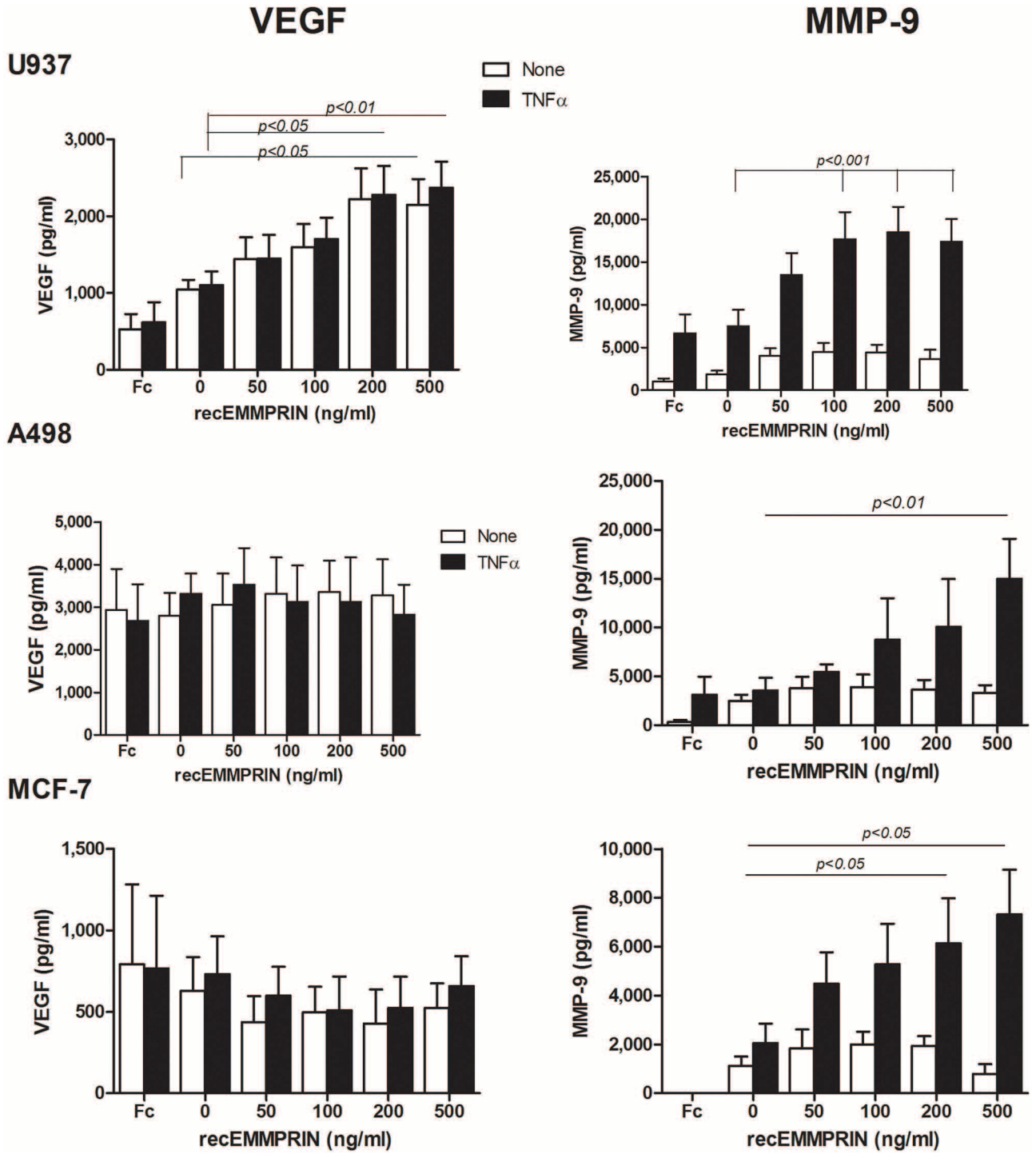

FIGURE 4 | Effect of recombinant EMMPRIN on the secretion of MMP-9, VEGF, and EMMPRIN. 5×10^5 A498, MCF-7 or U937 cells were incubated separately in a serum-free medium with or without TNFα (1 ng/ml) for 48 h, and with the addition of increasing amounts of recombinant EMMPRIN or the IgG Fc fragment (at 200 ng/ml). Concentrations of MMP-9 and VEGF were determined in the supernatants using ELISA ($n = 7$).

three possibilities resulted in similar inhibition of EMMPRIN protein production (about 5-folds for both A498 and MCF-7 cells, $p < 0.05$ and $p < 0.01$, respectively, **Figure 6C**). In A498 or MCF-7 co-cultures with the monocytic U937 cell line, the absence of EMMPRIN resulted in inhibition of VEGF secretion by 8–9-folds and 6–7-folds, respectively, ($p < 0.05$ and $p < 0.01$, respectively, relative to the TNF-stimulated co-cultures without knocking down EMMPRIN, **Figure 6B**). Likewise, in both A498

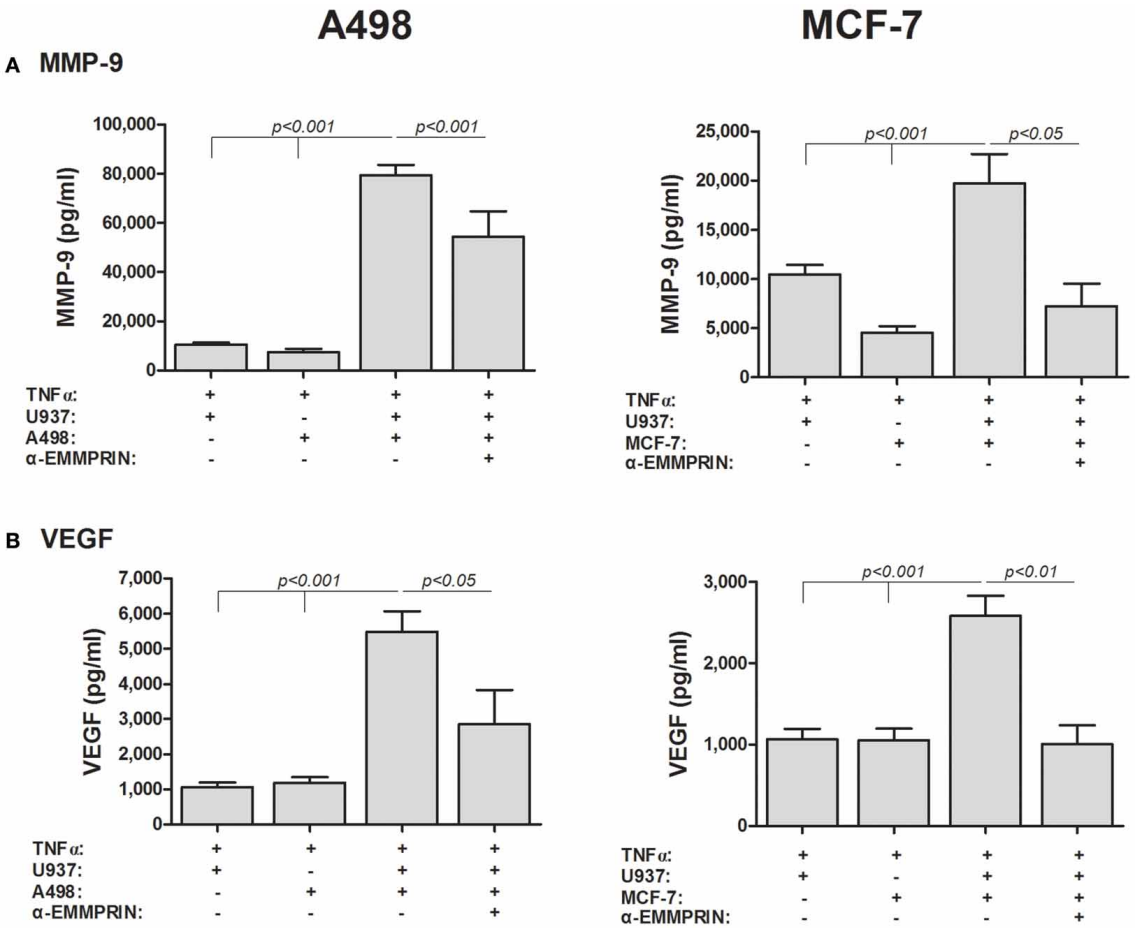

FIGURE 5 | EMMPRIN neutralization inhibits secretion of VEGF and MMP-9. 5×10^5 A498 or MCF-7 cells were incubated separately or in co-cultures with 2.5×10^5 U937 cells in a serum-free medium supplemented with TNFα (1 ng/ml) for 48 h, with or without the addition of anti-EMMPRIN (2 ng/ml). Concentrations of **(A)** MMP-9 and **(B)** VEGF were determined in the supernatants using ELISA ($n = 7$).

and MCF-7 co-cultures, reduced EMMPRIN expression inhibited secretion of MMP-9 by about 3-folds ($p < 0.05$, **Figure 6A**).

SOLUBLE EMMPRIN IS SUFFICIENT TO INDUCE MMP-9 AND VEGF

Although we have shown that EMMPRIN is required for the maximal induction of VEGF and MMP-9 in co-cultures of tumor cells with monocytes, it remains unclear whether the effect is mediated by the membranal or the soluble protein. The dose-dependent effect of the recombinant EMMRPIN (**Figure 4**) was a first indication that the soluble protein mediates the effect. We further reasoned that soluble EMMPRIN found in the supernatant would be sufficient to induce secretion of VEGF and MMP-9 in the opposite cell type. We therefore, collected sups from TNFα-stimulated single cultures of each of the tumor cells, and incubated them diluted in medium at a ratio of 1:4 with U937 cells, with or without the presence of TNFα (**Figure 7**). Relative to the TNFα-stimulated U937 single culture, the A498- or MCF-7-derived sups non-significantly increased MMP-9 secretion from U937 cells by 1.44- and 1.26-folds, respectively, but anti-EMMPRIN specifically inhibited it by 3–4-folds ($p < 0.05$). Similarly, the A498- or MCF-7-derived sups increased VEGF secretion from U937 cells by 2.5-folds ($p < 0.01$), and anti-EMMPRIN specifically inhibited it by 2–3-folds ($p < 0.05$). In contrast, the reciprocal incubation of U937-derived sups with both TNFα-stimulated tumor cells (**Figure 8**) did not affect MMP-9 secretion, and elevated VEGF secretion by about 2.5-folds ($p < 0.001$). However, this effect on VEGF was not specific to EMMPRIN, as indicated by the lack of response to anti-EMMPRIN.

To finally prove that soluble EMMPRIN can mediate MMP-9 and VEGF induction in co-cultures, we incubated the two cell types in Boyden-modified chambers, where the inserts had a 0.3 μm pore size that prevented cell–cell contact. As before, the co-incubation of the A498 or the MCF-7 tumor cells with the U937 monocytic cell line in a mixture that allows cell–cell contact resulted in increase of both MMP-9 and VEGF in the supernatants, whereas the separate incubation of the cells in the inserts did not change this effect (**Figure 9**).

SOLUBLE EMMPRIN IS PRO-ANGIOGENIC

In addition to the ability of EMMPRIN to induce VEGF and MMP-9, we asked if EMMPRIN has direct pro-angiogenic

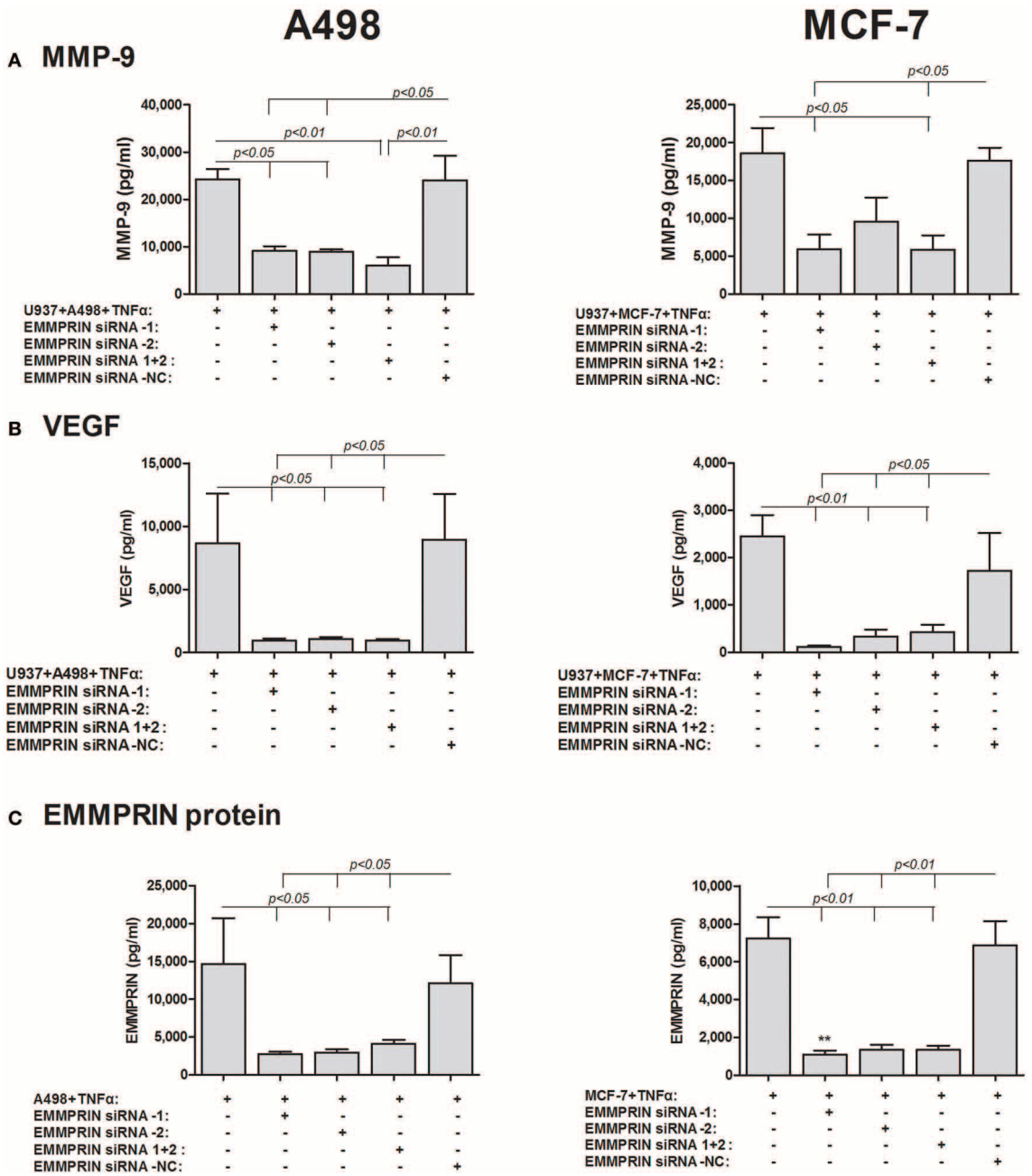

FIGURE 6 | Effect of EMMPRIN siRNA on the secretion of MMP-9, VEGF and EMMPRIN. 0.6×10^5 A498 or MCF-7 cells were incubated separately or in co-cultures with 0.3×10^6 U937 cells in a serum-free medium supplemented with TNFα (1 ng/ml) for 48 h. Tumor cells were first transfected with 5 nM of each of the two different EMMPRIN siRNA molecules, their combination or the negative control (NC), or left untreated. Concentrations of **(A)** MMP-9, **(B)** VEGF, and **(C)** EMMPRIN were determined in the supernatants using ELISA ($n = 5$).

effect on endothelial cells. Since endothelial cells migrate as one sheet, and migration and proliferation are inseparable, the distances between the two sides of the wound and their enhanced rate of closure over time reflect a pro-angiogenic activity. **Figures 10A,B** demonstrate that recombinant EMMPRIN (200 ng/ml) increased migration/proliferation of EaHy926 cells by 2.7-folds, although this trend did not reach significance. In a complementary experiment (**Figures 10C,D**), supernatants derived from A498 tumor cells that were transfected with EMMPRIN siRNA demonstrated a 7-fold ($p < 0.05$) decreased ability to repair the wound compared to untreated supernatants. Similar results were observed for MCF-7-derived supernatants (data not shown), suggesting a requirement for EMMPRIN presence.

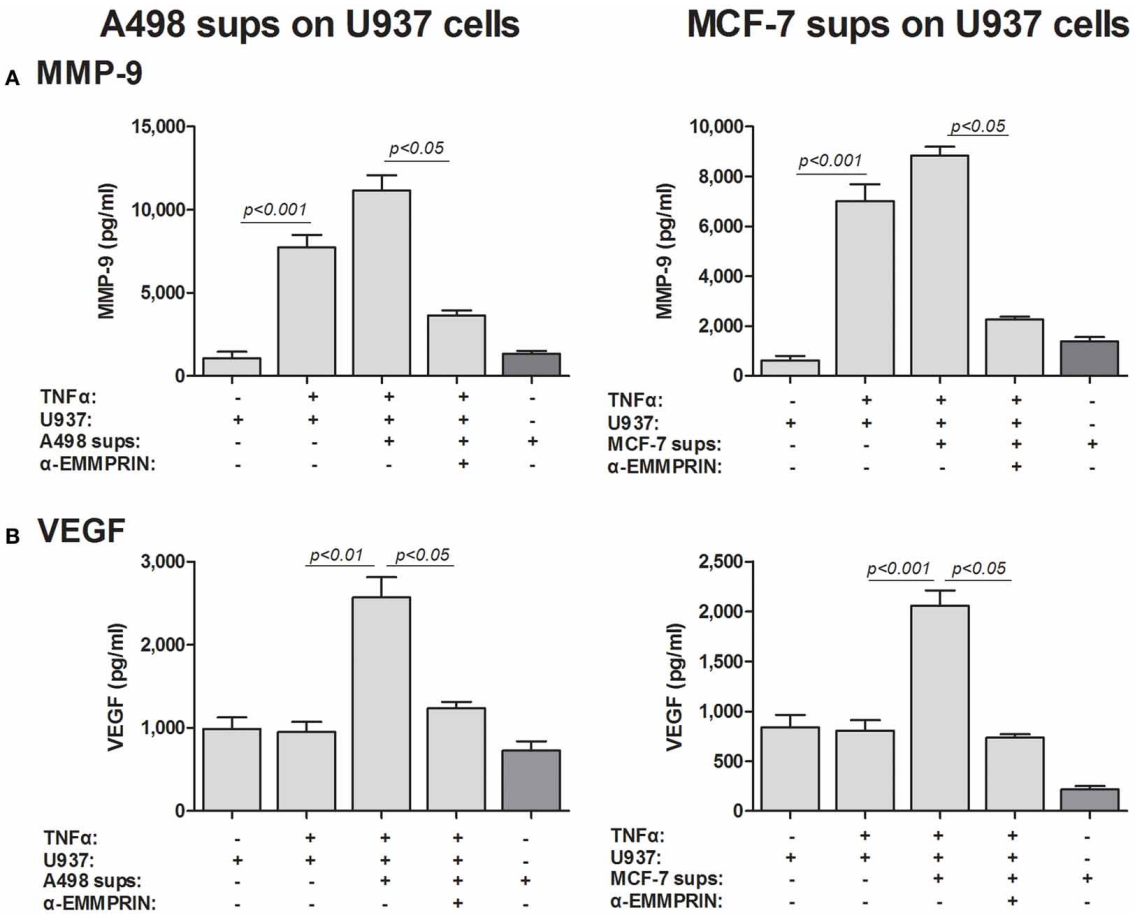

FIGURE 7 | Effect of cancer cell supernatants on monocyte expression of VEGF and MMP-9 is mediated through soluble EMMPRIN. 2×10^5 U937 cells were separately incubated in the presence of TNFα (1 ng/ml) for 48 h with or without the addition of diluted (1:4) supernatants derived from A498 or MCF-7 cells, and with the neutralizing anti-EMMPRIN antibody (2 ng/ml). Concentrations of **(A)** MMP-9 and **(B)** VEGF were determined by ELISA ($n = 5$).

To evaluate the pro-angiogenic effects of EMMPRIN *in vivo* we used the Matrigel plug assay, where liquefied Matrigel mixed with medium or with recombinant EMMPRIN (200 ng/ml) was injected subcutaneously. The plugs were harvested after 7 days and their gross morphology is presented (**Figure 10E**). The control plugs are clear and transparent with almost no visible blood vessels, whereas plugs that were mixed with recombinant EMMPRIN are opaque, reddish and clearly show formation of functional blood vessel network, strongly suggesting increased angiogenesis.

DISCUSSION

EMMPRIN has been shown before to be pro-angiogenic, through its ability to induce MMPs and VEGF. Here we demonstrate some novel points: (a) Expression of EMMPRIN is increased in tumor cells upon co-culturing with macrophages; (b) EMMPRIN is post-transcriptionally regulated by miRNA-146a; (c) Soluble EMMPRIN retains its biological activity, is required for maximal induction of both VEGF and MMP-9, and is generated by a serine protease that is yet to be identified; (d) soluble EMMPRIN alone has a pro-angiogenic activity and a direct effect on endothelial cells both *in vitro* and *in vivo*.

Enhanced EMMPRIN expression has been described before in many types of tumor tissues, but was usually not accompanied by detection of EMMPRIN expression on stromal cells (Zhong et al., 2008; Liang et al., 2009; Omi et al., 2012; Pinheiro et al., 2012; Lu et al., 2013), suggesting that stromal cell expression of EMMPRIN *in vivo* was very low and below the levels of antibody detection. Studies *in vitro* demonstrated that tumor cells enhance their expression of EMMPRIN when co-cultured with other cell types, such as fibroblasts (Tang et al., 2004; Sato et al., 2009) or endothelial cells (Caudroy et al., 2002; Bougaten et al., 2009), but expression of EMMPRIN in monocytes/macrophages co-cultured with tumor cells was hardly investigated. Here we show that EMMPRIN is constitutively expressed, although in low levels, on both cell types. The interactions of monocytes with tumor cells in co-cultures *in vitro* lead to increased membranal expression of the EMMPRIN protein in both cell types, but mostly in the tumor cells. Thus, in our system monocytes behave similarly to other stromal cells, and it is the tumor cells that are mostly responsible for the overexpression of EMMPRIN.

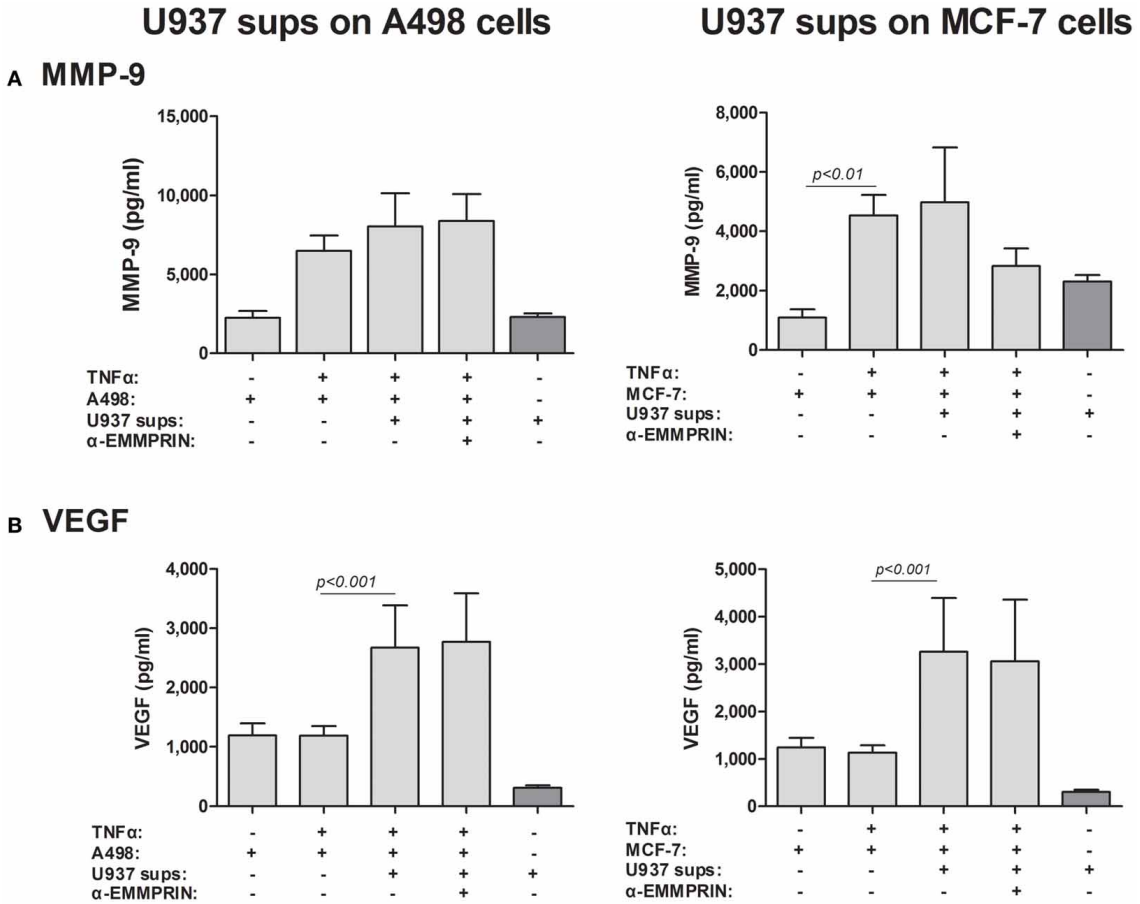

FIGURE 8 | Effect of monocyte supernatants on cancer cell expression of VEGF and MMP-9. 2×10^5 A498 or MCF-7 cells were separately incubated in the presence of TNFα (1 ng/ml) for 48 h with or without the addition of diluted (1:4) supernatants derived from U937 cells, and with the neutralizing anti-EMMPRIN antibody (2 ng/ml). Concentrations of **(A)** MMP-9 and **(B)** VEGF were determined by ELISA ($n = 5$).

The molecular mechanism responsible for this elevated expression of EMMPRIN in co-cultures remains unknown, although EGFR and angiotensin II were implicated in its expression in fibroblasts and macrophages, respectively (Weidle et al., 2010; Yang et al., 2010), but not in tumor cells. We have shown that EMMPRIN mRNA in the tumoral cell lines remains unchanged, and has even decreased in the U937 cells, whereas the protein expression was enhanced. This suggested post-transcriptional or translational regulation, and placed microRNA as possible regulators. We have chosen to look at miR-146a, as it plays a major role in both inflammatory processes and in regulation of cancer (Baltimore et al., 2008), and was previously implicated in the regulation of tumor cell-macrophage interactions (Perske et al., 2010). We first demonstrated that miR-146a is increased both in the two tumor cells and in the monocytic cells during co-culture, and then that its neutralization specifically inhibited secretion of EMMPRIN protein. These results further support a role for miR-146a in the interactions between tumor cells and macrophages, and suggest that increased miR-146a enhances, rather than inhibits translation of EMMPRIN. This was unexpected, as microRNAs usually inhibit protein translation. However, it is possible that miR-146a could indirectly regulate another protein that either controls EMMPRIN translation or interacts with EMMPRIN to enhance its degradation. Both possibilities may result in enhanced EMMPRIN expression. Alternatively, since different algorithms (e.g., miRanda, TargetScan) predict that miR-146a can directly bind to EMMPRIN mRNA, it is possible that miR-146a cooperates with other microRNAs, resulting in complex and non-linear effects. It is also noteworthy that microRNAs do not always inhibit translation, and in viruses they have been shown to regulate their enhanced replication, as is the case for the liver-specific miR-122 and hepatitis C virus (Jopling, 2008), suggesting an additional mode of action for microRNAs. The question of which signal elevates the expression of miR-146a in co-culture in both cell types merits further investigation.

In addition to the elevated membranal expression of EMMPRIN, co-cultures also increase soluble EMMPRIN, particularly in the A498 and U937 co-cultures. Previous studies have suggested three possible mechanisms that may generate soluble EMMPRIN. One mechanism is the generation of exosomes from multivesicular bodies undergoing exocytosis that include

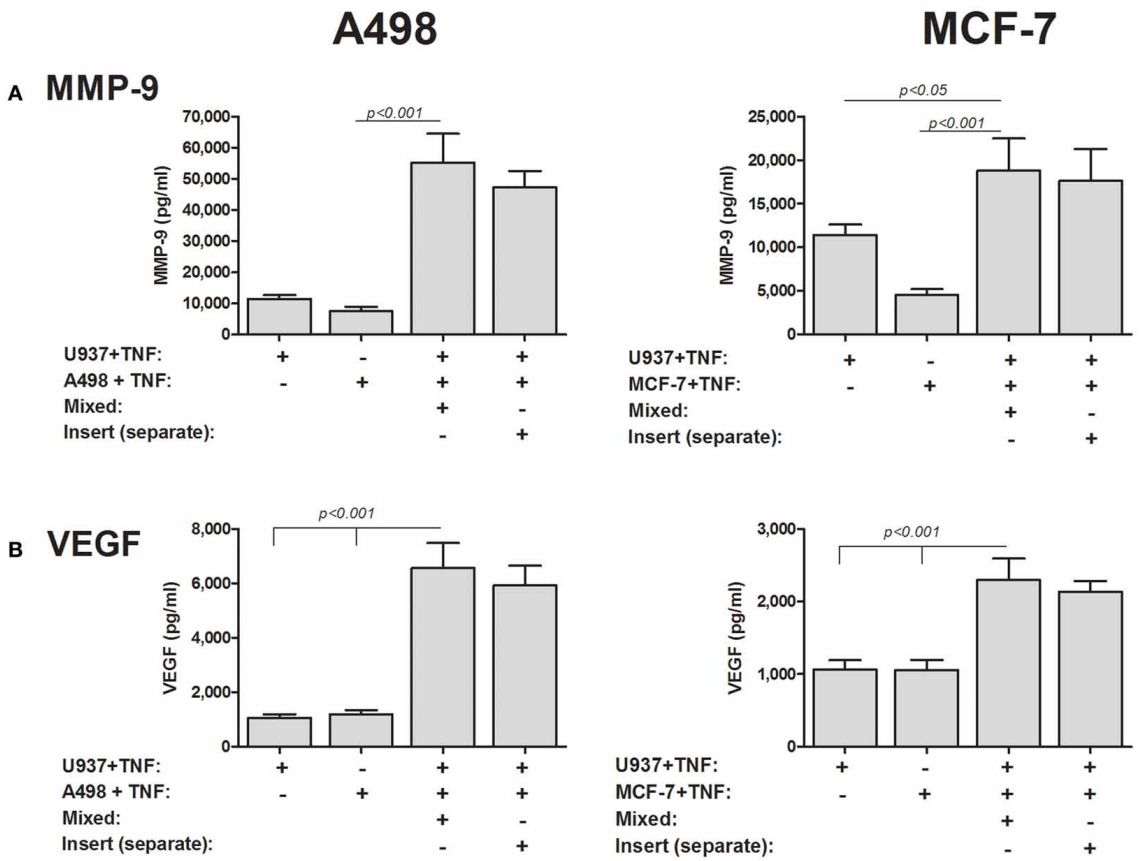

FIGURE 9 | Cell–cell contact is not required for induction of MMP-9 and VEGF. 2×10^5 A498 or MCF-7 cells or 1×10^5 U937 cells were incubated with TNFα (1 ng/ml) for 48 h, separately, in a mixed co-culture where cell–cell contact was allowed, or in inserts with a small pore size (0.3 μm) that precluded cell migration and cell–cell contact. Concentrations of **(A)** MMP-9 and **(B)** VEGF were determined by ELISA ($n = 8$).

EMMPRIN (Keller et al., 2009). Another possibility is the secretion of EMMPRIN in microvesicles that are shed off from the surface, particularly from lipid rafts (Millimaggi et al., 2007). This microvesicle-associated EMMPRIN could enhance MMPs production in endothelial cells, as well as increase their migration and ability to form tube-like structures (Millimaggi et al., 2007). A third mechanism for the secretion of EMMPRIN suggests that the surface protein is cleaved by a protease. In fact, MT1-MMP was found to cleave EMMPRIN in the linker region connecting the two Ig-like domains, leading to a reduced ability of the surface protein, but not the secreted protein, to induce MMPs in fibroblasts (Egawa et al., 2006). Our results rule out the possibility of alternative splicing yielding a soluble EMMPRIN protein, as well as the formation of relatively large structures such as microvesicles or exosomes that carry EMMPRIN, as EMMPRIN was not found in the pellets after ultracentrifugation. Our use of a wide range of protease inhibitors ruled out the involvement of most proteases. Specifically, the use of phenanthroline which inhibits zinc metalloproteinases such as MT1-MMP, suggested that MMPS may not be involved in the proteolytic cleavage of EMMPRIN. In contrast, we found that aprotinin, which inhibits several serine proteases, could decrease the accumulation of EMMPRIN in the supernatants, indicating that a serine protease that is yet to be identified is responsible for a shedding off of surface EMMPRIN in our system. The identification of the specific serine protease awaits further investigation, and plasmin or kalikrein, which are inhibited by aprotinin, seem like good candidates.

We also demonstrate that soluble EMMPRIN, whether synthetic (recombinant EMMPRIN) or produced in our co-culture system, was able to induce both MMP-9 and VEGF from the U937 monocytic cells. The experiments where recombinant EMMPRIN, supernatants derived from one cell type and added to another cell type and cells co-cultured using 0.3 μm inserts—all proved that soluble EMMPRIN was sufficient and that it retained its pro-angiogenic activity. Directionality of EMMPRIN activity was disclosed in the experiments where A498- or MCF-7-derived supernatants were added to U937 cells and demonstrated increased MMP-9 and VEGF secretion that was reduced by anti-EMMPRIN. The reciprocal experiment where U937-derived supernatants were added to the tumor cells resulted in increased VEGF, but not MMP-9 secretion, and even this was not specific, as anti-EMMPRIN could not inhibit this effect. It is also noteworthy that other factors found locally in the tumor microenvironment could induce MMP-9 and VEGF. Among these factors, hypoxia and presence of pro-inflammatory cytokines (e.g., TNFα) can

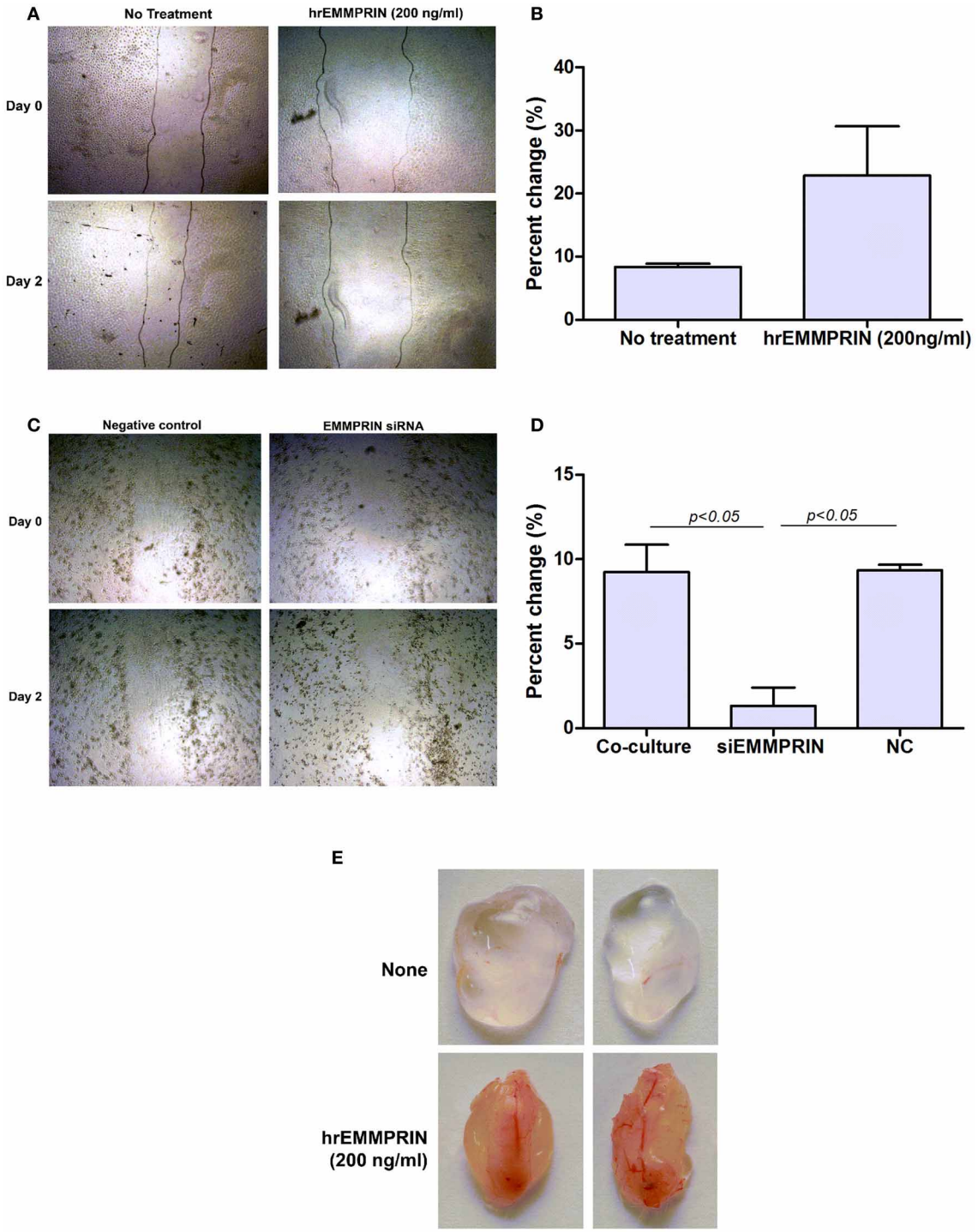

FIGURE 10 | Soluble EMMPRIN has pro-angiogenic properties. 2×10^6 Eahy926 cells were grown to confluency in 24-well plates, and a wound assay was performed by making a scratch, as described in the methods. **(A)** A representative photograph of the scratched area in a wound assay at the beginning (0 h) and at the end (48 h) of the experiment (magnification X40) while adding recombinant EMMPRIN (200 ng/ml). Gray lines depict the cell front. **(B)** Rate of wound closure was measured by comparing the distance between the two sides of the scratch using image analysis software at the beginning and at the end of the experiment, and calculating the percent of change ($n = 3$). **(C)** A representative photograph of scratched area in a wound assay when adding diluted (1:4) supernatants derived from the co-cultures or co-cultures where A498 cells were first transfected with EMMPRIN siRNA or its negative control (NC), and **(D)** analysis of its rate of wound closure ($n = 3$). **(E)** Matrigel plug assay was carried out as described in the methods, and plugs were removed and photographed after 7 days.

induce VEGF and MMP-9 through the activity of the HIF and NF-κB transcription factors (Pages and Pouyssegur, 2005; Yan and Boyd, 2007). EMMPRIN is not redundant, however, and it has a role in maximizing secretion of VEGF and MMP-9, as we show by specifically targeting EMMPRIN, either by siRNA or by anti-EMMPRIN.

Thus, increased tumoral EMMPRIN expression results in generation of soluble EMMPRIN, which probably binds to its ligand on monocytes (either a yet unknown ligand, or EMMPRIN itself through homophilic interactions), and induces secretion of MMP-9 and VEGF from monocytes. This is also in agreement with the fact that monocytes and macrophages are better producers of these proteins than tumor cells.

Finally, EMMPRIN pro-angiogenic activity is not limited to induction of MMP-9 and VEGF. Recent studies have shown that EMMPRIN may directly contribute to the regulation of the angiogenic process by up-regulating soluble forms of VEGF and VEGFR2 in endothelial cells (Bougaten et al., 2009; Pinheiro et al., 2012). We therefore examined the effects of EMMPRIN on endothelial cells *in vitro* using the wound assay. We show that recombinant EMMPRIN could accelerate the rate of wound closure, and when EMMPRIN expression in tumor cells was silenced by siRNA, wound repair was inhibited. Likewise, addition of EMMPRIN to matrigel plugs resulted in growth of blood vessels into the plug, demonstrating that EMMPRIN directly affects endothelial cells *in vivo* as well. Thus, EMMPRIN is directly involved in endothelial migration/proliferation, and the mechanisms that are responsible for this action should be further explored.

In conclusion, we show that co-cultures of tumor cells and macrophages induce EMMPIN expression, as well as production of MMP-9 and VEGF, mostly by the macrophages. Secreted EMMPRIN, which is generated in our system by a serine protease that is yet to be identified, is functional and sufficient to stimulate macrophages to produce VEGF and MMP-9. Furthermore, EMMPRIN has an additional direct pro-angiogenic effect on endothelial cells, suggesting that in the tumoral microenvironment angiogenesis is orchestrated by the effects of EMMPRIN in this triage of tumor cell-macrophage-endothelial cell interactions, and emphasizing the importance of such interactions. Additional questions, such as the deciphering of the signaling pathway that is responsible for the increased EMMPRIN expression and enhanced miR-146a expression merit further investigation.

ACKNOWLEDGMENTS

This study was supported by a grant from the Rappaport Family Institute for Research in the Medical Sciences. We wish to thank Profs. Nitza Lahat and Haim Bitterman for useful advice and critical reading of the manuscript.

REFERENCES

Ali, K. S., Ali, H. Y., and Jubrael, J. M. (2012). Concentration levels of IL-10 and TNFalpha cytokines in patients with human papilloma virus (HPV) DNA(+) and DNA(-) cervical lesions. *J. Immunotoxicol.* 9, 168–172. doi: 10.3109/1547691X.2011.642419

Balkwill, F. (2009). Tumour necrosis factor and cancer. *Nat. Rev. Cancer* 9, 361–371. doi: 10.1038/nrc2628

Baltimore, D., Boldin, M. P., O'connell, R. M., Rao, D. S., and Taganov, K. D. (2008). MicroRNAs: new regulators of immune cell development and function. *Nat. Immunol.* 9, 839–845. doi: 10.1038/ni.f.209

Belton, R. J. Jr., Chen, L., Mesquita, F. S., and Nowak, R. A. (2008). Basigin-2 is a cell surface receptor for soluble basigin ligand. *J. Biol. Chem.* 283, 17805–17814. doi: 10.1074/jbc.M801876200

Blot, E., Chen, W., Vasse, M., Paysant, J., Denoyelle, C., Pille, J. Y., et al. (2003). Cooperation between monocytes and breast cancer cells promotes factors involved in cancer aggressiveness. *Br. J. Cancer* 88, 1207–1212. doi: 10.1038/sj.bjc.6600872

Bougaten, F., Quemener, C., Kellouche, S., Naimi, B., Podgorniak, M. P., Millot, G., et al. (2009). EMMPRIN promotes angiogenesis through HIF-2{alpha} mediated regulation of soluble VEGF isoforms and their receptor VEGFR-2. *Blood* 114, 5547–5556. doi: 10.1182/blood-2009-04-217380

Caudroy, S., Polette, M., Nawrocki-Raby, B., Cao, J., Toole, B. P., Zucker, S., et al. (2002). EMMPRIN-mediated MMP regulation in tumor and endothelial cells. *Clin. Exp. Metastasis* 19, 697–702. doi: 10.1023/A:1021350718226

Charles, K. A., Kulbe, H., Soper, R., Escorcio-Correia, M., Lawrence, T., Schultheis, A., et al. (2009). The tumor-promoting actions of TNF-alpha involve TNFR1 and IL-17 in ovarian cancer in mice and humans. *J. Clin. Invest.* 119, 3011–3023. doi: 10.1172/JCI39065

De Palma, M., Murdoch, C., Venneri, M. A., Naldini, L., and Lewis, C. E. (2007). Tie2-expressing monocytes: regulation of tumor angiogenesis and therapeutic implications. *Trends Immunol.* 28, 519–524. doi: 10.1016/j.it.2007.09.004

Egawa, N., Koshikawa, N., Tomari, T., Nabeshima, K., Isobe, T., and Seiki, M. (2006). Membrane type 1 matrix metalloproteinase (MT1-MMP/MMP-14) cleaves and releases a 22-kDa extracellular matrix metalloproteinase inducer (EMMPRIN) fragment from tumor cells. *J. Biol. Chem.* 281, 37576–37585. doi: 10.1074/jbc.M606993200

Egeblad, M., and Werb, Z. (2002). New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer* 2, 161–174. doi: 10.1038/nrc745

Elamin, I., Zecevic, R. D., Vojvodic, D., Medenica, L., and Pavlovic, M. D. (2008). Cytokine concentrations in basal cell carcinomas of different histological types and localization. *Acta Dermatovenerol. Alp. Panonica Adriat.* 17, 55–59.

Gordon, S., and Martinez, F. O. (2010). Alternative activation of macrophages: mechanism and functions. *Immunity* 32, 593–604. doi: 10.1016/j.immuni.2010.05.007

Hagemann, T., Robinson, S. C., Schulz, M., Trumper, L., Balkwill, F. R., and Binder, C. (2004). Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-alpha dependent up-regulation of matrix metalloproteases. *Carcinogenesis* 25, 1543–1549. doi: 10.1093/carcin/bgh146

Hollborn, M., Stathopoulos, C., Steffen, A., Wiedemann, P., Kohen, L., and Bringmann, A. (2007). Positive feedback regulation between MMP-9 and VEGF in human RPE cells. *Invest. Ophthalmol. Vis. Sci.* 48, 4360–4367. doi: 10.1167/iovs.06-1234

Jopling, C. L. (2008). Regulation of hepatitis C virus by microRNA-122. *Biochem. Soc. Trans.* 36, 1220–1223. doi: 10.1042/BST0361220

Keller, S., Konig, A. K., Marme, F., Runz, S., Wolterink, S., Koensgen, D., et al. (2009). Systemic presence and tumor-growth promoting effect of ovarian carcinoma released exosomes. *Cancer Lett.* 278, 73–81. doi: 10.1016/j.canlet.2008.12.028

Lahat, N., Bitterman, H., Weiss-Cerem, L., and Rahat, M. A. (2011). Hypoxia increases membranal and secreted HLA-DR in endothelial cells, rendering them T-cell activators. *Transpl. Int.* 24, 1018–1026. doi: 10.1111/j.1432-2277.2011.01304.x

Lewis, C., and Murdoch, C. (2005). Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies. *Am. J. Pathol.* 167, 627–635. doi: 10.1016/S0002-9440(10)62038-X

Liang, Y. X., He, H. C., Han, Z. D., Bi, X. C., Dai, Q. S., Ye, Y. K., et al. (2009). CD147 and VEGF expression in advanced renal cell carcinoma and their prognostic value. *Cancer Invest.* 27, 788–793. doi: 10.1080/07357900802709167

Lu, Q., Lv, G., Kim, A., Ha, J. M., and Kim, S. (2013). Expression

and clinical significance of extracellular matrix metalloproteinase inducer, EMMPRIN/CD147, in human osteosarcoma. *Oncol. Lett.* 5, 201–207.

Mantovani, A., Allavena, P., and Sica, A. (2004). Tumour-associated macrophages as a prototypic type II polarised phagocyte population: role in tumour progression. *Eur. J. Cancer* 40, 1660–1667. doi: 10.1016/j.ejca.2004.03.016

Mantovani, A., Sozzani, S., Locati, M., Allavena, P., and Sica, A. (2002). Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* 23, 549–555. doi: 10.1016/S1471-4906(02)02302-5

Millimaggi, D., Mari, M., D'ascenzo, S., Carosa, E., Jannini, E. A., Zucker, S., et al. (2007). Tumor vesicle-associated CD147 modulates the angiogenic capability of endothelial cells. *Neoplasia* 9, 349–357. doi: 10.1593/neo.07133

Mosser, D. M., and Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8, 958–969. doi: 10.1038/nri2448

Murdoch, C., Giannoudis, A., and Lewis, C. E. (2004). Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood* 104, 2224–2234. doi: 10.1182/blood-2004-03-1109

Murdoch, C., Muthana, M., Coffelt, S. B., and Lewis, C. E. (2008). The role of myeloid cells in the promotion of tumour angiogenesis. *Nat. Rev. Cancer* 8, 618–631. doi: 10.1038/nrc2444

Murdoch, C., Muthana, M., and Lewis, C. E. (2005). Hypoxia regulates macrophage functions in inflammation. *J. Immunol.* 175, 6257–6263.

Nabeshima, K., Iwasaki, H., Koga, K., Hojo, H., Suzumiya, J., and Kikuchi, M. (2006). Emmprin (basigin/CD147): matrix metalloproteinase modulator and multifunctional cell recognition molecule that plays a critical role in cancer progression. *Pathol. Int.* 56, 359–367. doi: 10.1111/j.1440-1827.2006.01972.x

Omi, Y., Shibata, N., Okamoto, T., Obara, T., and Kobayashi, M. (2012). The role of CD147 in the invasiveness of follicular thyroid carcinoma cells. *Thyroid* 22, 383–394. doi: 10.1089/thy.2010.0426

Owen, J. L., Iragavarapu-Charyulu, V., Gunja-Smith, Z., Herbert, L. M., Grosso, J. F., and Lopez, D. M. (2003). Up-regulation of matrix metalloproteinase-9 in T lymphocytes of mammary tumor bearers: role of vascular endothelial growth factor. *J. Immunol.* 171, 4340–4351.

Pages, G., and Pouyssegur, J. (2005). Transcriptional regulation of the Vascular Endothelial Growth Factor gene–a concert of activating factors. *Cardiovasc. Res.* 65, 564–573. doi: 10.1016/j.cardiores.2004.09.032

Perske, C., Lahat, N., Levin, S. S., Bitterman, H., Hemmerlein, B., and Rahat, M. A. (2010). Loss of inducible nitric oxide synthase expression in the mouse renal cell carcinoma cell line RENCA is mediated by microRNA miR-146a. *Am. J. Pathol.* 177, 2046–2054. doi: 10.2353/ajpath.2010.091111

Pinheiro, C., Longatto-Filho, A., Soares, T. R., Pereira, H., Bedrossian, C., Michael, C., et al. (2012). CD147 immunohistochemistry discriminates between reactive mesothelial cells and malignant mesothelioma. *Diagn. Cytopathol.* 40, 478–483. doi: 10.1002/dc.22821

Sato, T., Ota, T., Watanabe, M., Imada, K., Nomizu, M., and Ito, A. (2009). Identification of an active site of EMMPRIN for the augmentation of matrix metalloproteinase-1 and -3 expression in a co-culture of human uterine cervical carcinoma cells and fibroblasts. *Gynecol. Oncol.* 114, 337–342. doi: 10.1016/j.ygyno.2009.04.004

Serafini, P., Borrello, I., and Bronte, V. (2006). Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin. Cancer Biol.* 16, 53–65. doi: 10.1016/j.semcancer.2005.07.005

Tang, Y., Kesavan, P., Nakada, M. T., and Yan, L. (2004). Tumor-stroma interaction: positive feedback regulation of extracellular matrix metalloproteinase inducer (EMMPRIN) expression and matrix metalloproteinase-dependent generation of soluble EMMPRIN. *Mol. Cancer Res.* 2, 73–80.

Tang, Y., Nakada, M. T., Kesavan, P., McCabe, F., Millar, H., Rafferty, P., et al. (2005). Extracellular matrix metalloproteinase inducer stimulates tumor angiogenesis by elevating vascular endothelial cell growth factor and matrix metalloproteinases. *Cancer Res.* 65, 3193–3199.

Tang, Y., Nakada, M. T., Rafferty, P., Laraio, J., McCabe, F. L., Millar, H., et al. (2006). Regulation of vascular endothelial growth factor expression by EMMPRIN via the PI3K-Akt signaling pathway. *Mol. Cancer Res.* 4, 371–377. doi: 10.1158/1541-7786.MCR-06-0042

Weidle, U. H., Scheuer, W., Eggle, D., Klostermann, S., and Stockinger, H. (2010). Cancer-related issues of CD147. *Cancer Genomics Proteomics* 7, 157–169.

Yan, C., and Boyd, D. D. (2007). Regulation of matrix metalloproteinase gene expression. *J. Cell. Physiol.* 211, 19–26. doi: 10.1002/jcp.20948

Yan, L., Zucker, S., and Toole, B. P. (2005). Roles of the multifunctional glycoprotein, emmprin (basigin; CD147), in tumour progression. *Thromb. Haemost.* 93, 199–204.

Yang, L. X., Liu, H., Guo, R. W., Ye, J., Wang, X. M., Qi, F., et al. (2010). Angiotensin II induces EMMPRIN expression in THP-1 macrophages via the NF-kappaB pathway. *Regul. Pept.* 163, 88–95. doi: 10.1016/j.regpep.2010.04.012

Yurchenko, V., Constant, S., Eisenmesser, E., and Bukrinsky, M. (2010). Cyclophilin-CD147 interactions: a new target for anti-inflammatory therapeutics. *Clin. Exp. Immunol.* 160, 305–317. doi: 10.1111/j.1365-2249.2010.04115.x

Zhong, W. D., Han, Z. D., He, H. C., Bi, X. C., Dai, Q. S., Zhu, G., et al. (2008). CD147, MMP-1, MMP-2 and MMP-9 protein expression as significant prognostic factors in human prostate cancer. *Oncology* 75, 230–236. doi: 10.1159/000163852

Zhou, J., Zhu, P., Jiang, J. L., Zhang, Q., Wu, Z. B., Yao, X. Y., et al. (2005). Involvement of CD147 in overexpression of MMP-2 and MMP-9 and enhancement of invasive potential of PMA-differentiated THP-1. *BMC Cell Biol.* 6:25. doi: 10.1186/1471-2121-6-25

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 March 2013; paper pending published: 19 April 2013; accepted: 23 June 2013; published online: 12 July 2013.

*Citation: Amit-Cohen B-C, Rahat MM and Rahat MA (2013) Tumor cell-macrophage interactions increase angiogenesis through secretion of EMMPRIN. Front. Physiol. **4**:178. doi: **10.3389/fphys.2013.00178***

This article was submitted to Frontiers in Vascular Physiology, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 17 July 2013
doi: 10.3389/fphys.2013.00157

The irradiated tumor microenvironment: role of tumor-associated macrophages in vascular recovery

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Radiotherapy is an important modality used in the treatment of more than 50% of cancer patients in the US. However, despite sophisticated techniques for radiation delivery as well as the combination of radiation with chemotherapy, tumors can recur. Thus, any method of improving the local control of the primary tumor by radiotherapy would produce a major improvement in the curability of cancer patients. One of the challenges in the field is to understand how the tumor vasculature can regrow after radiation in order to support tumor recurrence, as it is unlikely that any of the endothelial cells within the tumor could survive the doses given in a typical radiotherapy regimen. There is now considerable evidence from both preclinical and clinical studies that the tumor vasculature can be restored following radiotherapy from an influx of circulating cells consisting primarily of bone marrow derived monocytes and macrophages. The radiation-induced influx of bone marrow derived cells (BMDCs) into tumors can be prevented through the blockade of various cytokine pathways and such strategies can inhibit tumor recurrence. However, the post-radiation interactions between surviving tumor cells, recruited immune cells, and the remaining stroma remain poorly defined. While prior studies have described the monocyte/macrophage inflammatory response within normal tissues and in the tumor microenvironment, less is known about this response with respect to a tumor after radiation therapy. The goal of this review is to summarize existing research studies to provide an understanding of how the myelomonocytic lineage may influence vascular recovery within the irradiated tumor microenvironment.

Keywords: radiation, macrophages, vasculogenesis, angiogenesis, blood vessels, tumor growth

TUMOR-ASSOCIATED MACROPHAGES

Infiltrating leukocytes are a common finding in solid tumors, first described by Virchow in 1863 and confirmed in modern studies (Wood and Gollahon, 1977; Milas et al., 1987; Balkwill and Mantovani, 2001). Tumor-associated macrophages (TAMs) are recruited to tumors and can promote tumor growth, survival, and may result in resistance to therapeutic treatments (De Palma and Lewis, 2013). As tumors mature, they acquire a heterogeneous, infiltrative population of bone marrow-derived cells (BMDCs), including a diverse array of myelomonocytic cells: neutrophils, dendritic cells, myeloid derived suppressor cells and monocytes/macrophages (Akashi et al., 2000; Nagaraj and Gabrilovich, 2010). The extent of TAM infiltration appears to correlate with a poor clinical prognosis and an increase in tumor burden (Takanami et al., 1999; Shieh et al., 2009; Toge et al., 2009).

TAMs originate as circulating monocytes recruited to tumors by cytokine gradients produced by tumor cells as well as the tumor stroma (Mantovani et al., 1992). A diverse array of cytokines and growth factors has been demonstrated to stimulate macrophage recruitment to tumors (Balkwill, 2004; Allavena et al., 2008). Initially, TAMs were felt to have anti-tumor properties; however, Mantovani et al. found that isolated macrophages from a weakly immunogenic sarcoma cell line were able to stimulate tumor cell growth *in vitro* (Mantovani, 1978). TAMs have now been found by many investigators to also promote tumor growth *in vivo*, often by producing a proangiogenic environment (Folkman, 1974; Polverini et al., 1977; Lin and Pollard, 2004).

As a simplified paradigm, macrophages are frequently considered to be polarized toward two specific phenotypes; however, it is important to realize there are many macrophage phenotypes with different specialized functions (Qian and Pollard, 2010). Classically activated macrophages (M1) are "pro-inflammatory" cells designed to protect the host from pathogenic infections. M1 macrophages are stimulated by LPS and IFN-gamma to produce IL-12, IL-6, inducible NO synthase (iNOS), and TNF-alpha (Modolell et al., 1995). These M1 populations have an enhanced ability to generate reactive oxygen species, upregulate phagocytosis, and have enhanced functionality as antigen presenting cells (Martinez et al., 2009). In contrast, M2 macrophages (alternatively activated) are considered "anti-inflammatory" as they promote tissue repair through IL-4, IL-13 and prostaglandin signaling and result in the production of IL-10 and TGF-beta (Corraliza et al., 1995; Mantovani et al., 2009). M2 populations are able to suppress cytokine production and reduce activation of T-cells, decrease antigen presenting ability, promote angiogenesis, stimulate extracellular matrix degradation, and enhance cell survival (Murdoch et al., 2008; Lu et al., 2011). Increased arginase I (Arg I) expression is often used as a marker of the M2 phenotype

due to changes in arginine metabolism away from NO generation to polyamine production (Ho and Sly, 2009). Many studies have found that the tumor microenvironment preferentially polarizes TAMs to the M2 phenotype (Gabrilovich et al., 2012; Ruffell et al., 2012).

The activation or polarization toward a particular macrophage phenotype appears to be dependent on the cytokine milieu, the presence of specific growth factors, and the level of hypoxia within the tumor microenvironment (Munder et al., 1998; Goerdt et al., 1999; Gordon, 2003; Mosser, 2003; Stout et al., 2005). However, it should be noted that macrophages can also simultaneously produce M1 as well as M2-related cytokines and that the expression is highly dependent on the tumor type, stage, and location as well as the host microenvironment. Additionally, the dynamic tumor environment may constantly shift the ratio of macrophage phenotypes depending on the current environmental conditions (Murdoch et al., 2004; Pollard, 2004). For example, an unregulated M1 population could result in a shift toward a chronic inflammatory state, while an uncontrolled M2 population could result in severe immunosuppression (Mantovani et al., 2004; Condeelis and Pollard, 2006). Furthermore, lack of M1 signals (i.e., downregulated tumor/stromal production of IL-4, IL-10, and IL-13) drive TAMs toward the M2 phenotype (Mantovani et al., 2004; Pollard, 2004; Solinas et al., 2009).

In summary, TAMs are bone marrow derived monocytic cells with unique functional subsets that are recruited to tumors by cytokine gradients and frequently differentiate into the M2 macrophage phenotype. TAM infiltration can result in an immunosuppressed environment, the promotion of proangiogenic pathways, and consequently, enhanced tumor growth and tumor cell survival.

HYPOXIA AND THE PROANGIOGENIC ROLE OF TAMs

The tumor microenvironment is often transiently or chronically in a state of low oxygen tension (Vaupel and Mayer, 2007). To survive in a hypoxic environment, tumors must establish a functional vascular network (See **Figure 1**). Tumors frequently adapt to hypoxia by preventing the degradation of hypoxia-induced transcription factor complexes (i.e., HIF1 and HIF2) resulting in their stabilization and subsequent transcription of genes that promote tumor survival, including proangiogenic cytokines (Giaccia et al., 2004; Keith et al., 2012). Using primary human macrophages, Fang et al. demonstrated that HIF1 and HIF2 co-regulate many hypoxia-related genes; however, by using siRNA specific knockdown studies of HIF1 and HIF2, they found that each of these genes can target certain hypoxia-associated genes independently (Fang et al., 2009).

TAMs commonly associate in necrotic, low oxygenated areas of tumors (Leek et al., 1996; Burke et al., 2003; Lewis and Murdoch,

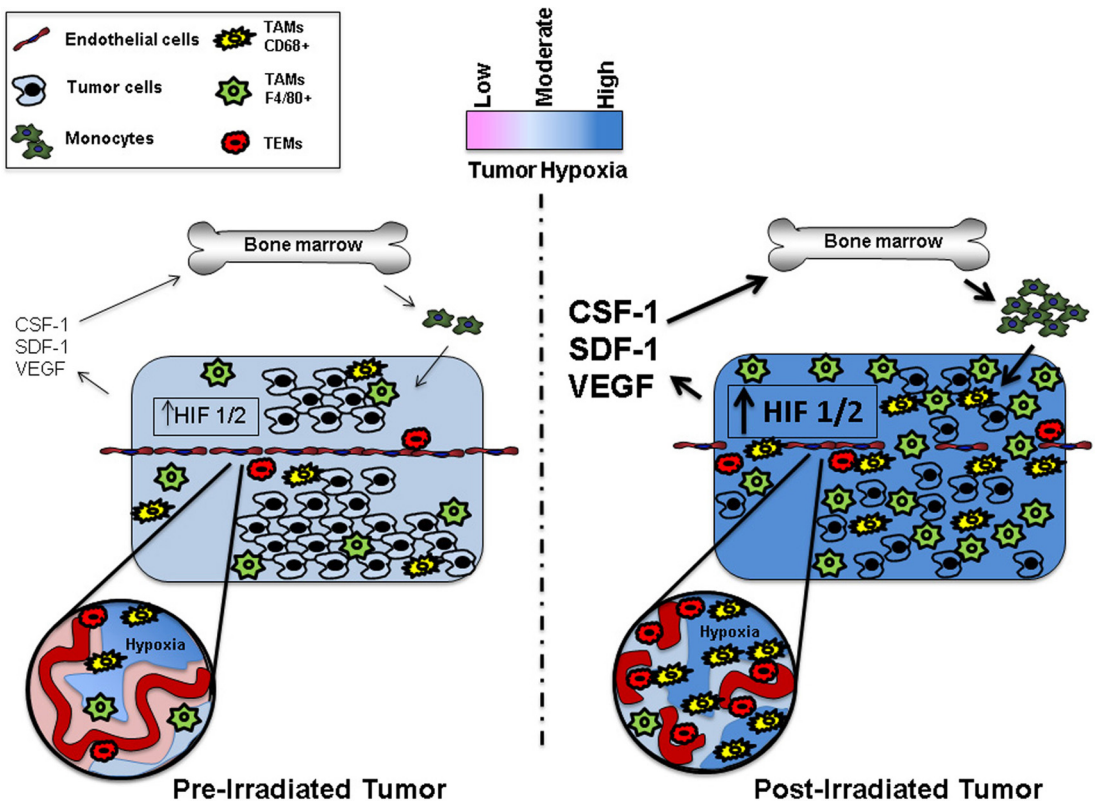

FIGURE 1 | Representation ofthe microenvironment of pre- and postirradiated tumors. The post-irradiated tumor has increased levels of hypoxia, upregulated HIF 1/2 signaling, and expression of a diverse spectrum of cytokines as well as a greater recruitment and influx of bone marrow derived TAMs. TEMs commonly associate with the vasculature, while CD68+ TAMs frequently localize to areas of severe hypoxia.

2005). Hypoxic environments increase expression of the CXCR4 receptor on TAMs and increase the chemotactic response to its ligand, stromal cell-derived factor-1 (SDF-1/CXCL-12) (Schioppa et al., 2003). Localization of TAMs to hypoxic regions is mediated by cytokine gradients resulting from hypoxia-induced HIF1/2 stabilization (Talks et al., 2000; Murdoch et al., 2004; Jin et al., 2006; Knowles and Harris, 2007; Han et al., 2008). Cramer et al. demonstrated that HIF1 expression was required for myeloid cell motility and invasiveness (Cramer et al., 2003). Furthermore, Du et al. demonstrated that intracranial implants of HIF1-deficient glioma cells had reduced levels of infiltrating monocytes within tumors as well as reduced levels of tumor SDF-1 and MMP-9 protein expression (Du et al., 2008).

Modulation of HIF1/2 has been demonstrated to affect macrophage phenotype as well as function. However, Werno et al. demonstrated, using a macrophage lineage HIF1 knockout model, that TAM infiltration of tumors was not dependent on HIF1, but HIF1 was necessary to promote the polarization of TAMs to the M2 phenotype (Werno et al., 2010). Consistent with this, Doedens and colleagues showed that whereas hypoxia suppressed T-cell activation within tumors and resulted in tumor progression, T-cell suppression was reduced in a HIF1 macrophage lineage specific knockout model and resulted in decreased tumor growth (Doedens et al., 2010). Both HIF1 and HIF2 appear to be important regulators of the M1 and M2 polarization phenotypes. Takeda et al. reported that classical activation cytokines (IFN-gamma, LPS) increased HIF1 mRNA, but strongly repressed HIF2 mRNA production, and, conversely, IL-4, the alternative activation cytokine, resulted in an increase of HIF2 mRNA (Takeda et al., 2010). Furthermore, M2-polarized macrophages demonstrated an upregulation of HIF2 mRNA. HIF2 was also found to regulate Arg I protein expression; however, in contrast, HIF1 stabilization increased iNOS expression. Deletion of HIF2 in mouse macrophages resulted in the inability to generate an appropriate inflammatory response and murine tumors implanted in the HIF2 depleted macrophage mouse model demonstrated decreased TAM infiltration as well as decreased levels of the CSFR1 and CXCR4 receptors (Imtiyaz et al., 2010). Hypoxia-induced HIF1/2 activation and the resulting differential effects on TAMs remains a complicated, highly regulated system playing a significant role in tumor progression and survival.

With respect to angiogenesis, increased macrophage infiltration in tumors is associated with a higher vascular density in breast, glioma, bladder, and esophageal tumors (Leek et al., 1996; Nishie et al., 1999; Hanada et al., 2000; Koide et al., 2004). TAMs promote tumor angiogenesis and vascularization by releasing proangiogenic cytokines such as VEGF and the matrix metalloproteinases (Lewis and Pollard, 2006). A study using breast tumor spheroids found increased VEGF levels and increased vascular connections when incubated with a macrophage population (Bingle et al., 2006). Similarly, Lewis et al. found that VEGF mRNA was upregulated in macrophages associated with human breast cancer (Lewis et al., 2000). Inhibition of VEGF and VEGFR2 by monoclonal antibodies decreased macrophage infiltration of *in vivo* pancreatic tumors (Dineen et al., 2008). Interestingly, Stockmann et al. used a VEGF-A macrophage specific lineage knockout in the MMTV-PyMT breast cancer model and demonstrated similar levels of tumor-associated macrophage infiltration, decreased tumor VEGFR2 activation, and a decrease in the length of tumor blood vessels; however, overall tumor growth was actually enhanced (Stockmann et al., 2008). These results indicate differential effects of tumor-produced VEGF-A compared to macrophage-produced VEGF-A on the vascular network. Further experiments demonstrated that tumor cell death was enhanced by chemotherapy in tumor-bearing mice lacking myeloid-specific VEGF-A, suggesting that targeting the proangiogenic function of TAMs could sensitize tumors to cytotoxic therapy. Further interplay of macrophages and endothelial cells was recently demonstrated by He et al. who demonstrated that BM-derived hematopoietic cells incubated *in vitro* with immortalized endothelial cell layers resulted in the generation of M2-like macrophage colonies (He et al., 2012). Modulation of the extracellular matrix also impacts the development of vascular networks. Coussens et al. demonstrated upregulated matrix metalloproteinase-9 (MMP-9) production by bone marrow derived TAMs and increased tumor angiogenesis (Coussens et al., 2000). In a related study, Hao et al. found that BMDCs were recruited to areas of high VEGF expression, expressed elevated levels of MMP-9, and that capillary development was greatly reduced in MMP-9 knockout mice (Hao et al., 2008). Thus, the activation of macrophages within a hypoxic environment results in the release of proangiogenic cytokines and extracellular matrix modulating factors.

A subpopulation of tumor-associated macrophages/monocytes has been identified that express the Tie2 angiopoietin receptor and are defined as Tie2-expressing monocytes (TEMs) (De Palma et al., 2005). De Palma et al. using a suicide gene strategy showed that the selective killing of TEMs, prevented angiogenesis, and slowed tumor growth in mouse xenografts (De Palma et al., 2003). Importantly, TEM elimination did not significantly reduce the overall number of TAMs, indicating that TEMs are a small subpopulation of macrophages/monocytes. Venneri et al. demonstrated that a TEM population was present in human cancers and that these cells were responsive *in vitro* to chemotactic migratory stimulation by Ang2, the ligand for the Tie2 receptor (Venneri et al., 2007). Furthermore, co-injection studies of TEMs with human glioma cells resulted in more vascularized tumor xenografts in contrast to co-injection with TEM-depleted monocyte populations. Of note, TEMs associated with tumors were found to express the Tie2 receptor at elevated levels over those of circulating TEMs. Finally, tumor overexpression of Ang2, resulted in increased accumulation of TEMs within the tumor microenvironment (Coffelt et al., 2010). Hypoxia was also demonstrated to upregulate Tie2 expression in TEMs and downregulate TNF-alpha and IL-12 levels, known anti-angiogenic cytokines (Murdoch et al., 2007). Hypoxia induced by vascular disrupting agents produced an increase in tumor SDF-1 expression as well as increased infiltration of CXCR4+ TEMs (Welford et al., 2011). While TAMs infiltrate the hypoxic and necrotic regions of tumors, TEMs are more frequently localized around tumor blood vessels, possess greater proangiogenic qualities, and can function as an immunosuppressive cell, similar

to the M2 macrophage phenotype (De Palma et al., 2005; Pucci et al., 2009; Coffelt et al., 2011).

In addition to the Tie2 population, other proangiogenic TAM subsets within tumors have been identified including: CD11b+VEGFR1+ hematopoietic cells and CD11c+MCH-II+ dendritic cell precursors (Hattori et al., 2002; Yang et al., 2004; De Palma and Naldini, 2006). Similarly, CD11b+Gr-1+ myeloid cells were also found to mediate resistance to anti-VEGF therapies (Shojaei et al., 2007); however, this population may be related more toward neutrophils rather than macrophages (Shojaei and Ferrara, 2008). Movadehi et al. found that MHC II^{hi} and MHC II^{low} subsets of TAMs were associated with M1 and M2 phenotypes, respectively (Movahedi et al., 2010). Furthermore, using the chorioallantoic membrane (CAM) assay of angiogenesis, MHC II^{low} TAMs had a two-fold higher vessel count compared to MHC II^{hi} TAMs, demonstrating the enhanced proangiogenic ability of MHC II^{low} TAMs.

TAMs are comprised of several distinct subpopulations and are recruited to hypoxic regions of tumors via cytokine signaling gradients. In turn, they secrete growth factors to promote blood vessel formation and proteinases that remodel the tumor vascular network. Restoration of the vascular supply can result in tumor survival, proliferation, and potentially, an increased risk of metastasis.

INTRINSIC RADIATION RESPONSE OF MONOCYTES/MACROPHAGES

While previous studies found that stimulated monocytes/macrophages are innately resistant to radiotherapy, as they were demonstrated to be post-mitotic cells, Jenkins et al. reported that the activation of M2-polarized macrophages resulted in a higher cell proliferation (Hildebrandt et al., 1998; Jenkins et al., 2011). This result suggests that within the acute response to ionizing radiation, M2 macrophages may actually be more sensitive to radiation-induced DNA damage and result in cell death, in contrast to quiescent M1-polarized macrophages.

In addition to the intrinsic radiosensitivity of particular M1 or M2 TAM subsets, the influence of ionizing radiation on macrophage function may be an even more important factor in tumor survival. Early *in vitro* research found that radiation interferes with the recognition and degradation of antigens and results in the failure of macrophages to generate antibody responses against these targets (Donaldson et al., 1956; Nelson and Becker, 1959; Pribnow and Silverman, 1967). Similarly, Geiger et al. reported that macrophages of irradiated mice were unable to stimulate antibody production against *Shigella*, however, the phagocytic activity of irradiated macrophages was not impaired (Geiger and Gallily, 1974). In contrast, Lambert et al. demonstrated that *in vitro* radiation resulted in the priming of the macrophage cell line RAW 264.7 and upregulated MHC Class I molecules (Lambert and Paulnock, 1987). Additionally, radiation was found to augment antibody-dependent cell-mediated cytotoxicity (ADCC) in the murine macrophage cell line J774 (Duerst and Werberig, 1991). Other studies have also confirmed that radiation induces the activation of macrophages *in vitro* and *in vivo* through increased rates of phagocytosis, lysosomal enzyme production, and H_2O_2 production as well as the retained capacity to respond to cytokines (Sablonniere et al., 1983; Gallin et al., 1985; Gallin and Green, 1987; Hester and Coggin, 1989). Thus, while the antigen-presenting functions of macrophages are disrupted by radiation, their innate phagocytic function remains intact.

With respect to cytokine production, radiation-exposed macrophages have increased IL-1beta mRNA expression, upregulated TNF-alpha production, are able to potentiate nitric oxide production by interferon-gamma, and release a variety of growth factors (i.e., PDGF, IGF-1) (Sherman et al., 1991; O'Brien-Ladner et al., 1993; Iwamoto and McBride, 1994; Nemoto et al., 1995; Thornton et al., 1996; Vodovotz et al., 1999; McKinney et al., 2000). Therefore, the modulation of cytokine production by ionizing radiation may influence the macrophage polarization phenotype and function. Coates et al. found that macrophages from irradiated C57BL/6 mice demonstrated enhanced M2 activity while irradiated macrophages from CBA/CaJ mice had increased M1 activity (Coates et al., 2008). This result suggested that ionizing radiation can induce a phenotypic polarization shift, but overall, macrophage polarization is dependent on the background genetic environment.

The above studies demonstrate that ionizing radiation directly affects macrophage function. The understanding of the intrinsic radiation response of macrophages, including disrupted antigen recognition, modulation of macrophage polarization to an immunosuppressive phenotype, and the production of proangiogenic cytokines may result in the identification of signaling pathways that could be targeted to generate a more radiosensitive subpopulation of macrophages, and ultimately, an increase in tumor responsiveness to radiation therapy.

TAMS AND THE IRRADIATED MICROENVIRONMENT

Radiosensitivity describes the *in vitro* response of cells to ionizing radiation, a property that depends critically on the ability of the cells to repair DNA as well as the activation of other intrinsic survival pathways. The radioresponse of tumors is defined as the *in vivo* change in tumor size after radiation therapy. Several components of the tumor microenvironment that can greatly affect the radioresponse of tumors are: the level of tissue oxygenation, the sensitivity of tumor endothelial cells to radiation, activation of tumor stroma to express survival factors, and immune cell infiltration of the tumor.

An influential study from the joint laboratories of Fuks and Kolesnick proposed that the radiation sensitivity of tumors to dose fractions of 10 Gy or more was governed by the sensitivity of tumor endothelial cells to apoptosis (Garcia-Barros et al., 2003). However, earlier data from the Suit laboratory had shown that the radiation dose to control 50% of transplanted tumors in mice did not depend on the radiation sensitivity of the tumor stroma (Budach et al., 1993). This apparent contradiction can be explained by the different assays of tumor response used—growth delay in the Fuks/Kolesnick study and tumor control (TCD50) by the Suit lab. Indeed, the dual contribution of tumor cell radiosensitivity and stromal sensitivities was demonstrated by Gerweck et al. using the growth delay assay (Ogawa et al., 2007). Several additional reports have also demonstrated that radiation results in the reduction of blood vessel density (Song et al., 1974; Timke et al., 2008; Zeng et al., 2008; Kioi et al., 2010).

In addition to the intrinsic sensitivity of the tumor cells at the time of irradiation, the effects on the tumor stroma can also produce events that regulate tumor radioresponse. For example, the destruction of the vasculature by ionizing radiation causes hypoxic conditions which results in the activation of HIF-1, stimulation of cytokine signaling cascades, and the recruitment of macrophages and immune cells (Moeller et al., 2004; Li et al., 2007; Kioi et al., 2010). This can also occur in normal tissues as well: alveolar macrophages in the selectively irradiated mouse lung were shown to increase at 8 weeks post-treatment indicating the local organ repopulation of macrophages through tissue resident precursors or from bone marrow progenitor cells (Gross, 1977; Peel and Coggle, 1980). Similarly, Johnston et al. found that after 15 Gy of thoracic radiation, macrophages and lymphocytes were elevated within irradiated normal tissues at 16 and 24 weeks post-treatment (Johnston et al., 2004).

Milas et al. found that the tumor-associated macrophage content varied widely between *in vivo* tumor implants, but there was a trend toward increased macrophage content and reduced local tumor radiocurability (Milas et al., 1987). A second study confirmed that a high macrophage content in tumors was able to overcome the growth delay seen in pre-irradiated tumor beds implying the importance to TAMs for tumor angiogenesis (Milas, 1990). Similar studies have also demonstrated tumor infiltration of BMDCs after treatment with ionizing radiation (Stephens et al., 1978; Jung et al., 1990; Chen et al., 2009). Using a prostate cancer cell line in mouse xenografts, Tsai et al. demonstrated that radiation-induced TAM accumulation occurred 1–2 weeks after treatment and that irradiated TAMs expressed elevated Arg I levels suggesting an M2 phenotype (Tsai et al., 2007). Additionally, when irradiated TAMs were co-injected with tumor cells, the resulting tumors demonstrated enhanced growth rates compared to samples co-injected with unirradiated TAMs. With respect to the clinical setting, Baeten et al. found increased CD68+ macrophages in tumor biopsy samples of rectal cancer patients after radiotherapy and Kioi et al. demonstrated an increase in CD11b+ myeloid cells in glioblastomas recurring after radiation (Baeten et al., 2006; Kioi et al., 2010).

Ahn et al. demonstrated that radiation treatment of MT1A2 mouse mammary tumors results in an influx of CD11b+ cells expressing high levels in MMP-9 in either irradiated tumors or tumors grown in a pre-irradiated tumor bed (Ahn and Brown, 2008). Additionally, the expression of MMP-9 by CD11b+ myelomonocytes was necessary for vascular restoration and tumor growth in irradiated tissues. Finally, selective depletion of CD11b+ cells by a monoclonal antibody inhibited tumor growth in pre-irradiated tissues (Ahn et al., 2010). Taken together, these data demonstrate that TAMs promote tumor growth and stimulates early tumor regrowth through improved blood vessel formation.

The influx of TAMs after radiation appears to be the result of increased levels of the transcription factor HIF-1, secondary to increased tumor hypoxia after irradiation. Using an dual inhibitor of both HIF-1 and HIF-2, Kioi et al. found a decrease in the number of radiation-induced BMDC infiltration (mostly CD11b+ cells) in an orthotopic mouse xenograft model of human glioblastoma (Kioi et al., 2010). Similarly, treatment of mice with irradiated tumors using carrageenan, to deplete systemic monocytes/macrophages, also resulted in decreased tumor infiltration of CD11b+ cells after radiation treatment. Further, ionizing radiation induced elevated levels of the downstream HIF-1 target, SDF-1, within U251 tumor xenografts. Blocking the interaction of SDF-1 and its receptor CXCR4, by using the CXCR4 inhibitor AMD3100 or a CXCR4 neutralizing antibody, resulted in decreased tumor perfusion and an enhanced radioresponse of the glioma xenograft model. Interestingly, Kozin et al. also showed an increase in CD11b+ myeloid cells in irradiated tumors and demonstrated that whole body radiation (depleting the bone marrow compartment) combined with the local irradiation of a tumor site resulted in improved local tumor control compared to local radiation alone (Kozin et al., 2010). Additionally, an infusion of myeloid progenitor cells improved tumor regrowth after local radiation. Similar to the study by Kioi et al. SDF-1 was also found to be upregulated in irradiated tumor tissues and blocking the SDF-1/CXCR4 interaction with AMD3100 inhibited tumor re-growth after radiation. Both studies found that Tie2+ BMDCs were significantly increased in tumors after local radiation and that these cells, while localized to the vasculature, were not incorporated into tumor vessel walls.

Chiang et al. found that CD68+ TAMs accumulate in hypoxic regions of certain tumors, but this is dependent on the tumor type as well as the local microenvironment (Chiang et al., 2012). However, after radiation therapy, CD11b+ myeloid cells were distributed into distinct spatial locations: CD68+ TAMs were found in areas of central hypoxia, while F4/80+ TAMs were found on the edge of hypoxic regions adjacent to necrotic regions. They proposed that radiation therapy may activate specific factors to localize or retain CD68+ TAMs into anoxic or hypoxic regions. Finally, they determined that the radiation-activated CD68+ TAMs expressed Arg I, indicating a polarization toward the M2 phenotype of macrophage, and that TAM recruitment was dependent on SDF-1. An additional study has indicated that tumors implanted into pre-irradiated fields grow slower than in unirradiated control tissues (i.e., the "tumor bed effect") and demonstrate an aggregation of CD68+ TAMs in hypoxic regions (Chen et al., 2011). Furthermore, when BMDCs were injected systemically into mice with tumors grown in a pre-irradiated field, they incorporated specifically into the tumor vasculature of the low blood vessel density regions.

In addition to the SDF-1/CXCR4 pathway enhanced by radiation-induced tumor hypoxia, the CSF-1/CSF1R signaling complex has also been recently implicated in recruitment of myeloid cells to growing tumors and in promoting the radiation-induced monocytic infiltration of tumors. Dorsch et al. demonstrated that transfection of the human CSF-1 gene into a synergetic mouse model resulted in increased TAM infiltration of the tumor (Dorsch et al., 1993). Another study determined that the CSF-1 ligand could stimulate monocytes to produce VEGF and form microtubule structures *in vitro* (Eubank et al., 2003). Using a small molecule inhibitor to the receptor of CSF-1, Priceman et al. found that the CSF-1/CSF1R pathway was necessary for the recruitment of TAMs, promoted tumor progression, and the release of proangiogenic cytokines (Priceman et al., 2010).

Recently, Xu et al. demonstrated that radiation increased TAM accumulation in tumors, upregulated *in vivo* tumor expression of CSF-1 and interestingly, in irradiated prostate cancer patients, found that serum levels of CSF-1 were also increased (Xu et al., 2013). A selective inhibitor of the CSF-1 receptor combined with radiation therapy suppressed tumor growth compared to radiation alone. They proposed that the mechanism for the increased CSF-1 expression in tumors was by radiation-induced DNA damage resulting in the activation and translocation of the ABL kinase into the cell nucleus, binding to the CSF-1 gene promoter, and the enhancement of CSF-1 gene transcription.

CONCLUSIONS

Following tumor irradiation, DNA damage, cell death, and increased tumor hypoxia promotes the production of VEGF, SDF-1, and CSF-1 resulting in the recruitment, infiltration, and retention of monocytes/macrophages within tumors. The recruited heterogeneous populations of TAMs release proangiogenic cytokines and metalloproteinases to promote blood vessel formation within tumors. The level of hypoxia appears to distribute particular TAM subgroups to specific regions of the tumor. While the TEM subset is frequently localized to the perivascular niche, other subpopulations of TAMs are divided across necrotic, peri-necrotic, and low oxygen tension regions. Additionally, M2 macrophage polarization appears to be the dominant phenotype within hypoxic tumors.

Radiation is a unique therapy modality as it causes DNA damage and enhances tumor hypoxia, but only within a targeted region. Radiation-induced recruitment of TAMs appears to occur in a similar manner as that caused by tumor hypoxia, is partially dependent on the SDF-1/CXCR4 and CSF-1/CSFR signaling pathways, and promotes polarization toward the M2 phenotype. Thus, the accumulation of radiation-induced TAMs within a tumor may result in the increased production of proangiogenic cytokines, the recovery of the vascular network, and consequently, tumor regrowth.

While the generalized process of TAM recruitment has been identified, many unanswered questions and challenges remain. First, the heterogeneous population of TAMs needs to be clearly identified both by phenotypic markers and function. Which markers clearly define the subpopulations of TAMs? Are the radiation-induced TAM populations different from the tumor resident TAMs? Furthermore, does TAM infiltration of irradiated tumors change over time (i.e., an acute response and/or a chronic response)? What are the functions of the specific TAM subgroups (i.e., cytokine release, extracellular matrix remodeling, or immunosuppression)? Second, several cytokine-related signaling pathways have been implicated in the recruitment of TAMs to irradiated tumor sites; however, much more research is needed. For example, what are the specific intracellular and extracellular signaling pathways driving TAM recruitment and distribution within a tumor? And, does radiation merely enhance hypoxic signaling or does it generate its own unique signaling network? Thus, the radiation-induced signaling pathways driving TAM recruitment, distribution, and function remain to be fully elucidated. Thirdly, retrospective clinical data suggests that increased macrophage infiltration of tumors is often a poor prognostic feature. Could the subtype of TAM infiltration into tumors be used as a more specific prognostic tool? Would it be possible to stratify patients based on the subtype of TAM infiltration (pre or post-radiation) to certain risk groups or even select for certain treatment strategies? Additional studies are needed to correlate clinical outcomes with the biological data in order to answer these questions.

Finally, evidence supports that TAMs promote tumor growth and survival. By understanding which TAM subsets are most beneficial to the tumor and by defining the intra- and extracellular pathways, novel therapies can be developed to disrupt TAM recruitment and function. Therefore, ablation of TAM infiltration within tumors may be a unique strategy to enhance the effectiveness of radiation therapy by decreasing angiogenic signaling, disrupting vascular recovery, reducing local tumor recurrence rates, and decreasing the risk of invasion and metastasis.

REFERENCES

Ahn, G. O., and Brown, J. M. (2008). Matrix metalloproteinase-9 is required for tumor vasculogenesis but not for angiogenesis: role of bone marrow-derived myelomonocytic cells. *Cancer Cell* 13, 193–205. doi: 10.1016/j.ccr.2007.11.032

Ahn, G. O., Tseng, D., Liao, C. H., Dorie, M. J., Czechowicz, A., and Brown, J. M. (2010). Inhibition of Mac-1 (CD11b/CD18) enhances tumor response to radiation by reducing myeloid cell recruitment. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8363–8368. doi: 10.1073/pnas.0911378107

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I. L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 404, 193–197. doi: 10.1038/35004599

Allavena, P., Sica, A., Solinas, G., Porta, C., and Mantovani, A. (2008). The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. *Crit. Rev. Oncol. Hematol.* 66, 1–9. doi: 10.1016/j.critrevonc.2007.07.004

Baeten, C. I., Castermans, K., Lammering, G., Hillen, F., Wouters, B. G., Hillen, H. F., et al. (2006). Effects of radiotherapy and chemotherapy on angiogenesis and leukocyte infiltration in rectal cancer. *Int. J. Radiat. Oncol. Biol. Phys.* 66, 1219–1227. doi: 10.1016/j.ijrobp.2006.07.1362

Balkwill, F. (2004). Cancer and the chemokine network. *Nat. Rev. Cancer* 4, 540–550. doi: 10.1038/nrc1388

Balkwill, F., and Mantovani, A. (2001). Inflammation and cancer: back to Virchow? *Lancet* 357, 539–545. doi: 10.1016/S0140-6736(00)04046-0

Bingle, L., Lewis, C. E., Corke, K. P., Reed, M. W., and Brown, N. J. (2006). Macrophages promote angiogenesis in human breast tumour spheroids *in vivo*. *Br. J. Cancer* 94, 101–107. doi: 10.1038/sj.bjc.6602901

Budach, W., Taghian, A., Freeman, J., Gioioso, D., and Suit, H. D. (1993). Impact of stromal sensitivity on radiation response of tumors. *J. Natl. Cancer Inst.* 85, 988–993.

Burke, B., Giannoudis, A., Corke, K. P., Gill, D., Wells, M., Ziegler-Heitbrock, L., et al. (2003). Hypoxia-induced gene expression in human macrophages: implications for ischemic tissues and hypoxia-regulated gene therapy. *Am. J. Pathol.* 163, 1233–1243. doi: 10.1016/S0002-9440(10)63483-9

Chen, F. H., Chiang, C. S., Wang, C. C., Fu, S. Y., Tsai, C. S., Jung, S. M., et al. (2011). Vasculatures in tumors growing from preirradiated tissues: formed by vasculogenesis and resistant to radiation and antiangiogenic therapy. *Int. J. Radiat. Oncol. Biol. Phys.* 80, 1512–1521. doi: 10.1016/j.ijrobp.2011.02.055

Chen, F. H., Chiang, C. S., Wang, C. C., Tsai, C. S., Jung, S. M., Lee, C. C., et al. (2009). Radiotherapy decreases vascular density and causes hypoxia with macrophage aggregation in TRAMP-C1 prostate tumors. *Clin.*

Cancer Res. 15, 1721–1729. doi: 10.1158/1078-0432.CCR-08-1471

Chiang, C. S., Fu, S. Y., Wang, S. C., Yu, C. F., Chen, F. H., Lin, C. M., et al. (2012). Irradiation promotes an m2 macrophage phenotype in tumor hypoxia. *Front. Oncol.* 2:89. doi: 10.3389/fonc.2012.00089

Coates, P. J., Rundle, J. K., Lorimore, S. A., and Wright, E. G. (2008). Indirect macrophage responses to ionizing radiation: implications for genotype-dependent bystander signaling. *Cancer Res.* 68, 450–456. doi: 10.1158/0008-5472.CAN-07-3050

Coffelt, S. B., Chen, Y. Y., Muthana, M., Welford, A. F., Tal, A. O., Scholz, A., et al. (2011). Angiopoietin 2 stimulates TIE2-expressing monocytes to suppress T cell activation and to promote regulatory T cell expansion. *J. Immunol.* 186, 4183–4190. doi: 10.4049/jimmunol.1002802

Coffelt, S. B., Tal, A. O., Scholz, A., De Palma, M., Patel, S., Urbich, C., et al. (2010). Angiopoietin-2 regulates gene expression in TIE2-expressing monocytes and augments their inherent proangiogenic functions. *Cancer Res.* 70, 5270–5280. doi: 10.1158/0008-5472.CAN-10-0012

Condeelis, J., and Pollard, J. W. (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 124, 263–266. doi: 10.1016/j.cell.2006.01.007

Corraliza, I. M., Soler, G., Eichmann, K., and Modolell, M. (1995). Arginase induction by suppressors of nitric oxide synthesis (IL-4, IL-10 and PGE2) in murine bone-marrow-derived macrophages. *Biochem. Biophys. Res. Commun.* 206, 667–673. doi: 10.1006/bbrc.1995.1094

Coussens, L. M., Tinkle, C. L., Hanahan, D., and Werb, Z. (2000). MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 103, 481–490.

Cramer, T., Yamanishi, Y., Clausen, B. E., Forster, I., Pawlinski, R., Mackman, N., et al. (2003). HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell* 112, 645–657.

De Palma, M., and Lewis, C. E. (2013). Macrophage regulation of tumor responses to anticancer therapies. *Cancer Cell* 23, 277–286. doi: 10.1016/j.ccr.2013.02.013

De Palma, M., and Naldini, L. (2006). Role of haematopoietic cells and endothelial progenitors in tumour angiogenesis. *Biochim. Biophys. Acta* 1766, 159–166. doi: 10.1016/j.bbcan.2006.06.003

De Palma, M., Venneri, M. A., Galli, R., Sergi Sergi, L., Politi, L. S., Sampaolesi, M., et al. (2005). Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell* 8, 211–226. doi: 10.1016/j.ccr.2005.08.002

De Palma, M., Venneri, M. A., Roca, C., and Naldini, L. (2003). Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nat. Med.* 9, 789–795. doi: 10.1038/nm871

Dineen, S. P., Lynn, K. D., Holloway, S. E., Miller, A. F., Sullivan, J. P., Shames, D. S., et al. (2008). Vascular endothelial growth factor receptor 2 mediates macrophage infiltration into orthotopic pancreatic tumors in mice. *Cancer Res.* 68, 4340–4346. doi: 10.1158/0008-5472.CAN-07-6705

Doedens, A. L., Stockmann, C., Rubinstein, M. P., Liao, D., Zhang, N., DeNardo, D. G., et al. (2010). Macrophage expression of hypoxia-inducible factor-1 alpha suppresses T-cell function and promotes tumor progression. *Cancer Res.* 70, 7465–7475. doi: 10.1158/0008-5472.CAN-10-1439

Donaldson, D. M., Marcus, S., Gyi, K. K., and Perkins, E. H. (1956). The influence of immunization and total body x-irradiation on intracellular digestion by peritoneal phagocytes. *J. Immunol.* 76, 192–199.

Dorsch, M., Hock, H., Kunzendorf, U., Diamantstein, T., and Blankenstein, T. (1993). Macrophage colony-stimulating factor gene transfer into tumor cells induces macrophage infiltration but not tumor suppression. *Eur. J. Immunol.* 23, 186–190. doi: 10.1002/eji.1830230129

Du, R., Lu, K. V., Petritsch, C., Liu, P., Ganss, R., Passegue, E., et al. (2008). HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer Cell* 13, 206–220. doi: 10.1016/j.ccr.2008.01.034

Duerst, R., and Werberig, K. (1991). Cells of the J774 macrophage cell line are primed for antibody-dependent cell-mediated cytotoxicity following exposure to gamma-irradiation. *Cell. Immunol.* 136, 361–372.

Eubank, T. D., Galloway, M., Montague, C. M., Waldman, W. J., and Marsh, C. B. (2003). M-CSF induces vascular endothelial growth factor production and angiogenic activity from human monocytes. *J. Immunol.* 171, 2637–2643.

Fang, H. Y., Hughes, R., Murdoch, C., Coffelt, S. B., Biswas, S. K., Harris, A. L., et al. (2009). Hypoxia-inducible factors 1 and 2 are important transcriptional effectors in primary macrophages experiencing hypoxia. *Blood* 114, 844–859. doi: 10.1182/blood-2008-12-195941

Folkman, J. (1974). Tumor angiogenesis. *Adv. Cancer Res.* 19, 331–358.

Gabrilovich, D. I., Ostrand-Rosenberg, S., and Bronte, V. (2012). Coordinated regulation of myeloid cells by tumours. *Nat. Rev. Immunol.* 12, 253–268. doi: 10.1038/nri3175

Gallin, E. K., and Green, S. W. (1987). Exposure to gamma-irradiation increases phorbol myristate acetate-induced H2O2 production in human macrophages. *Blood* 70, 694–701.

Gallin, E. K., Green, S. W., and Sheehy, P. A. (1985). Enhanced activity of the macrophage-like cell line J774.1 following exposure to gamma radiation. *J. Leukoc. Biol.* 38, 369–381.

Garcia-Barros, M., Paris, F., Cordon-Cardo, C., Lyden, D., Rafii, S., Haimovitz-Friedman, A., et al. (2003). Tumor response to radiotherapy regulated by endothelial cell apoptosis. *Science* 300, 1155–1159. doi: 10.1126/science.1082504

Geiger, B., and Gallily, R. (1974). Effect of X-irradiation on various functions of murine macrophages. *Clin. Exp. Immunol.* 16, 643–655.

Giaccia, A. J., Simon, M. C., and Johnson, R. (2004). The biology of hypoxia: the role of oxygen sensing in development, normal function, and disease. *Genes Dev.* 18, 2183–2194. doi: 10.1101/gad.1243304

Goerdt, S., Politz, O., Schledzewski, K., Birk, R., Gratchev, A., Guillot, P., et al. (1999). Alternative versus classical activation of macrophages. *Pathobiology* 67, 222–226. doi: 10.1159/000028096

Gordon, S. (2003). Alternative activation of macrophages. *Nat. Rev. Immunol.* 3, 23–35. doi: 10.1038/nri978

Gross, N. J. (1977). Alveolar macrophage number: an index of the effect of radiation on the lungs. *Radiat. Res.* 72, 325–332.

Han, Z. B., Ren, H., Zhao, H., Chi, Y., Chen, K., Zhou, B., et al. (2008). Hypoxia-inducible factor (HIF)-1 alpha directly enhances the transcriptional activity of stem cell factor (SCF) in response to hypoxia and epidermal growth factor (EGF). *Carcinogenesis* 29, 1853–1861. doi: 10.1093/carcin/bgn066

Hanada, T., Nakagawa, M., Emoto, A., Nomura, T., Nasu, N., and Nomura, Y. (2000). Prognostic value of tumor-associated macrophage count in human bladder cancer. *Int. J. Urol.* 7, 263–269.

Hao, Q., Liu, J., Pappu, R., Su, H., Rola, R., Gabriel, R. A., et al. (2008). Contribution of bone marrow-derived cells associated with brain angiogenesis is primarily through leukocytes and macrophages. *Arterioscler. Thromb. Vasc. Biol.* 28, 2151–2157. doi: 10.1161/ATVBAHA.108.176297

Hattori, K., Heissig, B., Wu, Y., Dias, S., Tejada, R., Ferris, B., et al. (2002). Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat. Med.* 8, 841–849. doi: 10.1038/nm740

He, H., Xu, J., Warren, C. M., Duan, D., Li, X., Wu, L., et al. (2012). Endothelial cells provide an instructive niche for the differentiation and functional polarization of M2-like macrophages. *Blood* 120, 3152–3162. doi: 10.1182/blood-2012-04-422758

Hester, R. B., and Coggin, J. H. Jr. (1989). Tumorigenic sublethal whole-body X-irradiation of RFM mice enhances cell-mediated cytotoxicity while transiently depressing T- and B-lymphocytes. *Mol. Biother.* 1, 244–249.

Hildebrandt, G., Seed, M. P., Freemantle, C. N., Alam, C. A., Colville-Nash, P. R., and Trott, K. R. (1998). Mechanisms of the anti-inflammatory activity of low-dose radiation therapy. *Int. J. Radiat. Biol.* 74, 367–378.

Ho, V. W., and Sly, L. M. (2009). Derivation and characterization of murine alternatively activated (M2) macrophages. *Methods Mol. Biol.* 531, 173–185. doi: 10.1007/978-1-59745-396-7_12

Imtiyaz, H. Z., Williams, E. P., Hickey, M. M., Patel, S. A., Durham, A. C., Yuan, L. J., et al. (2010). Hypoxia-inducible factor 2alpha regulates macrophage function in mouse models of acute and tumor inflammation. *J. Clin. Invest.* 120, 2699–2714. doi: 10.1172/JCI39506

Iwamoto, K. S., and McBride, W. H. (1994). Production of 13-hydroxyoctadecadienoic acid and tumor necrosis factor-alpha by murine peritoneal macrophages in response to irradiation. *Radiat. Res.* 139, 103–108.

Jenkins, S. J., Ruckerl, D., Cook, P. C., Jones, L. H., Finkelman, F. D., van Rooijen, N., et al. (2011). Local

macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science* 332, 1284–1288. doi: 10.1126/science.1204351

Jin, D. K., Shido, K., Kopp, H. G., Petit, I., Shmelkov, S. V., Young, L. M., et al. (2006). Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes. *Nat. Med.* 12, 557–567. doi: 10.1038/nm1400

Johnston, C. J., Williams, J. P., Elder, A., Hernady, E., and Finkelstein, J. N. (2004). Inflammatory cell recruitment following thoracic irradiation. *Exp. Lung Res.* 30, 369–382. doi: 10.1080/01902140490438915

Jung, H., Kruger, H. J., Brammer, I., Zywietz, F., and Beck-Bornholdt, H. P. (1990). Cell population kinetics of the rhabdomyosarcoma R1H of the rat after single doses of X-rays. *Int. J. Radiat. Biol.* 57, 567–589.

Keith, B., Johnson, R. S., and Simon, M. C. (2012). HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. *Nat. Rev. Cancer* 12, 9–22. doi: 10.1038/nrc3183

Kioi, M., Vogel, H., Schultz, G., Hoffman, R. M., Harsh, G. R., and Brown, J. M. (2010). Inhibition of vasculogenesis, but not angiogenesis, prevents the recurrence of glioblastoma after irradiation in mice. *J. Clin. Invest.* 120, 694–705. doi: 10.1172/JCI40283

Knowles, H. J., and Harris, A. L. (2007). Macrophages and the hypoxic tumour microenvironment. *Front. Biosci.* 12, 4298–4314.

Koide, N., Nishio, A., Sato, T., Sugiyama, A., and Miyagawa, S. (2004). Significance of macrophage chemoattractant protein-1 expression and macrophage infiltration in squamous cell carcinoma of the esophagus. *Am. J. Gastroenterol.* 99, 1667–1674. doi: 10.1111/j.1572-0241.2004.30733.x

Kozin, S. V., Kamoun, W. S., Huang, Y., Dawson, M. R., Jain, R. K., and Duda, D. G. (2010). Recruitment of myeloid but not endothelial precursor cells facilitates tumor regrowth after local irradiation. *Cancer Res.* 70, 5679–5685. doi: 10.1158/0008-5472.CAN-09-4446

Lambert, L. E., and Paulnock, D. M. (1987). Modulation of macrophage function by gamma-irradiation. Acquisition of the primed cell intermediate stage of the macrophage tumoricidal activation pathway. *J. Immunol.* 139, 2834–2841.

Leek, R. D., Lewis, C. E., Whitehouse, R., Greenall, M., Clarke, J., and Harris, A. L. (1996). Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res.* 56, 4625–4629.

Lewis, C., and Murdoch, C. (2005). Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies. *Am. J. Pathol.* 167, 627–635. doi: 10.1016/S0002-9440(10)62038-X

Lewis, C. E., and Pollard, J. W. (2006). Distinct role of macrophages in different tumor microenvironments. *Cancer Res.* 66, 605–612. doi: 10.1158/0008-5472.CAN-05-4005

Lewis, J. S., Landers, R. J., Underwood, J. C., Harris, A. L., and Lewis, C. E. (2000). Expression of vascular endothelial growth factor by macrophages is up-regulated in poorly vascularized areas of breast carcinomas. *J. Pathol.* 192, 150–158. doi: 10.1002/1096-9896(2000)9999:9999<::AID-PATH687>3.0.CO;2-G

Li, F., Sonveaux, P., Rabbani, Z. N., Liu, S., Yan, B., Huang, Q., et al. (2007). Regulation of HIF-1alpha stability through S-nitrosylation. *Mol. Cell* 26, 63–74. doi: 10.1016/j.molcel.2007.02.024

Lin, E. Y., and Pollard, J. W. (2004). Role of infiltrated leucocytes in tumour growth and spread. *Br. J. Cancer* 90, 2053–2058. doi: 10.1038/sj.bjc.6601705

Lu, T., Ramakrishnan, R., Altiok, S., Youn, J. I., Cheng, P., Celis, E., et al. (2011). Tumor-infiltrating myeloid cells induce tumor cell resistance to cytotoxic T cells in mice. *J. Clin. Invest.* 121, 4015–4029. doi: 10.1172/JCI45862

Mantovani, A. (1978). Effects on *in vitro* tumor growth of murine macrophages isolated from sarcoma lines differing in immunogenicity and metastasizing capacity. *Int. J. Cancer* 22, 741–746.

Mantovani, A., Allavena, P., and Sica, A. (2004). Tumour-associated macrophages as a prototypic type II polarised phagocyte population: role in tumour progression. *Eur. J. Cancer* 40, 1660–1667. doi: 10.1016/j.ejca.2004.03.016

Mantovani, A., Bottazzi, B., Colotta, F., Sozzani, S., and Ruco, L. (1992). The origin and function of tumor-associated macrophages. *Immunol. Today* 13, 265–270. doi: 10.1016/0167-5699(92)90008-U

Mantovani, A., Sica, A., Allavena, P., Garlanda, C., and Locati, M. (2009). Tumor-associated macrophages and the related myeloid-derived suppressor cells as a paradigm of the diversity of macrophage activation. *Hum. Immunol.* 70, 325–330. doi: 10.1016/j.humimm.2009.02.008

Martinez, F. O., Helming, L., and Gordon, S. (2009). Alternative activation of macrophages: an immunologic functional perspective. *Annu. Rev. Immunol.* 27, 451–483. doi: 10.1146/annurev.immunol.021908.132532

McKinney, L. C., Aquilla, E. M., Coffin, D., Wink, D. A., and Vodovotz, Y. (2000). Ionizing radiation potentiates the induction of nitric oxide synthase by interferon-gamma and/or lipopolysaccharide in murine macrophage cell lines. Role of tumor necrosis factor-alpha. *Ann. N.Y. Acad. Sci.* 899, 61–68.

Milas, L. (1990). Tumor bed effect in murine tumors: relationship to tumor take and tumor macrophage content. *Radiat. Res.* 123, 232–236.

Milas, L., Wike, J., Hunter, N., Volpe, J., and Basic, I. (1987). Macrophage content of murine sarcomas and carcinomas: associations with tumor growth parameters and tumor radiocurability. *Cancer Res.* 47, 1069–1075.

Modolell, M., Corraliza, I. M., Link, F., Soler, G., and Eichmann, K. (1995). Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. *Eur. J. Immunol.* 25, 1101–1104. doi: 10.1002/eji.1830250436

Moeller, B. J., Cao, Y., Li, C. Y., and Dewhirst, M. W. (2004). Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules. *Cancer Cell* 5, 429–441.

Mosser, D. M. (2003). The many faces of macrophage activation. *J. Leukoc. Biol.* 73, 209–212.

Movahedi, K., Laoui, D., Gysemans, C., Baeten, M., Stange, G., Van den Bossche, J., et al. (2010). Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. *Cancer Res.* 70, 5728–5739. doi: 10.1158/0008-5472.CAN-09-4672

Munder, M., Eichmann, K., and Modolell, M. (1998). Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4+ T cells correlates with Th1/Th2 phenotype. *J. Immunol.* 160, 5347–5354.

Murdoch, C., Giannoudis, A., and Lewis, C. E. (2004). Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood* 104, 2224–2234. doi: 10.1182/blood-2004-03-1109

Murdoch, C., Muthana, M., Coffelt, S. B., and Lewis, C. E. (2008). The role of myeloid cells in the promotion of tumour angiogenesis. *Nat. Rev. Cancer* 8, 618–631. doi: 10.1038/nrc2444

Murdoch, C., Tazzyman, S., Webster, S., and Lewis, C. E. (2007). Expression of Tie-2 by human monocytes and their responses to angiopoietin-2. *J. Immunol.* 178, 7405–7411.

Nagaraj, S., and Gabrilovich, D. I. (2010). Myeloid-derived suppressor cells in human cancer. *Cancer J.* 16, 348–353. doi: 10.1097/PPO.0b013e3181eb3358

Nelson, E. L., and Becker, J. R. (1959). The effect of whole-body x irradiation on the bactericidal activity of phagocytic cells. I. Survival of Pseudomonas aeruginosa within phagocytes from peritoneal exudates of mice. *J. Infect. Dis.* 104, 13–19.

Nemoto, K., Ishihara, H., Tanaka, I., Suzuki, G., Tsuneoka, K., Yoshida, K., et al. (1995). Expression of IL-1 beta mRNA in mice after whole body X-irradiation. *J. Radiat. Res.* 36, 125–133.

Nishie, A., Ono, M., Shono, T., Fukushi, J., Otsubo, M., Onoue, H., et al. (1999). Macrophage infiltration and heme oxygenase-1 expression correlate with angiogenesis in human gliomas. *Clin. Cancer Res.* 5, 1107–1113.

O'Brien-Ladner, A., Nelson, M. E., Kimler, B. F., and Wesselius, L. J. (1993). Release of interleukin-1 by human alveolar macrophages after *in vitro* irradiation. *Radiat. Res.* 136, 37–41.

Ogawa, K., Boucher, Y., Kashiwagi, S., Fukumura, D., Chen, D., and Gerweck, L. E. (2007). Influence of tumor cell and stroma sensitivity on tumor response to radiation. *Cancer Res.* 67, 4016–4021. doi: 10.1158/0008-5472.CAN-06-4498

Peel, D. M., and Coggle, J. E. (1980). The effect of X irradiation on alveolar macrophages in mice. *Radiat. Res.* 81, 10–19.

Pollard, J. W. (2004). Tumour-educated macrophages promote tumour progression and metastasis. *Nat. Rev. Cancer* 4, 71–78. doi: 10.1038/nrc1256

Polverini, P. J., Cotran, P. S., Gimbrone, M. A. Jr., and Unanue, E. R. (1977). Activated macrophages induce vascular proliferation. *Nature* 269, 804–806.

Pribnow, J. F., and Silverman, M. S. (1967). Studies on the radiosensitive phase of the primary antibody response in rabbits. I. The role of the macrophage. *J. Immunol.* 98, 225–229.

Priceman, S. J., Sung, J. L., Shaposhnik, Z., Burton, J. B., Torres-Collado, A. X., Moughon, D. L., et al. (2010). Targeting distinct tumor-infiltrating myeloid cells by inhibiting CSF-1 receptor: combating tumor evasion of antiangiogenic therapy. *Blood* 115, 1461–1471. doi: 10.1182/blood-2009-08-237412

Pucci, F., Venneri, M. A., Biziato, D., Nonis, A., Moi, D., Sica, A., et al. (2009). A distinguishing gene signature shared by tumor-infiltrating Tie2-expressing monocytes, blood "resident" monocytes, and embryonic macrophages suggests common functions and developmental relationships. *Blood* 114, 901–914. doi: 10.1182/blood-2009-01-200931

Qian, B. Z., and Pollard, J. W. (2010). Macrophage diversity enhances tumor progression and metastasis. *Cell* 141, 39–51. doi: 10.1016/j.cell.2010.03.014

Ruffell, B., Affara, N. I., and Coussens, L. M. (2012). Differential macrophage programming in the tumor microenvironment. *Trends Immunol.* 33, 119–126. doi: 10.1016/j.it.2011.12.001

Sablonniere, B., Nicolas, J., Neveux, Y., and Drouet, J. (1983). Effect of whole-body irradiation on phagocytic activity of rat alveolar macrophages. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.* 44, 575–584.

Schioppa, T., Uranchimeg, B., Saccani, A., Biswas, S. K., Doni, A., Rapisarda, A., et al. (2003). Regulation of the chemokine receptor CXCR4 by hypoxia. *J. Exp. Med.* 198, 1391–1402. doi: 10.1084/jem.20030267

Sherman, M. L., Datta, R., Hallahan, D. E., Weichselbaum, R. R., and Kufe, D. W. (1991). Regulation of tumor necrosis factor gene expression by ionizing radiation in human myeloid leukemia cells and peripheral blood monocytes. *J. Clin. Invest.* 87, 1794–1797. doi: 10.1172/JCI115199

Shieh, Y. S., Hung, Y. J., Hsieh, C. B., Chen, J. S., Chou, K. C., and Liu, S. Y. (2009). Tumor-associated macrophage correlated with angiogenesis and progression of mucoepidermoid carcinoma of salivary glands. *Ann. Surg. Oncol.* 16, 751–760. doi: 10.1245/s10434-008-0259-6

Shojaei, F., and Ferrara, N. (2008). Refractoriness to antivascular endothelial growth factor treatment: role of myeloid cells. *Cancer Res.* 68, 5501–5504. doi: 10.1158/0008-5472.CAN-08-0925

Shojaei, F., Wu, X., Malik, A. K., Zhong, C., Baldwin, M. E., Schanz, S., et al. (2007). Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. *Nat. Biotechnol.* 25, 911–920. doi: 10.1038/nbt1323

Solinas, G., Germano, G., Mantovani, A., and Allavena, P. (2009). Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. *J. Leukoc. Biol.* 86, 1065–1073. doi: 10.1189/jlb.0609385

Song, C. W., Sung, J. H., Clement, J. J., and Levitt, S. H. (1974). Vascular changes in neuroblastoma of mice following x-irradiation. *Cancer Res.* 34, 2344–2350.

Stephens, T. C., Currie, G. A., and Peacock, J. H. (1978). Repopulation of gamma-irradiated Lewis lung carcinoma by malignant cells and host macrophage progenitors. *Br. J. Cancer* 38, 573–582.

Stockmann, C., Doedens, A., Weidemann, A., Zhang, N., Takeda, N., Greenberg, J. I., et al. (2008). Deletion of vascular endothelial growth factor in myeloid cells accelerates tumorigenesis. *Nature* 456, 814–818. doi: 10.1038/nature07445

Stout, R. D., Jiang, C., Matta, B., Tietzel, I., Watkins, S. K., and Suttles, J. (2005). Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J. Immunol.* 175, 342–349.

Takanami, I., Takeuchi, K., and Kodaira, S. (1999). Tumor-associated macrophage infiltration in pulmonary adenocarcinoma: association with angiogenesis and poor prognosis. *Oncology* 57, 138–142. doi: 10.1159/000012021

Takeda, N., O'Dea, E. L., Doedens, A., Kim, J. W., Weidemann, A., Stockmann, C., et al. (2010). Differential activation and antagonistic function of HIF-{alpha} isoforms in macrophages are essential for NO homeostasis. *Genes Dev.* 24, 491–501. doi: 10.1101/gad.1881410

Talks, K. L., Turley, H., Gatter, K. C., Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J., et al. (2000). The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. *Am. J. Pathol.* 157, 411–421.

Thornton, S. C., Walsh, B. J., Bennett, S., Robbins, J. M., Foulcher, E., Morgan, G. W., et al. (1996). Both *in vitro* and *in vivo* irradiation are associated with induction of macrophage-derived fibroblast growth factors. *Clin. Exp. Immunol.* 103, 67–73.

Timke, C., Zieher, H., Roth, A., Hauser, K., Lipson, K. E., Weber, K. J., et al. (2008). Combination of vascular endothelial growth factor receptor/platelet-derived growth factor receptor inhibition markedly improves radiation tumor therapy. *Clin. Cancer Res.* 14, 2210–2219. doi: 10.1158/1078-0432.CCR-07-1893

Toge, H., Inagaki, T., Kojimoto, Y., Shinka, T., and Hara, I. (2009). Angiogenesis in renal cell carcinoma: the role of tumor-associated macrophages. *Int. J. Urol.* 16, 801–807. doi: 10.1111/j.1442-2042.2009.02377.x

Tsai, C. S., Chen, F. H., Wang, C. C., Huang, H. L., Jung, S. M., Wu, C. J., et al. (2007). Macrophages from irradiated tumors express higher levels of iNOS, arginase-I and COX-2, and promote tumor growth. *Int. J. Radiat. Oncol. Biol. Phys.* 68, 499–507. doi: 10.1016/j.ijrobp.2007.01.041

Vaupel, P., and Mayer, A. (2007). Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metastasis Rev.* 26, 225–239. doi: 10.1007/s10555-007-9055-1

Venneri, M. A., De Palma, M., Ponzoni, M., Pucci, F., Scielzo, C., Zonari, E., et al. (2007). Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. *Blood* 109, 5276–5285. doi: 10.1182/blood-2006-10-053504

Vodovotz, Y., Coffin, D., DeLuca, A. M., McKinney, L., Cook, J. A., Wink, D., et al. (1999). Induction of nitric oxide production in infiltrating leukocytes following *in vivo* irradiation of tumor-bearing mice. *Radiat. Oncol. Investig.* 7, 86–97. doi: 10.1002/(SICI)1520-6823(1999)7:2<86::AID-ROI4>3.0.CO;2-L

Welford, A. F., Biziato, D., Coffelt, S. B., Nucera, S., Fisher, M., Pucci, F., et al. (2011). TIE2-expressing macrophages limit the therapeutic efficacy of the vascular-disrupting agent combretastatin A4 phosphate in mice. *J. Clin. Invest.* 121, 1969–1973. doi: 10.1172/JCI44562

Werno, C., Menrad, H., Weigert, A., Dehne, N., Goerdt, S., Schledzewski, K., et al. (2010). Knockout of HIF-1alpha in tumor-associated macrophages enhances M2 polarization and attenuates their pro-angiogenic responses. *Carcinogenesis* 31, 1863–1872. doi: 10.1093/carcin/bgq088

Wood, G. W., and Gollahon, K. A. (1977). Detection and quantitation of macrophage infiltration into primary human tumors with the use of cell-surface markers. *J. Natl. Cancer Inst.* 59, 1081–1087.

Xu, J., Escamilla, J., Mok, S., David, J., Priceman, S. J., West, B. L., et al. (2013). Abrogating the protumorigenic influences of tumor-infiltrating myeloid cells byCSF1R signaling blockade improves the efficacy of radiotherapy in prostatecancer. *Cancer Res.* 73, 2782–2794. doi: 10.1158/0008-5472.CAN-12-3981

Yang, L., DeBusk, L. M., Fukuda, K., Fingleton, B., Green-Jarvis, B., Shyr, Y., et al. (2004). Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* 6, 409–421. doi: 10.1016/j.ccr.2004.08.031

Zeng, L., Ou, G., Itasaka, S., Harada, H., Xie, X., Shibuya, K., et al. (2008). TS-1 enhances the effect of radiotherapy by suppressing radiation-induced hypoxia-inducible factor-1 activation and inducing endothelial cell apoptosis. *Cancer Sci.* 99, 2327–2335. doi: 10.1111/j.1349-7006.2008.00943.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 March 2013; accepted: 11 June 2013; published online: 17 July 2013.

*Citation: Russell JS and Brown JM (2013) The irradiated tumor microenvironment: role of tumor-associated macrophages in vascular recovery. Front. Physiol. **4**:157. doi: 10.3389/fphys.2013.00157*

This article was submitted to Frontiers in Vascular Physiology, a specialty of Frontiers in Physiology.