Université de Montréal

Pro-inflammatory and angiogenic activities of VEGF and angiopoietins in murine sponge/Matrigel model

par

Tharsika Sinnathamby

Département de pharmacologie

Faculté de Médecine

Mémoire présenté à la Faculté des Études Supérieures en vue de l'obtention du grade de

Maître ès sciences (M.Sc.) en pharmacologie

Juin 2014

Université de Montréal

Faculté des Études Supérieures

Ce mémoire intitulé :

Pro-inflammatory and angiogenic activities of VEGF and angiopoietins in murine sponge/Matrigel model

a été évalué par un jury composé des personnes suivantes :

Jonathan LEDOUX , Ph.D.

Président-rapporteur

Martin G. SIROIS, Ph.D.

Directeur de Recherche

Jean-François GAUCHAT, Ph.D.

Membre du jury

RÉSUMÉ

La dérégulation de la formation et l'intégrité des vaisseaux sanguins peut conduire à un état pathologique tel qu'observé dans de nombreuses maladies ischémiques telles que: la croissance de tumeur solide, l'arthrite rhumatoïde, le psoriasis, les rétinopathies et l'athérosclérose. Par conséquent, la possibilité de moduler l'angiogenèse régionale chez les patients souffrant d'ischémie est cliniquement pertinente. Un élément clé dans l'induction de l'angiogenèse pathologique est une inflammation qui précède et accompagne la formation des nouveaux vaisseaux. Ce phénomène est démontré par l'augmentation de la perméabilité vasculaire et le recrutement de monocytes/ macrophages et cellules polynucléaires (neutrophiles). En collaboration avec d'autres groupes, nous avons montré que différents facteurs de croissance tels que le facteur de croissance endothélial vasculaire et les angiopoïétines peuvent non seulement promouvoir l'angiogenèse mais aussi induire diverses étapes connexes au processus de la réaction inflammatoire, y compris la synthèse et la libération des médiateurs inflammatoires et la migration des neutrophiles. Les objectifs de notre étude étaient d'adresser si le *vascular endothelial growth factor* (VEGF) et les angiopoïétines (Ang1 et Ang2) sont capables de promouvoir la formation des nouveaux vaisseaux sanguins au fil du temps et d'identifier la présence de différentes cellules inflammatoires dans ce processus. Des éponges d'alcool polyvinylique stérilisées et imbibées de Matrigel appauvri en facteur de croissance (contenant PBS, VEGF, Ang1 ou Ang2 (200 ng/200 µl)) ont été insérées sous la peau de souris C57/Bl6 anesthésiées. Les éponges ont ensuite été retirées aux jours 4, 7, 14 ou 21 après la procédure pour des analyses histologiques, immunohistologiques et cytométriques. La formation des nouveaux vaisseaux a été validée par la coloration au Trichrome de Masson et des analyses histologiques et immunohistologiques contre les cellules endothéliales (anti-CD31). De plus, la maturation des vaisseaux a été démontrée par la coloration séquentielle contre les cellules endothéliales (anti-CD31) et musculaires lisses (anti-alpha-actine). Nous avons effectué la même procédure pour caractériser le recrutement de neutrophiles (anti-MPO), et de macrophages (anti-F4/80). Afin de mieux délimiter la présence de différents sous-ensembles de leucocytes recrutés dans les éponges, nous avons utilisé une technique de cytométrie en flux sur des préparations de cellules isolées à partir de ces éponges. Nous avons observé que le VEGF et les angiopoïétines

favorisent le recrutement de cellules endothéliales et la formation de nouveaux vaisseaux plus rapidement qu'en présence de PBS. Une fois formé au jour 7, ces nouveaux vaisseaux restent stables en nombre, et ne subissent pas une réorganisation importante de leur surface. Ces vaisseaux maturent grâce au recrutement et au recouvrement par les cellules musculaires lisses des néovaisseaux. En outre, le micro-environnement angiogénique est composé de cellules inflammatoires, principalement de neutrophiles, macrophages et quelques cellules de type B et T. Donc, le VEGF, l'Ang1 et l'Ang2 induisent séparément la formation et la stabilisation de nouveaux vaisseaux sanguins, ainsi que le recrutement de cellules inflammatoires avec des puissances différentes et une action temps-dépendante dans un modèle d'éponge/Matrigel.

Mots-clés: VEGF, angiopoïétines, angiogenèse, maturation, inflammation, neutrophiles, macrophages

SUMMARY

A deregulation in blood vessel formation and integrity can lead to a pathological state as seen in many ischemic diseases such as tumor growth, rheumatoid arthritis, psoriasis, retinopathies and atherosclerosis. Therefore, the possibility to modulate regional angiogenesis in patients suffering from ischemia is clinically relevant. One key feature in the induction of pathological angiogenesis is that inflammation precedes and accompanies the formation of neovessels as evidenced by increased vascular permeability and the recruitment of monocytes/macrophages and neutrophils. Along with other groups, we have previously shown that selected growth factors, namely vascular endothelial growth factor (VEGF) and angiopoietins (Ang1 and Ang2) can not only promote angiogenesis but can also induce inflammatory responses, including the synthesis/release of inflammatory mediators and neutrophil migration. The objectives of our study were to address how VEGF and angiopoietins are capable of promoting the formation of neovessels over time and to identify the presence of different inflammatory cells in this event. Sterilized polyvinyl alcohol (PVA) sponges soaked in growth factor-depleted Matrigel containing PBS, VEGF, Ang1 or Ang2 (200 ng/200 μl) were subcutaneously inserted into anesthetized C57/Bl6 mice. The sponges were then removed at day 4, 7, 14 or 21 post-procedure for histological, immunohistological (IHC) and flow cytometric analyses. The formation of neovessels was validated by Masson's Trichrome staining and by IHC against endothelial cells (anti-CD31) and its maturation was elucidated by sequential IHC staining against endothelial cells and smooth muscle cells (anti-alpha-actin). Likewise, we performed IHC to characterize the recruitment of neutrophils (anti-MPO), and macrophages (anti-F4/80). To better delineate the presence of different leukocyte subsets recruited in the sponges, we utilized multicolor flow cytometry procedure on single cell preparation from the sponges. We observed that both VEGF and angiopoietins favors the recruitment of endothelial cells and the formation of new vessels more rapidly as compared to PBS. Once formed by day 7, these neovessels remain stable in number, do not undergo reorganization in their cross sectional area and mature through the recruitment and ensheathing of smooth muscle cells. In addition, the angiogenic micro-environment is comprised of inflammatory cells, mainly neutrophils, macrophages, and sparsly T and B cells. Hence, VEGF, Ang1 and Ang2 individually promote the formation and stabilisation of

neovessels and the venue of inflammatory cells with different potency in a temporal dependant manner in a sponge/Matrigel model.

Key words: VEGF, angiopoietins, angiogenesis, maturation, inflammation, neutrophils, macrophages

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

Ang:	angiopoietin
APCs:	antigen presenting cells
COX-2:	cyclooxygenase-2
CXCL:	(C-X-C motif) ligand
DC:	dendritic cell
EC:	endothelial cell
EGF:	epidermal growth factor
FGF:	fibroblast growth factor
HUVEC:	human umbilical vein endothelial cell
ICAM and VCAM:	intracellular and vascular cellular adhesion molecules, respectively
IL:	interleukin
LPS:	lipopolysaccharide
MHCII:	major histocompatibility class II
MMP:	metalloproteinase
MPO:	myeloperoxidase
NO:	nitric oxide
NRP:	neuropilin
PAF:	platelet-activating factor
PDGF:	platelet-derived growth factor
PECAM-1:	platelet endothelial-cell adhesion molecule-1
PI3K:	phosphoinositide 3-kinase
PlGF:	placenta growth factor
RTK:	receptor tyrosine kinase
SMC:	smooth muscle cell
Tie:	tyrosine kinase with immunoglobulin and epidermal growth factor homology domains
TNF:	tumor necrosis factor

VEGF:	vascular endothelial growth factor
VEGFR:	vascular endothelial growth factor receptor
WPB:	Weibel-Palade body

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my project supervisor, Dr. Martin G. Sirois, for giving me the wonderful opportunity to explore the intricate world of research. His patience, guidance, active participation and effort were essential to the birth of this document and to my formation as a student in scientific research. Thank you very much for your encouragements and motivations.

I am also grateful to Dr. Cheolho Cheong for his guidance as a collaborator of this project and for showing me new techniques in obtaining the objective of this thesis. All of his advices and suggestions have been greatly appreciated.

I owe my sincere thanks to the Department of pharmacology and to the Faculty of graduate studies of Université de Montréal for providing me with high quality education. I am especially indebted to thank the committee members, Dr. Jonathan Ledoux, Dr. Martin G. Sirois and Dr. Jean-François Gauchat, who are evaluating my thesis. Similarly, I would like to thank the Montreal Heart Institute for providing me a wonderful platform for a bright future. Moreover, I gratefully acknowledge the funding sources, Montreal Heart Institute and the department of pharmacology of Université de Montréal, which have made my M.Sc. work possible.

I would like to extent my appreciation to my fellow lab mates: Pauly, Elizabeth, Damien, Marie-Elaine and Diana for providing me a cordial working environment during these years. Thank you Pauly and Damien for the humor and the friendly sarcasms that always makes me laugh. Inevitably, the small everyday chit chats with Elizabeth and the coffee breaks with Diana will always be remembered. Finally, I devote my special gratitude to Marie-Elaine for her ideas in optimizing my staining and for her help during my experiments.

I would like to equally thank my parents for their love, care, guidance, encouragement and invaluable support throughout my academic trajectory. Their sacrifices have brought me to

where I am today. I would also like to thank my brother and sister for being my best friends all my life and for listening to both my enthusiasms and my disappointments. I love you guys.

Above all, I thank God for his many blessings.

I would like to dedicate my thesis to my parents.

1.0 INTRODUCTION

The endothelium of the vascular system is a disseminated organ forming a physical barrier between the vessel lumen and the extracellular space. It is comprised of quiescent endothelial cells (ECs) interconnected by junctional proteins and ensheathed by perivascular cells *(e.g.* pericytes and vascular smooth muscle cells (SMCs); Figure 1). ECs play a pivotal role in maintaining tissue homeostasis, which includes the controlling of vasomotor tone and cellular and molecular trafficking across quiescent cells, and in the maintenance of blood fluidity and vascular permeability [1] . In addition, ECs have been shown to participate as key players in tissue vascularization following injury in a process termed angiogenesis. They are also implicated in physiological inflammatory response where their surface adhesion glycoprotein (glycocalyx) expression allows transendothelial migration of blood cells and plasma proteins to the site of infection or injury [2]. Thus, the endothelium is not merely a static physical barrier but indeed an active cell system.

Apart from its various functions, ECs show remarkable heterogeneity in structure, time and space allowing the endothelium to mold itself based on the needs of the underlying tissue. In fact, the phenotype of ECs can differ between different organs, between different segments of a vascular loop and yet between adjacent cells in the same organ. Thus, the constant response of the endothelium to an array of agonists and environmental challenges can lead to, under certain circumstances, a perturbed state and contribute to the development of numerous vascular diseases involving angiogenesis (reviewed in [1]).

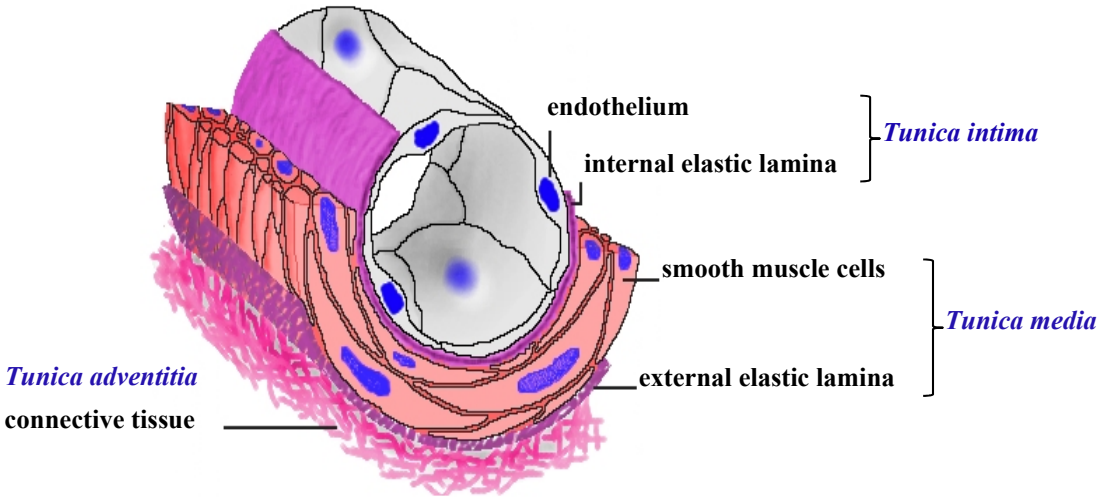


Figure 1: Anatomy of blood vessels. Blood vessels of the vascular network are comprised of three concentric layers (tunics). The inner layer called *tunica intima* is composed of endothelial cells and a thin layer of supporting connective tissue. The middle muscular and/or elastic layer, termed *tunica media*, is comprised of perivascular cells (pericytes and smooth muscle cells). Finally, the outer layer is comprised of fibrous connective tissue and is called *tunica adventitia*. (Adapted from http://www.siumed.edu/~dking2/crr/cvguide.htm Copyright © 2005, Board of Trustees, Southern Illinois University)

1.1 Angiogenesis

1.1.1 Origin and mechanism of blood vessel formation

The cardiovascular system, comprised of the heart and the circulatory system (the network of blood vessels), is the first functional system to develop in vertebrate embryo [3]. The luminal surface of the circulatory system is comprised of ECs derived from the mesoderm germ layer during development. Precisely, hemangioblasts in the yolk sac differentiate from mesodermal

progenitor cells giving rise to hematopoietic stem cells and angioblasts. Angioblasts in turn proliferates, migrates and differentiates into ECs assembling the primitive *de novo* vascular plexus of veins and arteries – a process termed vasculogenesis. Subsequent sprouting ensures expansion of the vascular network of larger vessels ramifying into smaller ones, known as angiogenesis (Figure 2). The latter is a tightly regulated process that occurs throughout life, from *utero* to old age, ensuring adequate oxygen and nutrient supply to all body cells.

Classic angiogenesis, or sprouting angiogenesis is a multistep process as detailed by Dr. Folkman in 1971 [4]. Overall, the steps in angiogenesis include enzymatic degradation of capillary basement membrane, EC proliferation, directed migration of ECs, EC tube formation and perivascular cell stabilization [5]. In fact, ECs are equipped with oxygen sensor for the maintenance of vascular homeostasis. Hence, local hypoxia due to poor tissue perfusion is detected by these sensors and initiates the angiogenic process. Firstly, the perivascular cells covering the pre-existing vessel detach from the basement membrane by metalloproteinase (MMP)-mediated proteolytic degradation. This loosens the EC junctions and dilates the nascent vessel. Simultaneously, the release of growth factors and inflammatory cytokines by ECs and inflammatory cells increases the permeability of EC layer causing the plasma proteins to extravasate and lay down a provisional extracellular matrix scaffold. Next, the proteases that are present at the angiogenic site remodel the extracellular matrix into an angio-competent milieu. One EC, known as the tip cell, is selected to lead the tip towards the angiogenic signal. The neighbors of the tip cell become stalk cells, which divide to elongate the stalk and establish the lumen. Interestingly, the tip cells are equipped with filopodia to sense environmental cues while the stalk cells release molecules to convey spatial information

about their position to their neighbors. Finally, the newly formed vessels become functional, mature and stable through the ensheathing of perivascular cells, entering a quiescent state. Once functional, occlusions in arteries can cause a pressure difference in the arterioles. This calls for the growth of collateral arteries or "natural bypass" from pre-existing arterio-arteriolar anastomoses to overcome the shear forces in a process termed arteriogenesis [6]. Although, angiogenesis remains a necessity under physiological circumstances, this complex process involving multiple factors and exerting specific activities at different phases, becomes a culprit under numerous pathological conditions.

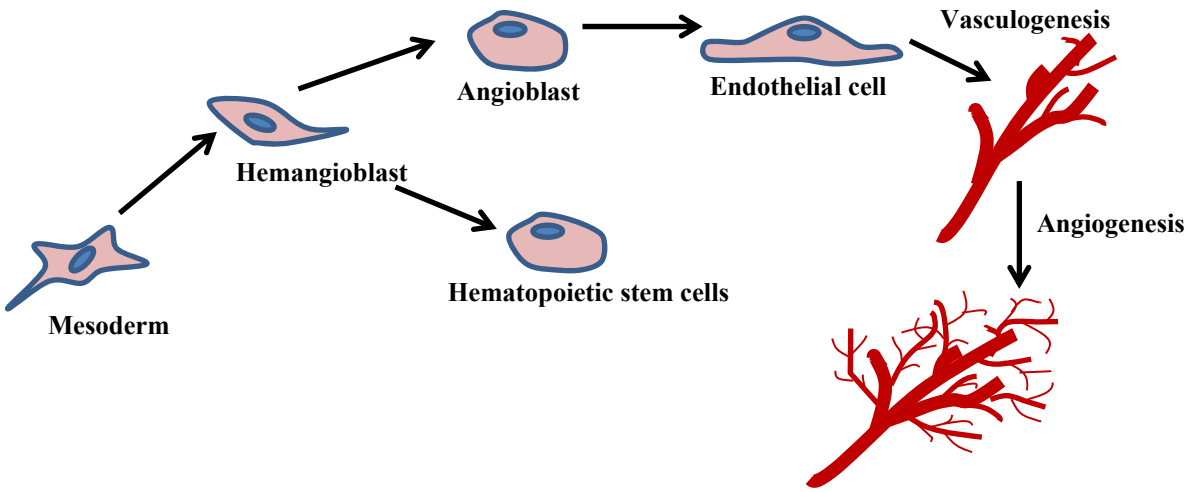


Figure 2: Schematic overview of vasculogenesis and angiogenesis. Extraembryonic mesoderm, located in the blood islands of the yolk sac, differentiates into hemangioblast giving rise to angioblast and hematopoietic stem cell lineages. Following the commitment to EC lineage, angioblasts gather and rearrange to form capillary-like tubes forming the primary circulatory network, termed vasculogenesis. Angiogenesis is the formation of neovessels from preexisting vessels which occurs both during embryogenesis and during an organism's life.

1.1.2 Physiological angiogenesis

In vertebrate development, angiogenesis plays a pivotal role in nourishing the growing organs with oxygen and in providing instructive trophic signals to promote organ morphogenesis [7]. Though angiogenesis continues to contribute to organ and somatic growth after birth, most blood vessels remain in a quiescent state as of adulthood. In fact, EC turnover in healthy adults is remarkably low. It is only reactivated under certain conditions namely during wound healing, intense physical activity and menstrual cycle.

In wound healing, a natural restorative response to tissue injury, angiogenesis takes place during the proliferative phase where it provides the necessary nutrients to sustain the newly formed tissue [8]. Likewise, during prolonged physical exercises, the increased oxygen demand in skeletal muscles is compensated in the long-term through the formation of new blood vessels [9]. Finally, physiological angiogenesis is fundamental to the female reproductive system (ovaries, uterus) and it is the only organ in adult humans which undergoes a regular cycle of growth and regression of blood vessels. Indeed, during the menstrual cycle, angiogenesis generally takes place in the growing *corpus luteum* and the endometrium. When fertilization does not occur, the endometrial lining is shed along with the newly formed blood vessels. However, upon fertilization, the placenta takes over the angiogenic process sustaining the developing embryo throughout pregnancy [10].

1.1.3 Pathological angiogenesis

Angiogenesis is a tightly regulated process that requires the maintenance of a balance between pro-angiogenic (stimulatory) and anti-angiogenic (inhibitory) factors. A perturbation in this equilibrium can result in either excessive angiogenesis or insufficient angiogenesis leading to a pathological state [11]. Numerous disorders have been associated with excessive angiogenesis including solid tumor, psoriasis, rheumatoid arthritis, retinopathy and atherosclerosis. High fat diet has also been demonstrated to promote angiogenesis in adipose tissue which in turn stimulates adipogenesis, the generation of adipocytes; hence, creating a vicious cycle [12, 13]. Alternately, other disorders have been identified for abnormal vessel regression and maturation such as purpura, scleroderma and nephropathy. Moreover, local loss of blood supply in patients results in tissue ischemia leading to death or disability.

Presently, several medications have been identified to inhibit angiogenesis [14-16] but efforts to therapeutically generate new blood vessels have not been as successful. Therefore, understanding the molecular mechanism of angiogenesis is crucial for the development of therapeutic strategies to combat these inflammatory, malignant and ischemic disorders.

1.2 Regulation of angiogenesis

Over the years, a plethora of endogenous mediators, including growth factors (vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-α and -β, fibroblast growth factor (FGF), epidermal growth factor (EGF) and angiopoietins), matrix

metalloproteinases (*e.g.* MMP-2, MMP-7, MMP-9 and MMP-12), cytokines (*e.g.* tumor necrosis factor; TNF-α), chemokines (interleukins; IL-8), and integrins (*e.g.* $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_5$ and $\alpha_v\beta_3$) have been discovered to regulate angiogenesis. Among these angiogenic factors, VEGF and angiopoietins are well-established to exhibit both distinct and overlapping expression patterns that collaborate to regulate the different stages of physiological angiogenesis. The following sections will summarize the properties and functions of these growth factors and their places in the angiogenic process.

1.2.1 Vascular endothelial growth factor (VEGF)

In 1989, VEGF (initially identified as vascular permeability factor (VPF) [17]) was isolated and identified [18] as a potent, diffusible and EC-specific mitogen. Its discovery arose the hypothesis that it may possess a significant role in the regulation of physiological and pathological growth of blood vessels [18-20]. Indeed, it is presently well-established that VEGF is the most potent, versatile and ubiquitous vascular growth factor known to date.

The VEGF family is comprised of seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and placenta growth factor (PlGF) all containing a highly preserved VEGF homology domain. VEGF-A, being the first one to be identified is a homodimeric glycoprotein of approximately 45 kDa [18]. In healthy adults, the VEGF-A mRNA is highly expressed in lung, kidney, heart and adrenal gland and marginally expressed in liver, spleen and gastric mucosa [21]. It is also expressed by cultured SMCs [22], macrophages [23] and ECs [24]. At the cellular level, VEGF-A functions as a major paracrine

regulator of the angiogenic response, which modulates EC proliferation, migration, sprouting and survival [25].

The human gene coding for VEGF-A is localized on chromosome 6p21.3 [26] and consists of eight exons separated by seven introns [27, 28]. Alternative exon splicing of this gene results in the generation of four major isoforms: VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189} and VEGF-A_{206} [27, 28] (Figure 3). Other less frequent splice variants such as VEGF-A_{145} [29], VEGF-A_{183} [30], VEGF-A_{162} [31] and VEGF-A_{165b} [32] have also been reported. Exons 1 to 5 are conserved in all isoforms. The splice variation among the isoforms depends on the presence or absence of exon 6 and 7 which encode distinct heparin binding domains. The acidic polypeptide, VEGF-A_{121}, lacking both exons 6 and 7 does not bind to heparin and is highly diffusible [33, 34]. The highly basic variants, $VEGF_{189}$ and $VEGF_{206}$ contain both exon 6 and exon 7 and bind heparin with high affinity [33]; hence they are completely sequestered in the extracellular matrix. $VEGF_{165}$ being the predominant isoform [33] lacks exon 6 but contains exon 7 in its coding sequence. It is only moderately diffusible while a significant amount of it remains bound to the cell surface of the extracellular matrix [35]. The difference in the diffusibility of these splice variants create a gradient of VEGF expression that is responsible for guiding and shaping the vascular network during angiogenesis.

Although structurally similar to VEGF-A, the other members of the VEGF family with their splice variants is differentially expressed in many cell types and display different biological activities. For example, PlGF in adults is a master switch in pathological angiogenesis. It is predominantly expressed in the placenta, heart and lungs [36]. Indeed, mice

lacking PlGF gene are phenotypically inert but show impaired angiogenesis, plasma extravasation and collateral growth during ischemia, inflammation, wound healing and cancer [37]. In contrast, PlGF overexpressed transgenic mice produce increased vascularization, inflammation and vascular permeability in the skin [38, 39]. VEGF-B is abundantly expressed in adult myocardium, skeletal muscle and pancreas [40]. Its expression is also observed in developing heart, brown fat, muscle and spinal cord during mouse embryogenesis. VEGF-B is postulated to be involved in controlling VEGF-A bioavailability; its exact function however, is unknown [21]. The expression of VEGF-C is exclusive in regions of developing lymphatic vessels and in lymph nodes [42, 43]; hence, its function is associated with the lymphatic system during development. In adult tissues however, VEGF-C is expressed in heart, placenta, ovary, small intestine and thyroid gland [21]. VEGF-D is largely identified in the lung and skin during embryogenesis [44] and in the lung, heart, skeletal muscle, colon and small intestine in adults. In humans, expression of VEGF-D is used as a prognostic marker for lymphatic metastasis [45]. Lastly, the VEGF-E isoform is encoded by Orf virus and is involved in inducing pathological angiogenesis in virus-infected lesions [46]. The later splice variant does not have a mammalian homologue.

The VEGF isoforms stimulate various cellular responses by binding to VEGF receptors. VEGF receptors belong to class V receptor tyrosine kinases (RTK) and is comprised of VEGFR-1 (also known as Flt-1; 180kDa), VEGFR-2 (also known as KDR or Flk-1; 200-230kDa) and VEGFR-3 (also known as Flt-4; 195kDa). Each receptor is constructed of seven immunoglobulin-like domains in the extracellular domain, a single transmembrane domain and a consensus tyrosine kinase sequence containing intracellular domain [47]. The signaling

by VEGF receptors is initiated upon the binding of a covalently linked VEGF dimer to the extracellular receptor domain. This interaction promotes receptor homo- and heterodimerization (VEGFR-1 with VEGFR-2 and VEGFR-2 with VEGFR-3) followed by kinase activation and autophosphorylation of specific tyrosine residues located in the intracellular juxtamembrane domain, the kinase insert domain, and the carboxylic tail of the receptor [48]. Subsequently, a variety of signaling molecules interact at specific sites of the VEGF receptor dimers to activate distinct downstream cellular pathways [49].

Among the three receptors, VEGFR-2 is considered to be the major mediator of many physiological effects of VEGF-A on ECs. It is expressed on vascular ECs, lymphatic cells, megakaryocytes and haematopoietic stem cells [50] and binds VEGF-A, the processed form of VEGF-C and VEGF-D, and VEGF-E. VEGFR-2 signaling is modulated through co-receptors termed heparin sulfated proteoglycans, which also interact with several isoforms of VEGF [51]. In fact, neuropilin-1 (NRP-1), one such modulator, is the primary co-receptor of VEGF-A/VEGFR-2 ligand/receptor complex [52]. Although, this putative receptor was previously identified to bind collapsin/semaphorin family mediating neuronal guidance, it is now evident that NRP-1 can also amplify the effectiveness of VEGF-A/VEGFR-2 signal transduction [53]. NRP-1 is highly expressed in vascular ECs and its presence in these cells promotes VEGFR-2 phosphorylation and activation. Indeed, our laboratory has demonstrated that NRP-1 enhances VEGF-A/VEGFR-2-mediated EC migration and proliferation, and platelet activating factor (PAF) synthesis, a pro-inflammatory molecule [54].

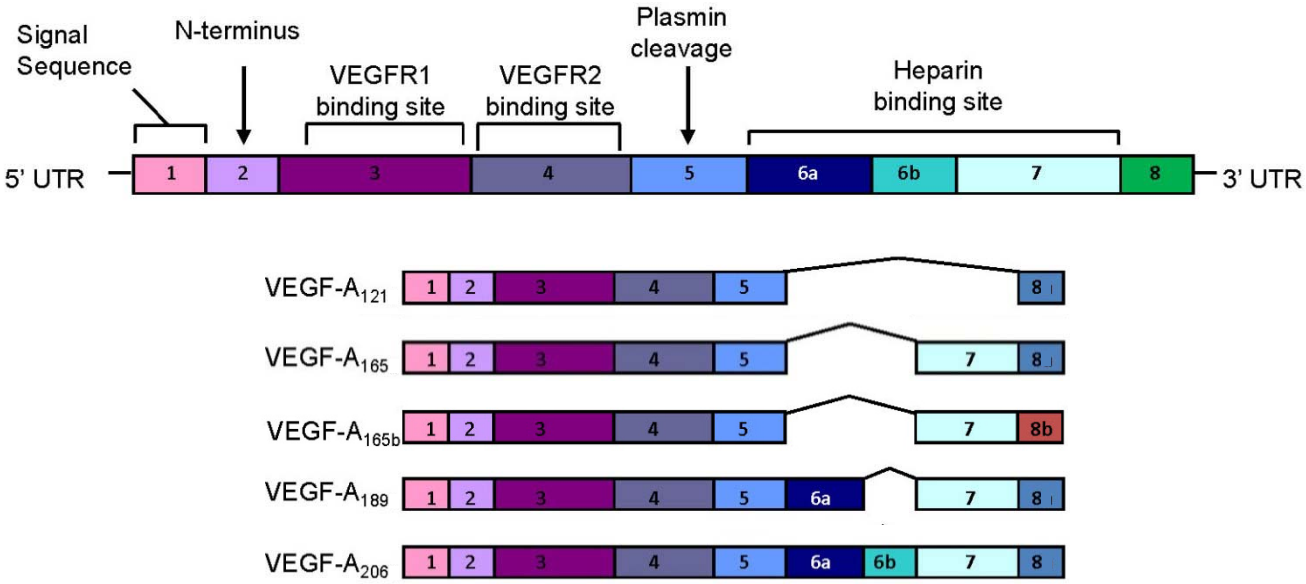


Figure 3: Schematic representation of predominant isoforms of VEGF-A in humans. The different exon (indicated with numbers) compositions as a result of alternative splicing gives rise to the various isoforms of VEGF-A. Exons 1 to 5 are conserved in all VEGF-A splice variants. Exon 3 is implicated in the homodimerization of VEGF-A and in its interaction with VEGFR-1 while exon 4 is required for the binding of VEGF-A onto VEGFR-2. Exons 6 and 7 are both involved in binding heparin. Exon 7 additionally links VEGF-A onto their co-receptor neuropilin-1 (NRP-1). Finally, exon 8b is present only on inhibitory isoforms of VEGF-A. (Adapted from Fearnley, G.W. et *al.* 2013 [41])

1.2.1.1 VEGF in angiogenesis

VEGF-A (here after referred to VEGF) has been revealed to induce angiogenesis in a variety of *in vivo* and *in vitro* models. Knockout mice containing one allele of VEGF exhibit a number of development anomalies such as defective vascularization in several organs and reduced

nucleated red blood cell number within the blood islands in the yolk sac [55]. Consequently, these mice die within the first few days of development. Yet more, partial inhibition of VEGF using inducible gene targeting in early postnatal life leads to stunted body growth and impaired organ development leading to death in mice [56]. Similarly in adults, VEGF elicits pronounced angiogenesis in various *in vivo* models including the rabbit cornea [57], the primate iris [58] and the Matrigel plug in mice [59]. Concomitantly, *in vitro* studies revealed the ability of VEGF to promote the growth of vascular ECs derived from arteries, veins and lymphatics [18, 19, 60-64].

It is now evident that VEGF regulates and orchestrates the angiogenic cascade (EC proliferation, survival, migration and permeability) through activated VEGFR-2 post-receptor signaling pathways. The binding of VEGF-A to VEGFR-2 results in the autophosphorylation of the following tyrosine residues in the intracellular domain of the receptor: Tyr951 and Tyr996 (located in the kinase domain), Tyr1054 and Tyr1059 (located in the kinase domain), and Tyr1175 and Tyr1214 (located in the C-terminal tail) (Figure 4). Phosphorylation of Tyr951 and Tyr1175 creates a binding site for VEGFR-associated protein (VRAP), and Sck [65] and PLCγ_1 [66], respectively, which subsequently activate Raf *via* protein kinase C (PKC) in a Ras-independent manner [67]. This in turn induces the activation of the extracellular regulated kinase (ERK) pathway (p42/44 mitogen activated protein kinase (MAPK)) leading to gene transcription required for EC proliferation. VEGFR-2 also activates phosphoinositide 3-kinase (PI3K), which results in an increase of lipid phosphatidylinositol (3,4,5)P_3 (PIP_3), conducting the activation of protein kinase B (Akt/PKB) and small GTP-binding protein, Rac. The Akt/PKB pathway, 1) promotes EC survival by inhibiting pro-apoptotic factors such as B-

cell lymphoma-2 associated death promoter homologue (BAD) and caspase-9 [68], and 2) increases vascular permeability and cellular migration through the activation of endothelial nitric oxide synthase (eNOS) [69, 70]. Rac [71], on the other hand, along with other adapter proteins including p38MAPK [72] and focal kinase (FAK) with its substrate paxillin [73] have been demonstrated to promote cellular migration. In addition, the activation of VEGFR-2 on ECs promotes the production of PAF by ECs, which apart from its role during inflammation (involved in promoting the rolling and adhesion of leukocytes) also potentiate the migration of cultured ECs and increase vascular permeability [54]. Although the exact mechanism is not known, Src family members interact with activated VEGFR-2 and induce vascular permeability in mice [74].

In addition to its vast activities on the endothelium *via* VEGFR-2, VEGF is also responsible for guiding and shaping of the vascular tree during the angiogenic process [75-77]. It does so through the differential spatial distribution of VEGF isoforms in the extracellular space which creates a gradient of VEGF expression. Such gradient is crucial for selecting the tip cells and the stalk cells during the sprouting of nascent blood vessels. This patterning is controlled at the level of transcription, isoform splicing and cell surface retention. For instance, the splice variants that bind heparin remain on the cell surface or in the extracellular matrix, while the splice variants lacking retention motif diffuse away from the surface. Furthermore, the transcriptional level of VEGF determines the site of sprouting. For instance, tip cell formation and sprouting only occurs at regions of highest VEGF concentration *via* the induction of gene expression of the NOTCH signaling pathway ligand Delta-like ligand 4 (Dll4) [78, 79]. Dll4 expression is restricted to developing arteries and at the tip of vascular

sprouts [80, 81] and it is almost never expressed on the neighboring stalk cells. Together, the differential expression of Dll4 mediated by VEGF gradient favors the appropriate formation of filopodia, sprouting and branching of nascent vessels creating functional blood vessels at the end of an angiogenic response.

1.2.2 Angiopoietins

Angiopoietins, identified in mid 1990s, are a novel class of angiogenic growth factors that exert a crucial regulatory role in the maintenance of vascular integrity and quiescence. In fact, the different cellular functions of angiopoietins (responsible for the assembling and disassembling of the EC lining of blood vessels) in concert with VEGF coordinate precise morphogenic events in angiogenesis.

1.2.2.1 Structure, localization and function of Ang1 and Ang2

The angiopoietin protein family is comprised of four members, Ang1, Ang2, Ang3 and Ang4 (Figure 5), all of which having a size of about 500 amino acids [83-85]. Structurally, the angiopoietins are composed of two domains: a fibrinogen-like C-terminal domain and an alpha-helical rich coiled-coil N-terminal domain [86]. The receptor binding sequence located in the fibrinogen-like domain of these growth factors enables their binding to the receptor **t**yrosine kinase with **i**mmunoglobulin and **e**pidermal growth factor homology domains 2 (Tie2) while the coiled-coil motif promotes angiopoietin oligomerisation, a prerequisite for Tie2 receptor activation [86].

The human gene coding for Ang1 and Ang2, the predominant isoforms, is localized on chromosome 8q22 and 8q23, respectively [87, 88]. Alternative exon splicing of the Ang1 gene results in the generation of four isoforms: Ang1-1.5 kb (the prototypic form), Ang1-1.3 kb, Ang1-0.9 kb and Ang1-0.7 kb [89]. The Ang1 splice variants, Ang1-1.3 kb, Ang1-0.9 kb and Ang1-0.7 kb, bind Tie2 with similar potency but do not activate Tie2 signaling. Instead, they serve as a dominant negative regulator of the full length Ang1 (Ang1-1.5 kb) activity, which both binds and activates Tie2 [89]. Similarly, alternative exon splicing of the Ang2 gene gives rise to a splice variant of 443 amino acids ($Ang2_{443}$) [90]. It lacks part of its coiled-coil motif and as a consequence renders it incapable of inducing Tie2 autophosphorylation upon its binding. $Ang2_{443}$ nonetheless, pre-occupies the angiopoietin binding site on Tie2 and prevents the binding of the full length Ang1 and Ang2 to their receptor; hence, sequestering Tie2 activation [90]. Recently, an additional isoform of Ang2 ($Ang2_{2B}$) with a partial truncated amino terminal coiled-coil domain has also been identified in chicken as a result of 5' intron alternative splicing [91].

In adults, Ang1 is constitutively expressed in pericytes, SMCs and fibroblasts [83]. Its expression in these cells enables them to regulate the adjacent endothelium through a paracrine interaction. In addition, Ang1 expression has also been noted in neuronal cells [92] and in some types of tumor cells [92, 93]. During embryonic development however, Ang1 expression is temporal dependent. It is first expressed in the myocardium and later in mesenchymal cells surrounding the developing vessels [84, 94, 95]. Recently, our laboratory discovered the expression of Ang1 in the cytoplasm of neutrophils [96]. In contrast, presynthesized Ang2 is almost exclusively stored within the storage granules of ECs termed

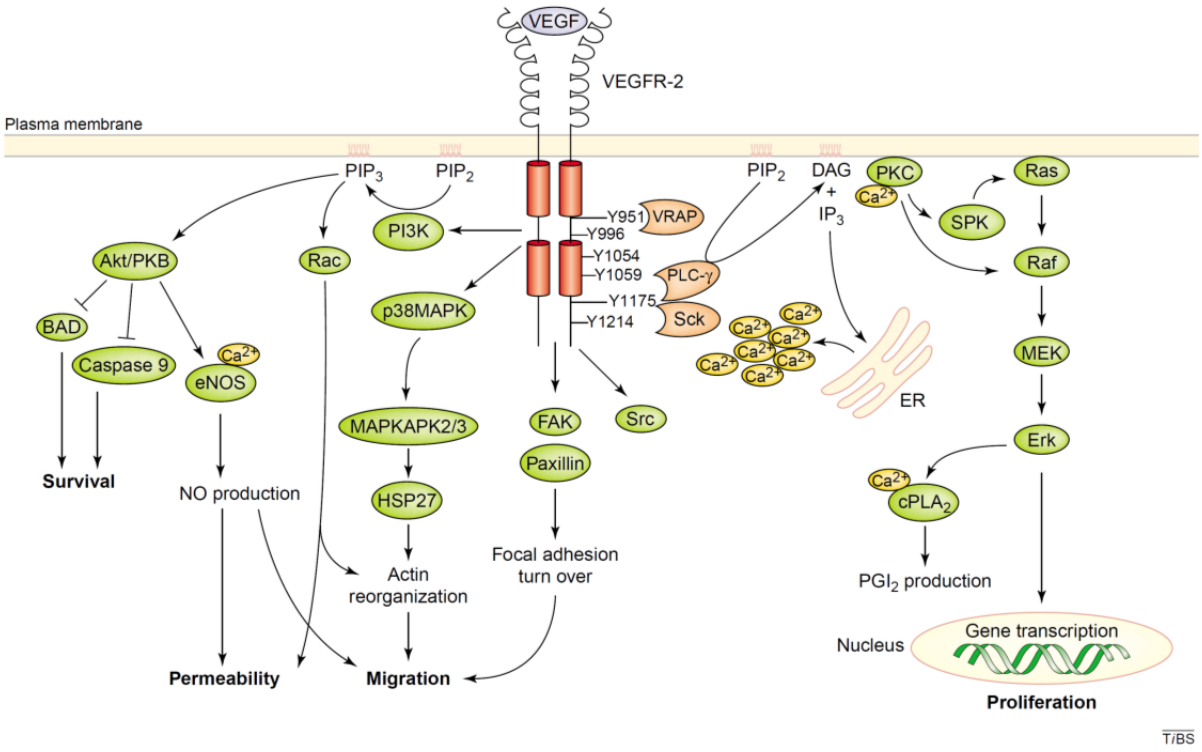


Figure 4: Schematic representation of the intracellular pathways activated through VEGFR-2. Upon the activation of VEGFR-2 receptors on ECs, adopter proteins including VEGFR-associated protein (VRAP), Sck and PLC-γ bind specific tyrosine residues on the receptors *via* their SH2 domain. Their binding in turn phosphorylates and activates the aforementioned adaptor proteins. Activation of PLC-γ induces the second messengers, DAG and IP_3. While DAG activates PKC, IP_3 induces the release of Ca^{2+} and in turn act on the endoplasmic reticulum. In addition, other proteins are also activated by VEGFR-2 such as Src, PI3K, FAK and p38MAPK. Together, these signaling pathways promote EC migration, proliferation, survival and vascular permeability. (Adapted from Cross, M.J. et *al.* 2003 [82])

Weibel-Palade bodies (WPB) that allows the rapid release of Ang2 in response to various stimuli [97]. The release of Ang2 by activated ECs operates as a built-in autocrine switch, which promotes the transition of the quiescent endothelium to an activated state. For instance, under physiological conditions, Ang2 is only strongly upregulated in regions undergoing vascular remodeling such as during the menstrual cycle and wound healing [98]. Their expression is also elevated in tumor cells [99-101] and in ECs of tumor blood vessels [93, 102, 103]. Furthermore, marginal level of Ang2 expression has been detected in Kaposi's sarcoma cells [104] and in Muller cells of the retina [105].

Although the well-characterized ligands, Ang1 and Ang2, bind their receptor Tie2 at the same site with similar affinities, Ang2 was originally identified as a competitive antagonist of Ang1/Tie2 ligand/receptor signaling axis in ECs [97, 106-111]. It is now apparent that the biology of Ang2 is less straight-forward; it can behave both, as an agonist or as an antagonist of Tie2 signaling depending on the concentration and the spatial-temporal context it is in. Indeed, Ang2 can activate Tie2 receptor on ECs promoting its migration, survival and capillary tube formation at high concentration [112] or after prolonged incubation [113]. Interestingly, recent studies in our laboratory have identified other agonistic activities of Ang2/Tie2 complex both on ECs (*e.g.* promotes PAF synthesis, P-selectin translocation, neutrophil adhesion) and on neutrophils (*e.g.* promotes PAF synthesis, CD11b/CD18 integrin activation and its migration) following a short incubation and at same concentration as Ang1 [115-118]. We have observed that neutrophils treated with either Ang1 or Ang2 alone for 7.5 minutes promotes PAF synthesis at a concentration of 10^{-9} M [115]. Nonetheless, on normal vascular endothelium, Ang2 acts as a Tie2 antagonist to destabilize the endothelium and

empowers the initiation of the angiogenic response [95, 119-121]. Ang1 on the other hand, is an agonistic ligand of the Tie2 receptor where its binding to Tie2 contributes to blood vessel maturation and stability. In addition to the same agonistic effects exerted by Ang2 on ECs and neutrophils, Ang1 also promotes the survival of ECs and neutrophils, inhibits VEGF-mediated adhesion molecule (intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin) increase on ECs and promotes the synthesis of IL-8 and IL-1β [122, 123]. In fact, the group of Yu, X. et *al* identified a short loop within the angiopoietin fibrinogen domain, which may potentially confer the differential agonistic and antagonistic properties of these biologically important molecules [124].

1.2.2.2 Structure, localization and function of Ang3 and Ang4

In 1999, Valenzuela et *al*. reported the existence of Ang3 and Ang4, inter-species homologues found in mice and humans, respectively [85]. These orthologues, sharing 65% amino acid homology, are found at the same gene locus in mice and humans [85]. Similar to Ang1 and Ang2, the protein structure of Ang3 and Ang4 contains an N-terminal coiled-coil domain and a C-terminal fibrinogen domain. Both Ang3 and Ang4 are predominately synthesized as dimers connected by disulfide bonds, which resemble the native structure of Ang2 [83, 85]. Although, much less is known about them till this date, the expression of Ang3 has been identified to be distributed in multiple tissues in mice while the expression of Ang4 has been identified to be restricted to the lungs in humans [85]. However, the expression of Ang4 can be significantly increased in response to hypoxia and to various growth factors [125,

126]. Likewise, Ang3 expression in rats has also been demonstrated to be enhanced in lungs, brain and heart in response to hypoxia [127].

Ang3 and Ang4 exert similar activities upon their binding to Tie2 receptor [128]. Initially, Ang3 was characterized as an antagonist of Tie2 based on the observation that it, when expressed in a chimeric form, inhibited the activity of Ang1 in humans [85]. However, an *in vivo* study using murine corneal micropocket assay revealed that these orthologs (Ang3 and Ang4) both have the capacity to induce angiogenesis [128]; hence, classifying them as Tie2 receptor agonists [85]. In fact, it is now accepted that the action of Ang3 is context-dependent which can act both as an agonist and as an antagonist while Ang4 is an agonistic ligand of Tie2.

1.2.2.3 Structure, localization and regulation of Tie receptors

In the early 1990s, a novel class of RTK, termed Tie receptors, was identified consisting of two members, Tie1 (135 kDa) and Tie2 (150 kDa) [129-131]. These receptors of ≈1100 amino acids consist of an extracellular, transmembrane and an intracellular/cytoplasmic domain and are expressed on both vascular and lymphatic EC surface. The extracellular amino-terminal domain of Tie receptors is comprised of three EGF-like cysteine rich repeats, two immunoglobulin-like domains flanking the EGF-like repeats and three fibronectin-type III repeats. The intracellular portion of Tie receptors contains two highly conserved tyrosine kinase domains with 76% sequence homology that allows the activation of various downstream effectors following its autophoshorylation [129-133]. Although structurally

similar, the extracellular portions of these receptors demonstrate numerous differences (sequence homology of 33%) [133].

During the early stages of development, Tie1 is expressed in differentiating angioblasts of the head mesenchyme, in the splanchnopleura (a layer of embryonic cells lining the walls of the visceral organs formed by the association of mesoderm and endoderm), dorsal aorta and in migrating ECs of the developing heart [134]. In adults, Tie1 expression is predominantly observed on EC surface along with Tie2 [135]. While the angiopoietins have been identified as the ligands of Tie2 receptor, Tie1 remains as an orphan receptor. Nonetheless, Tie1 has been demonstrated to modulate Tie2 mediated signaling through intracellular heterotypic interaction [135]. Tie1 on its own does not activate downstream intracellular signaling. In agreement with this notion, Tie1 undergoes proteolytic cleavage in presence of VEGF and inflammatory cytokines (*e.g.* TNF-α) releasing its soluble extracellular domain. This portion of the receptor then binds Tie2 forming the Tie1:Tie2 complex and thereby modulates the downstream signaling pathways [136, 137]. Yet, Kontos et *al.* recently identified the existence of a chimeric form of Tie1, c-fms-Tie1, which upon ligand stimulation results in Tie1 autophosphorylation and activation of downstream P13K/Akt pathway similar to Tie2; thus, suggesting that Tie1 receptors may potentially exert biological activities under certain circumstances [138].

Tie2 receptors are expressed in embryonic and adult endothelium [139], haemopoietic endothelial progenitors [129], lens epithelial cells [130], leukemia cells [131, 132, 140], eosinophils [141] and in circulating human neutrophils [117, 118]. In addition, Tie2 positive

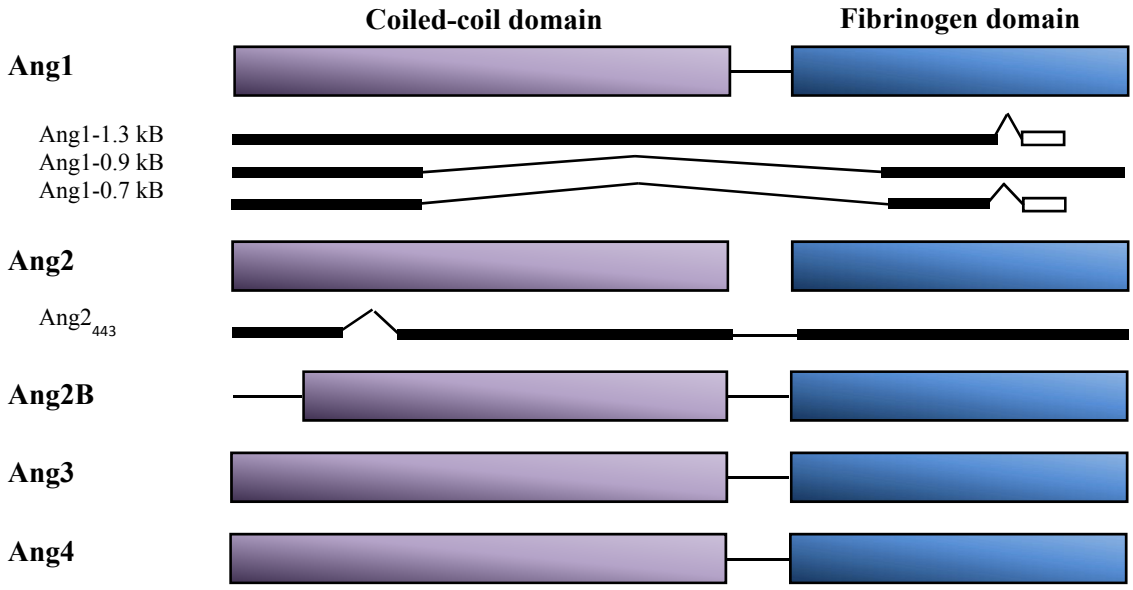


Figure 5: Schematic representation of angiopoietins and their isoforms. The angiopoietin superfamily consisting of Ang1, Ang2, Ang3 and Ang4 are structurally related with a coiled-coil domain and a fibrinogen domain. Four splice variants of Ang1 have been identified; Ang1-1.5 kB (the full length), Ang1-1.3 kB, Ang1-0.9 kB and Ang1-0.7 kB. Ang2 on the other hand are found in their full form or as $Ang2_{443}$ lacking part of its coiled-coil domain. Ang2B is a splice variant observed in chicken. Ang3 and Ang4 are inter-species homologues found in mice and humans, respectively. (Adapted from Jones, N. et *al.* 2001 [114])

subpopulation of monocytes, termed Tie2-expressing monocytes (TEM), has been identified to exist in concert with tumor-associated macrophages during tumor formation [142]. The expression of Tie2 is induced by hypoxia and pro-inflammatory mediators (*e.g.* TNF-α and IL-1β) [143] and is noted to be highly upregulated in tumoral angiogenesis [139, 144, 145]. Activation of Tie2 receptor follows the same basic steps as all other RTKs (*e.g.* VEGFRs). The binding of Ang1 *per se* to Tie2 with a binding constant of ≈3 nM leads to receptor

dimerization followed by rapid activation of the cytoplasmic kinase domain through autophosphorylation of specific tyrosine residues, which subsequently activates intracellular signaling pathways [139, 146].

1.2.2.4 Ang1 and Ang2 in developmental angiogenesis: genetic evidence

Over the years, numerous studies utilizing transgenic mouse models have provided concrete evidence that the angiopoietin/Tie signalling pathway is essential for vessel remodelling and stabilisation during angiogenesis. Indeed, conventional knockout of Ang1 ($Ang1^{-/-}$) in mice resulted in the formation of dilated vasculature lacking complexity (reduced numbers of small vessel formation and diminished branching) [94]. The nascent blood vessels were prone to ruptures as they were constructed with fewer ECs and failed to anchor onto the extracellular matrix [94]. As a result, $Ang1^{-/-}$ mice died very early during embryogenesis. Conversely, transgenic mice overexpressing Ang1 in the skin produced large vessels (presumably capillaries) with marked improvement in vascular integrity; the newly formed vessels were ensheathed by pericytes [147, 148]. Moreover, as mice deficient of Tie2 receptor ($Tie2^{-/-}$) produced similar phenotype as $Ang1^{-/-}$ mice, it is now well-established that Ang1 is an agonistic ligand of Tie2 [149-152]. Furthermore, double-transgenic mice overexpressing both Ang1 and VEGF in the skin of mice did not only produce highly structured vessels in greater numbers and diameter but the vessels were covered by pericytes and SMCs, and lacked leakiness and permeability which were absent in mice expressing VEGF alone [153]. Together, these results concluded that VEGF and Ang1 regulates angiogenesis at distinct

levels where VEGF induces vessel sprouting and growth while Ang1 through Tie2 mediates remodelling and maturation of newly formed blood vessels.

Ang2, being the antagonistic member of the angiopoietin family at the receptor level (under certain conditions) during angiogenesis also behaves to counteract Ang1 activity in transgenic mice models. For instance, mice overexpressing Ang2 were embryonic lethal and possessed similar phenotypes as mice deficient in Ang1 or Tie2 expression [84]. In agreement with these observations, Ang2 was demonstrated to disrupt the interaction between EC monolayer and SMCs in culture [154]. Ang2-null mice in contrast, were born normal but developed chylous ascites (an accumulation of milky chyle in the peritoneal cavity as a result of lymphatic disruption [155]) within the first few days postnatal. In fact, depending on the genetic background of the mice, Ang2-deficient mice died within the first 14 days of their life or developed normally to adulthood but with persistent vascular defects (such as impaired responses to inflammatory challenges). Hence, it was concluded that the release of Ang2 by ECs induces vascular regression.

The effects of the angiopoietins are not solely limited to the vascular system. It is now evident that the angiopoietins also have redundant roles in lymphatic vascular development through a process termed lymphangiogenesis [95]. The lymphatic vascular system is the body's second vascular system that is involved in the maintenance of normal tissue fluid homeostasis, immune surveillance, and absorption of fatty acids and lipid soluble vitamins in the gut. Although, very little is known about the role of Ang1 during lymphangiogenesis, mice overexpressing Ang1 has been shown to induce the formation of large lymphatic vessels in

increased numbers [156] either directly *via* Tie2 [157] or indirectly *via* VEGF-C/VEGFR3 [156]; Ang1 induces lymphatic vessel enlargement, sprouting and proliferation. Interestingly, Ang2 has been demonstrated to play a crucial role in keeping the lymphatic vessels under a quiescent state. For instance, Ang2 null mice have been shown to develop severe lymphatic defects [95], but minor blood vessel defects. The vessels formed in these mice failed to remodel or form a structured network and did not support the ensheathing of SMCs. Thus, Ang2 acts as an agonist on lymphatic vessels but also as an antagonist on blood vessels [95, 158].

1.2.2.5 Ang1 and Ang2 on the endothelium

While both Ang1 and Ang2 bind to the same site in the extracellular domain of Tie2, only Ang1 was initially characterized as the principal ligand of Tie2 receptor. Yet, it is now evident that Ang1 and Ang2 can multimerize prior to their binding onto Tie2, which promotes Tie2 receptor dimerization and subsequently activates the autophosphorylation of adjacent tyrosine residues on the carboxyl terminal of the receptor [159, 160]. The activation of Tie2 by Ang1 promotes vessel survival, migration and reorganization, inhibits vascular leakage and suppresses inflammatory gene expression.

Autophosphorylation of the tyrosine kinase domain on Tie2 receptor leads to the phosphorylation of p85 subunit of PI3K, which activates Akt and in turn phosphorylates and inhibits the forkhead transcription factor, FKHR, in ECs. FKHR in the endothelium is responsible for inducing EC apoptosis through the phosphorylation and inactivation of pro-

apoptotic factors including BAD and pro-caspase-9 [114, 161-164]. It also promotes the expression of genes involved in vascular destabilization and remodeling such as Ang2 [114, 161-163, 165]. Thus, the inhibitory effect of Ang1 on FKHR prevents EC apoptosis and vascular destabilization. In addition, Akt activation leads to an increased expression of survivin, a classical inhibitor of apoptosis, and promotes cell survival [166, 167]. Along with its anti-apoptotic and stabilization effects, the PI3K/Akt signaling pathway promotes blood vessel maturation. It maintains vascular quiescence by enhancing the interaction between perivascular cells and the endothelium which thereby suppresses inflammatory phenotypes. Concurrently, the NF-κB pathway is inhibited upon the activation of Tie2 by Ang1 through A20-binding inhibitor of NF-κB activation-2 (ABIN-2) which simultaneously exerts protective effect on the endothelium by preventing EC apoptosis [168, 169]. Ang2 on the other hand, antagonize the anti-apoptotic effect of Ang1. It induces EC apoptosis leading to the regression of the vasculature through the recruitment of macrophages.

Furthermore, Ang1 signaling stimulates Tie2-dependent EC migration *via* the adaptor protein Dok-R [170, 171]. The recruitment of Dok-R to the activated Tie2 receptor (p-Tyr1107) requires both the phosphotyrosine binding (PTB) and pleckstrin homology domains located on the adaptor protein. Phosphorylated Dok-R then creates interaction site for Nck and the serine kinase, p21-activating kinase (Pak), leading to the activation of EC migration and the reorganization of the cytoskeleton [146]. In addition, the activation of Tie2 on ECs induces the phosphorylation of focal adhesion kinase (FAK) which leads to the phosphorylation and activation of paxilline and p42/44 MAPK (ERK) [172]. Their activation in turn contributes to EC migration. In fact, blocking Tie2 activation inhibits ERK mediated Ang1 activation of EC

migration [173]. Other mediators including the adaptor protein ShcA, the GTPases RhoA and Rac1, growth factor receptor bound-2 (Grb2), Grb-7, p85 subunit of PI3K, eNOS and SH2 domain containing phosphatase (SHP2) are also recruited to Tie2 in ECs which have been identified to exert an important role not only in promoting EC migration but also in promoting EC proliferation and differentiation in the presence of Ang1 [146, 170, 174, 175]. SHP2 in particular additionally functions as a negative regulator of Tie2 phosphorylation [170, 176].

Ang1 also plays an important role in the reorganization of ECs into tubule-like structures. In fact, Ang1-stimulated reorganization of cultured ECs into tubules and their invasion into 3D matrices is Tie2-dependent [177]. Reorganization of ECs was not observed in ECs lacking Tie2 but was rescued by Tie2 activating antibody in the rat aortic ring assay [178]. Furthermore, the use of inhibitors and dominant-negative constructs has indicated a number of signaling intermediates involved in Ang1 induced reorganization of endothelium and motility including PI3K, SchA, focal adhesion kinase, and endothelial NO synthase [172, 179-181]. Consistent with such remodeling effects, Ang1 stimulates the production of proteases, including plasmin and matrix metalloproteases, which decrease the EC-substratum interaction allowing the ECs to reshape the vessel lumen [182]. Ang2 has also been demonstrated to promote vascular remodeling by inducing an inflammatory response [183]. Such response activates the endothelium and thereby increases vascular permeability and renders the endothelium susceptible to inflammatory cytokine. In fact, long-term incubation of EC by Ang2 activates PI3K/Akt pathway, which promotes EC survival, sprouting and migration [184].

Ang1 regulates EC-cell and cell-matrix interactions through which it is well-known to exert anti-inflammatory biological activities. The intercellular expression of VE-cadherins on ECs forms a complex, which can be broken by VEGF, decreasing vascular permeability. This effect can in fact be antagonized by Ang1. It is mediated through the translocation of Tie2 to EC surface in response to Ang1, which subsequently interact with Tie1 and activate the signaling pathways reducing vascular permeability. Similarly, Ang1, under inflammatory conditions is capable of reducing vascular permeability. It promotes a tight interaction between adhesion molecules at EC junctions (PECAM-1 and VE-cadherins).

It is well established that Ang1 exerts its function during the later phase of angiogenesis in promoting vessel maturation [147, 185]. However, few studies demonstrate that Ang1 can also promote EC chemotaxis [186] and induce EC sprouting under appropriate conditions [177, 186, 187]. The mitogenic effect of Ang1 on the other hand remains controversial. While Kanda, S et *al.* demonstrated that Ang1 can induce EC proliferation [188], several other groups have shown otherwise, that Ang1 has few or no mitogenic activity on EC [177, 186, 189].

1.2.2.6 The role of Ang1 and Ang2 in angiogenesis

The angiopoietin/Tie2 signaling axis is a key interaction during sprouting angiogenesis, which regulates the transition of ECs from a quiescent state to an activated state. In fact, the constitutive expression of Ang1 in adult perivascular cells covering the vasculature functions as a default pathway to maintain the quiescence resting state of the endothelium. It does so by

clustering with homotypic Tie2 complexes present at inter-EC-cell junctions and initiating signaling pathways promoting cell-cell adhesion, anti-permeability and cell survival. Interestingly, Ang1 does not only protect and seal the endothelium but also limits the endothelium from being activated by exogenous cytokines. For instance, Ang1 has been shown under certain experimental conditions to inhibit VEGF-induced blood vessel formation and adhesion molecule expression in order to avoid vascular homeostasis perturbation [119, 190].

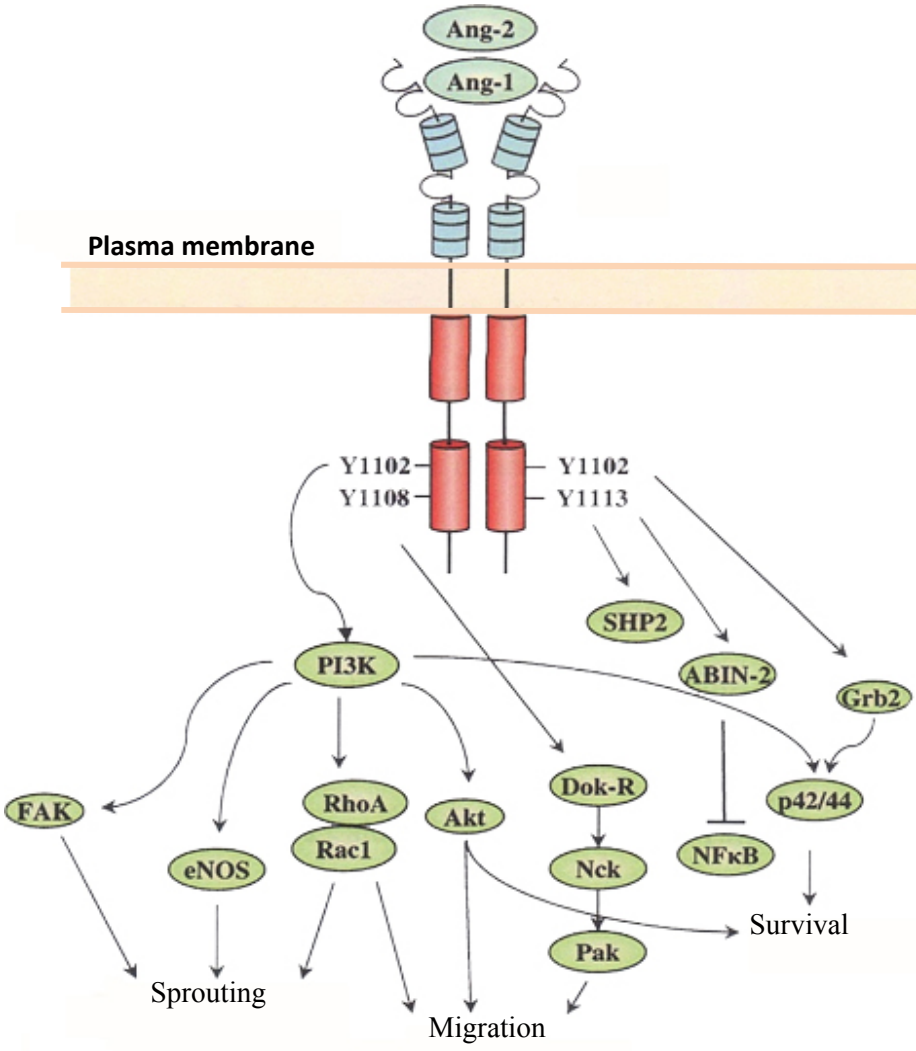


Figure 6: Schematic representation of the intracellular pathways activated through Tie2. The binding of Ang1 and Ang2 onto Tie2 induces the dimerization and autophosphorylation of the receptor. Phosphorylated tyrosine activates the PI3K pathway which subsequently activates Akt and RhoA/Rac1 implicated in cellular migration. FAK and eNOS also activated by PI3K leads to vessel sprouting. In addition, the pro-survival activity of angiopoietins is mediated by the p42/44 MAPK which is activated by both PI3K and Grb2/Ras/Raf pathways. Finally, the anti-inflammatory activity of Ang1 is mediated through the inhibiton of NFκB. (Adapted from Brindle, N.P. et *al.* 2006 [197])

This state is however antagonized by Ang2 in vessels undergoing angiogenesis. While almost undetectable in quiescent vasculature, Ang2 expression is dramatically upregulated at the transcriptional level and stored in the Weibel-Palade bodies by the pro-angiogenic stimuli including growth factors (*e.g.* FGF-2 and VEGF), cytokines (*e.g.* TNF) and environmental cues (hypoxia, high glucose levels and superoxides) [92, 109, 191-193]. They are then rapidly released in response to phorbal esters, thrombin and histamine leading to the destabilization of the quiescent endothelium (loosening of the attachment between ECs and perivascular cells) [84]. The unstable endothelium facilitates the infiltration of proteases, cytokines and angiogenic myeloid cells, which consequently primes the vasculature to respond to numerous exogenous stimuli for a robust angiogenic response in presence of VEGF [194, 195]. Interestingly, in absence of VEGF, high Ang2 levels lead to vessel regression and not to destabilization as it occurs during the menstrual cycle. Hence, Ang2 functions as a built-in switch controlling the transition of the resting quiescent endothelium towards the activated responsive endothelium. Indeed, it is the Ang1:Ang2 ratio which determines the functional status of the vasculature [196]. In resting state, a vasculature remains quiescent as the Ang1:Ang2 ratio is in favor of Ang1. Following EC activation, this ratio is locally shifted in favor of Ang2. The ECs of unstable vessels may die in the absence of VEGF or migrate and proliferate, initiating the angiogenic cascade in presence of VEGF. Pericytes use the developing sprouts as migration guidance cues and cover the newly formed vessel [198, 199]. Subsequently, the endothelium switches back to the quiescent state in the absence of additional stimulus.

1.3 Inflammation

Inflammation or an inflammatory response is a natural defensive reaction in response to tissue damage caused by micro-organisms, noxious stimuli, nutritive deficiency and pro-inflammatory mediators (secreted by apoptotic, necrotic and damaged cells). The maintenance of tissue integrity is vital to human health as we are continuously exposed to such molecular threats. Under physiological conditions, the inflammatory response is tightly regulated by soluble proteins (cytokines, chemokines and growth factors) that create an interactive network between inflammatory cells and vascular ECs within the affected site. The clinical manifestation of such complex system of chemical cues and cell interactions include *rubor* (redness), *calore* (heat), *dolore* (pain), *tumor* (swelling) and *functio laesa* (loss of function). When inflammation is not properly regulated, the balance between pro- and anti-inflammatory molecules is shifted away from the initiation of the healing process and towards the occurrence of persisting inflammation. In fact, inflammation is classified as either acute or chronic.

An inflammatory response to any cellular insult begins acutely, that is, it occurs rapidly and is of short duration (within few days). Regardless of the nature of the initial trigger, the series of events involved in acute inflammation can be categorized into different phases. Firstly, a variety of chemical mediators cause local microvessel dilation at the site of injury to increase blood flow (causing erythema and heat release). These mediators equally increase local vessel permeability in order to allow the recruitment of proteins and leukocytes (*e.g.* neutrophils and monocytes/macrophages) to the site of infection (causing swelling of the

tissue or edema). In concert, mast cells, platelets, ECs, dendritic cells (DCs), macrophages and other tissue resident cells also release chemoattractants to recruit neutrophils to the affected area. The latter takes place *via* local biosynthetic changes of adhesion molecules in ECs allowing the transmigration of neutrophils from the circulation to the site of action. Monocytes follow later, and once in the tissue they undergo anatomical and functional changes that transform them into macrophages. These leukocytes (neutrophils and macrophages) then destroy invading microbes and clear cellular debris through the process of phagocytosis. Finally, the local acute inflammatory response, in the absence of further inflammatory stimuli, is ceased with tissue repair.

If the stimulus persists, inflammation can last days, months or even years and is said to be chronic. In fact, chronic inflammation is abnormal and does not benefit the body. It is primarily mediated by monocytes/macrophages, lymphocytes and plasmocytes through the enhanced production of proteolytic enzymes, reactive oxygen species, MMPs, serine proteases and growth factors and is characterized by increased fibroblast proliferation, collagen deposition, fibrosis and angiogenesis. Yet, the inflammatory and morphological profiles of chronic inflammation differ significantly depending on the nature of the initial trigger. Nonetheless, persistent aggressive stimuli in chronic inflammation leads to a pathological state as observed in rheumatoid arthritis, atherosclerosis, ischemia and solid tumors [200, 201].

1.3.1 Regulation of inflammation

As briefly mentioned above, neutrophils and monocytes/macrophages play a crucial role in coordinating the inflammatory response (reviewed in [202]). They arise in a process termed myelopoiesis and are of haematopoietic pluripotent stem cell origin. These stem cells in the bone marrow give rise to myeloid (neutrophils, basophils, eosinophils, monocytes/ macrophages and DCs) and lymphoid lineages (T cells, B cells, DCs, and natural killer (NK) cells). Following tissue injury or infection, an "emergency myelopoiesis" takes place in humans, which generates large pools of neutrophils and monocytes in the bone marrow beyond the normal requirement of a healthy person.

1.3.1.1 Polymorphonuclear leukocytes (PMNs)

Morphologically, neutrophils are classified as PMNs and granulocytes together with basophils and eosinophils due to their multi-lobulated nucleus and the presence of specific granules in the cytoplasm. These leukocytes can be histologically distinguished based on the staining capacity of their granules to different dyes. While basophil granulocytes are stained by basic dyes, eosinophil and neutrophil granulocytes are stained by acidic and neutral dyes, respectively. Basophils are the least numerous of the granulocytes and account for about 0.01-0.3% of circulating leukocytes. They are involved in mediating hypersensitivity reactions (*e.g.* allergic reactions) of the immune system. Specifically, when activated, basophils release histamine (a pro-inflammatory mediator) and other enzymes that lead to a vast array of allergic symptoms. Eosinophils on the other hand account for 2-4% of circulating leukocytes

and are the major participant in the development of allergic inflammation. They also function as cytotoxic effector cells against parasitic infections [203]. Lastly, neutrophils, the well-studied leukocyte, function as the body's primary line of defense against invading pathogens (*e.g.* bacteria) in order to safeguard the body from infections.

Neutrophils represent about 50-70% of total leukocytes and are the most abundant inflammatory cell in the blood circulation. In humans, the neutrophil count represents approximately 100-700 million cells/100 mL of blood with a daily production of 0.8-1.6 x 10^9 neutrophils/kg of body mass. Such ample production of neutrophils is required in order to maintain a constant neutrophil concentration in the blood as their half-life is relatively short (10-24 hours) [204]. However, under an inflammatory state, the number of neutrophils increases significantly with an increased life span as they are one of the first responders to tissue injury and infection. This is made possible as neutrophils under steady state belong to one of two pools in the circulation; the circulating pool, which consists of neutrophils that are freely circulating or the marginated pool, which consists of neutrophils that are bound to the endothelium of small vessels [205]. Upon an inflammatory response, the marginated pool of neutrophils, serving as a reserve, is quickly mobilized to the circulating pool increasing the concentration of neutrophils in the blood.

In healthy adults, circulating neutrophils exist in a resting state in order to protect the host tissue from damage by accidental release of neutrophil toxic intracellular contents. The resting neutrophils become activated *via* a two-stage process. First, they become primed by agents such as bacterial products and cytokines or chemokines (e.g. granulocyte-macrophage

colony-stimulating factor (GM-CSF), TNF-α, IL-8 and IFN-γ) [206] and then they get mobilized to the site of infection or inflammation where they encounter activating signals to trigger bacterial killing.

The migration of neutrophils from the circulation across the endothelium (also referred to as neutrophil extravasation) to the site of inflammation can be divided into three stages: rolling, firm adhesion and transmigration [207]. Neutrophil extravasation occurs mainly in the post-capillary venules as the low haemodynamic shear force in these regions along with the expression of selectins on neutrophils facilitate the tethering of the neutrophils onto the activated endothelium. This slows down the flow of neutrophils and enables it to move in a position close to the endothelium and away from the central blood stream. The selectin adhesion family is comprised of L-selectin, P-selectin and E-selectin. L-selectin is constitutively expressed on neutrophils and they bind the immunoglobulin superfamily proteins glyCAM and MadCAM on ECs. This mediates weak constitutive neutrophil-endothelium interaction under normal conditions. However, the expression of L-selectin is significantly increased during inflammation following P-selectin translocation to the endothelium. P-selectin is normally stored in the Weibel Palade body of ECs and is rapidly translocated to the cell surface following the release of an inflammatory stimulus such as histamine, thrombin or phorbol esters [208-210]. The primary ligand of P-selectin is P-selectin glycoprotein ligand-1 (PSGL-1) which is constitutively expressed on all leukocytes. The binding of P-selectin to PSGL-1 activates the p42/44 MAPK and induces the release of IL-8 by leukocytes, which recruits more leukocytes [211, 212]. Finally, E-selectin has only been shown to be expressed on activated ECs upon the inflammatory stimulus lipopolysaccharide

(LPS), TNF-α and IL-1β [213]. It binds ESL-1 found on leukocyte cell surface. Together the expression of selectins along with their ligands contributes to the rolling of neutrophils along the endothelium and favors the transient attachment of the neutrophils onto the endothelium for a firm binding. Furthermore, the rolling of neutrophils induces the release of pro-inflammatory mediators such as IL-8 and PAF which directly activates another class of adhesion proteins, the β_2 integrins immunoglobulin superfamily. Integrins are usually expressed in a low-affinity binding state until cellular stimulation. The activation of β_2 integrins leads to a conformational change increasing their affinity to their ligands, ICAM-1 and ICAM-2, on ECs. Indeed, ICAM-1 is constitutively expressed in marginal amounts on EC surface but its expression is increased significantly upon pro-inflammatory stimuli while ICAM-2 is constitutively expressed on EC surface. In fact, it is the β_2 integrins with ICAM-1 or ICAM-2 interaction that is responsible for the firm adhesion of the leukocytes onto the endothelium and the transmigration of the neutrophils into the extracellular environment. They do so by passing through the junctions between neighboring ECs while interacting with surface ligands expressed on ECs (*e.g.* platelet endothelial-cell adhesion molecule-1 (PECAM-1)).

Once the neutrophils have left the circulation and passed through the endothelium, they migrate towards the inflamed tissue along a chemotactic gradient. At the site of infection, the primed neutrophils actively synthesize and secrete cytokines, chemokines, leukotrienes and prostaglandins to recruit other leukocytes. In particular, activated neutrophils have been reported to synthesize IL-8, IL-1, IL-1ra, IL-6, IL-12, TGF-β and TNF-α [214] where many of these have been shown to have the potential to activate both neutrophils and other

inflammatory/immune cells including monocytes, macrophages, NK cells, lymphocytes and immature DCs [216].

Furthermore, neutrophil granules contain a multitude of antimicrobial and cytotoxic substances that get released to destroy the invading microorganism. In fact, neutrophils possess three different types of granules: azurophil granules, specific granules and gelatinase granules [217]. The azurophil granules are characterized by their content of hydrolytic and bacterial proteins such as elastase, bacteriacidal permeability-increasing proteins, defensins and myeloperoxidase (MPO). MPO in particular, is a peroxidase enzyme which produces hypochlorous acid (HOCl) form hydrogen peroxide (H_2O_2) during the neutrophil's respiratory burst (the rapid release of reactive oxygen species from neutrophils). This phenomenon aids the degradation of internalized particle of bacteria in neutrophils. Similarly, the specific granules are characterized by the presence of lactoferine and the gelatinase granules contain high concentration of gelatinase. Although, neutrophil granulation shows heterogeneity, they all contain lysozymes and high cytotoxic potentials. Furthermore, since these granules get exocytosed during degranulation, the membrane of granules fuses with the plasma membrane and furnish the cell with new receptors and other functional proteins. One such example is the β_2 integrin found in neutrophils, which get incorporated into the plasma membrane by such mechanism during the extravasation of neutrophils [218].

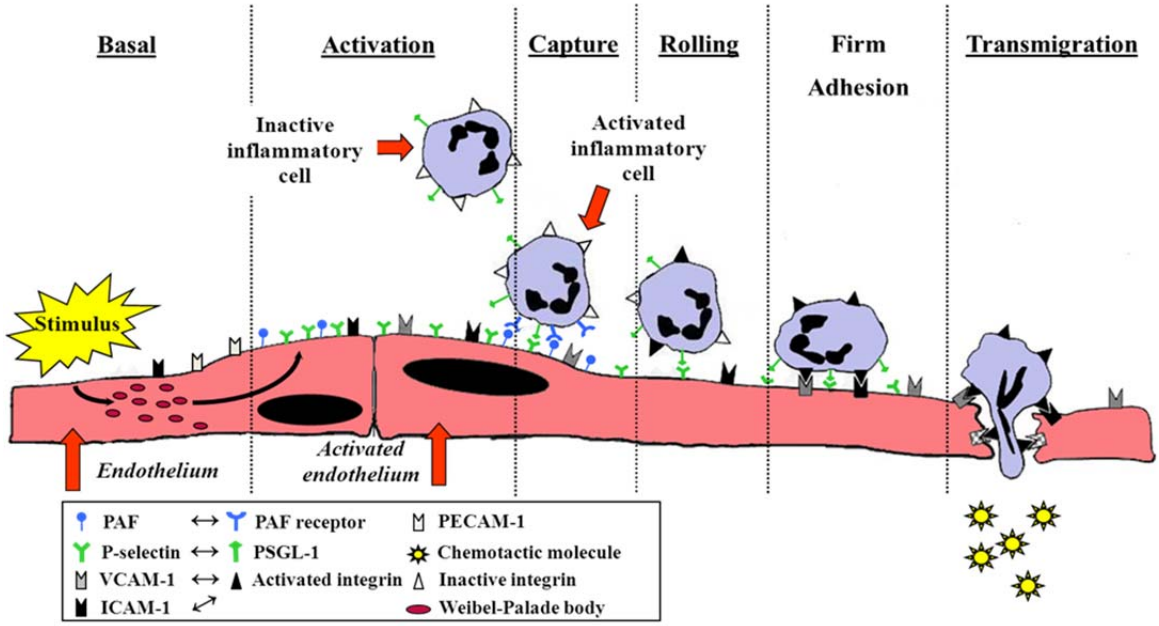


Figure 7: Steps in leukocyte infiltration. Upon stimulus by pro-inflammatory mediators, ECs become activated producing different classes of adhesion molecules such as the selectins and the immunoglobulin superfamily proteins both on neutrophils and on ECs. This in turn allows the neutrophils to interact with the endothelium in a transient manner facilitating its rolling. Following firm adhesion of the neutrophils onto the ECs mediated by integrins further allows the transmigration of the neutrophils along the chemoattractant gradient created in the extracellular space. (Adapted from Kreiglstein, C.F. et *al.* 2001 [215])

1.3.1.2 Mononuclear phagocytes

Unlike neutrophils, the mononuclear phagocytic system consisting primarily of monocytes, macrophages and DCs exhibit extensive heterogeneity with respect to morphology, biochemistry, surface antigen expression, secretory products and functions. Together, these

cells constitute ≈5-10% of total blood leukocytes in humans. Monocytes, which are in fact the precursor of macrophages and DCs, get released into the bloodstream during development where they circulate for several days before entering peripheral tissues. Once out of the circulation, monocytes give rise to a variety of tissue resident macrophages and to specialized cells (*e.g.* DCs) throughout the body. Functionally, although monocytes are involved in mediating host antimicrobial defence [219], their primary role is to replenish the pool of tissue resident macrophages and DCs both in steady state and during an inflammatory response. In fact, monocytes have been demonstrated to be implicated in many inflammatory diseases, including atherosclerosis [220].

Macrophages are tissue resident phagocytic cells present in both lymphoid and nonlymphoid organs. They are immune effector cells that exhibit essential roles in maintaining steady state tissue homeostasis *via* the clearance of cellular debris (subsequent to an inflammatory response *per se*) and apoptotic cells [221]. In addition to their central roles, tissue resident macrophages also fulfill tissue specific functions through their manifestation as Kupffer cells (in the liver), microglia (in central nervous system), Langerhans cells (in the epidermis and dermis of the skin), osteoclasts (in the bone), alveolar macrophages (in lungs), and splenic marginal zone and metallophilic macrophages (in the spleen) in different tissue types. Moreover, like other effector cells, macrophages can belong to various phenotypic subsets depending on their microenvironment or the stimuli they are exposed to. In fact, two distinct states of polarized activation for macrophages have been proposed: the classically activated (M1) macrophages and the alternately activated (M2) macrophages [222]. M1 macrophages with pro-inflammatory cytokine profile (expressing TNF-α, IL-1, IL-6, IL-12

and IL-23) arise following stimulation with IFN-γ alone or in concert with bacterial moieties, such as LPS or TNF-α [223]. This subset of macrophages also increases their concentration of superoxide anions, oxygen radicals and nitrogen radicals in order to promote their killing activities [224, 225]. In contrast, M2 macrophages with anti-inflammatory cytokine profile are polarized by distinct stimuli and can be subdivided into M2a (expressing IL-10, TGF-β and IL-1ra), M2b (expressing IL-1, IL-6, IL-10 and TNF-α), and M2c (expressing IL-10 and TGF-β) macrophages. M2a macrophages are stimulated by IL-4 or IL-13, while M2b macrophages are induced by LPS or IL-1ra and M2c macrophages by IL-10 or TGF-β. All M2 macrophage subsets express scavenger receptors (SRs), mannose receptors (MRs), and IL-10, which allows them to participate in parasitic clearance, tissue remodelling, immune modulation, and tumor progression [225].

These differentially polarized macrophages also express different chemokines. For instance, M1 macrophages express chemokine (C-X-C motif) ligand 9 (CXCL9), CXCL10 and CXCL5 which ultimately promote the recruitment of immune cells and improve their capacity to kill intracellular pathogens [226]. In contrast, M2 macrophages downregulate CXCL9, CXCL10 and CXCL5 chemokines expression [227, 228]. M2a macrophages promote the expression of chemokine (C-C motif) ligand 24 (CCL24), CCL17 and CCL22 which specifically bind chemokine (C-C motif) receptor 3 (CCR3) and CCR4 and accelerate the recruitment of eosinophils, basophils and T-cells. Similarly, M2b macrophages secrete CCL1 which upon its binding to CCR1 promotes the infiltration of eosinophils and different subsets of T-cells. Finally, M2c macrophages promote the accumulation of eosinophils and T-cells through the secretion of CXCL13, CCL16 and CCL18 combined with CXCR5, CCR1 and

CCR8. All in all, the recruitment of eosinophils and T cells (Th2 cells, regulatory T-cells, and naïve T-cells) through the secretion of numerous chemokines induce the initiation of the immune response (reviewed in [229]).

Besides their heterogeneity and plasticity, macrophages overall, are crucial to tissue defense during inflammation where their function (cytotoxic, phagocytic and secretory) is determined by the physiological environment. Firstly, macrophages are well-known for their scavenging role in the clearance of micro-organisms, foreign particles and altered-self materials (apoptotic cells, senescence erythrocytes and inflammatory products) during inflammation [230]. This activity which subsequently relies on phagocytosis and intracellular degradation is essential for tissue maintenance, defense and repair. Moreover, upon microbial ingestion a microbicidal function is usually involved.

Phagocytosis, a highly selective process, involves recognition and engulfment of micro-organisms and cellular debris. Recognition of such materials during infection, inflammation and wound repair requires specific interaction between surface proteins (ligands) to be ingested and the plasma membrane (phagocytic receptors) of macrophages. In fact, there are at least three different types of phagocytic receptors involved in the recognition of the particles, including Fc IgG receptors, complement phagocytosis receptors and lectin receptors [231]. In addition, the pro-inflammatory mediators found at the site of inflammation further stimulate phagocytosis for an efficient ingestion but also limits the tissue destructive processes accompanying phagocytosis.

Following phagocytosis, a variety of hydrolytic and proteolytic enzymes initiates intracellular degradation of the ingested particles. In fact, the predominant hydrolase enzymes that exert potent digestive capacity in activated macrophages are acid phosphatase (AP) and tartrate-resistant acid phosphatase (TRAP). Moreover, in microbial infection, the sequestered microbe in the macrophages is exposed to oxygen radicals, nutritive deprivation, low pH, and digestive enzymes. Such harsh conditions lead to the disruption of their cellular structure resulting in the death and degradation of the invading pathogen. Free radicals such as reactive oxygen intermediates (hydroxyl radicals and hydrogen peroxide) and reactive nitrogen (nitric oxide (NO)) intermediates are also involved in attacking pathogens that are susceptible to oxidizing agents [232]. Lastly, an oxygen-independent killing mechanism has been identified in activated macrophages which rely solely on defensins. Defensins are a class of anti-pathogenic peptides which orchestrate the formation of ion-permeable cannels in bacterial cell membrane [233]. Such release in turn leads to the shutdown of cellular functions of the pathogen.

In addition to their phagocytic function, activated macrophages are traditional antigen presenting cells (APCs) that mediates cytotoxic activities. In fact, macrophages increase surface expression of major histocompatibility class II (MHCII) proteins which are key molecules that display foreign antigens to T-lymphocytes [234]. This cross talk between macrophages and T-lymphocytes in turn initiates the specific immune response. MHCII proteins are also expressed on the surface membrane of B-cells, Langerhans cells and DCs. However, dendritic cells are the most efficient antigen presenting cell type to activate T-lymphocytes. They are 100-fold more potent than macrophages as APCs [235, 236]. Antigen

presentation by macrophages involves a series of events. Foreign antigens are internalized by phagocytosis and usually undergo intracellular processing, after which the antigenic fragment is displayed with the MHCII molecules on the surface of macrophages. The expression of such antigens is recognized by specific T-lymphocytes and initiates the immune response involving active killer cells. It is important to note that expression of MHCII on macrophages is transitory; activated macrophages expressing MHCII eventually loses its antigen presenting capabilities. Interestingly, the ratio of class II molecule-positive to negative macrophages varies widely between different tissues. For example, peritoneal macrophages and spleen white pulp macrophages are mainly negative, whereas spleen red pulp macrophages are predominantly positive [237].

Finally, macrophages elucidate potent secretory functions. They secrete a multitude of mediators ranging in biological activities from the induction of cell growth to cell death. These mediators themselves can possess multiple functions or can join together to mediate a single activity. Macrophages are a major source of many cytokines involved in inflammation, haematopoiesis, angiogenesis (discussed latter) and many other homeostatic processes. Few examples of the cytokines released by activates macrophages include interleukins, interferons, TGF-β and TNF-α and TNF-β [238]. Many of these cytokines are involved in the recruitment of other leukocytes to the site of injury.

1.4 Angiogenesis and inflammation

Considerable evidences up to date suggest that angiogenesis and inflammation are not independent events as was initially thought but are codependent processes involving common molecular mechanisms [239]. In fact, inflammation has been identified to be crucial in the initiation and the accompaniment of angiogenesis in numerous pathological circumstances. Supporting this notion, the exhaustingly studied pro-angiogenic growth factors (VEGF, Ang1 and Ang2) have been shown to exert inflammatory properties while the vice versa is also true for the inflammatory cells (neutrophils and monocytes/macrophages).

At the molecular level, oxygen tension in response to systemic stimuli or local tissue injury leads to the loosening of the endothelial monolayer driven by excess VEGF, NO and other cellular mediators; a hallmark of inflammation [240]. This increased vascular permeability permits the plasma components and inflammatory cells (*e.g* neutrophils and monocytes/macrophages) to exit the bloodstream into the extracellular space, initiating the acute inflammatory response. Resulting inflammation yields a microenvironment comprising of numerous growth factors, proteases and cytokines that subsequently induce the angiogenic cascade either directly and/or indirectly leading to the creation of a highly vascularized granulation tissue. Under normal physiology, the homeostatic control is restored upon the resolution of the inflammatory response and the regression of the newly formed vasculature. However, in the absence of vascular regression, positive feedback mechanisms operating between the blood vessels and the inflammatory infiltrate sustain the new vasculature and further impair the inflammatory response. Such is the case in a number of chronic

inflammatory disorders with distinct etiopathogenic origin including atherosclerosis, psoriasis, rheumatoid arthritis, Crohn's disease, diabetes and cancer. Recently, the previously established non-inflammatory disorders such as osteoarthritis and obesity have also been displayed to involve inflammation and angiogenesis in an exacerbated manner [241].

1.4.1 The paradox of angiogenic growth factors in inflammation

1.4.1.1 VEGF

VEGF, when secreted locally, is a potent and powerful vascular permeabilizing agent that allows the escape of solute rich fluid and leukocytes from the circulation into the extravascular space; a hallmark of inflammation [242, 243]. In any event, vascular permeability is a characteristic property of the vessel wall and depends on a number of variables such as physical properties of the fluid or cell being transported (size and charge), gradient between compartments (pressure and concentration) and the mode of transportation (channels or vesicles). Nonetheless, ECs in response to VEGF undergo marked structural and molecular changes that increase the permeability of the vasculature.

One of the first structural changes that takes place soon after VEGF administration is the formation of a cluster of linked caveolar vesicles through ECs that join to link the vascular luminal and abluminal surfaces [244]. Such structure, termed vesicular vacuolar organelles (VVOs), creates a passageway for the movement of fluid and cells across the vessel wall. Similar to these processes, transcellular gaps (running close to but not at the EC junction) and

intercellular gaps (at the cell junction) have also been identified to be created in frogs' and rats' vessels in response to VEGF [245]. Although, it is not known whether these latter processes are VVOs or whether they are entirely a separate structure, it is evident together with other i*n vivo* and *in vitro* studies that VEGF administration promotes the formation of *fenestrea* (large holes) in EC barrier promoting vascular leakage of inflammatory cells [246].

Furthermore, VEGF also affects the integrity of the endothelium through junctional changes [247]. Upon VEGF stimulation, tight junctions and adherens junctions between adjacent ECs are disturbed, leading to the loosening of the cell-cell contacts between ECs. Consequently, the micro-sized gaps created between ECs permit the extravasation of leukocytes across the vasculature [245]. In concert, VEGF induces EC expression and translocation of adhesion molecules (P-selectin, E-selectin, ICAM-1 and VCAM-1) to further favor the recruitment and activation of leukocytes onto the endothelium. The recruited leukocytes subsequently, release PAF in the interstitial fluid, which acts both in an autocrine and paracrine manner on ECs to trigger the inflammatory cascade.

1.4.1.2 Angiopoietins

ECs that line blood vessels form a physical barrier that separates the vessel lumen from the extracellular space. As previously mentioned, increased permeability of such EC barrier is a hallmark of inflammation. Interestingly, apart from being a potent angiogenic factor, Ang1 has been shown to also behave as an anti-inflammatory mediator on ECs by sealing the endothelium against vascular leakage. In fact, a study using transgenic mice overexpressing

Ang1 protected the blood vessels against vascular leakage when challenged with inflammatory agents [248]; suggesting that Ang1 exerts anti-inflammatory properties.

Over the years, various groups have tested the role of Ang1 in different models of inflammation both *in vivo* and *in vitro*. It is now well-established that the anti-inflammatory actions of Ang1 include the suppression of vascular leakage, the downregulation of EC adhesion molecule expression and the inhibition of leukocyte adhesion and transmigration across the endothelium. Bringing up some experimental evidences, Ang1 significantly reduces the permeability of human umbilical vein EC (HUVEC) in culture when induced with thrombin or VEGF [249, 250]. Furthermore, Ang1 reduces TNF-α induced leukocyte transmigration on EC surface, downregulates the expression of E-selectins on EC surface, decreases vascular endothelial cadherin and PECAM-1 basal level phosphorylation and inhibits VEGF induced adhesion molecule expression (e.g. ICAM-1 and VCAM-1) on the endothelium [190, 251]. While the signaling mechanism involved in the anti-inflammatory actions of Ang1 remains undetermined, PI3K and NFκB pathways have been shown to be involved in the regulation of Ang1 mediated inflammatory gene expression [190, 252].

Although, substantial evidences show that Ang1 mediates anti-inflammatory effects on ECs, other studies have shown contradictory findings and argue that Ang1 mediates pro-inflammatory responses. In fact, both Ang1 and Ang2 exert pro-inflammatory functions on ECs with similar potency. Consistently, angiopoietins can prolong EC survival; however, the effect of Ang2 on neutrophil *via*bility depends on the experimental conditions [106, 112, 162, 167, 253, 254]. While some groups argue that the prolonged survival of ECs in presence of

Ang2 is established *via* the PI3K pathway [112, 253], others believe that it does so through its pro-apoptotic activity on ECs. The pro-survival effect of Ang2 may be mediated through VE-cadherins [120] or through the activation of apoptotic factors [254]. Furthermore, our laboratory has demonstrated that the angiopoietins induce endothelial synthesis of PAF [115] and the translocation of P-selectins [116, 117]. Increased expression of P-selectins recruits neutrophils and facilitate their transmigration into the tissue. Moreover, Ang1 and Ang2 potentiate the pro-inflammatory effect of cytokines and growth factors on the endothelium. For instance, Ang2 in presence of TNF-α favor the adhesion of leukocytes onto ECs [255]. Furthermore, when combined with VEGF in a murine model, Ang1 promotes the formation of blood vessels large numbers with a greater diameter while when Ang2 is combined with VEGF, the vessels are longer in length [119].

Moreover, Ang2 triggers an inflammatory response by activating the endothelium and inducing vascular permeability [256]. This was further supported by experiments studying inflammation in Ang2-deficient mice [255]. The mice in this study were unable to elicit an acute inflammatory response following intraperitoneal injection of thioglycolate or *Staphylococcus* aureus. Detailed mechanistic analyses revealed that the Ang2-deficient mice were not capable of expressing cytokine-inducible adhesion molecules on their luminal cell surface after inflammatory activation. Hence, Ang2 does not affect EC adhesion molecule expression directly. Instead, it primes the quiescent endothelium to control the responsiveness to inflammatory cytokines [255, 257].

Angiopoietins also exert pro-inflammatory activities on neutrophils. Our laboratory noted that both Ang1 and Ang2 individually exert similar capacity to promote neutrophil adhesion [116, 117]. However, when combined together, Ang1 and Ang2 possessed an additive effect on neutrophil adhesion but no difference in P-selectin translocation [117]. As neutrophil adhesion requires the translocation of P-selectins, these observations suggested that the angiopoietins might directly act on neutrophils. Indeed, we demonstrated for the first time the expression of Tie2 receptors on neutrophils [117]. Along with other groups, we have subsequently demonstrated that Ang1 and Ang2 exert various pro-inflammatory activities on neutrophils [117, 258, 259]. For example, they induce PAF synthesis and CD11b/CD18 (β2-integrin) activation [117]. The latter is an adhesion molecule that involved in linking the neutrophils onto the endothelium. The angiopoietins can also directly induce the migration of neutrophils and potentiate the effect of IL-8 on neutrophil migration [213].

1.4.2 The paradox of inflammatory cells in angiogenesis

1.4.2.1 Neutrophils

Neutrophils have traditionally been thought to provide the first line of defense of the innate immune system by phagocytizing invading microorganisms. Recent evidence however demonstrates that neutrophils play important role in physiological angiogenesis. For instance, during the proliferative stage of the menstrual cycle, neutrophils liberate VEGF that mediates the growth and proliferation of the endometrial tissue [260]. This observation was further supported in a murine study where depletion of mice neutrophil using anti-Gr1 antibody

inhibited the proliferation of ECs in the endometrium as compared to control mice [261]. Other *in vivo* models of angiogenesis revealed the requirement of neutrophils in neovascularization in other tissues. For instance, Gr1 mediated neutrophil depletion in C57 black mice implanted with Matrigel containing CXCL1 or IL-8, the most potent chemotactic factors of neutrophils, produced a significant reduction in blood vessel formation in the skin [262]. Similarly, neutrophil depletion in Swiss Webster mice implanted with slow releasing pellets of pro-angiogenic factor, FGF-2, into the corneas inhibited the angiogenic process [263].

Importantly, human biopsies of solid tumor (e.g colon adenocarninoma, bronchioloalveolar carcinoma, myxofibrosarcoma, gastric sarcoma and melanoma) displayed massive infiltration of neutrophils. Moreover, a high positive correlation between elevated numbers of neutrophil and increased intra-tumoral microvessel density was detected in patients with high grade malignant tumor [264-267] and correlating with poor prognosis [268]. Thus, neutrophils are also implicated in pathological angiogenesis. This initial observation was tested using the chick embryo chorioallantoic membrane (CAM) assay implanted with pro-angiogenic factors FGF2, VEGF or HT1080 tumor cells for ten days. The results showed a fourfold increase in heterophil (the chicken analogue of neutrophil) infiltration at the studied region in presence of the angiogenic factors and tumor cells as compared to no treatment control [269]. Yet, treating the implants with an anti-inflammatory cortisone disrupting inflammation significantly reduced the number of heterophils recruited and the formation of novel vessels [269]. Although the exact mechanism through which tumor associated neutrophils mediate or modulate angiogenesis has not been fully elucidated, several

hypotheses have been raised to explain this mechanism. Some suggest that the release of MMPs, in particular MMP-9, by neutrophils induce proteolysis of the ECM liberating angiogenic factors such as VEGF and FGF2 [270]. Once released, these factors act upon nearby ECs to prompt vascularization. Others suggest that the activated neutrophils can directly secrete a multitude of soluble pro-angiogenic factors such as VEGF that may influence the angiogenic switch within tumors stimulating EC migration, proliferation and differentiation. Finally, it is also believed that neutrophils may even stimulate angiogenesis through intimate cell-to-cell interactions with ECs [271].

1.4.2.2 Monocytes and macrophages

Monocytes/macrophages infiltration is often preceded and accompanied during *in vivo* angiogenesis as observed in tissue repair, remodeling and tumor growth. Indeed, the abolishment of monocytes drastically reduces angiogenesis in wound healing [272] while chemoattractant-mediated stimulation of monocyte recruitment promotes angiogenesis and the formation of collateral vessels [273]. It is suggested that the increased neovascularization in atherosclerotic plaques is due to the accumulation of monocytes/macrophages [274]. Furthermore, macrophages play a pivotal role in tumor angiogenesis through the production of myriad of potent angiogenic cytokines and growth factors (*e.g.* VEGF, TGF-α and –β, FGF, EGF, angiopoietins and IL-8) and secretion of ECM degrading enzyme (*e.g* MMP-2, MMP-7, MMP-9 and MMP-12) [275, 276]. A positive correlation between macrophage infiltration and angiogenesis was observed in human gliomas, which were inversely proportional with prognosis. Detailed studies identified that M2 phenotypic macrophages were involved in

tumor angiogenesis. These macrophages were termed tumor associated macrophages (TAMs) [277]. Indeed, Bingle and his colleagues demonstrated that the presence of TAMs within a solid tumor contributes to the immediate initiation of angiogenesis; in the absence of TAMs tumor angiogenesis is delayed [278].

Based on these observations, subsequent studies examined the mechanism by which macrophages promote the formation of new vessels. It is now evident that macrophages are able to stimulate all phases of angiogenesis through their secretory products [275]. Firstly, macrophages require activation in order for it to become angiogenic [279]. This phenotype is achieved when exposed to low oxygen concentration, under wound-like concentrations of lactate, pyruvate or hydrogen ions or in response to cytokines (PAF) and monocytic chemotactic proteins. As the induction of angiogenesis requires dissolution of the capillary basement membrane enabling the release of soluble growth factors, macrophages have been identified to release proteases to fulfill this requirement. Indeed, macrophages release metalloproteinases (*e.g.* collagenase), serine proteases (*e.g.* t-PA and u-PA) and monokines (*e.g.* TGF-B, PDGF and IL-6), which together change the composition of the ECM and modify the responsiveness on the ECs to the different growth factors [280-282]. Along with these pro-angiogenic effects of macrophages, the released factors, other than proteases, are also involved in promoting the migration and proliferation of ECs [275]. Hence, macrophages provide cytokines for the initiation and maintenance of the angiogenic process.

1.5 Angiogenic model

One of the important technical problems faced by researchers studying angiogenesis is the inability to use an appropriate method for assessing angiogenesis as a whole. As angiogenesis involves numerous steps (e.g. EC migration, proliferation, differentiation and structural rearrangement) and encompasses various cell types including ECs, mural cells (pericytes and SMCs), the complexity of such process makes it a challenge in putting together a model encompassing all of these aspects of vascular formation. In fact, most of the available angiogenic assays only target the individual steps of angiogenesis [283]. Hence, an ideal assay would be one that is reliable, technically straightforward, easy to quantify and physiologically relevant (looks at the overall effect of a tested factor on the formation of new vessels).

Currently, a variety of *in vitro* and *in vivo* assays are being used in studying angiogenesis, each of which having strengths and weaknesses. *In vitro* methods, including cellular (proliferation, migration, tube formation) and organotypic (aortic ring) assays, are valuable in providing initial information but require multiple tests to obtain concluding interpretation [284]. *In vivo* methods on the other hand, are generally more difficult to quantify and time consuming to perform (Table I), but are essential in studying the complex vascular response as no *in vitro* models can fully achieve this response [285].

In 1988, Fajardo et *al.* described a new assay using polyvinyl alcohol sponges containing angiogenic factors and/or antagonists [286]. These sponges were subcutaneously introduced in the animal through a small, dorsal incision for the evaluation of host-derived blood vessel penetration and/or other cell infiltration. Presently, this angiogenic sponge model has been

modified in order to enable the introduction of live cells (*e.g.* tumor cells or inflammatory cells) into the center of the sponge. Once harvested, the sponge discs can be fixed, sectioned and stained. Histological examination of sponge sections showed increased neovascularization and distinctive cellular infiltration at the edges, including fibroblasts, ECs and leukocytes [287]. Hence, polyvinyl alcohol sponges provide a pro-inflammatory environment. A major disadvantage in inserting the sponges alone in mice is that it promotes fibrosis around the sponge as a foreign body response [288].

Recently, the Matrigel plug assay became the method of choice for many angiogenesis based studies. Matrigel is a laminin-rich mixture of basement membrane components isolated from Engelbreth-Holm-Swarm mouse tumor, which provides a suitable pro-angiogenic environment [288]. It was initially used to investigate capillary tube formation *in vitro*. Matrigel in liquid form at $4^{o}C$ is mixed with test cells or angiogenic factors and is subcutaneously injected into mice. Once in the host, Matrigel solidifies and forms a gel plug. The trapping of the growth factor in the Matrigel allows the slow release of the tested factor. These plugs in mice can be retrieved at different time points post-administration and examined histologically to determine the extent of neovessel formation. Quantification of the vessels in histologic sections may be tedious but accurate. In addition, measurement of the amount of hemoglobin content in the plug as the method of quantification is also widely used. However, the latter approach of quantification may be misleading as the blood content in the Matrigel plug may come from other sources during the retrieval of the plug [288].

In our laboratory, we have combined the Matrigel plug assay and the polyvinyl alcohol sponge angiogenesis system in order to create an *in vivo* system that provides both an angiogenic and a pro-inflammatory environment. This model, a novel variant of the existing sponge/Matrigel model is technically simple and does not induce non-specific immune response leading to fibrotic encapsulation of the implanted matrix.

Models	Advantages	Disadvantages
Corneal micropocket	• Used in rabbits, rats, and mice • Newly formed vessels are easily identified • Permits non-invasive observations and long-term monitoring • Quantitative	• Atypical angiogenesis • Expensive • Ethically questionable • Technically difficult, especially in mouse eye • Not a suitable technique to study tumor angiogenesis • Non-specific inflammatory response
Chick chorioallantoic membrane (CAM) assay	• Technically simple • Inexpensive • Permits non-invasive observation • Sprouting angiogenesis	• Visualization of the new vessels is difficult • Embryonic • Non-specific inflammatory reactions
Sponge/matrix implant	• Technically simple • Inexpensive • Can be used for time course experiments • Suitable for tumor angiogenic studies • Quantitative	• Time consuming • Encapsulated by granulation tissue • The composition of the sponges may make inter-experimental comparisons difficult • The tested factor may be retained in the sponge • Animals must be monitored
Matrigel plug	• Technically simple • Quantitative • Does not induce non-specific inflammatory response	• Difficult to make uniform 3D plugs • Analysis is tedious and time consuming • Expensive
Zebrafish	• Using whole animal • Technically simple • Allows gene analysis of vessel development • Quantitative • Fast assay	• Embryonic • Non-mammalian • Expensive

Table I: Advantages and disadvantages of the major *in vivo* angiogenesis assay. (Adapted from Norrby, K. et *al*. 2006 [288])

1.6 Project reasoning and purpose

Angiogenesis is a fundamental process in which multiple factors exert specific activities at different phases [289]. The early stage of this process is strictly dependent on VEGF which is well-documented for its ability to promote the growth and sprouting of ECs derived from arteries, veins and lymphatics [18, 19, 60-64]. In fact, invasion of ECs and formation of capillary like structure by VEGF into collagen gel has been reported in the past [64, 290]. Furthermore, VEGF elicits strong angiogenic and mitogenic responses in a variety of *in vivo* models including the chick chorioallantoic membrane [19, 62], the rabbit cornea [57], the matrigel plug in mice [58, 291], the primate iris and many others. However, in pre-clinical and clinical testing, VEGF-induced angiogenesis lacked vascular basement membrane and α-smooth muscle actin-cbpositive pericytes leading to the regression of the newly formed vessels with time. Indeed, the presence of VEGF in the late phase of angiogenesis was observed to be not only obsolete but also detrimental for vessel functionality and pericyte regeneration [292, 293]. The discovery of angiopoietins opened a new era in the research of therapeutic angiogenesis. Among the regulators of vessel maturation, Ang1 is essential during the latter phase of angiogenesis where it supports EC survival and maintains the integrity of the endothelium. In contrast, Ang2, almost exclusively produced by ECs promotes blood vessel destabilization and regression in the absence of survival factors (e.g. VEGF and FGF-2). Recently, biopsy observations of different pathologies have shown that inflammation precedes and accompanies pathological angiogenesis. Moreover, a panel of experts analyzed numerous clinical trial reports in coronary angiogenesis and argued that inflammation is much more important in inducing angiogenesis than tissue ischemia [294]. Consistent with these

reports, our laboratory has identified the inflammatory properties of VEGF, Ang1 and Ang2, suggesting that the inflammatory response is a crucial event in angiogenesis. However, inflammation was regarded as a confounding factor in the search for angiogenic models. Thus, we hypothesized that an establishment of a murine model that is both angiogenic and pro-inflammatory would provide a suitable environment for the formation of functional vessels mediated by selected growth factors. The purpose of this project is to: 1) elucidate the individual capacity of VEGF, Ang1 and Ang2 to promote blood vessel formation in a spatio-temporal dependent manner, 2) examine whether this model allows maturation of the newly formed vessels in presence of the growth factors, and 3) identify the presence of different inflammatory cells accompanying the angiogenic process, in a timely and sequential study.

2.0 ARTICLE

VEGF and angiopoietins promote inflammatory cell recruitment

and mature blood vessel formation in murine sponge/Matrigel model

Tharsika SINNATHAMBY[1,2], Tae Jin YUN[3,4], Marie-Élaine CLAVET-LANTHIER[1],

Cheolho CHEONG[3,4,5] & Martin G. SIROIS[1,2*]

[1]Research Center, Montreal Heart Institute, [2]Department of pharmacology, Faculty of medicine, Université de Montréal and [3]Laboratory of cellular physiology and immunology, Institut de Recherches Cliniques de Montréal (IRCM) and [4]Division of experimental medicine, Department of medicine, McGill University and [5]Department of microbiology, infectiology and immunology, Faculty of medicine, Université de Montréal, Montréal, Canada.

[*]Correspondence to:

Martin G. Sirois

Research Center, Montreal Heart Institute

5000 Belanger Street, Montréal, Canada H1T 1C8

Phone #: 514-376-3330 ext. 3583

Fax #: 514-376-1355

Running title: Growth factor mediated angiogenesis

Key words: angiopoietins, angiogenesis, neovessel maturation, inflammatory cells, VEGF

Number of figures: 6

Contract grant sponsor: Canadian Institutes of Health Research

Contract grant number: MOP-97943 (MGS) and MOP-125933 (CC)

ABSTRACT

A key feature in the induction of pathological angiogenesis is that inflammation precedes and accompanies the formation of neovessels as evidenced by increased vascular permeability and the recruitment of inflammatory cells. Previously, we and other groups have shown that selected growth factors, namely vascular endothelial growth factor (VEGF) and angiopoietins (Ang1 and Ang2) do not only promote angiogenesis but can also induce inflammatory response. Herein, given a pro-inflammatory environment, we addressed the individual capacity of VEGF and angiopoietins to promote the formation of mature neovessels and to identify the different types of inflammatory cells accompanying the angiogenic process over time. Sterilized polyvinyl alcohol (PVA) sponges soaked in growth factor-depleted Matrigel mixed with PBS, VEGF, Ang1 or Ang2 (200 ng/200 μl) were subcutaneously inserted into anesthetized mice. Sponges were removed at day 4, 7, 14 or 21 post-procedure for histological, immunohistological (IHC) and flow cytometry analyses. As compared to PBS-treated sponges, the three growth factors promoted the recruitment of inflammatory cells, mainly neutrophils and macrophages, and to a lesser extent, T- and B-cells. In addition, they were more potent and more rapid in the recruitment of endothelial cells (ECs) and in the formation and maturation (ensheating of smooth muscle cells around ECs) of neovessels. Thus, the autocrine/paracrine interaction among the different inflammatory cells in combination with VEGF, Ang1 or Ang2 provides a suitable microenvironment for the formation and maturation of blood vessels.

INTRODUCTION

The ramification of novel blood vessels from pre-existing vascular network, termed angiogenesis, is a coordinated sequence of cellular events consisting of sprouting, endothelial cell (EC) proliferation, directed migration of ECs, EC tube formation and perivascular stabilization [Carmeliet and Jain, 2011]. Such multistep process is tightly regulated through the maintenance of a balance between soluble pro-angiogenic (stimulatory) and anti-angiogenic (inhibitory) factors [Liekens et al., 2001; Noonan et al., 2008]. A local perturbation of this equilibrium can result in either excessive or insufficient angiogenesis leading to a variety of diseases. With the identification of several pro-angiogenic molecules, potential therapeutic interference with vessel formation is being studied as promising tool for clinical applications [Griffioen and Molema, 2000]. For instance, while therapeutic inhibition of angiogenesis may be beneficial in diseases associated to excessive neovessel growth (e.g. solid tumor, rheumatoid arthritis, diabetic retinopathy, atherosclerosis and psoriasis) [Hanahan and Folkman, 1996], stimulation of angiogenesis may be beneficial in conditions associated with insufficient formation of new vasculature (e.g. tissue damage after reperfusion of ischemic tissue and cardiac failure) [de Muinck and Simons, 2004].

Vascular endothelial growth factor (VEGF) is a well-studied growth factor that effectively promotes neovessel sprouting and growth in the initial phase of angiogenesis [Carmeliet and Jain, 2011; de Muinck and Simons, 2004]. Upon discovery, its high angiogenic potential arose the hypothesis that VEGF monotherapy may be sufficient to promote therapeutic angiogenesis. However, in both pre-clinical and clinical testing, although VEGF

monotherapy was successful in promoting the formation of blood vessels, they lacked vascular basement membrane and/or the ensheathing of α-smooth muscle actin (α-SMA)-positive pericytes and smooth muscle cells (SMCs), leading to the regression of newly formed vessels [de Muinck and Simons, 2004; Dor et al., 2002; Henry et al., 2003]. Thus, VEGF-orientated clinical trials did not support the expected beneficial outcome in patients [Simons et al., 2000; Stewart et al., 2009]. The discovery of a novel class of EC-specific ligands termed angiopoietins (Ang1 and Ang2) showed their capacity through the activation of Tie2 receptor to modulate the maturation and stabilization of newly formed vessels. For instance, while Ang1 in the late phase of angiogenesis plays an important role in promoting vascular maturation and contributing to enhance the integrity of EC barrier, Ang2 is identified to have the capacity to destabilize pre-existing vessels prior to VEGF-induced angiogenesis [Davis et al., 1996; Maisonpierre et al., 1997; Thurston et al., 2000]. Furthermore, Ang1 has also been demonstrated to have the capacity to promote *in vivo* angiogenesis and both Ang1 and Ang2 have the potential to increase EC migration and sprouting under certain experimental conditions [Mochizuki et al., 2002; Teichert-Kuliszewska et al., 2001]. Nonetheless, the angiopoietins themselves exert low mitogenic or proliferative activity on ECs [Davis et al., 1996], suggesting that VEGF and angiopoietins exhibit distinct and overlapping expression patterns which collaborate to regulate the different stages of physiological angiogenesis. Hence, a single pro-angiogenic factor may not be sufficient and effective in orchestrating all stages of the angiogenic process and a combination of pro-angiogenic mediators (*e.g.* growth factors with cytokines) may be required in the formation of stable blood vessels. In agreement with such premise, the emerging relationship between leukocyte infiltration and angiogenesis attracted a lot of attention over the last years.

Proliferating tissue in rheumatoid arthritis, psoriasis and solid tumors *per se*, contains an abundance of inflammatory cells (neutrophils, monocytes/macrophages and dendritic cells) that promote pathological angiogenesis either directly and/or indirectly leading to the creation of a highly vascularized granulation tissue [Costa et al., 2007]. The angiogenic events, in these pathologies, further support the inflammatory response, creating a vicious cycle. In accordance with these observations, clinical trial reports referring to coronary angiogenesis suggested that inflammation is an important stimulus in the induction of the angiogenic cascade [Simons et al., 2000] and very little angiogenesis takes place in the absence of inflammation [Jones et al., 1999]. We, in parallel with other groups, have demonstrated that VEGF and angiopoietins, in addition to being angiogenic factors, are also potent inflammatory regulators; once again indicating the necessity of inflammation in the accompaniment of angiogenesis [Dumas et al., 2012; Maliba et al., 2008; Neagoe et al., 2009; Neagoe et al., 2012]. During the last years, we have shown that Ang1 and Ang2, acting on Tie2 receptor, are capable of promoting the synthesis of platelet activating factor (PAF), a potent pro-inflammatory mediator, in both ECs and neutrophils. Upon its synthesis, PAF promotes neutrophil upregulation of β_2-integrin complex (CD11b/CD18) contributing to neutrophil adhesion and their migration onto activated ECs [Lemieux et al., 2005; Maliba et al., 2006]. In addition, we have reported the capacity of Ang1 to promote the synthesis and release of IL-1 and IL-8 [Dumas et al., 2012; Haddad and Sirois, 2014] which are both involved during inflammation and angiogenesis [Qazi et al., 2011; Voronov et al., 2007]. Nonetheless, the exact link between inflammation and angiogenesis such as the type and the temporal role of the recruited inflammatory cells during angiogenesis remains unanswered.

Various *in vivo* models using biomaterials (*e.g.* polyvinyl alcohol sponges) and/or Matrigel have been used extensively to analyze the angiogenic capacity of growth factors, cytokines, chemokines and non-protein mediators in a number of different hosts [Norrby, 2006]. Yet, many of these studies either did not look at the maturation of blood vessels, a crucial event in the stabilisation of nascent blood vessels, or the newly formed vessels were identified to be immature (lacking the ensheathing of SMCs). Hence, in the current study, we utilized a novel variant of the sponge/Matrigel angiogenic model such that the pro-inflammatory sponges were pre-incubated in growth factor depleted Matrigel containing the tested growth factor prior to subcutaneous implantation into wild type mice in order to 1) assess the individual pro-angiogenic capacity of VEGF, Ang1 and Ang2 to promote the formation and the maturation of neovessels, and 2) to identify the different inflammatory cells accompanying angiogenesis in a spatio-temporal manner.

MATERIAL & METHODS

Mice

C57BL/6 and BALB/c mice, 10-11 weeks old, were purchased from Charles River Laboratories (Montréal, Canada) and *CD115*$^{gfp/+}$and *Zbtb46*$^{gfp/+}$ mice were purchased from Jackson laboratories (Bar Harbor, ME). All animal experiments were approved by the ethical animal care committees of the Montreal Heart Institute and Institut de Recherches Cliniques de Montréal.

Sponge preparation and implantation

Sterilized polyvinyl alcohol (PVA) sponges (6 mm diameter x 2 mm width) were soaked in 200 µl of growth factor depleted Matrigel (BD Biosciences, Mississauga, Canada) containing PBS or 200 ng of VEGF (PeproTech, Rocky Hill, NJ), Ang1 or Ang2 (R&D Systems, Minneapolis, MN) for 20 minutes at 4^{o}C. Subsequently, the sponges were incubated for 20 minutes at 37^{o}C prior to implantation. Under anesthesia with 2% isoflurane USP, two sponges treated with the same growth factors were inserted subcutaneously through two 1 cm orthogonal incisions in the dorsa of the animals. The incisions were then clipped for closure, and the mice were subcutaneously injected with an analgesic agent (0.1 ml of Anaphen; 1 mg/ml). The mice were sacrificed under anaesthesia at day 4, 7, 14 or 21 post-procedure.

Histology and immunohistochemistry analyses

The harvested sponge implants were fixed in 10% formalin PBS-buffered solution, embedded into paraffin blocks and sectioned sagittally (6 μm-thick). The sections were stained with Masson's trichrome reagent for a global overview of cellular invasion in the implants. Immunohistological stainings were performed using the avidin-biotin complex for the validation of angiogenesis and inflammatory cells infiltration as previously described [Lemieux et al., 2005; Marchand et al., 2002]. The primary antisera used in this study were: ECs specific goat anti-mouse CD31 (Santa Cruz Biotechnology Inc, CA), SMCs specific mouse anti-mouse α-SMA (Sigma-Aldrich, Steinheim, Germany; clone 1A4), neutrophils specific rabbit anti-mouse myeloperoxydase (MPO) (Thermo Scientific, Rockford, IL) and macrophages specific rat anti-mouse F4/80 (Biolegend, San Diego, CA; clone BM8).

To assess the maturation of neovessels, a sequential double immunohistochemistry (IHC) staining was performed. The sponge sections underwent first round of IHC using the primary antisera anti-CD31 and host specific biotinylated secondary antibody. Peroxidase was developed by the DAB substrate. The tissues underwent a second round of IHC protocol with the primary antisera anti-α-SMA and host specific biotinylated secondary antibody. Alpha(α)-SMA expression was detected in turquoise using Vina green chromogen (Biocare Medical Inc, Concord, CA).

Microscopy and quantification

Images were collected using a brightfield microscope and were analysed using Image-Pro Plus software. Images of selected regions of highest positive signal were acquired under 200x magnification of each stained section (endothelial cells, neutrophils, macrophages and smooth muscle cells). These selected regions were then quantified using the color segmentation method. Thresholds were empirically set to select pixels by analysing a test set of 10 images per batch of staining. The selected pixels represented the expression of the stained cell. These empirically determined thresholds were recorded in a macro and were applied to all images that were analysed. The number of pixels counted by the macro was recorded in mm^2. The Matrigel area was measured using Image Pro's calibrated area measurement tool in mm^2. The percent occupancy of studied cells in the Matrigel from each sponge was calculated by taking the mean of: (area of counted pixels (mm^2)/area of Matrigel (mm^2)) x 100 of five randomly selected images per sponge. The mean microvessel density was expressed as the absolute number of microvessels counted/area of Matrigel (mm^2). The cross-sectional area occupied by these blood vessels was also simultaneously measured. The vessel maturation index was measured as: ((number of α-SMA-positive vessels/number of CD31-positive vessels) x 100).

Sponge single cell preparation and flow cytometry analysis

Single cell suspensions were isolated from sponges and spleen as previously described [Choi et al., 2011]. Briefly, the sponges and corresponding spleens were isolated from C57BL/6 mice, minced and incubated for 60 min at 37^{o}C in an enzyme mixture. Following the blockage

of Fc receptors using culture supernatant of 2.4G2 hybdridoma, the cells were stained with fluorophore-conjugated antibodies. The stained cells were acquired using LSR Fortessa (Becton Dickinson, Mississauga, Canada) and were analyzed using FlowJo (Tree Star Inc., Ashland, OR). The monoclonal antibodies used in both flow cytometry analysis and FACS were anti-mouse CD45, CD64, CD3, CD19, Ly6G (clone: 1A8), MHCII, CD11c and corresponding isotype controls were purchased from BioLegend.

Analysis of phagocytosis

Sponge and splenic $CD45^+CD11c^+MHCII^+$ cells isolated from C57BL/6 mice were incubated with 0.00134% of 0.50 μm Fluoresbrite® YG Microspheres (Polysciences, Inc., Warrington, PA) for 30 min at 37^{o}C. The cells were then labeled with monoclonal antibodies against CD45, CD11c, MHCII and CD19 and analyzed by flow cytometry.

Mixed leukocyte reactions

Sponge and splenic $CD45^+$ cells were FACS (Beckman Coulter MoFlo, Mississauga, Canada) sorted into $CD11c^+MHCII^+$ cell population and $CD11c^-MHCII^-Ly6G^+$ neutrophils from C57BL/6 mice. For proliferative analysis, splenic T-cells were isolated from BALB/c mice by excluding $B220^+$, $F4/80^+$, $CD49b^+$, $I\text{-}Ab^+$ cells using anti-rat IgG Dynabeads (Invitrogen, Burlington, Canada). These allogenic T-cells were subsequently labeled with carboxyfluorescein diacetate-succinimidyl ester (CFSE) and were combined with isolated stimulator cells (splenic $CD11c^+MHCII^+$ cells, sponge $CD11c^+MHCII^+$ cells and neutrophils;

stimulator: T-cell ratio of 1:10) in microtest wells at 5,000 of stimulator to 50,000 T-cells/well. Four days later, T-cell proliferation was evaluated by CFSE dilution in flow cytometry.

Statistical analyses

Results are presented as the mean ± SEM and all comparisons were made between each conditions at corresponding days by analysis of variance (ANOVA) followed by a Bonferroni t-test. Differences were considered significant at p-values less than 0.05.

RESULTS

VEGF and angiopoietins promote blood vessel formation

Previous studies have demonstrated that VEGF and angiopoietins play precise, complementary and coordinated roles in angiogenesis. In the present study, we wanted to assess the individual pro-angiogenic activities of VEGF and angiopoietins in a novel variant of the sponge/Matrigel angiogenic model. To monitor vascularization and to examine the angiogenic microenvironment in the sponges, we performed histological analysis using Masson's trichrome staining of the sponges at different time points from day 4 to day 21. Sponges containing PBS followed the time-course of the host foreign body response in terms of cellular infiltration and neovessels formation (Figure 1A and C). In contrast, sponges loaded with VEGF, Ang1 or Ang2 (200 ng/200 μl) elicited a robust invasion of various cell types into the Matrigel giving rise to a highly vascularized matrix by day 7 (Figure 1B, D-F).

Based on endothelial cell-specific CD31 IHC detection (Figure 2A; upper left insert), for a more comprehensive analysis, sponges containing PBS, VEGF, Ang1 or Ang2 showed marginal amount of EC recruitment by day 4 (Figure 2A). However, in the presence of any one of the tested growth factors, this effect became significant by day 7 and 14 with an increase of ≈3-5 fold as compared to PBS-treated sponges. At day 21, the percentage of CD31 expression in PBS-treated sponges became comparable to VEGF and Ang2 treated sponges, whereas Ang1 continuously maintained its capacity to recruit ECs. These recruited ECs took their neovessel structure (lumen formation) by day 7 in presence of VEGF, Ang1 or Ang2

(Figure 2B). Once formed by day 7, the microvessel density remained stable, ranging from 50 to 70 vessels/mm^2 of Matrigel. Although delayed in time, the microvessel density under PBS-treatment became comparable (≈50 vessels/mm^2 of Matrigel) to the growth factor-treated sponges by day 14 (Figure 2B). Finally, in presence of Ang2 the average cross-sectional area occupied by the neovessels formed by day 14 to 21 (>250 μm^2) was greater than the area of vessels formed in presence of VEGF or Ang1 (≈150-225 μm^2). However, under PBS treatment, the primary vessels formed by day 14, were smaller (≈100 μm^2) but underwent remodeling and nearly doubled by day 21 (Figure 2C).

VEGF and angiopoietins promote blood vessel maturation

Vessel maturation is critical in angiogenesis, as the stability of an induced vasculature is dependent on the mural cell association to prevent vessel regression [Bergers and Song, 2005]. We thus, wanted to elucidate the temporal sequel of VEGF and angiopoietins mediated maturation of neovessels given a pro-inflammatory environment. The recruitment of SMCs was detected based on α-SMA protein expression by day 7 in all tested conditions (Figure 3A). Yet, treatment with VEGF and angiopoietins individually triggered a more rapid and pronounced recruitment of SMCs, producing a ≈10-12-fold increase as compared to PBS-treated group. By day 14, the number of SMCs detected under growth factor stimulation plateaued, while the venue of SMCs under PBS-treatment caught up yet remaining ≈2.5-3-fold lower to what was mediated by VEGF and the angiopoietins. We also observed that by day 21, the area covered by SMCs in presence of VEGF or Ang1 was maintained whereas it partially declined under PBS or Ang2 treatment (Figure 3A).

To assess whether the SMCs remained sparse into the Matrigel and/or associated with neovessels, we performed double IHC staining against CD31 and α-SMA proteins. We observed a common inflection point by day 7 in presence of the different growth factors, favoring the formation of neovessels, the migration of SMCs and the surrounding of SMCs around the neovessels as compared to PBS-treated sponges (Figure 3B). Sponges harvested at day 4 under all of the tested treatments only supported the recruitment of ECs and not of SMCs (Figure 3A). By day 14 and 21, the maturing blood vessels were covered with multiple layers of SMCs for all conditions (Figure 3B). However, although the number of neovessels surrounded by at least a single layer of SMCs by day 7 was ≈60-70% and reached up to 80% by day 14 or 21 under growth factor treatments, it plateaued to about 40% by day 14 in PBS-treated sponges (Figure 3C).

VEGF and angiopoietins mediated angiogenesis is accompanied by inflammatory cells

Inflammatory cells, namely neutrophils and monocytes/macrophages participate in the angiogenic process through the secretion of pro- and anti-inflammatory cytokines by controlling EC activation, migration and proliferation [El Awad et al., 2000; Lingen, 2001; Voronov et al., 2003]. Using anti-MPO antibody (Figure 4A; upper right insert), we observed significant recruitment of neutrophils by day 7 in presence of VEGF, which peaked by day 14 covering about ≈2% of total surface area, and then faded away by day 21. In addition, VEGF was more potent as compared to the angiopoietins to promote the recruitment of neutrophils by day 14. Ang2 showed a significant peak (≈1%) in neutrophil recruitment by day 7 and its potency decreased gradually over time. On the other hand, Ang1 showed a mild effect on

neutrophil recruitment as compared to PBS-treated sponges (Figure 4A). We looked at the recruitment of monocytes/macrophages based on F4/80 selective protein detection (Figure 4B; upper right insert). The three growth factors individually tended to have a peak recruitment of macrophages by day 14, which was massive and significant under VEGF treatment (≈1%) as compared to PBS and angipoietins-treated animals (Figure 4B).

Characterization of inflammatory and immune cells in sponges by FACS analyses

To delineate the different leukocyte subsets recruited in the sponges, we utilized multicolor flow cytometry procedure on single cell preparation from sponges. $CD45^+$ leukocytes in the sponges were primarily comprised of $CD11c^+MHCII^+$ cells, $CD11c^-MHCII^-Ly6G^+$ cells (neutrophils), $CD11c^-MHCII^-CD3^+$ cells (T-cells) and $CD11c^-MHCII^+CD19^+$ cells (B-cells) (Figure 5A). The fate of $CD11c^+MHCII^+$ cells at this point remained to be investigated. Previous studies demonstrated that dentritic cells (DCs) constitutively express the hematopoietic markers CD45, CD11c and MHCII in lymphoid tissues such as spleen and lymph nodes. Nonetheless, this marker expression profile on its own is not sufficient to define classical DCs (cDCs) in nonlymphoid tissues. In fact, high and similar levels of CD11c and MHCII expression have been observed in both cDCs and in macrophages [Gautier et al., 2012]. Thus, we performed marker analyses, genetic and functional studies to specifically identify the $CD11c^+MHCII^+$ cell population in the sponges as cDCs and/or macrophages. Recently, *Zbtb46* was identified as a selectively expressed transcription factor by cDCs but not by monocytes, macrophages and other lymphoid and myeloid lineages (e.g. neutrophils, T-cells and B-cells) [Satpathy et al., 2012]. Therefore, we harvested sponge cells from $Zbtb46^{+/+}$

(WT) and *Zbtb46*$^{gfp/+}$ mice and analyzed for GFP expression. As anticipated, CD45^{+} cell population containing splenic DCs were GFP^{+} while neutrophils recruited in the sponges were devoid of GFP expression. In contrast to splenic DCs, CD11c^{+}MHCII^{+} cell population isolated from the sponges lacked expression of GFP (Figure 5B). Next, FACS-sorted CD11c^{+}MHCII^{+} cells from sponges and spleens along with neutrophils were tested for their ability to stimulate allogenic T-cells. Only splenic DCs were strong stimulators of T-cell proliferation (Figure 5C). T-cells alone, neutrophils from sponges and spleen and CD11c^{+}MHCII^{+} cells from sponges did not induce allogenic T-cell proliferation. These results confirm that CD11c^{+}MHCII^{+} cells in the sponges do not possess DCs functional characteristics.

In contrast, CD11c^{+}MHCII^{+} cells from PBS, VEGF, Ang1 and Ang2-treated sponges were positive for F4/80 marker while the neutrophils from the corresponding sponges were negative (Figure 6A). Although in the past, F4/80 served as a reliable marker of macrophages, additional analysis of a panel of surface markers is now required to define macrophage population. Recently, the surface marker CD64 expression was identified as a reliable marker of mature tissue macrophages [Gautier et al., 2012]. Interestingly, in our study, we observed that the CD11c^{+}MHCII^{+} cells isolated from the sponges were all CD64^{+}. Moreover, they also expressed CD68 and CD206, two additional markers of macrophages [Gautier et al., 2012] (Figure 6B). To test whether the CD11c^{+}MHCII^{+} cells recruited in the sponges had the phagocytic activity of macrophages, we analyzed their phagocytic capacity by flow cytometry. Neutrophils from the sponges took up 0-2 beads/cell while all sponge MHCII^{+}CD11c^{+} cells were highly phagocytic (>3 beads uptake/cell). B-cells isolated from the sponges served as our

negative control and it provided no phagocytic activity (Figure 6C). To further confirm these isolated cells from the sponges as macrophages, we used transgenic *CD115*$^{gfp/+}$ mice, which express MCSF-1R, a receptor for macrophage-colony stimulating factor [Sasmono et al., 2007]. Indeed, the CD11c^{+}MHCII^{+} cells isolated from the sponges implanted in *CD115*$^{gfp/+}$ mice were GFP positive. However, B-cells (negative control) from the transgenic *CD115*$^{gfp/+}$ and WT mice were GFP negative (Figure 6D). Taken together, these results demonstrate that the MHCII^{+}CD11c^{+} cells present in the sponges are macrophages and not DCs.

DISCUSSION

Compelling studies have demonstrated the direct participation of neutrophils and monocytes/macrophages in the induction of inflammatory response prior to the initiation of pathological angiogenesis. Indeed, the release of pro-inflammatory cytokines and growth factors provides a suited autocrine/paracrine milieu to fully support blood vessels formation [Aplin et al., 2006; Gong and Koh, 2010; Lin et al., 2006; Schruefer et al., 2005]. As we have previously illustrated the pro-inflammatory activities of VEGF and angiopoietins, we were led to address their capacity to promote inflammatory response associated to *in vivo* angiogenesis. In the present study, we utilized a novel variant of the murine sponge/Matrigel angiogenic assay to evaluate the sequel of host-derived blood vessel formation and inflammatory cell infiltration into the sponges. Herein, we demonstrate that VEGF, Ang1 and Ang2 individually are highly potent and efficacious in recruiting ECs, SMCs and inflammatory cells (mainly neutrophils and macrophages, and sparsely T- and B-cells). More importantly, these tested growth factors given individually were not only capable to favor the formation of neovessels but also their maturation as observed by the coordinated ensheating of SMCs around the neovessels and the presence of circulating red blood cells in the vessel lumen. Hence, this study suggests the potential contribution of both inflammatory cells and angiogenic growth factors to fully support blood vessel formation and their maturation.

Recent efforts in clinical trials focus on localized therapy for restoring blood flow in ischemic regions as tissue loss in these patients was localized [Simons et al., 2000]. While growth factor therapy remained a gold standard for the induction of local therapeutic

angiogenesis, translating this concept into an effective and safe therapy for patients became a challenge. Presently, bio-material based approaches is being successfully utilized in animal models to study the capacity of growth factors, cytokines/chemokines and nonprotein mediators to promote blood vessel formation [Andrade et al., 1997] . One such method is the subcutaneous implantation of PVA sponges in mice, which promotes a robust infiltration of inflammatory cells, providing a pro-inflammatory environment, and giving rise to a highly vascularized sponge matrix. However, due to continuous inflammation, these newly formed vessels were postulated to be fragile, permeable and dilated with no indication of neovessel maturation (lack of SMCs ensheathing) [Andrade et al., 1997]. A major disadvantage of such matrix implantation is that it induces non-specific inflammatory host response and thus limits to acute studies [Staton et al., 2009]. Later, the Matrigel plug assay became the widely used model for studies involving *in vivo* testing for angiogenesis, as it provides a natural environment for the formation of neovessels without inducing non-specific immune response [Staton et al., 2009]. Yet, although Matrigel injection containing VEGF in mice successfully promoted the formation of neovessels, the model did not lead to the maturation of the newly formed vessels [Tengood et al., 2010]. As inflammation is an important stimulus for the induction of new vessel growth, we hypothesized that the combination of both these approaches might fulfill the required environment to favor the formation and maturation of neovessels. The classical sponge/Matrigel model, encompassing both the sponge model and the Matrigel assay, requires the subcutaneous injection of Matrigel containing the protein of interest, 20-30 minutes prior to the surgical introduction of PVA sponges [Akhtar et al., 2002; Norrby, 2006]. This method has been identified to provide variable amount of test compound within the implants and to trigger the fibrotic encapsulation of the sponges [Norrby, 2006]. In

our variation of the sponge/Matrigel model, we have soaked PVA sponges into Matrigel containing the tested growth factors prior to the surgical implantation. We observed that our technique was simple, less time consuming, that each sponge implant contained equal volume of the tested growth factors and it did not induce non-specific immune response.

We observed an early onset of EC migration in the sponges within the first 4 days and a significant number of blood vessel formation by day 7 under VEGF or angiopoietin stimulation, thus, challenging the classical role of angiopoietins in angiogenesis. Interestingly, the amount of ECs migrated into the sponges kept increasing up to day 14 or 21, while the number of blood vessels once formed by day 7 remained stable, suggesting that the model itself exerts a restrain on the maximal capacity of blood vessel formation even in presence of free ECs. Our data is in line with previous studies reporting the pro-angiogenic and mitogenic activities of VEGF in various *in vivo* models including the chick chorioallantoic membrane [Plouet et al., 1989], the rabbit cornea [Phillips et al., 1994] and the primate iris [Tolentino et al., 1996]. However, the capacity of angiopoietins to initiate the angiogenic cascade remains controversial. For instance, while some *in vivo* reports demonstrated that Ang1 alone is unable to induce angiogenesis but can potentiate VEGF mediated angiogenic response [Asahara et al., 1998; Chae et al., 2000], others showed that Ang1 can promote a robust neovascularization in Matrigel implants [Babaei et al., 2003]. The implication of Ang2 in angiogenesis is tied with VEGF where it promotes destabilization of pre-existing blood vessels in the absence of VEGF [Holash et al., 1999; Lobov et al., 2002]. Yet, other studies reported that Ang2 alone can induce vascular remodeling and angiogenesis in absence of VEGF [Kim et al., 2000b; Mochizuki et al., 2002]. Our study illustrates that the pro-inflammatory environment itself is

sufficient to initiate the angiogenic cascade and the addition of the tested growth factors further allows this effect to be more potent and efficient.

Interestingly, we also observed the venue and the ensheathing of SMCs around neovessels by day 7 in presence of the tested growth factors. Indeed, all three growth factors promoted the maturation of blood vessels with equal potency. Although, our result is consistent with the stabilizing effect of Ang1 on vascular endothelium, it is also in contradiction with the proposed role for VEGF and Ang2 during angiogenesis. In fact, VEGF and angiopoietins are incapable of directly activating SMCs. Yet, they can promote the activation of ECs and support the migration of inflammatory cells (e.g. macrophages and neutrophils) which can promote the release of various growth factors and cytokines (e.g. FGF, VEGF, Ang1, interleukins (IL-1β, IL-8 and -10) and CXCL1) [Dinarello, 2009; Gaudry et al., 1997; Neagoe et al., 2009; Noonan et al., 2008]. Ang2 in particular, has been shown to possess pro-inflammatory characteristics on both ECs and neutrophils [Fiedler and Augustin, 2006; Fiedler et al., 2006; Kim and Koh, 2011; Lemieux et al., 2005]. In addition, neutrophils and macrophages can equally trigger the release of numerous metalloproteinases, neutrophil elastase and reactive oxygen species (ROS), which can facilitate extracellular matrix degradation, favoring the migration and proliferation of ECs and SMCs (reviewed in [van Hinsbergh et al., 2006]). In addition, the presence of neutrophils and macrophages in the sponges at day 7 during the recruitment of SMCs may initiate a paracrine compensation pathway in order to trigger the maturation event. Interestingly, from the histological sections, we observed that the newly formed vessels in presence of VEGF, Ang1 or Ang2 were "functional" based on the presence red blood cells in the neovessels and that they appeared to

be non-leaky. Vascular permeability study must be conducted in order to confirm this later statement. However, since not all the neovessels formed in the sponges are necessarily matured at any given time, it is thus, not possible to confirm the absence of vascular leakiness. Furthermore, we also observed that the blood vessels once formed undergo no or marginal diameter remodeling. Ang1 in the past has been identified to play an important role in the reorganization of EC into tubule-like structures during angiogenesis by stimulating the production of proteases. Plasmin and matrix metalloproteases, examples of such proteases, decrease the EC-substratum interaction allowing the ECs to reshape the vessel lumen [Kim et al., 2000a]. However, in our study, upon the formation of neovessels by day 7 (with growth factors), we did not observe additional remodeling over time. This may be due to the rapid maturation of the newly formed vessels taking place simultaneous to blood vessel formation which may prevent further unrestricted enlargement of the growing vessels [Hoeben et al., 2004]. As for the PBS-treated sponges, the delayed recruitment of SMCs may explain the slight remodeling of the area of occupancy that took place between day 14 and 21. Together, VEGF, Ang1 and Ang2 alone are capable of mediating the maturation process in the presence of a pro-inflammatory environment suggesting that inflammation plays a major role in the angiogenic process.

This notion is further strengthened as observed under various pathological conditions. For instance, suppression of inflammatory response by genetic abnormalities, pathophysiological processes, or pharmacotherapy produce adverse effects in the ability of the host to induce new vessel growth [Jones et al., 1999]; hence inflammation, once considered to be a homeostatic response protecting the body from invading pathogens, is now been shown to

function as a critical stimulus for neovessel growth. Neutrophils being the most abundant leukocyte in the circulation have been demonstrated to play important roles during pathological angiogenesis. Although, the exact mechanism through which tumor associated neutrophils mediate or modulate angiogenesis has not been fully elucidated, the importance of neutrophils in tumor angiogenesis has been noted from human biopsies [Nozawa et al., 2006; Van den Steen et al., 2000]. Similarly, increased macrophage infiltration in various types of cancer correlates positively with vascularity, tumor stage and malignancy [Chen et al., 2003; Torisu et al., 2000]. Once again the exact function of the macrophages in the tumor environment remains a nuance.

Likewise, although we did not study the exact roles of inflammatory cells in angiogenesis, we observed the presence of neutrophils, macrophages, and sparsely T- and B-cells, in the tissue section by IHC and/or flow cytometry. Surprisingly, the presence of neutrophils, expected to be one of the first cells recruited at the site of inflammation, was still observed at latter time points raising the question whether we have continuous recruitment of neutrophils in sponges or if they have been differentiated to other cell types. Recently, it was suggested that neutrophils could differentiate into neutrophil-DC hybrids with DC-like properties in the setting of experimentally induced inflammatory lesions in mice [Geng et al., 2013; Matsushima et al., 2013]. DCs are professional antigen presenting cells, which reside in peripheral tissues in an immature state. Upon microbial contact and stimulation by inflammatory cytokines, it possesses a unique ability to induce both primary and secondary T- and B-cell responses. It is now clear that DCs express a wide array of pro- and anti-inflammatory mediators that mediate a significant role in those pathophysiological settings

characterized by DC activation and angiogenesis [Sozzani et al., 2007]. Thus, we hypothesized that neutrophils may differentiate into neutrophil-DC hybrids in our system. However, $CD11c^{+}MHCII^{+}$ cells, which we initially thought to be DCs, turned out to be neither DCs nor neutrophil-DC hybrid cells. These cells 1) did not express DC-lineage transcriptional factor Zbtb46 and 2) did not stimulate allogenic T-cells in MLR assay. Instead, they expressed macrophage specific markers including F4/80, CD68, CD206 and CD115/mCSF1R and were highly phagocytic. Hence, our sponge/Matrigel model, apart from B and T-cells, contains two major inflammatory cell populations: neutrophils and macrophages.

In summary, our murine sponge/Matrigel model in presence of the pro-angiogenic growth factors (VEGF, Ang1 or Ang2) allowed the formation of new vessels and more importantly, it led to their maturation. Moreover, the recruitment of inflammatory cells in the Matrigel by the provided growth factors further accelerated these processes with greater potency. Thus, such pro-inflammatory/angiogenic model along with the growth factors may provide a suited autocrine/paracrine environment capable of triggering and supporting the formation and maturation of neovessels, illustrating the necessity of inflammation in the creation of mature blood vessels. Further studies will be needed through selected depletion of neutrophils and monocytes/macrophages to delineate the role of these cells in such angiogenic model.

ACKNOWLEDGEMENTS

This work was supported by grants from the Canadian Institutes of Health Research (CIHR; MOP-97943 (MGS) and MOP-125933 (CC)) and from the Heart and Stroke Foundation of Quebec (MGS). Cheolho Cheong is is a Chercheur-Boursier Junior of Fonds de Recherche du Québec-Santé.

REFERENCES

Akhtar N, Dickerson EB, Auerbach R. 2002. The sponge/Matrigel angiogenesis assay. Angiogenesis 5:75-80.

Andrade SP, Machado RD, Teixeira AS, Belo AV, Tarso AM, Beraldo WT. 1997. Sponge-induced angiogenesis in mice and the pharmacological reactivity of the neovasculature quantitated by a fluorimetric method. Microvascular research 54:253-61.

Aplin AC, Gelati M, Fogel E, Carnevale E, Nicosia RF. 2006. Angiopoietin-1 and vascular endothelial growth factor induce expression of inflammatory cytokines before angiogenesis. Physiol Genomics 27:20-8.

Asahara T, Chen D, Takahashi T, Fujikawa K, Kearney M, Magner M, Yancopoulos GD, Isner JM. 1998. Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization. Circulation research 83:233-40.

Babaei S, Teichert-Kuliszewska K, Zhang Q, Jones N, Dumont DJ, Stewart DJ. 2003. Angiogenic actions of angiopoietin-1 require endothelium-derived nitric oxide. The American journal of pathology 162:1927-36.

Bergers G, Song S. 2005. The role of pericytes in blood-vessel formation and maintenance. Neuro Oncol 7:452-64.

Carmeliet P, Jain RK. 2011. Molecular mechanisms and clinical applications of angiogenesis. Nature 473:298-307.

Chae JK, Kim I, Lim ST, Chung MJ, Kim WH, Kim HG, Ko JK, Koh GY. 2000. Coadministration of angiopoietin-1 and vascular endothelial growth factor enhances collateral vascularization. Arteriosclerosis, thrombosis, and vascular biology 20:2573-8.

Chen JJ, Yao PL, Yuan A, Hong TM, Shun CT, Kuo ML, Lee YC, Yang PC. 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. Clinical cancer research : an official journal of the American Association for Cancer Research 9:729-37.

Choi JH, Cheong C, Dandamudi DB, Park CG, Rodriguez A, Mehandru S, Velinzon K, Jung IH, Yoo JY, Oh GT, Steinman RM. 2011. Flt3 signaling-dependent dendritic cells protect against atherosclerosis. Immunity 35:819-31.

Costa C, Incio J, Soares R. 2007. Angiogenesis and chronic inflammation: cause or consequence? Angiogenesis 10:149-66.

Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD. 1996. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell 87:1161-9.

de Muinck ED, Simons M. 2004. Re-evaluating therapeutic neovascularization. J Mol Cell Cardiol 36:25-32.

Dinarello CA. 2009. Immunological and inflammatory functions of the interleukin-1 family. Annual review of immunology 27:519-50.

Dor Y, Djonov V, Abramovitch R, Itin A, Fishman GI, Carmeliet P, Goelman G, Keshet E. 2002. Conditional switching of VEGF provides new insights into adult neovascularization and pro-angiogenic therapy. The EMBO journal 21:1939-47.

Dumas E, Martel C, Neagoe PE, Bonnefoy A, Sirois MG. 2012. Angiopoietin-1 but not angiopoietin-2 promotes neutrophil viability: Role of interleukin-8 and platelet-activating factor. Biochimica et biophysica acta 1823:358-67.

El Awad B, Kreft B, Wolber EM, Hellwig-Burgel T, Metzen E, Fandrey J, Jelkmann W. 2000. Hypoxia and interleukin-1beta stimulate vascular endothelial growth factor production in human proximal tubular cells. Kidney Int 58:43-50.

Fiedler U, Augustin HG. 2006. Angiopoietins: a link between angiogenesis and inflammation. Trends in immunology 27:552-8.

Fiedler U, Reiss Y, Scharpfenecker M, Grunow V, Koidl S, Thurston G, Gale NW, Witzenrath M, Rosseau S, Suttorp N, Sobke A, Herrmann M, Preissner KT, Vajkoczy P, Augustin HG. 2006. Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nature medicine 12:235-9.

Gaudry M, Bregerie O, Andrieu V, El Benna J, Pocidalo MA, Hakim J. 1997. Intracellular pool of vascular endothelial growth factor in human neutrophils. Blood 90:4153-61.

Gautier EL, Shay T, Miller J, Greter M, Jakubzick C, Ivanov S, Helft J, Chow A, Elpek KG, Gordonov S, Mazloom AR, Ma'ayan A, Chua WJ, Hansen TH, Turley SJ, Merad M,

Randolph GJ. 2012. Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. Nat Immunol 13:1118-28.

Geng S, Matsushima H, Okamoto T, Yao Y, Lu R, Page K, Blumenthal RM, Ward NL, Miyazaki T, Takashima A. 2013. Emergence, origin, and function of neutrophil-dendritic cell hybrids in experimentally induced inflammatory lesions in mice. Blood 121:1690-700.

Gong Y, Koh DR. 2010. Neutrophils promote inflammatory angiogenesis via release of preformed VEGF in an in vivo corneal model. Cell Tissue Res 339:437-48.

Griffioen AW, Molema G. 2000. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. Pharmacol Rev 52:237-68.

Haddad LE, Sirois MG. 2014. Angiopoietin-1 upregulates de novo expression of IL-1beta and Il1-Ra, and the exclusive release of Il1-Ra from human neutrophils. PloS one 9:e88980.

Hanahan D, Folkman J. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86:353-64.

Henry TD, Annex BH, McKendall GR, Azrin MA, Lopez JJ, Giordano FJ, Shah PK, Willerson JT, Benza RL, Berman DS, Gibson CM, Bajamonde A, Rundle AC, Fine J, McCluskey ER. 2003. The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis. Circulation 107:1359-65.

Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA. 2004. Vascular endothelial growth factor and angiogenesis. Pharmacological reviews 56:549-80.

Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ. 1999. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science 284:1994-8.

Jones MK, Wang H, Peskar BM, Levin E, Itani RM, Sarfeh IJ, Tarnawski AS. 1999. Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. Nature medicine 5:1418-23.

Kim H, Koh GY. 2011. Ang2, the instigator of inflammation. Blood 118:4767-8.

Kim I, Kim HG, Moon SO, Chae SW, So JN, Koh KN, Ahn BC, Koh GY. 2000a. Angiopoietin-1 induces endothelial cell sprouting through the activation of focal adhesion kinase and plasmin secretion. Circulation research 86:952-9.

Kim I, Kim JH, Moon SO, Kwak HJ, Kim NG, Koh GY. 2000b. Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Oncogene 19:4549-52.

Lemieux C, Maliba R, Favier J, Theoret JF, Merhi Y, Sirois MG. 2005. Angiopoietins can directly activate endothelial cells and neutrophils to promote proinflammatory responses. Blood 105:1523-30.

Liekens S, De Clercq E, Neyts J. 2001. Angiogenesis: regulators and clinical applications. Biochemical pharmacology 61:253-70.

Lin YJ, Lai MD, Lei HY, Wing LY. 2006. Neutrophils and macrophages promote angiogenesis in the early stage of endometriosis in a mouse model. Endocrinology 147:1278-86.

Lingen MW. 2001. Role of leukocytes and endothelial cells in the development of angiogenesis in inflammation and wound healing. Arch Pathol Lab Med 125:67-71.

Lobov IB, Brooks PC, Lang RA. 2002. Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo. Proceedings of the National Academy of Sciences of the United States of America 99:11205-10.

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN,

Yancopoulos GD. 1997. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277:55-60.

Maliba R, Brkovic A, Neagoe PE, Villeneuve LR, Sirois MG. 2008. Angiopoietin-mediated endothelial P-selectin translocation: cell signaling mechanisms. Journal of leukocyte biology 83:352-60.

Maliba R, Lapointe S, Neagoe PE, Brkovic A, Sirois MG. 2006. Angiopoietins-1 and -2 are both capable of mediating endothelial PAF synthesis: intracellular signalling pathways. Cellular Signalling 18:1947-57.

Marchand GS, Noiseux N, Tanguay JF, Sirois MG. 2002. Blockade of in vivo VEGF-mediated angiogenesis by antisense gene therapy: role of Flk-1 and Flt-1 receptors. American journal of physiology. Heart and circulatory physiology 282:H194-204.

Matsushima H, Geng S, Lu R, Okamoto T, Yao Y, Mayuzumi N, Kotol PF, Chojnacki BJ, Miyazaki T, Gallo RL, Takashima A. 2013. Neutrophil differentiation into a unique hybrid population exhibiting dual phenotype and functionality of neutrophils and dendritic cells. Blood 121:1677-89.

Mochizuki Y, Nakamura T, Kanetake H, Kanda S. 2002. Angiopoietin 2 stimulates migration and tube-like structure formation of murine brain capillary endothelial cells through c-Fes and c-Fyn. Journal of cell science 115:175-83.

Neagoe PE, Brkovic A, Hajjar F, Sirois MG. 2009. Expression and release of angiopoietin-1 from human neutrophils: intracellular mechanisms. Growth factors 27:335-44.

Neagoe PE, Dumas E, Hajjar F, Sirois MG. 2012. Angiopoietin-1 but not angiopoietin-2 induces IL-8 synthesis and release by human neutrophils. Journal of cellular physiology 227:3099-3110.

Noonan DM, De Lerma Barbaro A, Vannini N, Mortara L, Albini A. 2008. Inflammation, inflammatory cells and angiogenesis: decisions and indecisions. Cancer metastasis reviews 27:31-40.

Norrby K. 2006. In vivo models of angiogenesis. Journal of cellular and molecular medicine 10:588-612.

Nozawa H, Chiu C, Hanahan D. 2006. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. Proceedings of the National Academy of Sciences of the United States of America 103:12493-8.

Phillips GD, Stone AM, Jones BD, Schultz JC, Whitehead RA, Knighton DR. 1994. Vascular endothelial growth factor (rhVEGF165) stimulates direct angiogenesis in the rabbit cornea. In vivo 8:961-5.

Plouet J, Schilling J, Gospodarowicz D. 1989. Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells. The EMBO journal 8:3801-6.

Qazi BS, Tang K, Qazi A. 2011. Recent advances in underlying pathologies provide insight into interleukin-8 expression-mediated inflammation and angiogenesis. International journal of inflammation 2011:908468.

Sasmono RT, Ehrnsperger A, Cronau SL, Ravasi T, Kandane R, Hickey MJ, Cook AD, Himes SR, Hamilton JA, Hume DA. 2007. Mouse neutrophilic granulocytes express mRNA encoding the macrophage colony-stimulating factor receptor (CSF-1R) as well as many other macrophage-specific transcripts and can transdifferentiate into macrophages in vitro in response to CSF-1. J Leukoc Biol 82:111-23.

Satpathy AT, Kc W, Albring JC, Edelson BT, Kretzer NM, Bhattacharya D, Murphy TL, Murphy KM. 2012. Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. J Exp Med 209:1135-52.

Schruefer R, Lutze N, Schymeinsky J, Walzog B. 2005. Human neutrophils promote angiogenesis by a paracrine feedforward mechanism involving endothelial interleukin-8. Am J Physiol Heart Circ Physiol 288:H1186-92.

Simons M, Bonow RO, Chronos NA, Cohen DJ, Giordano FJ, Hammond HK, Laham RJ, Li W, Pike M, Sellke FW, Stegmann TJ, Udelson JE, Rosengart TK. 2000. Clinical trials in coronary angiogenesis: issues, problems, consensus: An expert panel summary. Circulation 102:E73-86.

Sozzani S, Rusnati M, Riboldi E, Mitola S, Presta M. 2007. Dendritic cell-endothelial cell cross-talk in angiogenesis. Trends in immunology 28:385-92.

Staton CA, Reed MW, Brown NJ. 2009. A critical analysis of current in vitro and in vivo angiogenesis assays. Int J Exp Pathol 90:195-221.

Stewart DJ, Kutryk MJ, Fitchett D, Freeman M, Camack N, Su Y, Della Siega A, Bilodeau L, Burton JR, Proulx G, Radhakrishnan S. 2009. VEGF gene therapy fails to improve perfusion of ischemic myocardium in patients with advanced coronary disease: results of the NORTHERN trial. Molecular therapy : the journal of the American Society of Gene Therapy 17:1109-15.

Teichert-Kuliszewska K, Maisonpierre PC, Jones N, Campbell AI, Master Z, Bendeck MP, Alitalo K, Dumont DJ, Yancopoulos GD, Stewart DJ. 2001. Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2. Cardiovascular research 49:659-70.

Tengood JE, Kovach KM, Vescovi PE, Russell AJ, Little SR. 2010. Sequential delivery of vascular endothelial growth factor and sphingosine 1-phosphate for angiogenesis. Biomaterials 31:7805-12.

Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD. 2000. Angiopoietin-1 protects the adult vasculature against plasma leakage. Nature medicine 6:460-3.

Tolentino MJ, Miller JW, Gragoudas ES, Chatzistefanou K, Ferrara N, Adamis AP. 1996. Vascular endothelial growth factor is sufficient to produce iris neovascularization and neovascular glaucoma in a nonhuman primate. Archives of ophthalmology 114:964-70.

Torisu H, Ono M, Kiryu H, Furue M, Ohmoto Y, Nakayama J, Nishioka Y, Sone S, Kuwano M. 2000. Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma: possible involvement of TNFalpha and IL-1alpha. International journal of cancer. Journal international du cancer 85:182-8.

Van den Steen PE, Proost P, Wuyts A, Van Damme J, Opdenakker G. 2000. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. Blood 96:2673-81.

van Hinsbergh VW, Engelse MA, Quax PH. 2006. Pericellular proteases in angiogenesis and vasculogenesis. Arteriosclerosis, thrombosis, and vascular biology 26:716-28.

Voronov E, Carmi Y, Apte RN. 2007. Role of IL-1-mediated inflammation in tumor angiogenesis. Advances in experimental medicine and biology 601:265-70.

Voronov E, Shouval DS, Krelin Y, Cagnano E, Benharroch D, Iwakura Y, Dinarello CA, Apte RN. 2003. IL-1 is required for tumor invasiveness and angiogenesis. Proc Natl Acad Sci U S A 100:2645-50.

FIGURE LEGENDS

Figure 1. Pro-angiogenic and inflammatory activities of VEGF and angiopoietins in mice. The images illustrate representative scans (A and B) and representative histological sections (Masson's trichrome staining, 400X magnification) of PVA sponges soaked in growth factor depleted Matrigel containing PBS, VEGF, Ang1 or Ang2 (200 ng/200 μl) harvested at day 7 (C-F). Treatment with VEGF, Ang1 or Ang2 promoted a marked recruitment of numerous inflammatory cells, endothelial cells and mural cells in the region of Matrigel within the sponges and the formation of neovessels (black arrow) containing circulating red blood cells (star), indicative of functional blood vessels (B, D-F). Neovessel formation from endothelial cells (lumen formation) lacking circulating red blood cells was also observed (white arrow). In contrast, PBS treated sponges showed less cellular accumulation and no blood vessel formation by day 7 (A and C).

Figure 2. Effect of VEGF and angiopoietins on angiogenesis in a time-dependent manner. PVA sponges soaked in growth factor depleted Matrigel containing PBS, VEGF, Ang1 or Ang2 (200 ng/200 μl) were removed from the animals at day 4, 7, 14 or 21. Subsequently, IHC staining against endothelial cell specific CD31 protein was performed in order to assess the recruitment of endothelial cells (IHC insert; 1000X; A), microvessel density (B) and the average cross-sectional area occupied by the vessels (C) in the Matrigel. Data are represented as mean ± SEM of 4 to 10 independent experiments per condition. *$p< 0.05$, **$p< 0.01$, *** $p< 0.001$ as compared to PBS-treatment at corresponding days, N/D: not detectable.

Figure 3. VEGF and angiopoietins mediated SMC migration and neovascular maturation in the sponges. PVA sponges soaked in growth factor depleted Matrigel containing PBS, VEGF, Ang1 or Ang2 (200 ng/200 μl) were removed from the animals at day 4, 7, 14 or 21. Subsequently, IHC staining against α-SMA was performed in order to assess the venue of SMCs (A). The 4 panels represent immunohistogical snapshots illustrating the temporal evolution of mature blood vessels in the sponges in presence of the studied growth factors (Ang1) at days 4, 7, 14 and 21. Endothelial cells were stained with anti-CD31 (brown staining; thin arrow) and SMCs were stained with anti-α-SMA (turquoise staining; thick arrow) (1000X magnification). The neovessels were not only ensheathed by SMCs but also contained red blood cells (star) (B). The percentage of mature blood vessels was quantified as the number of neovessels surrounded by SMCs over the total number of blood vessels (C). Data are represented as mean ± SEM of 4 to 10 independent experiments per condition. *p< 0.05, **p< 0.01, *** p< 0.001 as compared to PBS-treatment at corresponding days, N/D: not detectable.

Figure 4. Identification of VEGF and angiopoietins mediated inflammatory cells influx in the sponges. Neutrophil (A) and macrophage (B) accumulation in the sponge implants were measured as MPO (IHC insert; 1000X; A) and F4/80 (IHC insert; 1000X; B) expression, respectively, in the Matrigel region of the sponges. VEGF, Ang1 and Ang2 mediated inflammatory cells (neutrophil and macrophage) recruitment was temporal-dependent with different potency. Data are represented as mean ± SEM of 4 to 10 independent experiments per condition. *p< 0.05, **p< 0.01, *** p< 0.001 as compared to PBS-treatment, §§p< 0.01 as compared to VEGF-treatment at corresponding days, N/D: not detectable.

Figure 5. $CD11c^{+}MHCII^{+}$ cells recruited in the sponges do not possess DC-characteristics. Single cell suspensions from sponges harvested from C57BL/6 mice were examined for surface expression of indicated markers. The data illustrates the expression profile of CD11c and MHCII, CD3 (T-cells), CD19 (B-cells) and Ly6G (neutrophils) within $CD45^{+}$ gated cell population (A). Representative histogram of GFP expression of $CD11c^{+}MHCII^{+}$ cells in spleen (n=2), VEGF-treated sponges and neutrophils (n=4) harvested from *Zbtb46*$^{+/+}$ (WT) and *Zbtb46*$^{gfp/+}$ mice (B). FACS purified $CD11c^{+}MHCII^{+}$ cells isolated from sponges (treated with VEGF or Ang1) and spleens along with neutrophils retrieved from C57BL/6 mice were co-cultured with T-cells purified from BALB/c mice ($CD3^{+}$CFSE-labled T-cells) in MLR. CFSE levels were analyzed four days later. Proliferation of allogenic T-cells results in a reduction of CFSE fluorescence intensity (n=4; C).

Figure 6. $CD11c^{+}MHCII^{+}$ cells recruited in the sponges are classical and non-classical macrophages. The images illustrate representative histogram (n=2-4 independent experiments per condition) for F4/80 expression of $CD11c^{+}MHCII^{+}$ cells and $CD11c^{-}MHCII^{-}$ $Ly6G^{+}$ neutrophils isolated from sponges pretreated with PBS, VEGF, Ang1 or Ang2 (A). $CD11c^{+}MHCII^{+}$ $CD64^{+}$ cells were stained for the intracellular markers CD68 and CD206 (B). Single cell suspensions isolated from VEGF treated sponge were bathed with 0.5 μm YG microspheres for 30 min at 37^{o}C and the uptake of these microspheres (phagocytosis) were analysed by flow cytometry (C). B-cells, neutrophils and $CD11c^{+}MHCII^{+}$ cells isolated from VEGF treated sponges harvested from *CD115*$^{gfp/+}$ and WT mice were analysed for GFP expression (D).

CONFLICT OF INTEREST DISCLOSURE

All the authors read the manuscript and none of them had conflict of interest pertaining to the content of the current study.

FIGURE 1

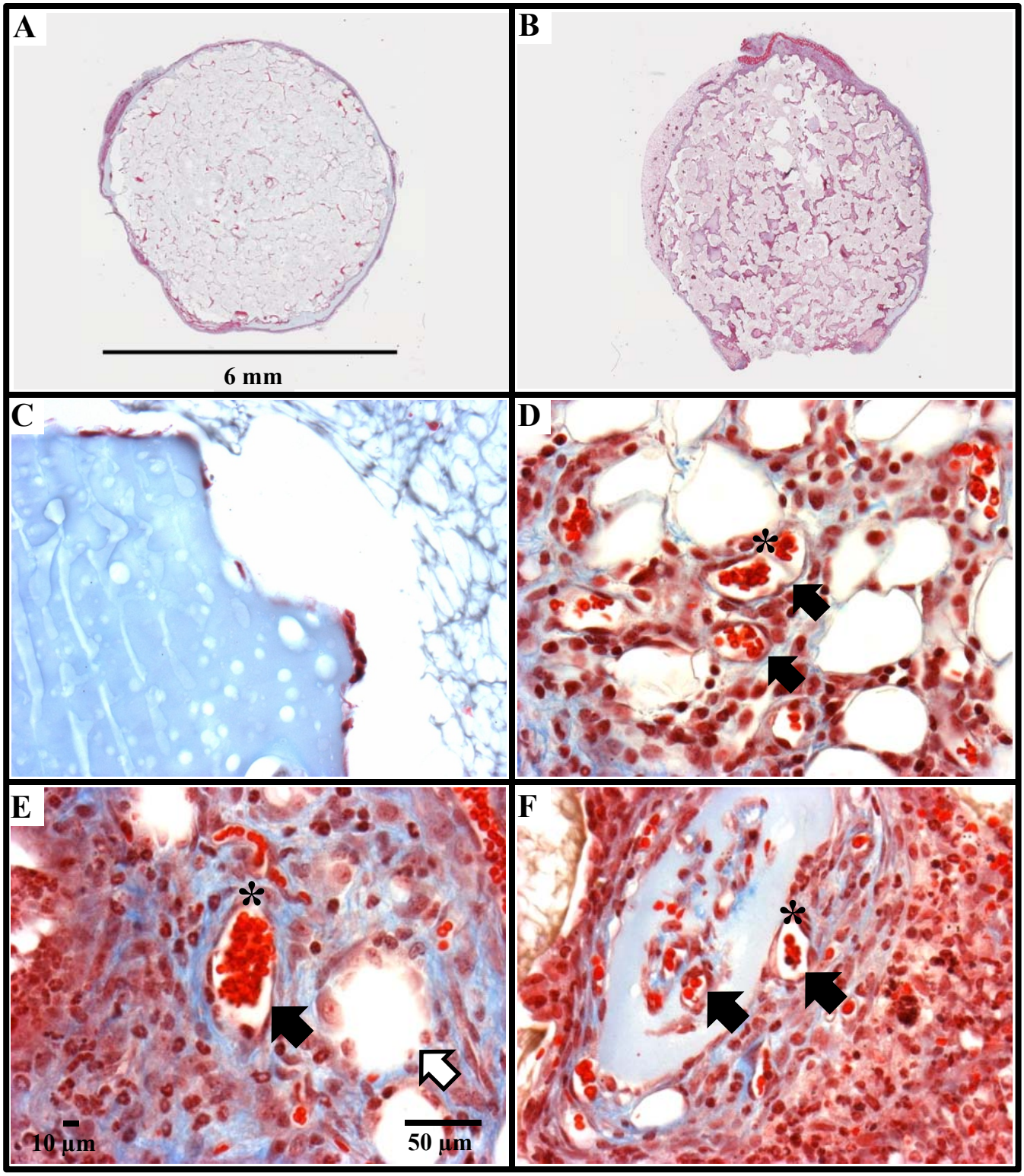


FIGURE 2

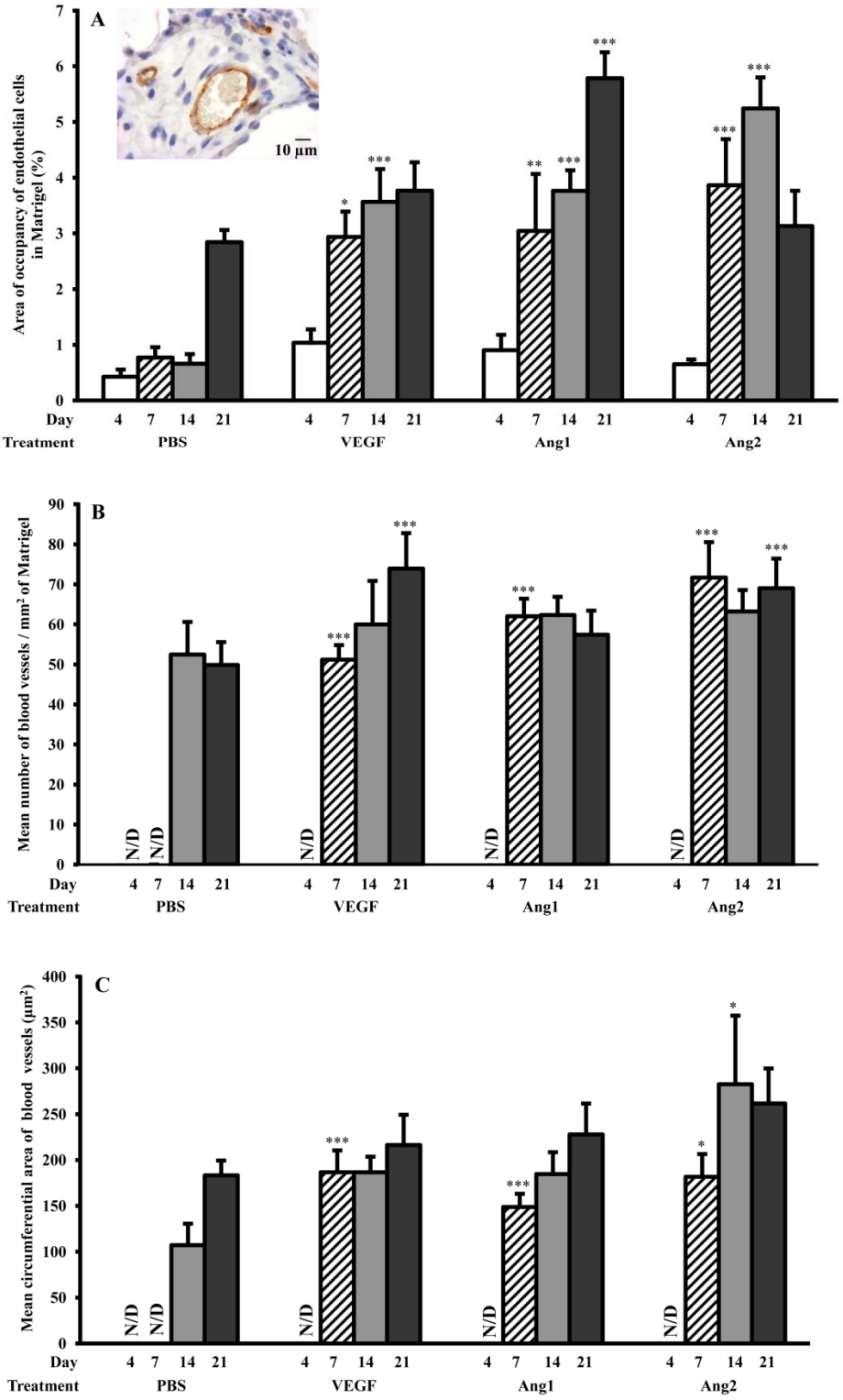

A
10 µm
Area of occupancy of endothelial cells in Matrigel (%)
B
Mean number of blood vessels / mm² of Matrigel
C
Mean circumferential area of blood vessels (µm²)
N/D
Day 4 7 14 21
Treatment PBS VEGF Ang1 Ang2

FIGURE 3

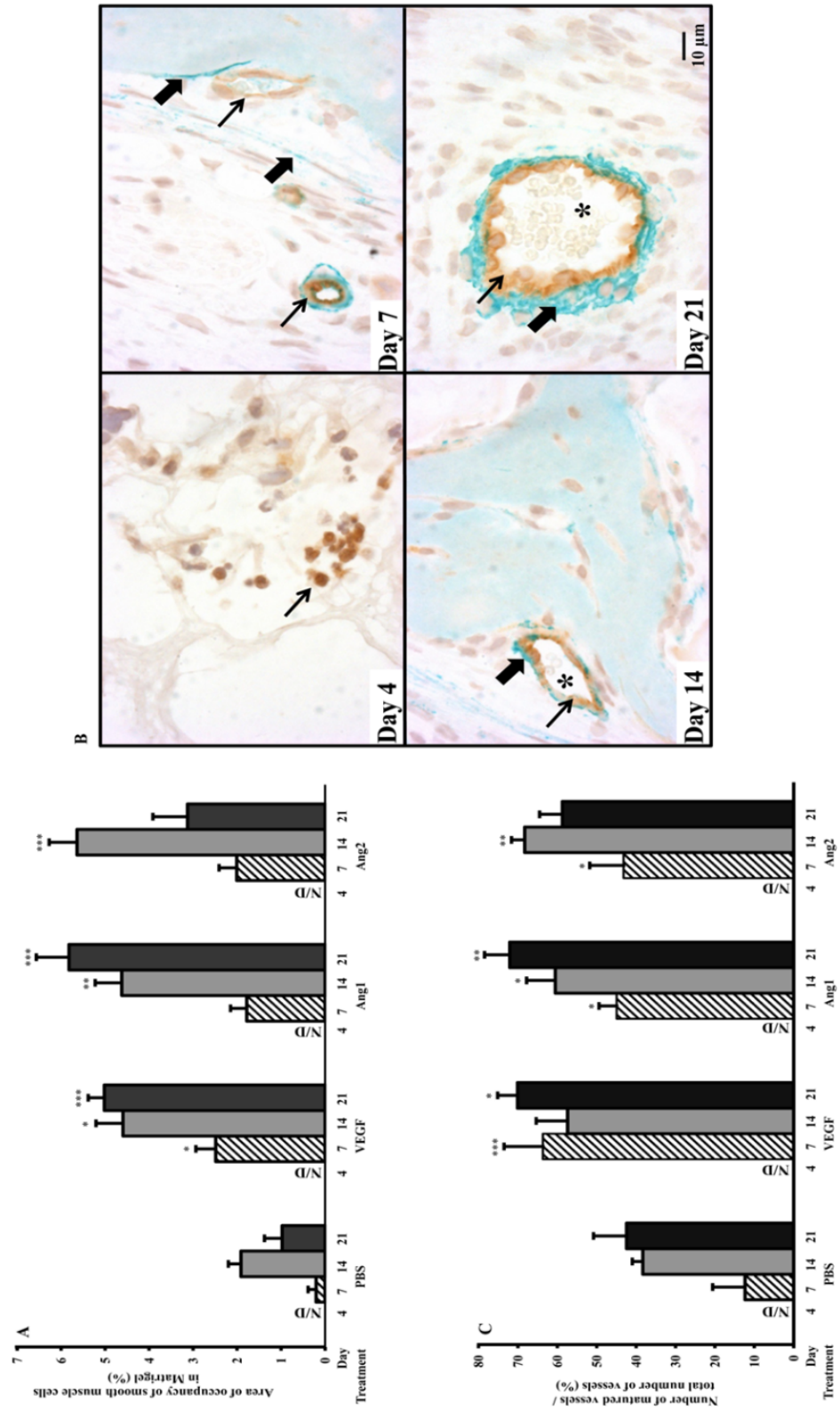

A
Area of occupancy of smooth muscle cells in Matrigel (%)
Day
Treatment
PBS
VEGF
Ang1
Ang2
N/D
B
Day 4
Day 7
Day 14
Day 21
10 µm
C
Number of matured vessels / total number of vessels (%)

FIGURE 4

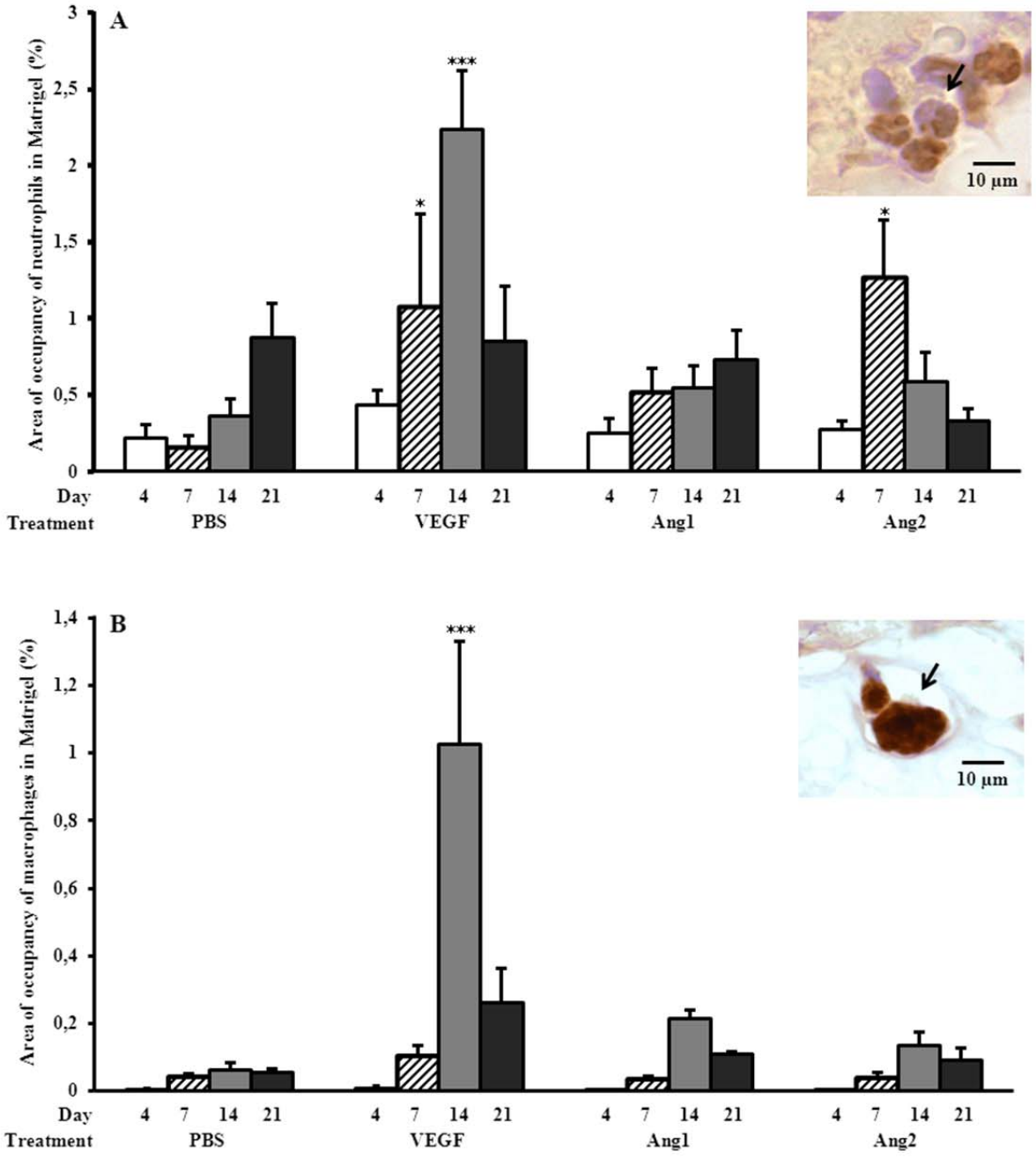

A
Area of occupancy of neutrophils in Matrigel (%)
3
2,5
2
1,5
1
0,5
0
*
***
*
10 µm
Day 4 7 14 21 4 7 14 21 4 7 14 21 4 7 14 21
Treatment PBS VEGF Angl Ang2
B
Area of occupancy of macrophages in Matrigel (%)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
***
10 µm
Day 4 7 14 21 4 7 14 21 4 7 14 21 4 7 14 21
Treatment PBS VEGF Angl Ang2

FIGURE 5

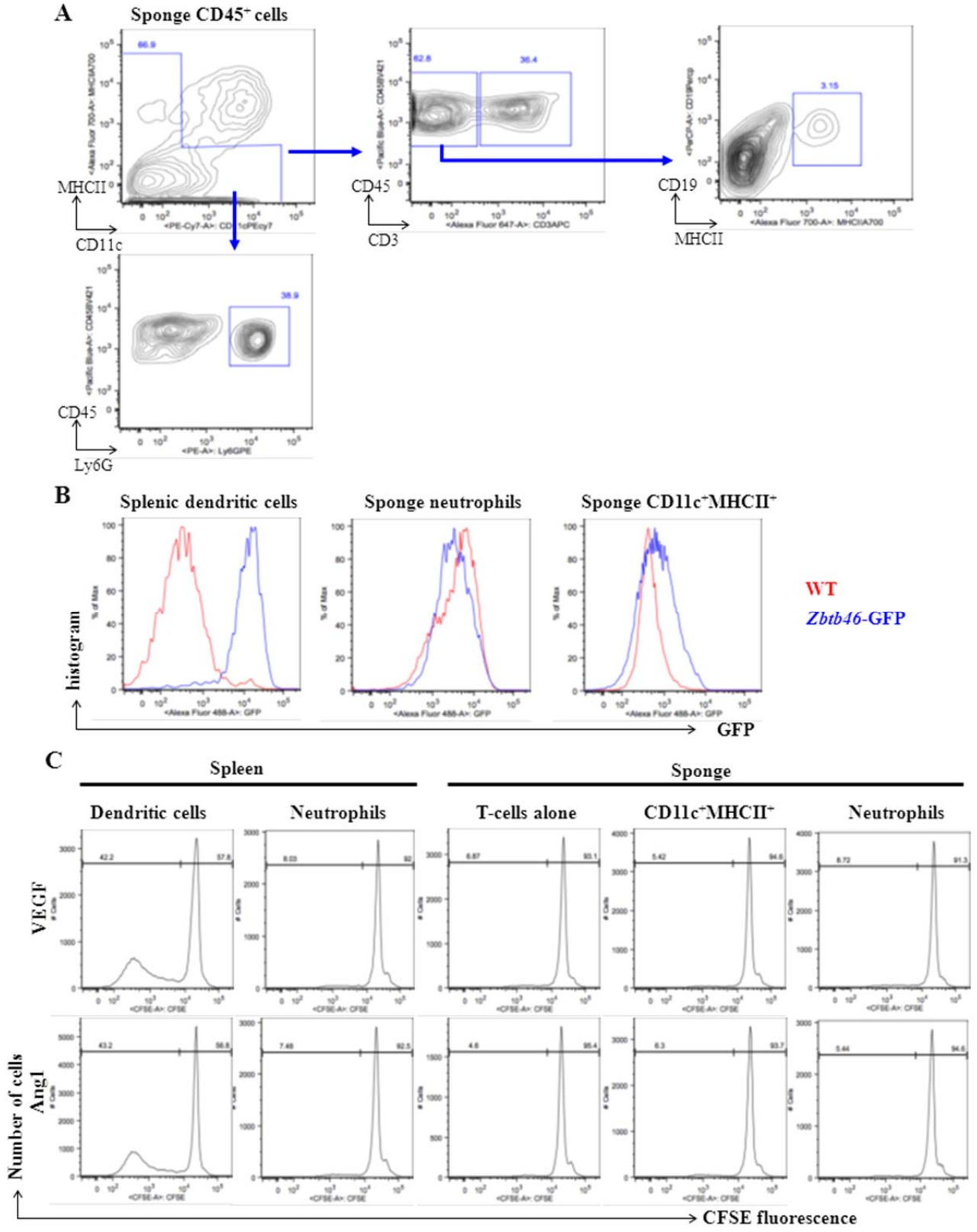
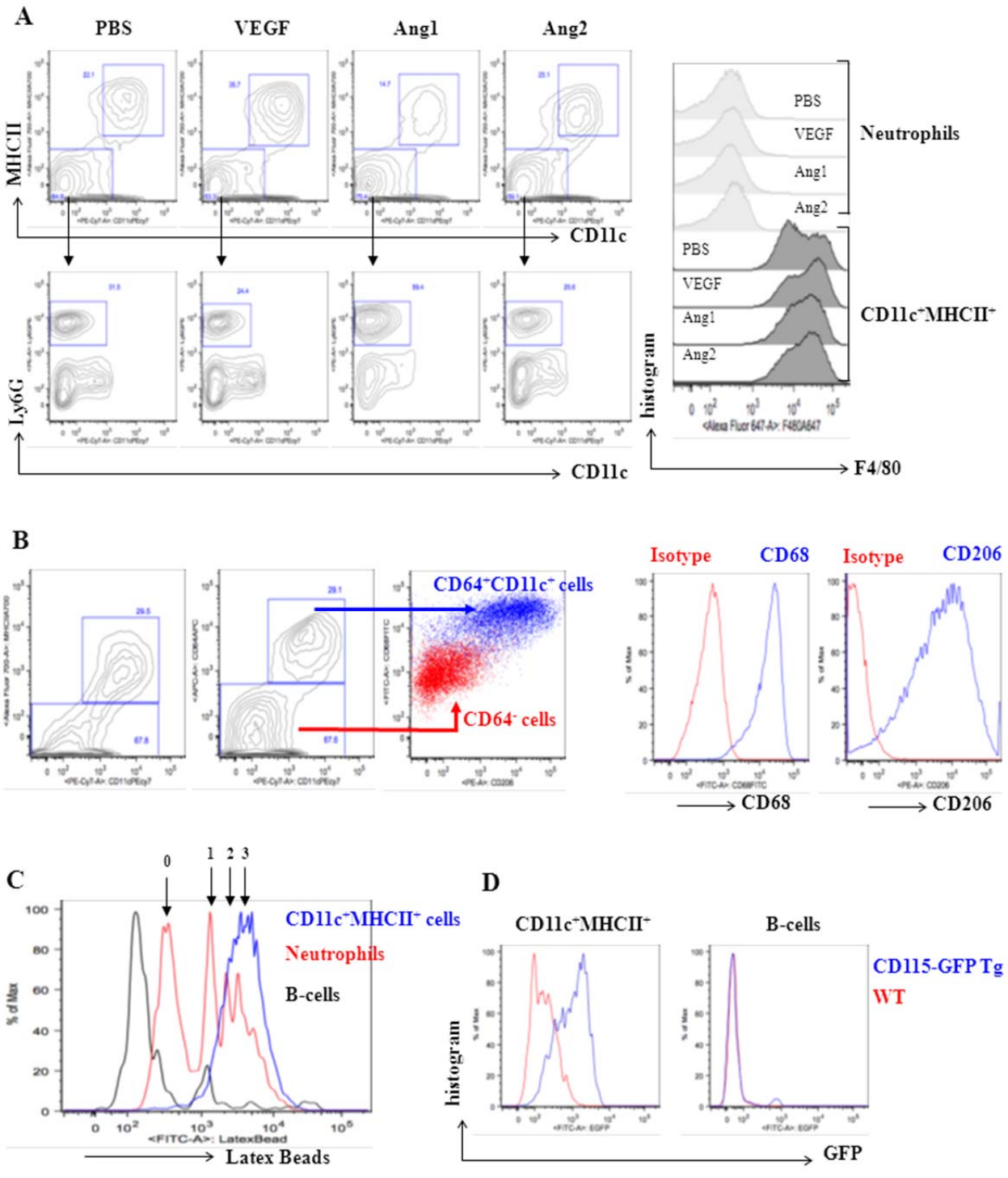

A
Sponge CD45+ cells
MHCII
CD11c
CD45
CD3
CD19
MHCII
CD45
Ly6G
B
Splenic dendritic cells
Sponge neutrophils
Sponge CD11c+MHCII-
histogram
GFP
WT
Zbtb46-GFP
C
Spleen
Sponge
Dendritic cells
Neutrophils
T-cells alone
CD11c+MHCII-
Neutrophils
VEGF
Angl
Number of cells
CFSE fluorescence

FIGURE 6


A
PBS
VEGF
Ang1
Ang2
MHCII
CD11c
Ly6G
CD11c
PBS
VEGF
Ang1
Ang2
Neutrophils
PBS
VEGF
Ang1
Ang2
CD11c⁺MHCII⁺
histogram
F4/80
B
CD64⁺CD11c⁺ cells
CD64⁺ cells
Isotype
CD68
Isotype
CD206
CD68
CD206
C
0
1
2
3
CD11c⁺MHCII⁺ cells
Neutrophils
B-cells
% of Max
Latex Beads
D
CD11c⁺MHCII⁺
B-cells
CD115-GFP Tg
WT
histogram
GFP

3.0 DISCUSSION

In an effort to elucidate the individual capacity of VEGF and angiopoietins to mediate angiogenesis and to identify the different inflammatory cells accompanying this process, we developed a novel variant of the murine sponge/Matrigel model. Firstly, we demonstrated that the tested growth factors (VEGF, Ang1 and Ang2) alone are highly potent and rapid in inducing the formation of mature and "functional" (presence of circulating red blood cells in the neovascular lumen) blood vessels. Moreover, the angiogenic process mediated by the three growth factors was primarily accompanied by the inflammatory leukocytes neutrophils and macrophages in a temporal-dependent manner. Marginal levels of lymphocytes were also detected in our study. Together, our findings reveal that VEGF, Ang1 and Ang2 individually, are not only capable of recruiting inflammatory cells and mediating the angiogenic process (EC proliferation and migration, and neovessel formation), but are also efficient in promoting the maturation of the neovessels; thus challenging the classical view of these growth factors having co-ordinated and complimentary roles in angiogenesis.

3.1 Suitability of the sponge/Matrigel model for angiogenic study

Recent efforts in clinical trials focus on localized therapy for restoring blood flow in ischemic regions as tissue loss in these patients are highly localized [294, 295]. Few of such attempts included the delivery of exogenous angiogenic factors through gene therapy and biochemical agents at the site of ischemia to promote angiogenesis [296-298]. Although, these methods initially produced therapeutic benefits, it however had limitations and produced no or negative

effects at later time point. While growth factor therapy remains a gold standard for the induction of local therapeutic angiogenesis, translating this concept into an effective and safe therapy for patients remains a challenge.

Presently, bio-material based approaches is being successfully utilized in animal models to study the capacity of cytokines, growth factors, chemokines and nonprotein mediators to promote blood vessel formation and inflammatory cells recruitment [296-298]. One such example is the subcutaneous implantation of polyvinyl alcohol (PVA) sponges in mice. This model has been shown to promote a robust invasion of inflammatory cells from the dermis into the sponge (providing a pro-inflammatory environment) and give rise to a highly vascularized sponge tissue [299]. However, a study using these PVA sponges postulated that the newly formed vessels in the sponges were fragile, permeable and dilated due to continuous inflammation in the angiogenic tissue; there was no indication of neovessel maturation (e.g. the presence of SMCs surrounding the newly formed vessels) [296]. A major disadvantage of such matrix implantation is that it induces non-specific inflammatory host response; hence, it limits to acute studies [283]. Later, the Matrigel plug (gelatinous protein mix) assay became the widely used model for studies involving *in vivo* testing for angiogenesis. The advantage of this model is that the growth factor depleted Matrigel provides a natural environment for the formation of neovessels without inducing non-specific immune response [300]. In fact, subcutaneous injection of this biologically inert substance containing VEGF in animal models successfully promoted the formation of neovessels but did not lead to their maturation, a crucial event in the stabilization of nascent blood vessels [59, 301].

In the present study, we combined both the PVA sponge model and the Matrigel plug assay in order to put forth a pro-inflammatory and angiogenic environment as we reasoned that inflammation (to some extent) might be an important stimulus for the creation of mature blood vessels. In our variation of the sponge/Matrigel model, we soaked the PVA sponges in growth-factor depleted Matrigel containing the tested growth factor prior to implantation; the sponges were completely coated with the mixture of Matrigel containing VEGF, Ang1 or Ang2. The only source of pro-angiogenic stimulus in our model came from the growth factors that we provided. As this approach mimicked the physiological environment, the Matrigel encapsulated sponges did not provoke non-specific immune response in our animals with time. In fact, the sponges remained intact with minimal fibrous covering.

Technique-wise, our model was very simple, easy to manipulate and less time-consuming as compared to other variants of the sponge/Matrigel assays. Furthermore, in contrast to Matrigel plugs, post-retrieval processing of the sponges for IHC and cellular quantification were very straightforward. Moreover, for the first time, we were able to confirm our findings using flow cytometric analysis of the sponges which allowed us to test numerous cell-specific markers simultaneously. Previous studies in the literature using the Matrigel and/or PVA sponge models usually performed histological techniques as their method of analysis.

3.2 Inflammatory cells potentiate the pro-angiogenic activities of VEGF and angiopoietins

Although, we did not study the exact roles of neutrophils and macrophages in angiogenesis, our experiments demonstrate that the presence of these leukocytes in our model enhances the pro-angiogenic activities of VEGF, Ang1 and Ang2 in the formation of mature blood vessels. Indeed, we have shown that VEGF, Ang1 and Ang2 individually are potent and rapid in inducing the recruitment of ECs and the formation of neovessels (lumen formation) as compared to PBS. Based on the literature, while the pro-angiogenic and mitogenic activities of VEGF have been demonstrated in various *in vivo* models [19, 57-59, 62], the capacity of angiopoietins to initiate the angiogenic cascade remains controversial. For instance, some *in vivo* reports demonstrate that Ang1 alone is unable to induce angiogenesis but can potentiate VEGF mediated angiogenic response [119, 302, 303] while other experiments show that Ang1 can in fact promote a robust neovascularization in Matrigel implants [59, 181, 304]. On the other hand, the implication of Ang2 in angiogenesis is normally tied with VEGF where it promotes destabilization of pre-existing blood vessels in the presence of VEGF [120, 305]. Notably, a recent study has demonstrated that Ang2 alone can induce vascular remodeling and angiogenesis in the absence of VEGF [112, 113, 306, 307]. Nonetheless, the expression of both VEGFRs and Tie2 on ECs along with the release of various growth factors, cytokines (e.g. FGF, VEGF, Ang1, IL-1β and -10), chemokines (IL-8) and CXCL1 (cytokine belonging to CXC chemokine family)) by neutrophils and macrophages may explain the capacity of exogenous VEGF and angiopoietins to trigger the angiogenic cascade in our model [96, 308-310]. In addition, the aforementioned leukocytes also release numerous metalloproteinases,

neutrophil elastase and reactive oxygen species which may function to degrade the extracellular space thereby facilitate the migration of ECs and SMCs. Consistent with our assumption, compelling studies do suggest that the recruitment of neutrophils and monocytes/macrophages during blood vessels formation release a variety of pro-inflammatory cytokines and growth factors to provide a suited autocrine and paracrine milieu to fully support this process. In fact, depletion of circulating neutrophils by anti-Gr1 therapy during the early stages of pancreatic carcinogenesis in mice significantly reduced the number of dysplastic islets that were undergoing angiogenesis [311]. Similarly, depletion of neutrophils with intraperitoneal injection of RB6-8C5 antibody inhibited corneal angiogenesis with reduced the protein levels of VEGF, macrophage inflammatory protein (MIP)-α and MIP-2 in mice [312]. As for the macrophages, its depletion in tumors has been shown to reduce tumor angiogenesis [313, 314]. All in all, although the pro-inflammatory environment provided by our model was sufficient to trigger the angiogenic cascade, the presence of the growth factors and the growth factor mediated recruitment of inflammatory cells enhanced this activity both in terms of potency and efficacy.

Interestingly, the number of blood vessels in our model once formed by day 7 did not significantly fluctuate in the growth factor treated sponges even in presence of free ECs. Either the potency of the growth factors may have declined with time or the sponge/Matrigel model itself may have exerted a restrain on the maximal capacity of blood vessel formation. This phenomenon is clinically beneficial, as we do not want to produce an excessive number of blood vessels at the region of ischemia when translating this approach to bedside.

Furthermore, numerous experiments up to date have shown that VEGF on its own is insufficient to complete angiogenesis [315]. The promising early angiogenic actions mediated by VEGF do not progress to produce the maturation events. Maturation of nascent vessels requires the recruitment of pericytes and SMCs that ensheath the vessels providing structural stabilization [316]. This is mediated by ECs that release platelet-derived growth factor-B (PDGF-B) attracting and stimulating the proliferation of PDGF-Rβ-expressing pericytes [317]. VEGF, in the maturation event, has been demonstrated to suppress PDGF-Rβ signaling in SMCs through the formation of PDGF-Rβ and activated VEGFR-2 complex [318]. In contrast to these findings, our results show that mice in presence of VEGF containing sponges induce SMC migration and promote maturation of the newly formed vessels. In fact, the maturation process occurred in parallel with the formation of blood vessels. The same effect was observed in sponges treated with Ang1 and Ang2. Although, this is consistent to the stabilizing effect of Ang1 on vascular endothelium, our results however, contradicts the proposed role of Ang2 in angiogenesis where it has been shown to destabilize pre-existing vessels. Ang1 expressed by perivascular and mural cells is capable through a paracrine activity of inducing the expression of heparin binding epidermal growth factor (HB-EGF) by ECs to stimulate SMCs migration [319, 320]. Similarly, Ang2 has been shown to be released by ECs and to possess pro-inflammatory characteristics on both ECs and neutrophils [117, 196, 255, 321]. Technically, based on previous studies, only Ang1 should be capable of promoting the maturation of blood vessels and not VEGF and Ang2. Yet, we observed that all three growth factors individually promoted the maturation of blood vessels with equal potency. Hence, the presence of neutrophils and macrophages initiates a paracrine compensation pathway in order to trigger the maturation events. A possible explanation could be due to the release of numerous MMPs

by neutrophils which may result in the degradation of ECM enabling SMCs to proliferate and migrate towards the nascent vessels. In addition, monocytes and macrophages have been demonstrated to release HB-EGF inducing the proliferation of SMCs [322], hence, this may be triggering the maturation event.

The newly formed blood vessels in presence of VEGF, Ang1 and Ang2 were "functional" as the blood vessels contained red blood cells in them and appeared to be non-leaky based on histological observations. Confirmation of vessel functionality requires the testing for vascular permeability. As the neovessels formed in the sponges are not all mature at a given time, it is thus not possible to confirm the absence of vascular leakiness. Furthermore, we also observed that the blood vessels once formed undergo no or marginal diameter remodeling. This may be due to the rapid ensheathing of SMCs around the newly formed vessels, which prevents further unrestricted enlargement of the growing vessels [21].

Bringing back the past experiments described in the previous section; our results suggest that the recruitment of inflammatory cells is crucial for the maturation of blood vessels as VEGF containing Matrigel plug assays (lacking inflammatory stimulus) were not successful in stabilizing the newly formed vessels [59, 301]. Yet, the autocrine/paracrine inflammatory response during angiogenesis must be controlled. For instance, the subcutaneous implantation of sponges, which provided a pro-inflammatory environment, was unsuccessful in creating mature blood vessels as it caused the encapsulation of the sponges by granulation tissue [296]. In the present study however, the recruitment of neutrophils and macrophages was temporal dependent where their presence declined by day 21 suggesting that their recruitment was

function-oriented. Hence, although inflammation (the recruitment of inflammatory cells) is an important stimulus for accompanying angiogenesis, persistent inflammation may be detrimental for the formation of functional vessels as the high nutritive demand of the inflammatory cells must be overcome by the creation of more vessels. In fact, human neutrophils have been demonstrated to express several DC markers when cultured with different combination of cytokines. For instance, the expression of DC markers, MHC II, CD40, CD86, CD1a, CD1b, and CD1c, were observed on neutrophil-committed precursors isolated from leukemia patients when cultured with GM-CSF, IL-4, and TNF-α [323]. Furthermore, similar results were obtained from neutrophils isolated from healthy individuals [324-327]. In mice, neutrophils isolated from chronic colitis lesions express MHC II and CD86 and have the capacity to present peptide antigen to CD4 T cells [328]. The presence of neutrophils at later time points in our study arose the questions whether we have continues recruitment of neutrophils in the sponges in presence of the growth factors or whether the initial recruitment of neutrophils transdifferentiate into DCs possessing neutrophil markers. Although, at first, we observed the presence of DC marker profile of $CD11C^{+}MHCII^{+}$ in the sponges containing the tested growth factors, careful marker analyses, genetic and functional studies demonstrated that that these double positive cells are macrophages and not from DC. Taken together, these results suggest that the neutrophils present in the sponges at later time point are not neutrophil-DC hybrid as they did not possess DC functionality but are continuously recruited by the growth factors which may be required for aiding the angiogenic process.

Finally, using flow cytometry we identified marginal levels of T- and B-cells in the sponges containing the tested growth factors. T- and B-cells are the key cellular players of the adaptive immune response. There are two broad classes of such response, the antibody response and the cell-mediated immune response, which are carried out by B-cells and T-cells, respectively. In antibody response, plasma B-cells, a subset of B-cells, are activated to secrete antibodies in the bloodstream which recognize specific foreign antigen. The binding of the antibody to the antigen inactivates viruses and microbial toxins. It is also responsible for immunological memory *via* memory B-cells, another subset of B-cells, such that when the host is exposed to the same pathogen again, these memory cells will quickly eliminate the pathogen. In cell-mediated immune response on the other hand, T cells react directly against foreign antigen that is presented on the surface of host cells or on antigen presenting cells mainly DCs and eliminate the infected cell. As our study at this point eliminates the presence of DCs in the sponges, it is difficult to reason that the angiogenic process in our model has promoted the recruitment of T- and B-cells. Yet, T- and B-cells are abundantly present in the bloodstream of mice; hence, this may explain why we observed the presence of T- and B-cells in the sponges using flow cytometry.

3.3 Future perspective

As the predominant inflammatory cells recruited in the sponge/Matrigel model are neutrophils and macrophages, further studies will be needed through the selected depletion of neutrophils and monocytes/macrophages to delineate the role of these cells in the angiogenic process. This will allow us to understand whether inflammation, or specifically the presence of neutrophils

and/or macrophages, is the missing puzzle that is required for the formation of mature blood vessels.

Currently, various methods have been made available to deplete neutrophils and monocytes from the circulation. For instance, neutrophil depletion may be attained through the injection of monoclonal anti-Ly6G (clone 1A8) antibody (500 μg/injection) every 48 hours in C57/Bl6 mice starting a day prior to the implantation of the sponges. This antibody has been shown to be effective in promoting selective depletion of circulating neutrophils [329-331]. Corresponding IgG isotype injection must be utilized as a control. Next, as for the role of macrophages, the convential approach to deplete monocytes and macrophages was either by clodronate liposomes or by antibodies against CSF1R but neither approach is specific for these cells [332, 333]. Recently, a transgenic mouse strain combining the macrophage colony-stimulating factor receptor gene (*Csf1r*) and lysozyme gene (*Lyz2*) ($Lysm^{Cre}$ x $Csf1r^{LsL\text{-}DTR}$) is made available that provides a complete loss of peripheral blood monocytes within 24 hours post-injection of 100 ng of diphteria toxin. For prolonged monocyte depletion, this injection can be repeated every 48 hours [334]. Thus this mouse strain can be utilized to implant our sponges in order to observe any changes in the absence of monocytes/macrophages.

As macrophages have been demonstrated to be key regulators of angiogenesis, it would be very interesting to examine the phenotype of the macrophages, whether they are M1 and/or M2 macrophages, recruited in our sponge/Matrigel model. Based on our results, we postulate that the recruitment of macrophages during the initial stages of angiogenesis may be of M1 phenotype which can then repolarize to M2 phenotype due to local microenvironmental

changes. Hence, such study may explain why we observed a temporal-dependent recruitment of macrophages in the sponges. In fact, previous studies have demonstrated that the phenotypes of macrophages are unstable and can change overtime. We can address this postulation by performing marker analysis that are specific to M1 and M2 macrophages on FACS-purified monocytes/macrophages from the sponges.

4.0 CONCLUSION

In summary, this study demonstrates that the sponge/Matrigel model together with the tested growth factors (VEGF, Ang1 and Ang2) provides a well-suited autocrine/paracrine environment to trigger and support the formation of mature blood vessels. In addition, the recruitment of inflammatory cells by these growth factors in a temporal-dependent manner further accelerates these processes with a greater potency. Thus, these two classes of pro-angiogenic growth factors can individually orchestrate angiogenesis with the companion of inflammation when provided a suitable platform.

5.0 BIBIOGRAPHY

1. Aird, W.C., *Endothelial cell heterogeneity.* Cold Spring Harbor perspectives in medicine, 2012. **2**(1): p. a006429.
2. Simionescu, N., M. Simionescu, and G.E. Palade, *Differentiated microdomains on the luminal surface of the capillary endothelium. I. Preferential distribution of anionic sites.* The Journal of cell biology, 1981. **90**(3): p. 605-13.
3. Ribatti, D., *Genetic and epigenetic mechanisms in the early development of the vascular system.* Journal of anatomy, 2006. **208**(2): p. 139-52.
4. Folkman, J., *Tumor angiogenesis.* Advances in cancer research, 1985. **43**: p. 175-203.
5. Carmeliet, P. and R.K. Jain, *Molecular mechanisms and clinical applications of angiogenesis.* Nature, 2011. **473**(7347): p. 298-307.
6. Heil, M. and W. Schaper, *Cellular mechanisms of arteriogenesis.* EXS, 2005(94): p. 181-91.
7. Coultas, L., K. Chawengsaksophak, and J. Rossant, *Endothelial cells and VEGF in vascular development.* Nature, 2005. **438**(7070): p. 937-45.
8. Nissen, N.N., et al., *Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing.* The American journal of pathology, 1998. **152**(6): p. 1445-52.
9. Booth, F.W. and D.B. Thomason, *Molecular and cellular adaptation of muscle in response to exercise: perspectives of various models.* Physiological reviews, 1991. **71**(2): p. 541-85.
10. Shimizu, T., et al., *Angiogenesis and microvasculature in the female reproductive organs: physiological and pathological implications.* Current pharmaceutical design, 2012. **18**(3): p. 303-9.
11. Carmeliet, P., *Manipulating angiogenesis in medicine.* Journal of internal medicine, 2004. **255**(5): p. 538-61.
12. Li, J., et al., *Gene expression profile of rat adipose tissue at the onset of high-fat-diet obesity.* American journal of physiology. Endocrinology and metabolism, 2002. **282**(6): p. E1334-41.
13. Rupnick, M.A., et al., *Adipose tissue mass can be regulated through the vasculature.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(16): p. 10730-5.
14. O'Reilly, M.S., et al., *Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma.* Cell, 1994. **79**(2): p. 315-28.
15. O'Reilly, M.S., et al., *Endostatin: an endogenous inhibitor of angiogenesis and tumor growth.* Cell, 1997. **88**(2): p. 277-85.
16. Kendall, R.L. and K.A. Thomas, *Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor.* Proceedings of the National Academy of Sciences of the United States of America, 1993. **90**(22): p. 10705-9.
17. Senger, D.R., et al., *Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid.* Science, 1983. **219**(4587): p. 983-5.

18. Ferrara, N. and W.J. Henzel, *Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells.* Biochemical and biophysical research communications, 1989. **161**(2): p. 851-8.
19. Leung, D.W., et al., *Vascular endothelial growth factor is a secreted angiogenic mitogen.* Science, 1989. **246**(4935): p. 1306-9.
20. Ferrara, N., et al., *The vascular endothelial growth factor family of polypeptides.* Journal of cellular biochemistry, 1991. **47**(3): p. 211-8.
21. Hoeben, A., et al., *Vascular endothelial growth factor and angiogenesis.* Pharmacological reviews, 2004. **56**(4): p. 549-80.
22. Williams, B., et al., *Angiotensin II increases vascular permeability factor gene expression by human vascular smooth muscle cells.* Hypertension, 1995. **25**(5): p. 913-7.
23. Berse, B., et al., *Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors.* Molecular biology of the cell, 1992. **3**(2): p. 211-20.
24. Namiki, A., et al., *Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells.* The Journal of biological chemistry, 1995. **270**(52): p. 31189-95.
25. Freeman, M.R., et al., *Peripheral blood T lymphocytes and lymphocytes infiltrating human cancers express vascular endothelial growth factor: a potential role for T cells in angiogenesis.* Cancer research, 1995. **55**(18): p. 4140-5.
26. Vincenti, V., et al., *Assignment of the vascular endothelial growth factor gene to human chromosome 6p21.3.* Circulation, 1996. **93**(8): p. 1493-5.
27. Houck, K.A., et al., *The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA.* Molecular Endocrinology, 1991. **5**(12): p. 1806-14.
28. Tischer, E., et al., *The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing.* The Journal of biological chemistry, 1991. **266**(18): p. 11947-54.
29. Poltorak, Z., et al., *VEGF145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix.* J Biol Chem, 1997. **272**(11): p. 7151-8.
30. Jingjing, L., et al., *Human Muller cells express VEGF183, a novel spliced variant of vascular endothelial growth factor.* Invest Ophthalmol Vis Sci, 1999. **40**(3): p. 752-9.
31. Lange, T., et al., *VEGF162, a new heparin-binding vascular endothelial growth factor splice form that is expressed in transformed human cells.* J Biol Chem, 2003. **278**(19): p. 17164-9.
32. Bates, D.O., et al., *VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma.* Cancer Res, 2002. **62**(14): p. 4123-31.
33. Houck, K.A., et al., *Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms.* The Journal of biological chemistry, 1992. **267**(36): p. 26031-7.
34. Ortega, N., H. Hutchings, and J. Plouet, *Signal relays in the VEGF system.* Front Biosci, 1999. **4**: p. D141-52.
35. Park, J.E., G.A. Keller, and N. Ferrara, *The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and*

bioactivity of extracellular matrix-bound VEGF. Molecular biology of the cell, 1993. **4**(12): p. 1317-26.
36. Persico, M.G., V. Vincenti, and T. DiPalma, *Structure, expression and receptor-binding properties of placenta growth factor (PlGF).* Current topics in microbiology and immunology, 1999. **237**: p. 31-40.
37. Carmeliet, P., et al., *Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions.* Nature medicine, 2001. **7**(5): p. 575-83.
38. Odorisio, T., et al., *Mice overexpressing placenta growth factor exhibit increased vascularization and vessel permeability.* Journal of cell science, 2002. **115**(Pt 12): p. 2559-67.
39. Oura, H., et al., *A critical role of placental growth factor in the induction of inflammation and edema formation.* Blood, 2003. **101**(2): p. 560-7.
40. Olofsson, B., et al., *Current biology of VEGF-B and VEGF-C.* Current opinion in biotechnology, 1999. **10**(6): p. 528-35.
41. Fearnley GW, S.G., Harrison MA, Wheatcroft SB, Tomlinson DC, Ponnambalam S. , *Vascular endothelial growth factor-A regulation of blood vessel sprouting in health and disease.* OA Biochemistry, 2013(1(1)).
42. Karkkainen, M.J., et al., *Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins.* Nature immunology, 2004. **5**(1): p. 74-80.
43. Kukk, E., et al., *VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development.* Development, 1996. **122**(12): p. 3829-37.
44. Farnebo, F., F. Piehl, and J. Lagercrantz, *Restricted expression pattern of vegf-d in the adult and fetal mouse: high expression in the embryonic lung.* Biochemical and biophysical research communications, 1999. **257**(3): p. 891-4.
45. He, Y., T. Karpanen, and K. Alitalo, *Role of lymphangiogenic factors in tumor metastasis.* Biochimica et biophysica acta, 2004. **1654**(1): p. 3-12.
46. Ogawa, S., et al., *A novel type of vascular endothelial growth factor, VEGF-E (NZ-7 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent mitotic activity without heparin-binding domain.* The Journal of biological chemistry, 1998. **273**(47): p. 31273-82.
47. Ortega, N., H. Hutchings, and J. Plouet, *Signal relays in the VEGF system.* Frontiers in bioscience : a journal and virtual library, 1999. **4**: p. D141-52.
48. Stuttfeld, E. and K. Ballmer-Hofer, *Structure and function of VEGF receptors.* IUBMB life, 2009. **61**(9): p. 915-22.
49. Schlessinger, J. and M.A. Lemmon, *SH2 and PTB domains in tyrosine kinase signaling.* Science's STKE : signal transduction knowledge environment, 2003. **2003**(191): p. RE12.
50. Katoh, O., et al., *Expression of the vascular endothelial growth factor (VEGF) receptor gene, KDR, in hematopoietic cells and inhibitory effect of VEGF on apoptotic cell death caused by ionizing radiation.* Cancer research, 1995. **55**(23): p. 5687-92.
51. Gitay-Goren, H., et al., *The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules.* The Journal of biological chemistry, 1992. **267**(9): p. 6093-8.

52. Soker, S., et al., *Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor.* Cell, 1998. **92**(6): p. 735-45.
53. Neufeld, G., et al., *The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis.* Trends in cardiovascular medicine, 2002. **12**(1): p. 13-9.
54. Bernatchez, P.N., et al., *Relative effects of VEGF-A and VEGF-C on endothelial cell proliferation, migration and PAF synthesis: Role of neuropilin-1.* Journal of cellular biochemistry, 2002. **85**(3): p. 629-39.
55. Carmeliet, P., et al., *Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele.* Nature, 1996. **380**(6573): p. 435-9.
56. Gerber, H.P., et al., *VEGF is required for growth and survival in neonatal mice.* Development, 1999. **126**(6): p. 1149-59.
57. Phillips, G.D., et al., *Vascular endothelial growth factor (rhVEGF165) stimulates direct angiogenesis in the rabbit cornea.* In vivo, 1994. **8**(6): p. 961-5.
58. Tolentino, M.J., et al., *Vascular endothelial growth factor is sufficient to produce iris neovascularization and neovascular glaucoma in a nonhuman primate.* Archives of ophthalmology, 1996. **114**(8): p. 964-70.
59. Marchand, G.S., et al., *Blockade of in vivo VEGF-mediated angiogenesis by antisense gene therapy: role of Flk-1 and Flt-1 receptors.* American journal of physiology. Heart and circulatory physiology, 2002. **282**(1): p. H194-204.
60. Connolly, D.T., et al., *Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis.* The Journal of clinical investigation, 1989. **84**(5): p. 1470-8.
61. Keck, P.J., et al., *Vascular permeability factor, an endothelial cell mitogen related to PDGF.* Science, 1989. **246**(4935): p. 1309-12.
62. Plouet, J., J. Schilling, and D. Gospodarowicz, *Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells.* The EMBO journal, 1989. **8**(12): p. 3801-6.
63. Conn, G., et al., *Amino acid and cDNA sequences of a vascular endothelial cell mitogen that is homologous to platelet-derived growth factor.* Proceedings of the National Academy of Sciences of the United States of America, 1990. **87**(7): p. 2628-32.
64. Pepper, M.S., et al., *In vitro angiogenic and proteolytic properties of bovine lymphatic endothelial cells.* Experimental cell research, 1994. **210**(2): p. 298-305.
65. Warner, A.J., et al., *The Shc-related adaptor protein, Sck, forms a complex with the vascular-endothelial-growth-factor receptor KDR in transfected cells.* The Biochemical journal, 2000. **347**(Pt 2): p. 501-9.
66. Takahashi, T., et al., *A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells.* The EMBO journal, 2001. **20**(11): p. 2768-78.
67. Takahashi, T., H. Ueno, and M. Shibuya, *VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells.* Oncogene, 1999. **18**(13): p. 2221-30.
68. Gerber, H.P., et al., *Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway.*

Requirement for Flk-1/KDR activation. The Journal of biological chemistry, 1998. **273**(46): p. 30336-43.
69. Fulton, D., et al., *Regulation of endothelium-derived nitric oxide production by the protein kinase Akt.* Nature, 1999. **399**(6736): p. 597-601.
70. Dimmeler, S., et al., *Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation.* Nature, 1999. **399**(6736): p. 601-5.
71. Eriksson, A., et al., *Small GTP-binding protein Rac is an essential mediator of vascular endothelial growth factor-induced endothelial fenestrations and vascular permeability.* Circulation, 2003. **107**(11): p. 1532-8.
72. Rousseau, S., et al., *p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells.* Oncogene, 1997. **15**(18): p. 2169-77.
73. Qi, J.H. and L. Claesson-Welsh, *VEGF-induced activation of phosphoinositide 3-kinase is dependent on focal adhesion kinase.* Experimental cell research, 2001. **263**(1): p. 173-82.
74. Eliceiri, B.P., et al., *Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability.* Molecular cell, 1999. **4**(6): p. 915-24.
75. Zachary, A.A., R.A. Montgomery, and M.S. Leffell, *Factors associated with and predictive of persistence of donor-specific antibody after treatment with plasmapheresis and intravenous immunoglobulin.* Human immunology, 2005. **66**(4): p. 364-70.
76. Rousseau, S., F. Houle, and J. Huot, *Integrating the VEGF signals leading to actin-based motility in vascular endothelial cells.* Trends in cardiovascular medicine, 2000. **10**(8): p. 321-7.
77. Lamalice, L., F. Houle, and J. Huot, *Phosphorylation of Tyr1214 within VEGFR-2 triggers the recruitment of Nck and activation of Fyn leading to SAPK2/p38 activation and endothelial cell migration in response to VEGF.* The Journal of biological chemistry, 2006. **281**(45): p. 34009-20.
78. Lobov, I.B., et al., *Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(9): p. 3219-24.
79. Liu, Z.J., et al., *Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis.* Molecular and cellular biology, 2003. **23**(1): p. 14-25.
80. Shutter, J.R., et al., *Dll4, a novel Notch ligand expressed in arterial endothelium.* Genes & development, 2000. **14**(11): p. 1313-8.
81. Claxton, S. and M. Fruttiger, *Periodic Delta-like 4 expression in developing retinal arteries.* Gene expression patterns : GEP, 2004. **5**(1): p. 123-7.
82. Cross, M.J., et al., *VEGF-receptor signal transduction.* Trends in biochemical sciences, 2003. **28**(9): p. 488-94.
83. Davis, S., et al., *Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning.* Cell, 1996. **87**(7): p. 1161-9.
84. Maisonpierre, P.C., et al., *Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis.* Science, 1997. **277**(5322): p. 55-60.

85. Valenzuela, D.M., et al., *Angiopoietins 3 and 4: diverging gene counterparts in mice and humans.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(5): p. 1904-9.
86. Procopio, W.N., et al., *Angiopoietin-1 and -2 coiled coil domains mediate distinct homo-oligomerization patterns, but fibrinogen-like domains mediate ligand activity.* The Journal of biological chemistry, 1999. **274**(42): p. 30196-201.
87. Grosios, K., et al., *Assignment of ANGPT4, ANGPT1, and ANGPT2 encoding angiopoietins 4, 1 and 2 to human chromosome bands 20p13, 8q22.3-->q23 and 8p23.1, respectively, by in situ hybridization and radiation hybrid mapping.* Cytogenetics and cell genetics, 1999. **84**(1-2): p. 118-20.
88. Cheung, A.H., R.J. Stewart, and P.A. Marsden, *Endothelial Tie2/Tek ligands angiopoietin-1 (ANGPT1) and angiopoietin-2 (ANGPT2): regional localization of the human genes to 8q22.3-q23 and 8p23.* Genomics, 1998. **48**(3): p. 389-91.
89. Huang, Y.Q., J.J. Li, and S. Karpatkin, *Identification of a family of alternatively spliced mRNA species of angiopoietin-1.* Blood, 2000. **95**(6): p. 1993-9.
90. Kim, I., et al., *Characterization and expression of a novel alternatively spliced human angiopoietin-2.* The Journal of biological chemistry, 2000. **275**(24): p. 18550-6.
91. Mezquita, J., et al., *Characterization of a novel form of angiopoietin-2 (Ang-2B) and expression of VEGF and angiopoietin-2 during chicken testicular development and regression.* Biochemical and biophysical research communications, 1999. **260**(2): p. 492-8.
92. Stratmann, A., W. Risau, and K.H. Plate, *Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis.* The American journal of pathology, 1998. **153**(5): p. 1459-66.
93. Zagzag, D., et al., *In situ expression of angiopoietins in astrocytomas identifies angiopoietin-2 as an early marker of tumor angiogenesis.* Experimental neurology, 1999. **159**(2): p. 391-400.
94. Suri, C., et al., *Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis.* Cell, 1996. **87**(7): p. 1171-80.
95. Gale, N.W., et al., *Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1.* Developmental cell, 2002. **3**(3): p. 411-23.
96. Neagoe, P.E., et al., *Expression and release of angiopoietin-1 from human neutrophils: intracellular mechanisms.* Growth factors, 2009. **27**(6): p. 335-44.
97. Fiedler, U., et al., *The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies.* Blood, 2004. **103**(11): p. 4150-6.
98. Goede, V., et al., *Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis.* Laboratory investigation; a journal of technical methods and pathology, 1998. **78**(11): p. 1385-94.
99. Koga, K., et al., *Expression of angiopoietin-2 in human glioma cells and its role for angiogenesis.* Cancer research, 2001. **61**(16): p. 6248-54.
100. Tanaka, S., et al., *Biologic significance of angiopoietin-2 expression in human hepatocellular carcinoma.* The Journal of clinical investigation, 1999. **103**(3): p. 341-5.

101. Torimura, T., et al., *Overexpression of angiopoietin-1 and angiopoietin-2 in hepatocellular carcinoma.* Journal of hepatology, 2004. **40**(5): p. 799-807.
102. Oliner, J., et al., *Suppression of angiogenesis and tumor growth by selective inhibition of angiopoietin-2.* Cancer cell, 2004. **6**(5): p. 507-16.
103. Zhang, L., et al., *Tumor-derived vascular endothelial growth factor up-regulates angiopoietin-2 in host endothelium and destabilizes host vasculature, supporting angiogenesis in ovarian cancer.* Cancer research, 2003. **63**(12): p. 3403-12.
104. Brown, L.F., et al., *Expression of Tie1, Tie2, and angiopoietins 1, 2, and 4 in Kaposi's sarcoma and cutaneous angiosarcoma.* The American journal of pathology, 2000. **156**(6): p. 2179-83.
105. Hackett, S.F., et al., *Angiopoietin 2 expression in the retina: upregulation during physiologic and pathologic neovascularization.* Journal of cellular physiology, 2000. **184**(3): p. 275-84.
106. Cohen, B., et al., *Leptin induces angiopoietin-2 expression in adipose tissues.* The Journal of biological chemistry, 2001. **276**(11): p. 7697-700.
107. Hammes, H.P., et al., *Angiopoietin-2 causes pericyte dropout in the normal retina: evidence for involvement in diabetic retinopathy.* Diabetes, 2004. **53**(4): p. 1104-10.
108. Kim, I., et al., *Tumor necrosis factor-alpha upregulates angiopoietin-2 in human umbilical vein endothelial cells.* Biochemical and biophysical research communications, 2000. **269**(2): p. 361-5.
109. Oh, H., et al., *Hypoxia and vascular endothelial growth factor selectively up-regulate angiopoietin-2 in bovine microvascular endothelial cells.* The Journal of biological chemistry, 1999. **274**(22): p. 15732-9.
110. Parikh, S.M., et al., *Excess circulating angiopoietin-2 may contribute to pulmonary vascular leak in sepsis in humans.* PLoS medicine, 2006. **3**(3): p. e46.
111. Sfiligoi, C., et al., *Angiopoietin-2 expression in breast cancer correlates with lymph node invasion and short survival.* International journal of cancer. Journal international du cancer, 2003. **103**(4): p. 466-74.
112. Kim, I., et al., *Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway.* Oncogene, 2000. **19**(39): p. 4549-52.
113. Teichert-Kuliszewska, K., et al., *Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2.* Cardiovascular research, 2001. **49**(3): p. 659-70.
114. Jones, N., et al., *Tie receptors: new modulators of angiogenic and lymphangiogenic responses.* Nature reviews. Molecular cell biology, 2001. **2**(4): p. 257-67.
115. Maliba, R., et al., *Angiopoietins-1 and -2 are both capable of mediating endothelial PAF synthesis: intracellular signalling pathways.* Cellular Signalling, 2006. **18**(11): p. 1947-57.
116. Maliba, R., et al., *Angiopoietin-mediated endothelial P-selectin translocation: cell signaling mechanisms.* Journal of leukocyte biology, 2008. **83**(2): p. 352-60.
117. Lemieux, C., et al., *Angiopoietins can directly activate endothelial cells and neutrophils to promote proinflammatory responses.* Blood, 2005. **105**(4): p. 1523-30.
118. Brkovic, A., et al., *Angiopoietin chemotactic activities on neutrophils are regulated by PI-3K activation.* Journal of leukocyte biology, 2007. **81**(4): p. 1093-101.

119. Asahara, T., et al., *Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization.* Circulation research, 1998. **83**(3): p. 233-40.
120. Lobov, I.B., P.C. Brooks, and R.A. Lang, *Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(17): p. 11205-10.
121. Visconti, R.P., C.D. Richardson, and T.N. Sato, *Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF).* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(12): p. 8219-24.
122. Dumas, E., et al., *Angiopoietin-1 but not angiopoietin-2 promotes neutrophil viability: Role of interleukin-8 and platelet-activating factor.* Biochimica et biophysica acta, 2012. **1823**(2): p. 358-67.
123. Haddad, L.E. and M.G. Sirois, *Angiopoietin-1 upregulates de novo expression of IL-1beta and Il1-Ra, and the exclusive release of Il1-Ra from human neutrophils.* PloS one, 2014. **9**(2): p. e88980.
124. Yu, X., et al., *Structural basis for angiopoietin-1-mediated signaling initiation.* Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(18): p. 7205-10.
125. Lund, E.L., et al., *Differential regulation of VEGF, HIF1alpha and angiopoietin-1, -2 and -4 by hypoxia and ionizing radiation in human glioblastoma.* International journal of cancer. Journal international du cancer, 2004. **108**(6): p. 833-8.
126. Yamakawa, M., et al., *Hypoxia-inducible factor-1 mediates activation of cultured vascular endothelial cells by inducing multiple angiogenic factors.* Circulation research, 2003. **93**(7): p. 664-73.
127. Abdulmalek, K., et al., *Differential expression of Tie-2 receptors and angiopoietins in response to in vivo hypoxia in rats.* American journal of physiology. Lung cellular and molecular physiology, 2001. **281**(3): p. L582-90.
128. Lee, H.J., et al., *Biological characterization of angiopoietin-3 and angiopoietin-4.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2004. **18**(11): p. 1200-8.
129. Dumont, D.J., et al., *tek, a novel tyrosine kinase gene located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors.* Oncogene, 1992. **7**(8): p. 1471-80.
130. Maisonpierre, P.C., et al., *Distinct rat genes with related profiles of expression define a TIE receptor tyrosine kinase family.* Oncogene, 1993. **8**(6): p. 1631-7.
131. Partanen, J., et al., *A novel endothelial cell surface receptor tyrosine kinase with extracellular epidermal growth factor homology domains.* Molecular and cellular biology, 1992. **12**(4): p. 1698-707.
132. Sato, T.N., et al., *Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system.* Proceedings of the National Academy of Sciences of the United States of America, 1993. **90**(20): p. 9355-8.

133. Schnurch, H. and W. Risau, *Expression of tie-2, a member of a novel family of receptor tyrosine kinases, in the endothelial cell lineage.* Development, 1993. **119**(3): p. 957-68.
134. Korhonen, J., et al., *The mouse tie receptor tyrosine kinase gene: expression during embryonic angiogenesis.* Oncogene, 1994. **9**(2): p. 395-403.
135. Marron, M.B., et al., *Evidence for heterotypic interaction between the receptor tyrosine kinases TIE-1 and TIE-2.* The Journal of biological chemistry, 2000. **275**(50): p. 39741-6.
136. Tsiamis, A.C., et al., *Vascular endothelial growth factor modulates the Tie-2:Tie-1 receptor complex.* Microvascular research, 2002. **63**(2): p. 149-58.
137. Yabkowitz, R., et al., *Inflammatory cytokines and vascular endothelial growth factor stimulate the release of soluble tie receptor from human endothelial cells via metalloprotease activation.* Blood, 1999. **93**(6): p. 1969-79.
138. Kontos, C.D., et al., *The endothelial receptor tyrosine kinase Tie1 activates phosphatidylinositol 3-kinase and Akt to inhibit apoptosis.* Molecular and cellular biology, 2002. **22**(6): p. 1704-13.
139. Wong, A.L., et al., *Tie2 expression and phosphorylation in angiogenic and quiescent adult tissues.* Circulation research, 1997. **81**(4): p. 567-74.
140. Muller, A., et al., *Expression of angiopoietin-1 and its receptor TEK in hematopoietic cells from patients with myeloid leukemia.* Leukemia research, 2002. **26**(2): p. 163-8.
141. Feistritzer, C., et al., *Expression and function of the angiopoietin receptor Tie-2 in human eosinophils.* The Journal of allergy and clinical immunology, 2004. **114**(5): p. 1077-84.
142. De Palma, M., et al., *Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors.* Cancer cell, 2005. **8**(3): p. 211-26.
143. Willam, C., et al., *Tie2 receptor expression is stimulated by hypoxia and proinflammatory cytokines in human endothelial cells.* Circulation research, 2000. **87**(5): p. 370-7.
144. Peters, K.G., et al., *Expression of Tie2/Tek in breast tumour vasculature provides a new marker for evaluation of tumour angiogenesis.* British journal of cancer, 1998. **77**(1): p. 51-6.
145. Takahama, M., et al., *Enhanced expression of Tie2, its ligand angiopoietin-1, vascular endothelial growth factor, and CD31 in human non-small cell lung carcinomas.* Clinical cancer research : an official journal of the American Association for Cancer Research, 1999. **5**(9): p. 2506-10.
146. Jones, N. and D.J. Dumont, *The Tek/Tie2 receptor signals through a novel Dok-related docking protein, Dok-R.* Oncogene, 1998. **17**(9): p. 1097-108.
147. Thurston, G., et al., *Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1.* Science, 1999. **286**(5449): p. 2511-4.
148. Suri, C., et al., *Increased vascularization in mice overexpressing angiopoietin-1.* Science, 1998. **282**(5388): p. 468-71.
149. Dumont, D.J., et al., *Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo.* Genes & development, 1994. **8**(16): p. 1897-909.

150. Sato, T.N., et al., *Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation.* Nature, 1995. **376**(6535): p. 70-4.
151. Patan, S., *TIE1 and TIE2 receptor tyrosine kinases inversely regulate embryonic angiogenesis by the mechanism of intussusceptive microvascular growth.* Microvascular research, 1998. **56**(1): p. 1-21.
152. Puri, M.C., et al., *Interaction of the TEK and TIE receptor tyrosine kinases during cardiovascular development.* Development, 1999. **126**(20): p. 4569-80.
153. Thurston, G., *Complementary actions of VEGF and angiopoietin-1 on blood vessel growth and leakage.* Journal of anatomy, 2002. **200**(6): p. 575-80.
154. Scharpfenecker, M., et al., *The Tie-2 ligand angiopoietin-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism.* Journal of cell science, 2005. **118**(Pt 4): p. 771-80.
155. Talluri, S.K., et al., *Chylous ascites.* North American journal of medical sciences, 2011. **3**(9): p. 438-40.
156. Tammela, T., et al., *Angiopoietin-1 promotes lymphatic sprouting and hyperplasia.* Blood, 2005. **105**(12): p. 4642-8.
157. Morisada, T., et al., *Angiopoietin-1 promotes LYVE-1-positive lymphatic vessel formation.* Blood, 2005. **105**(12): p. 4649-56.
158. Shimoda, H., et al., *Abnormal recruitment of periendothelial cells to lymphatic capillaries in digestive organs of angiopoietin-2-deficient mice.* Cell and tissue research, 2007. **328**(2): p. 329-37.
159. Kim, K.T., et al., *Oligomerization and multimerization are critical for angiopoietin-1 to bind and phosphorylate Tie2.* The Journal of biological chemistry, 2005. **280**(20): p. 20126-31.
160. Barton, W.A., et al., *Crystal structures of the Tie2 receptor ectodomain and the angiopoietin-2-Tie2 complex.* Nature structural & molecular biology, 2006. **13**(6): p. 524-32.
161. Hodous, B.L., et al., *Evolution of a highly selective and potent 2-(pyridin-2-yl)-1,3,5-triazine Tie-2 kinase inhibitor.* Journal of Medicinal Chemistry, 2007. **50**(4): p. 611-26.
162. Kim, I., et al., *Angiopoietin-1 regulates endothelial cell survival through the phosphatidylinositol 3'-Kinase/Akt signal transduction pathway.* Circulation research, 2000. **86**(1): p. 24-9.
163. Semones, M., et al., *Pyridinylimidazole inhibitors of Tie2 kinase.* Bioorganic & medicinal chemistry letters, 2007. **17**(17): p. 4756-60.
164. Cardone, M.H., et al., *Regulation of cell death protease caspase-9 by phosphorylation.* Science, 1998. **282**(5392): p. 1318-21.
165. Dumont, D.J., et al., *The endothelial-specific receptor tyrosine kinase, tek, is a member of a new subfamily of receptors.* Oncogene, 1993. **8**(5): p. 1293-301.
166. Harfouche, R., et al., *Mechanisms which mediate the antiapoptotic effects of angiopoietin-1 on endothelial cells.* Microvascular research, 2002. **64**(1): p. 135-47.
167. Papapetropoulos, A., et al., *Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway.* The Journal of biological chemistry, 2000. **275**(13): p. 9102-5.
168. Jeon, B.H., et al., *Tie-ing the antiinflammatory effect of angiopoietin-1 to inhibition of NF-kappaB.* Circulation research, 2003. **92**(6): p. 586-8.

169. Hughes, D.P., M.B. Marron, and N.P. Brindle, *The antiinflammatory endothelial tyrosine kinase Tie2 interacts with a novel nuclear factor-kappaB inhibitor ABIN-2.* Circulation research, 2003. **92**(6): p. 630-6.
170. Jones, N., et al., *A unique autophosphorylation site on Tie2/Tek mediates Dok-R phosphotyrosine binding domain binding and function.* Molecular and cellular biology, 2003. **23**(8): p. 2658-68.
171. Master, Z., et al., *Dok-R plays a pivotal role in angiopoietin-1-dependent cell migration through recruitment and activation of Pak.* The EMBO journal, 2001. **20**(21): p. 5919-28.
172. Kim, I., et al., *Angiopoietin-1 induces endothelial cell sprouting through the activation of focal adhesion kinase and plasmin secretion.* Circulation research, 2000. **86**(9): p. 952-9.
173. Tournaire, R., et al., *A short synthetic peptide inhibits signal transduction, migration and angiogenesis mediated by Tie2 receptor.* EMBO reports, 2004. **5**(3): p. 262-7.
174. Jones, N., et al., *Identification of Tek/Tie2 binding partners. Binding to a multifunctional docking site mediates cell survival and migration.* The Journal of biological chemistry, 1999. **274**(43): p. 30896-905.
175. Huang, L., et al., *GRB2 and SH-PTP2: potentially important endothelial signaling molecules downstream of the TEK/TIE2 receptor tyrosine kinase.* Oncogene, 1995. **11**(10): p. 2097-103.
176. Kontos, C.D., et al., *Tyrosine 1101 of Tie2 is the major site of association of p85 and is required for activation of phosphatidylinositol 3-kinase and Akt.* Molecular and cellular biology, 1998. **18**(7): p. 4131-40.
177. Koblizek, T.I., et al., *Angiopoietin-1 induces sprouting angiogenesis in vitro.* Current biology : CB, 1998. **8**(9): p. 529-32.
178. Hansbury, M.J., et al., *Production and characterization of a Tie2 agonist monoclonal antibody.* Angiogenesis, 2001. **4**(1): p. 29-36.
179. Chen, J.X., et al., *HSP90 and Akt modulate Ang-1-induced angiogenesis via NO in coronary artery endothelium.* Journal of applied physiology, 2004. **96**(2): p. 612-20.
180. Audero, E., et al., *Adaptor ShcA protein binds tyrosine kinase Tie2 receptor and regulates migration and sprouting but not survival of endothelial cells.* The Journal of biological chemistry, 2004. **279**(13): p. 13224-33.
181. Babaei, S., et al., *Angiogenic actions of angiopoietin-1 require endothelium-derived nitric oxide.* The American journal of pathology, 2003. **162**(6): p. 1927-36.
182. Asahara, T., et al., *Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization.* Circ Res, 1998. **83**(3): p. 233-40.
183. Kim, I., et al., *Angiopoietin-1 reduces VEGF-stimulated leukocyte adhesion to endothelial cells by reducing ICAM-1, VCAM-1, and E-selectin expression.* Circ Res, 2001. **89**(6): p. 477-9.
184. Yuan, H.T., et al., *Angiopoietin 2 is a partial agonist/antagonist of Tie2 signaling in the endothelium.* Molecular and cellular biology, 2009. **29**(8): p. 2011-22.
185. Cho, C.H., et al., *COMP-Ang1: a designed angiopoietin-1 variant with nonleaky angiogenic activity.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(15): p. 5547-52.

186. Witzenbichler, B., et al., *Chemotactic properties of angiopoietin-1 and -2, ligands for the endothelial-specific receptor tyrosine kinase Tie2.* The Journal of biological chemistry, 1998. **273**(29): p. 18514-21.
187. Hayes, A.J., et al., *Angiopoietin-1 and its receptor Tie-2 participate in the regulation of capillary-like tubule formation and survival of endothelial cells.* Microvascular research, 1999. **58**(3): p. 224-37.
188. Kanda, S., et al., *Angiopoietin 1 is mitogenic for cultured endothelial cells.* Cancer research, 2005. **65**(15): p. 6820-7.
189. Fujikawa, K., et al., *Role of PI 3-kinase in angiopoietin-1-mediated migration and attachment-dependent survival of endothelial cells.* Experimental cell research, 1999. **253**(2): p. 663-72.
190. Kim, I., et al., *Angiopoietin-1 reduces VEGF-stimulated leukocyte adhesion to endothelial cells by reducing ICAM-1, VCAM-1, and E-selectin expression.* Circulation research, 2001. **89**(6): p. 477-9.
191. Hegen, A., et al., *Expression of angiopoietin-2 in endothelial cells is controlled by positive and negative regulatory promoter elements.* Arteriosclerosis, thrombosis, and vascular biology, 2004. **24**(10): p. 1803-9.
192. Huang, Y.Q., et al., *Thrombin induces increased expression and secretion of angiopoietin-2 from human umbilical vein endothelial cells.* Blood, 2002. **99**(5): p. 1646-50.
193. Mandriota, S.J. and M.S. Pepper, *Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia.* Circulation research, 1998. **83**(8): p. 852-9.
194. Fiedler, U., et al., *Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation.* Nat Med, 2006. **12**(2): p. 235-9.
195. Pfaff, D., U. Fiedler, and H.G. Augustin, *Emerging roles of the Angiopoietin-Tie and the ephrin-Eph systems as regulators of cell trafficking.* J Leukoc Biol, 2006. **80**(4): p. 719-26.
196. Fiedler, U. and H.G. Augustin, *Angiopoietins: a link between angiogenesis and inflammation.* Trends in immunology, 2006. **27**(12): p. 552-8.
197. Brindle, N.P., P. Saharinen, and K. Alitalo, *Signaling and functions of angiopoietin-1 in vascular protection.* Circulation research, 2006. **98**(8): p. 1014-23.
198. Nicosia, R.F. and S. Villaschi, *Rat aortic smooth muscle cells become pericytes during angiogenesis in vitro.* Laboratory investigation; a journal of technical methods and pathology, 1995. **73**(5): p. 658-66.
199. Hashizume, H. and T. Ushiki, *Three-dimensional cytoarchitecture of angiogenic blood vessels in a gelatin sheet implanted in the rat skeletal muscular layers.* Archives of histology and cytology, 2002. **65**(4): p. 347-57.
200. Takahara, K., et al., *Autocrine/paracrine role of the angiopoietin-1 and -2/Tie2 system in cell proliferation and chemotaxis of cultured fibroblastic synoviocytes in rheumatoid arthritis.* Human pathology, 2004. **35**(2): p. 150-8.
201. Folkman, J., *Tumor angiogenesis: therapeutic implications.* The New England journal of medicine, 1971. **285**(21): p. 1182-6.
202. Medzhitov, R., *Origin and physiological roles of inflammation.* Nature, 2008. **454**(7203): p. 428-35.

203. Driss, V., et al., *[Eosinophil: a new effector of innate immunity?].* Medecine sciences : M/S, 2010. **26**(6-7): p. 621-6.
204. Kennedy, A.D. and F.R. DeLeo, *Neutrophil apoptosis and the resolution of infection.* Immunologic research, 2009. **43**(1-3): p. 25-61.
205. Athens, J.W., et al., *Leukokinetic studies. IV. The total blood, circulating and marginal granulocyte pools and the granulocyte turnover rate in normal subjects.* The Journal of clinical investigation, 1961. **40**: p. 989-95.
206. Hallett, M.B. and D. Lloyds, *Neutrophil priming: the cellular signals that say 'amber' but not 'green'.* Immunology today, 1995. **16**(6): p. 264-8.
207. Woodfin, A., M.B. Voisin, and S. Nourshargh, *Recent developments and complexities in neutrophil transmigration.* Current opinion in hematology, 2010. **17**(1): p. 9-17.
208. Bonfanti, R., et al., *PADGEM (GMP140) is a component of Weibel-Palade bodies of human endothelial cells.* Blood, 1989. **73**(5): p. 1109-12.
209. Hattori, R., et al., *Complement proteins C5b-9 induce secretion of high molecular weight multimers of endothelial von Willebrand factor and translocation of granule membrane protein GMP-140 to the cell surface.* The Journal of biological chemistry, 1989. **264**(15): p. 9053-60.
210. Hattori, R., et al., *Stimulated secretion of endothelial von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140.* The Journal of biological chemistry, 1989. **264**(14): p. 7768-71.
211. Hentzen, E., et al., *Hydrodynamic shear and tethering through E-selectin signals phosphorylation of p38 MAP kinase and adhesion of human neutrophils.* Annals of biomedical engineering, 2002. **30**(8): p. 987-1001.
212. Simon, S.I., et al., *Neutrophil tethering on E-selectin activates beta 2 integrin binding to ICAM-1 through a mitogen-activated protein kinase signal transduction pathway.* Journal of immunology, 2000. **164**(8): p. 4348-58.
213. Yao, L., et al., *Divergent inducible expression of P-selectin and E-selectin in mice and primates.* Blood, 1999. **94**(11): p. 3820-8.
214. Cassatella, M.A., *The production of cytokines by polymorphonuclear neutrophils.* Immunology today, 1995. **16**(1): p. 21-6.
215. Krieglstein, C.F. and D.N. Granger, *Adhesion molecules and their role in vascular disease.* American Journal of Hypertension, 2001. **14**(6 Pt 2): p. 44S-54S.
216. Scapini, P., et al., *The neutrophil as a cellular source of chemokines.* Immunological reviews, 2000. **177**: p. 195-203.
217. Borregaard, N. and J.B. Cowland, *Granules of the human neutrophilic polymorphonuclear leukocyte.* Blood, 1997. **89**(10): p. 3503-21.
218. Borregaard, N., O.E. Sorensen, and K. Theilgaard-Monch, *Neutrophil granules: a library of innate immunity proteins.* Trends in immunology, 2007. **28**(8): p. 340-5.
219. Serbina, N.V., et al., *Monocyte-mediated defense against microbial pathogens.* Annual review of immunology, 2008. **26**: p. 421-52.
220. Woollard, K.J. and F. Geissmann, *Monocytes in atherosclerosis: subsets and functions.* Nature reviews. Cardiology, 2010. **7**(2): p. 77-86.
221. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nature reviews. Immunology, 2008. **8**(12): p. 958-69.

222. Murray, P.J. and T.A. Wynn, *Obstacles and opportunities for understanding macrophage polarization.* Journal of leukocyte biology, 2011. **89**(4): p. 557-63.
223. Mantovani, A., et al., *Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes.* Trends in immunology, 2002. **23**(11): p. 549-55.
224. Fairweather, D. and D. Cihakova, *Alternatively activated macrophages in infection and autoimmunity.* Journal of autoimmunity, 2009. **33**(3-4): p. 222-30.
225. Sindrilaru, A., et al., *An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice.* The Journal of clinical investigation, 2011. **121**(3): p. 985-97.
226. Mantovani, A., et al., *The chemokine system in diverse forms of macrophage activation and polarization.* Trends in immunology, 2004. **25**(12): p. 677-86.
227. Li, Q. and I.M. Verma, *NF-kappaB regulation in the immune system.* Nature reviews. Immunology, 2002. **2**(10): p. 725-34.
228. Hu, X., et al., *Inhibition of IFN-gamma signaling by glucocorticoids.* Journal of immunology, 2003. **170**(9): p. 4833-9.
229. Hao, N.B., et al., *Macrophages in tumor microenvironments and the progression of tumors.* Clinical & developmental immunology, 2012. **2012**: p. 948098.
230. Peiser, L. and S. Gordon, *The function of scavenger receptors expressed by macrophages and their role in the regulation of inflammation.* Microbes and infection / Institut Pasteur, 2001. **3**(2): p. 149-59.
231. Aderem, A. and D.M. Underhill, *Mechanisms of phagocytosis in macrophages.* Annual review of immunology, 1999. **17**: p. 593-623.
232. Haidaris, C.G. and P.F. Bonventre, *A role for oxygen-dependent mechanisms in killing of Leishmania donovani tissue forms by activated macrophages.* Journal of immunology, 1982. **129**(2): p. 850-5.
233. Haynes, R.J., P.J. Tighe, and H.S. Dua, *Antimicrobial defensin peptides of the human ocular surface.* The British journal of ophthalmology, 1999. **83**(6): p. 737-41.
234. Baumgart, M., et al., *Differential expression of major histocompatibility complex class II genes on murine macrophages associated with T cell cytokine profile and protective/suppressive effects.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(12): p. 6936-40.
235. Steinman, R.M., *The dendritic cell system and its role in immunogenicity.* Annual review of immunology, 1991. **9**: p. 271-96.
236. Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of immunity.* Nature, 1998. **392**(6673): p. 245-52.
237. Gordon, S. and P.R. Taylor, *Monocyte and macrophage heterogeneity.* Nature reviews. Immunology, 2005. **5**(12): p. 953-64.
238. Cavaillon, J.M., *Cytokines and macrophages.* Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie, 1994. **48**(10): p. 445-53.
239. Jackson, J.R., et al., *The codependence of angiogenesis and chronic inflammation.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 1997. **11**(6): p. 457-65.
240. Imhof, B.A. and M. Aurrand-Lions, *Angiogenesis and inflammation face off.* Nature medicine, 2006. **12**(2): p. 171-2.

241. Costa, C., J. Incio, and R. Soares, *Angiogenesis and chronic inflammation: cause or consequence?* Angiogenesis, 2007. **10**(3): p. 149-66.
242. Glass, C.A., S.J. Harper, and D.O. Bates, *The anti-angiogenic VEGF isoform VEGF165b transiently increases hydraulic conductivity, probably through VEGF receptor 1 in vivo.* The Journal of physiology, 2006. **572**(Pt 1): p. 243-57.
243. Yang, R., et al., *Effects of vascular endothelial growth factor on hemodynamics and cardiac performance.* Journal of cardiovascular pharmacology, 1996. **27**(6): p. 838-44.
244. Feng, D., et al., *Vesiculo-vacuolar organelles and the regulation of venule permeability to macromolecules by vascular permeability factor, histamine, and serotonin.* The Journal of experimental medicine, 1996. **183**(5): p. 1981-6.
245. Michel, C.C. and C.R. Neal, *Openings through endothelial cells associated with increased microvascular permeability.* Microcirculation, 1999. **6**(1): p. 45-54.
246. Bates, D.O. and S.J. Harper, *Regulation of vascular permeability by vascular endothelial growth factors.* Vascular pharmacology, 2002. **39**(4-5): p. 225-37.
247. Weis, S., et al., *Src blockade stabilizes a Flk/cadherin complex, reducing edema and tissue injury following myocardial infarction.* The Journal of clinical investigation, 2004. **113**(6): p. 885-94.
248. Thurston, G., et al., *Angiopoietin-1 protects the adult vasculature against plasma leakage.* Nature medicine, 2000. **6**(4): p. 460-3.
249. Gamble, J.R., et al., *Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions.* Circulation research, 2000. **87**(7): p. 603-7.
250. Satchell, S.C., K.L. Anderson, and P.W. Mathieson, *Angiopoietin 1 and vascular endothelial growth factor modulate human glomerular endothelial cell barrier properties.* Journal of the American Society of Nephrology : JASN, 2004. **15**(3): p. 566-74.
251. Pizurki, L., et al., *Angiopoietin-1 inhibits endothelial permeability, neutrophil adherence and IL-8 production.* British journal of pharmacology, 2003. **139**(2): p. 329-36.
252. Kim, I., et al., *Angiopoietin-1 negatively regulates expression and activity of tissue factor in endothelial cells.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2002. **16**(1): p. 126-8.
253. Harfouche, R. and S.N. Hussain, *Signaling and regulation of endothelial cell survival by angiopoietin-2.* American journal of physiology. Heart and circulatory physiology, 2006. **291**(4): p. H1635-45.
254. Nag, S., et al., *Increased angiopoietin2 expression is associated with endothelial apoptosis and blood-brain barrier breakdown.* Laboratory investigation; a journal of technical methods and pathology, 2005. **85**(10): p. 1189-98.
255. Fiedler, U., et al., *Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation.* Nature medicine, 2006. **12**(2): p. 235-9.
256. Roviezzo, F., et al., *Angiopoietin-2 causes inflammation in vivo by promoting vascular leakage.* The Journal of pharmacology and experimental therapeutics, 2005. **314**(2): p. 738-44.
257. Pfaff, D., U. Fiedler, and H.G. Augustin, *Emerging roles of the Angiopoietin-Tie and the ephrin-Eph systems as regulators of cell trafficking.* Journal of leukocyte biology, 2006. **80**(4): p. 719-26.

258. Sturn, D.H., et al., *Angiopoietin affects neutrophil migration.* Microcirculation, 2005. **12**(5): p. 393-403.
259. DeBusk, L.M., et al., *Tie2 receptor tyrosine kinase, a major mediator of tumor necrosis factor alpha-induced angiogenesis in rheumatoid arthritis.* Arthritis and rheumatism, 2003. **48**(9): p. 2461-71.
260. Mueller, M.D., et al., *Neutrophils infiltrating the endometrium express vascular endothelial growth factor: potential role in endometrial angiogenesis.* Fertility and sterility, 2000. **74**(1): p. 107-12.
261. Heryanto, B., J.E. Girling, and P.A. Rogers, *Intravascular neutrophils partially mediate the endometrial endothelial cell proliferative response to oestrogen in ovariectomised mice.* Reproduction, 2004. **127**(5): p. 613-20.
262. Benelli, R., et al., *Neutrophils as a key cellular target for angiostatin: implications for regulation of angiogenesis and inflammation.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2002. **16**(2): p. 267-9.
263. Shaw, J.P., et al., *Polymorphonuclear neutrophils promote rFGF-2-induced angiogenesis in vivo.* The Journal of surgical research, 2003. **109**(1): p. 37-42.
264. Nielsen, B.S., et al., *92 kDa type IV collagenase (MMP-9) is expressed in neutrophils and macrophages but not in malignant epithelial cells in human colon cancer.* International journal of cancer. Journal international du cancer, 1996. **65**(1): p. 57-62.
265. Mentzel, T., et al., *The association between tumour progression and vascularity in myxofibrosarcoma and myxoid/round cell liposarcoma.* Virchows Archiv : an international journal of pathology, 2001. **438**(1): p. 13-22.
266. Eck, M., et al., *Pleiotropic effects of CXC chemokines in gastric carcinoma: differences in CXCL8 and CXCL1 expression between diffuse and intestinal types of gastric carcinoma.* Clinical and experimental immunology, 2003. **134**(3): p. 508-15.
267. Mhawech-Fauceglia, P., et al., *The source of APRIL up-regulation in human solid tumor lesions.* Journal of leukocyte biology, 2006. **80**(4): p. 697-704.
268. Bellocq, A., et al., *Neutrophil alveolitis in bronchioloalveolar carcinoma: induction by tumor-derived interleukin-8 and relation to clinical outcome.* The American journal of pathology, 1998. **152**(1): p. 83-92.
269. Zijlstra, A., et al., *Proangiogenic role of neutrophil-like inflammatory heterophils during neovascularization induced by growth factors and human tumor cells.* Blood, 2006. **107**(1): p. 317-27.
270. Coussens, L.M. and Z. Werb, *Matrix metalloproteinases and the development of cancer.* Chemistry & biology, 1996. **3**(11): p. 895-904.
271. Yasuda, M., et al., *Differential roles of ICAM-1 and E-selectin in polymorphonuclear leukocyte-induced angiogenesis.* American journal of physiology. Cell physiology, 2002. **282**(4): p. C917-25.
272. DiPietro, L.A. and P.J. Polverini, *Role of the macrophage in the positive and negative regulation of wound neovascularization.* Behring Institute Mitteilungen, 1993(92): p. 238-47.
273. Arras, M., et al., *Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb.* The Journal of clinical investigation, 1998. **101**(1): p. 40-50.
274. Ross, R., *Atherosclerosis is an inflammatory disease.* American heart journal, 1999. **138**(5 Pt 2): p. S419-20.

275. Sunderkotter, C., et al., *Macrophages and angiogenesis.* Journal of leukocyte biology, 1994. **55**(3): p. 410-22.
276. Levine, H.A., B.D. Sleeman, and M. Nilsen-Hamilton, *A mathematical model for the roles of pericytes and macrophages in the initiation of angiogenesis. I. The role of protease inhibitors in preventing angiogenesis.* Mathematical biosciences, 2000. **168**(1): p. 77-115.
277. Biswas, S.K., et al., *A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation).* Blood, 2006. **107**(5): p. 2112-22.
278. Bingle, L., N.J. Brown, and C.E. Lewis, *The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies.* The Journal of pathology, 2002. **196**(3): p. 254-65.
279. Steinman, R.M., *Cytokines amplify the function of accessory cells.* Immunology letters, 1988. **17**(3): p. 197-202.
280. Nathan, C.F., *Secretory products of macrophages.* The Journal of clinical investigation, 1987. **79**(2): p. 319-26.
281. Klimetzek, V. and C. Sorg, *Lymphokine-induced secretion of plasminogen activator by murine macrophages.* European journal of immunology, 1977. **7**(3): p. 185-7.
282. Nathan, C. and M. Sporn, *Cytokines in context.* The Journal of cell biology, 1991. **113**(5): p. 981-6.
283. Staton, C.A., M.W. Reed, and N.J. Brown, *A critical analysis of current in vitro and in vivo angiogenesis assays.* International journal of experimental pathology, 2009. **90**(3): p. 195-221.
284. Sharkey, A.M., et al., *Vascular endothelial growth factor expression in human endometrium is regulated by hypoxia.* The Journal of clinical endocrinology and metabolism, 2000. **85**(1): p. 402-9.
285. AlMalki, W.H., et al., *Assessment methods for angiogenesis and current approaches for its quantification.* Indian journal of pharmacology, 2014. **46**(3): p. 251-6.
286. Fajardo, L.F., et al., *The disc angiogenesis system.* Laboratory investigation; a journal of technical methods and pathology, 1988. **58**(6): p. 718-24.
287. Cimpean, A.M., D. Ribatti, and M. Raica, *A brief history of angiogenesis assays.* The International journal of developmental biology, 2011. **55**(4-5): p. 377-82.
288. Norrby, K., *In vivo models of angiogenesis.* Journal of cellular and molecular medicine, 2006. **10**(3): p. 588-612.
289. Gavard, J., V. Patel, and J.S. Gutkind, *Angiopoietin-1 prevents VEGF-induced endothelial permeability by sequestering Src through mDia.* Developmental cell, 2008. **14**(1): p. 25-36.
290. Pepper, M.S., et al., *Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro.* Biochemical and biophysical research communications, 1992. **189**(2): p. 824-31.
291. Mesri, E.A., H.J. Federoff, and M. Brownlee, *Expression of vascular endothelial growth factor from a defective herpes simplex virus type 1 amplicon vector induces angiogenesis in mice.* Circulation research, 1995. **76**(2): p. 161-7.
292. Shyu, K.G., H. Chang, and J.M. Isner, *Synergistic effect of angiopoietin-1 and vascular endothelial growth factor on neoangiogenesis in hypercholesterolemic rabbit model with acute hindlimb ischemia.* Life sciences, 2003. **73**(5): p. 563-79.

293. Zhou, Y.F., et al., *Effects of gene delivery on collateral development in chronic hypoperfusion: diverse effects of angiopoietin-1 versus vascular endothelial growth factor.* Journal of the American College of Cardiology, 2004. **44**(4): p. 897-903.
294. Simons, M., et al., *Clinical trials in coronary angiogenesis: issues, problems, consensus: An expert panel summary.* Circulation, 2000. **102**(11): p. E73-86.
295. Rissanen, T.T., I. Vajanto, and S. Yla-Herttuala, *Gene therapy for therapeutic angiogenesis in critically ischaemic lower limb - on the way to the clinic.* European journal of clinical investigation, 2001. **31**(8): p. 651-66.
296. Andrade, S.P., et al., *Sponge-induced angiogenesis in mice and the pharmacological reactivity of the neovasculature quantitated by a fluorimetric method.* Microvascular research, 1997. **54**(3): p. 253-61.
297. Andrade, S.P., et al., *Effects of platelet activating factor (PAF) and other vasoconstrictors on a model of angiogenesis in the mouse.* International journal of experimental pathology, 1992. **73**(4): p. 503-13.
298. Ford, H.R., et al., *Characterization of wound cytokines in the sponge matrix model.* Archives of surgery, 1989. **124**(12): p. 1422-8.
299. Sullivan, K.M., et al., *Fibulin-5 functions as an endogenous angiogenesis inhibitor.* Laboratory investigation; a journal of technical methods and pathology, 2007. **87**(8): p. 818-27.
300. Staton, C.A., M.W. Reed, and N.J. Brown, *A critical analysis of current in vitro and in vivo angiogenesis assays.* Int J Exp Pathol, 2009. **90**(3): p. 195-221.
301. Tengood, J.E., et al., *Sequential delivery of vascular endothelial growth factor and sphingosine 1-phosphate for angiogenesis.* Biomaterials, 2010. **31**(30): p. 7805-12.
302. Chae, J.K., et al., *Coadministration of angiopoietin-1 and vascular endothelial growth factor enhances collateral vascularization.* Arteriosclerosis, thrombosis, and vascular biology, 2000. **20**(12): p. 2573-8.
303. Zhu, W.H., A. MacIntyre, and R.F. Nicosia, *Regulation of angiogenesis by vascular endothelial growth factor and angiopoietin-1 in the rat aorta model: distinct temporal patterns of intracellular signaling correlate with induction of angiogenic sprouting.* The American journal of pathology, 2002. **161**(3): p. 823-30.
304. Marchand, G.S., et al., American journal of physiology. Heart and circulatory physiology, 2002. **282**(1): p. H194-204.
305. Holash, J., et al., *Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF.* Science, 1999. **284**(5422): p. 1994-8.
306. Mochizuki, Y., et al., *Angiopoietin 2 stimulates migration and tube-like structure formation of murine brain capillary endothelial cells through c-Fes and c-Fyn.* Journal of cell science, 2002. **115**(Pt 1): p. 175-83.
307. Eggert, A., et al., *High-level expression of angiogenic factors is associated with advanced tumor stage in human neuroblastomas.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2000. **6**(5): p. 1900-8.
308. Gaudry, M., et al., *Intracellular pool of vascular endothelial growth factor in human neutrophils.* Blood, 1997. **90**(10): p. 4153-61.
309. Noonan, D.M., et al., *Inflammation, inflammatory cells and angiogenesis: decisions and indecisions.* Cancer metastasis reviews, 2008. **27**(1): p. 31-40.
310. Dinarello, C.A., *Immunological and inflammatory functions of the interleukin-1 family.* Annual review of immunology, 2009. **27**: p. 519-50.

311. Nozawa, H., C. Chiu, and D. Hanahan, *Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(33): p. 12493-8.
312. Gong, Y. and D.R. Koh, *Neutrophils promote inflammatory angiogenesis via release of preformed VEGF in an in vivo corneal model.* Cell and tissue research, 2010. **339**(2): p. 437-48.
313. Lin, E.Y., et al., *Macrophages regulate the angiogenic switch in a mouse model of breast cancer.* Cancer research, 2006. **66**(23): p. 11238-46.
314. Kimura, Y.N., et al., *Inflammatory stimuli from macrophages and cancer cells synergistically promote tumor growth and angiogenesis.* Cancer science, 2007. **98**(12): p. 2009-18.
315. Engler, D.A., *Use of vascular endothelial growth factor for therapeutic angiogenesis.* Circulation, 1996. **94**(7): p. 1496-8.
316. Jain, R.K., *Molecular regulation of vessel maturation.* Nature medicine, 2003. **9**(6): p. 685-93.
317. Hellstrom, M., et al., *Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis.* The Journal of cell biology, 2001. **153**(3): p. 543-53.
318. Greenberg, J.I., et al., *A role for VEGF as a negative regulator of pericyte function and vessel maturation.* Nature, 2008. **456**(7223): p. 809-13.
319. Iivanainen, E., et al., *Angiopoietin-regulated recruitment of vascular smooth muscle cells by endothelial-derived heparin binding EGF-like growth factor.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2003. **17**(12): p. 1609-21.
320. Armulik, A., A. Abramsson, and C. Betsholtz, *Endothelial/pericyte interactions.* Circulation research, 2005. **97**(6): p. 512-23.
321. Kim, H. and G.Y. Koh, *Ang2, the instigator of inflammation.* Blood, 2011. **118**(18): p. 4767-8.
322. Miyagawa, J., et al., *Localization of heparin-binding EGF-like growth factor in the smooth muscle cells and macrophages of human atherosclerotic plaques.* The Journal of clinical investigation, 1995. **95**(1): p. 404-11.
323. Oehler, L., et al., *Neutrophil granulocyte-committed cells can be driven to acquire dendritic cell characteristics.* The Journal of experimental medicine, 1998. **187**(7): p. 1019-28.
324. Iking-Konert, C., et al., *Transdifferentiation of polymorphonuclear neutrophils: acquisition of CD83 and other functional characteristics of dendritic cells.* Journal of molecular medicine, 2001. **79**(8): p. 464-74.
325. Fanger, N.A., et al., *Activation of human T cells by major histocompatability complex class II expressing neutrophils: proliferation in the presence of superantigen, but not tetanus toxoid.* Blood, 1997. **89**(11): p. 4128-35.
326. Iking-Konert, C., et al., *Up-regulation of the dendritic cell marker CD83 on polymorphonuclear neutrophils (PMN): divergent expression in acute bacterial infections and chronic inflammatory disease.* Clinical and experimental immunology, 2002. **130**(3): p. 501-8.
327. Pliyev, B.K., et al., *Extracellular acidosis promotes neutrophil transdifferentiation to MHC class II-expressing cells.* Cellular immunology, 2011. **271**(2): p. 214-8.

328. Ostanin, D.V., et al., *Acquisition of antigen-presenting functions by neutrophils isolated from mice with chronic colitis.* Journal of immunology, 2012. **188**(3): p. 1491-502.
329. Wozniak, K.L., J.K. Kolls, and F.L. Wormley, Jr., *Depletion of neutrophils in a protective model of pulmonary cryptococcosis results in increased IL-17A production by gammadelta T cells.* BMC Immunol, 2012. **13**: p. 65.
330. Daley, J.M., et al., *Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice.* Journal of leukocyte biology, 2008. **83**(1): p. 64-70.
331. Soehnlein, O., et al., *Neutrophil secretion products pave the way for inflammatory monocytes.* Blood, 2008. **112**(4): p. 1461-71.
332. MacDonald, K.P., et al., *An antibody against the colony-stimulating factor 1 receptor depletes the resident subset of monocytes and tissue- and tumor-associated macrophages but does not inhibit inflammation.* Blood, 2010. **116**(19): p. 3955-63.
333. Chow, A., B.D. Brown, and M. Merad, *Studying the mononuclear phagocyte system in the molecular age.* Nat Rev Immunol, 2011. **11**(11): p. 788-98.
334. Schreiber, H.A., et al., *Intestinal monocytes and macrophages are required for T cell polarization in response to Citrobacter rodentium.* J Exp Med, 2013. **210**(10): p. 2025-39.

6.0 APPENDIX 1: Authorization, permission and the published manuscript in the Journal of Cellular Biochemistry

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Oct 22, 2014

This is a License Agreement between tharsika sinnathamby ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3493280375529
License date	Oct 20, 2014
Order Content Publisher	John Wiley and Sons
Order Content Publication	Journal of Cellular Biochemistry
Order Content Title	VEGF and angiopoietins promote inflammatory cell recruitment and mature blood vessel formation in murine sponge/Matrigel model
Licensed copyright line	© 2014 Wiley Periodicals, Inc.
Order Content Author	Tharsika Sinnathamby,Tae Jin Yun,Marie-Élaine Clavet-Lanthier,Cheolho Cheong,Martin G. Sirois
Order Content Date	Aug 21, 2014
Start page	n/a
End page	n/a
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	pro-inflammatory and angiogenic activities of VEGF and angiopoietins in murine sponge/Matrigel model
Expected completion date	Oct 2014
Expected size (number of pages)	130
Total	**0.00 CAD**

Terms and Conditions

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a"Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your Rightslink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.
- You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone basis),

non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this licence must be completed within two years of the date of the grant of this licence (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be used in any other manner or for any other purpose, beyond what is granted in the license. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Wiley Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Wiley Material. Any third party content is expressly excluded from this permission.

- With respect to the Wiley Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Wiley Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Wiley Materials without the prior permission of the respective copyright owner. You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted to you hereunder to any other person.

- The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley Companies, or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto.

- NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU

- WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.
- You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any

provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.
- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.
- These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.
- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.
- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.
- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

WILEY OPEN ACCESS TERMS AND CONDITIONS

Wiley Publishes Open Access Articles in fully Open Access Journals and in Subscription journals offering Online Open. Although most of the fully Open Access journals publish open access articles under the terms of the Creative Commons Attribution (CC BY) License only, the subscription journals and a few of the Open Access Journals offer a choice of Creative Commons Licenses:: Creative Commons Attribution (CC-BY) license Creative Commons Attribution Non-Commercial (CC-BY-NC) license and Creative Commons Attribution Non-Commercial-NoDerivs (CC-BY-NC-ND) License. The license type is clearly identified on the article.

Copyright in any research article in a journal published as Open Access under a Creative Commons License is retained by the author(s). Authors grant Wiley a license to publish the article and identify itself as the original publisher. Authors also grant any third party the right to use the article freely as long as its integrity is maintained and its original authors, citation details and publisher are identified as follows: [Title of Article/Author/Journal Title and Volume/Issue. Copyright (c) [year] [copyright owner as specified in the Journal]. Links to the final article on Wiley's website are encouraged where applicable.

The Creative Commons Attribution License

The Creative Commons Attribution License (CC-BY) allows users to copy, distribute and transmit an article, adapt the article and make commercial use of the article. The CC-BY license permits commercial and non-commercial re-use of an open access article, as long as the author is properly attributed.

The Creative Commons Attribution License does not affect the moral rights of authors, including without limitation the right not to have their work subjected to derogatory treatment. It also does not affect any other rights held by authors or third parties in the article, including without limitation the rights of privacy and publicity. Use of the article must not assert or imply, whether implicitly or explicitly, any connection with, endorsement or sponsorship of such use by the author, publisher or any other party associated with the article.

For any reuse or distribution, users must include the copyright notice and make clear to others that the article is made available under a Creative Commons Attribution license, linking to the relevant Creative Commons web page.

To the fullest extent permitted by applicable law, the article is made available as is and without representation or warranties of any kind whether express, implied, statutory or otherwise and including, without limitation, warranties of title, merchantability, fitness for a particular purpose, non-infringement, absence of defects, accuracy, or the presence or absence of errors.

Creative Commons Attribution Non-Commercial License
The Creative Commons Attribution Non-Commercial (CC-BY-NC) License permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.(see below)
Creative Commons Attribution-Non-Commercial-NoDerivs License
The Creative Commons Attribution Non-Commercial-NoDerivs License (CC-BY-NC-ND) permits use, distribution and reproduction in any medium, provided the original work is properly cited, is not used for commercial purposes and no modifications or adaptations are made. (see below)
Use by non-commercial users
For non-commercial and non-promotional purposes, individual users may access, download, copy, display and redistribute to colleagues Wiley Open Access articles, as well as adapt, translate, text- and data-mine the content subject to the following conditions:

- The authors' moral rights are not compromised. These rights include the right of "paternity" (also known as "attribution" - the right for the author to be identified as such) and "integrity" (the right for the author not to have the work altered in such a way that the author's reputation or integrity may be impugned).
- Where content in the article is identified as belonging to a third party, it is the obligation of the user to ensure that any reuse complies with the copyright policies of the owner of that content.
- If article content is copied, downloaded or otherwise reused for non-commercial research and education purposes, a link to the appropriate bibliographic citation (authors, journal, article title, volume, issue, page numbers, DOI and the link to the definitive published version on **Wiley Online Library**) should be maintained. Copyright notices and disclaimers must not be deleted.
- Any translations, for which a prior translation agreement with Wiley has not been agreed, must prominently display the statement: "This is an unofficial translation of an article that appeared in a Wiley publication. The publisher has not endorsed this translation."

Use by commercial "for-profit" organisations
Use of Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee. Commercial purposes include:

- Copying or downloading of articles, or linking to such articles for further redistribution, sale or licensing;
- Copying, downloading or posting by a site or service that incorporates advertising with such content;
- The inclusion or incorporation of article content in other works or services (other than normal quotations with an appropriate citation) that is then available for sale or licensing, for a fee (for example, a compilation produced for marketing purposes, inclusion in a sales pack)
- Use of article content (other than normal quotations with appropriate citation) by for-profit organisations for promotional purposes
- Linking to article content in e-mails redistributed for promotional, marketing or educational purposes;
- Use for the purposes of monetary reward by means of sale, resale, licence, loan, transfer or other form of commercial exploitation such as marketing products
- Print reprints of Wiley Open Access articles can be purchased from: corporatesales@wiley.com

Further details can be found on Wiley Online Library http://olabout.wiley.com/WileyCDA/Section/id-410895.html

Other Terms and Conditions:

v1.9

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

ARTICLE
Journal of Cellular Biochemistry 116:45–57 (2015)

Journal of Cellular Biochemistry

VEGF and Angiopoietins Promote Inflammatory Cell Recruitment and Mature Blood Vessel Formation in Murine Sponge/Matrigel Model

Tharsika Sinnathamby,[1,2] JinTae Yun,[3,4] Marie-Élaine Clavet-Lanthier,[1] Cheolho Cheong,[3,4,5] and Martin G. Sirois[1,2]*

[1]*Research Center, Montreal Heart Institute, Montréal, Canada*

[2]*Department of Pharmacology, Faculty of Medicine, Université de Montréal, Montréal, Canada*

[3]*Laboratory of Cellular Physiology and Immunology, Institut de Recherches Cliniques de Montréal (IRCM), Montréal, Canada*

[4]*Division of Experimental Medicine, Department of Medicine, McGill University, Montréal, Canada*

[5]*Department of Microbiology, Infectiology and Immunology, Faculty of Medicine, Université de Montréal, Montréal, Canada*

ABSTRACT

A key feature in the induction of pathological angiogenesis is that inflammation precedes and accompanies the formation of neovessels as evidenced by increased vascular permeability and the recruitment of inflammatory cells. Previously, we and other groups have shown that selected growth factors, namely vascular endothelial growth factor (VEGF) and angiopoietins (Ang1 and Ang2) do not only promote angiogenesis, but can also induce inflammatory response. Herein, given a pro-inflammatory environment, we addressed the individual capacity of VEGF and angiopoietins to promote the formation of mature neovessels and to identify the different types of inflammatory cells accompanying the angiogenic process over time. Sterilized polyvinyl alcohol (PVA) sponges soaked in growth factor-depleted Matrigel mixed with PBS, VEGF, Ang1, or Ang2 (200 ng/200 μl) were subcutaneously inserted into anesthetized mice. Sponges were removed at day 4, 7, 14, or 21 post-procedure for histological, immunohistological (IHC), and flow cytometry analyses. As compared to PBS-treated sponges, the three growth factors promoted the recruitment of inflammatory cells, mainly neutrophils and macrophages, and to a lesser extent, T- and B-cells. In addition, they were more potent and more rapid in the recruitment of endothelial cells (ECs) and in the formation and maturation (ensheating of smooth muscle cells around ECs) of neovessels. Thus, the autocrine/paracrine interaction among the different inflammatory cells in combination with VEGF, Ang1, or Ang2 provides a suitable microenvironment for the formation and maturation of blood vessels. J. Cell. Biochem. 116: 45–57, 2015.

KEY WORDS: VEGF; ANGIOPOIETINS; ANGIOGENESIS; NEOVESSEL MATURATION; INFLAMMATORY CELLS

The ramification of novel blood vessels from pre-existing vascular network, termed angiogenesis, is a coordinated sequence of cellular events consisting of sprouting, endothelial cell (EC) proliferation, directed migration of ECs, EC tube formation, and perivascular stabilization [Carmeliet and Jain, 2011]. Such multistep process is tightly regulated through the maintenance of a balance between soluble pro-angiogenic (stimulatory) and anti-angiogenic (inhibitory) factors [Liekens et al., 2001; Noonan et al., 2008]. A local perturbation of this equilibrium can result in either excessive or insufficient angiogenesis leading to a variety of diseases. With the identification of several pro-angiogenic molecules, potential therapeutic interference with vessel formation is being studied as promising tool for clinical applications [Griffioen and Molema, 2000]. For instance, while therapeutic inhibition of

Conflict of interest: All the authors read the manuscript and none of them had conflict of interest pertaining to the content of the current study.

Grant sponsor: Canadian Institutes of Health Research; Grant numbers: MOP-97943 (MGS), MOP-125933 (CC).

*Correspondence to: Martin G. Sirois, Research Center, Montreal Heart Institute, 5000 Belanger Street, Montréal H1T 1C8, Canada. E-mail: [redacted]

Manuscript Received: 29 July 2014; Manuscript Accepted: 15 August 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 21 August 2014

DOI 10.1002/jcb.24941 • © 2014 Wiley Periodicals, Inc.

angiogenesis may be beneficial in diseases associated to excessive neovessel growth (e.g., solid tumor, rheumatoid arthritis, diabetic retinopathy, atherosclerosis, and psoriasis) [Hanahan and Folkman, 1996], stimulation of angiogenesis may be beneficial in conditions associated with insufficient formation of new vasculature (e.g., tissue damage after reperfusion of ischemic tissue and cardiac failure) [de Muinck and Simons, 2004].

Vascular endothelial growth factor (VEGF) is a well-studied growth factor that effectively promotes neovessel sprouting and growth in the initial phase of angiogenesis [de Muinck and Simons, 2004; Carmeliet and Jain, 2011]. Upon discovery, its high angiogenic potential arose the hypothesis that VEGF monotherapy may be sufficient to promote therapeutic angiogenesis. However, in both pre-clinical and clinical testing, although VEGF monotherapy was successful in promoting the formation of blood vessels, they lacked vascular basement membrane and/or the ensheathing of α-smooth muscle actin (α-SMA)-positive pericytes and smooth muscle cells (SMCs), leading to the regression of newly formed vessels [Dor et al., 2002; Henry et al., 2003; de Muinck and Simons, 2004]. Thus, VEGF-orientated clinical trials did not support the expected beneficial outcome in patients [Simons et al., 2000; Stewart et al., 2009]. The discovery of a novel class of EC-specific ligands termed angiopoietins (Ang1 and Ang2) showed their capacity through the activation of Tie2 receptor to modulate the maturation and stabilization of newly formed vessels. For instance, while Ang1 in the late phase of angiogenesis plays an important role in promoting vascular maturation and contributing to enhance the integrity of EC barrier, Ang2 is identified to have the capacity to destabilize pre-existing vessels prior to VEGF-induced angiogenesis [Davis et al., 1996; Maisonpierre et al., 1997; Thurston et al., 2000]. Furthermore, Ang1 has also been demonstrated to have the capacity to promote in vivo angiogenesis and both Ang1 and Ang2 have the potential to increase EC migration and sprouting under certain experimental conditions [Mochizuki et al., 2002; Teichert-Kuliszewska et al., 2001]. Nonetheless, the angiopoietins themselves exert low mitogenic or proliferative activity on ECs [Davis et al., 1996], suggesting that VEGF and angiopoietins exhibit distinct and overlapping expression patterns which collaborate to regulate the different stages of physiological angiogenesis. Hence, a single pro-angiogenic factor may not be sufficient and effective in orchestrating all stages of the angiogenic process and a combination of pro-angiogenic mediators (e.g., growth factors with cytokines) may be required in the formation of stable blood vessels. In agreement with such premise, the emerging relationship between leukocyte infiltration and angiogenesis attracted a lot of attention over the last years.

Proliferating tissue in rheumatoid arthritis, psoriasis, and solid tumors per se, contains an abundance of inflammatory cells (neutrophils, monocytes/macrophages, and dendritic cells) that promote pathological angiogenesis either directly and/or indirectly leading to the creation of a highly vascularized granulation tissue [Costa et al., 2007]. The angiogenic events, in these pathologies, further support the inflammatory response, creating a vicious cycle. In accordance with these observations, clinical trial reports referring to coronary angiogenesis suggested that inflammation is an important stimulus in the induction of the angiogenic cascade [Simons et al., 2000] and very little angiogenesis takes place in the absence of inflammation [Jones et al., 1999]. We, in parallel with other groups, have demonstrated that VEGF and angiopoietins, in addition to being angiogenic factors, are also potent inflammatory regulators; once again indicating the necessity of inflammation in the accompaniment of angiogenesis [Maliba et al., 2008; Neagoe et al., 2009; Dumas et al., 2012; Neagoe et al., 2012]. During the last years, we have shown that Ang1 and Ang2, acting on Tie2 receptor, are capable of promoting the synthesis of platelet activating factor (PAF), a potent pro-inflammatory mediator, in both ECs and neutrophils. Upon its synthesis, PAF promotes neutrophil upregulation of β_2-integrin complex (CD11b/CD18) contributing to neutrophil adhesion and their migration onto activated ECs [Lemieux et al., 2005; Maliba et al., 2006]. In addition, we have reported the capacity of Ang1 to promote the synthesis and release of IL-1 and IL-8 [Dumas et al., 2012; Haddad and Sirois, 2014] which are both involved during inflammation and angiogenesis [Voronov et al., 2007; Qazi et al., 2011]. Nonetheless, the exact link between inflammation and angiogenesis such as the type and the temporal role of the recruited inflammatory cells during angiogenesis remains unanswered.

Various in vivo models using biomaterials (e.g., polyvinyl alcohol sponges) and/or Matrigel have been used extensively to analyze the angiogenic capacity of growth factors, cytokines, chemokines, and non-protein mediators in a number of different hosts [Norrby, 2006]. Yet, many of these studies either did not look at the maturation of blood vessels, a crucial event in the stabilization of nascent blood vessels, or the newly formed vessels were identified to be immature (lacking the ensheathing of SMCs). Hence, in the current study, we utilized a novel variant of the sponge/Matrigel angiogenic model such that the pro-inflammatory sponges were pre-incubated in growth factor depleted Matrigel containing the tested growth factor prior to subcutaneous implantation into wild type mice in order to: (1) assess the individual pro-angiogenic capacity of VEGF, Ang1, and Ang2 to promote the formation and the maturation of neovessels; and (2) to identify the different inflammatory cells accompanying angiogenesis in a spatio-temporal manner.

MATERIALS AND METHODS

MICE

C57BL/6 and BALB/c mice, 10–11 weeks old, were purchased from Charles River Laboratories (Montréal, Canada), *CD115*$^{gfp/+}$, and *Zbtb46*$^{gfp/+}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animal experiments were approved by the ethical animal care committees of the Montreal Heart Institute and Institut de Recherches Cliniques de Montréal.

SPONGE PREPARATION AND IMPLANTATION

Sterilized polyvinyl alcohol (PVA) sponges (6 mm diameter ×2 mm width) were soaked in 200 μl of growth factor depleted Matrigel (BD Biosciences, Mississauga, Canada) containing PBS or 200 ng of VEGF (PeproTech, Rocky Hill, NJ), Ang1, or Ang2 (R&D Systems, Minneapolis, MN) for 20 min at 4°C. Subsequently, the sponges were incubated for 20 min at 37°C prior to implantation. Under

anesthesia with 2% isoflurane USP, two sponges treated with the same growth factors were inserted subcutaneously through two 1 cm orthogonal incisions in the dorsa of the animals. The incisions were then clipped for closure, and the mice were subcutaneously injected with an analgesic agent (0.1 ml of Anaphen; 1 mg/ml). The mice were sacrificed under anaesthesia at day 4, 7, 14, or 21 post-procedure.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY ANALYSES

The harvested sponge implants were fixed in 10% formalin PBS-buffered solution, embedded into paraffin blocks and sectioned sagittally (6 μm thick). The sections were stained with Masson's trichrome reagent for a global overview of cellular invasion in the implants. Immunohistological stainings were performed using the avidin-biotin complex for the validation of angiogenesis and inflammatory cells infiltration as previously described [Marchand et al., 2002; Lemieux et al., 2005]. The primary antisera used in this study were: ECs specific goat anti-mouse CD31 (Santa Cruz Biotechnology Inc., CA), SMCs specific mouse anti-mouse α-SMA (Sigma–Aldrich, Steinheim, Germany; clone 1A4), neutrophils specific rabbit anti-mouse myeloperoxydase (MPO) (Thermo Scientific, Rockford, IL), and macrophages specific rat anti-mouse F4/80 (BioLegend, San Diego, CA; clone BM8).

To assess the maturation of neovessels, a sequential double immunohistochemistry (IHC) staining was performed. The sponge sections underwent first round of IHC using the primary antisera anti-CD31 and host specific biotinylated secondary antibody. Peroxidase was developed by the DAB substrate. The tissues underwent a second round of IHC protocol with the primary antisera anti-α-SMA and host specific biotinylated secondary antibody. α-SMA expression was detected in turquoise using Vina green chromogen (Biocare Medical Inc., Concord, CA).

MICROSCOPY AND QUANTIFICATION

Images were collected using a brightfield microscope and were analyzed using Image-Pro Plus software. Images of selected regions of highest positive signal were acquired under 200× magnification of each stained section (endothelial cells, neutrophils, macrophages, and smooth muscle cells). These selected regions were then quantified using the color segmentation method. Thresholds were empirically set to select pixels by analyzing a test set of 10 images per batch of staining. The selected pixels represented the expression of the stained cell. These empirically determined thresholds were recorded in a macro and were applied to all images that were analyzed. The number of pixels counted by the macro was recorded in mm^2. The Matrigel area was measured using Image Pro's calibrated area measurement tool in mm^2. The percent occupancy of studied cells in the Matrigel from each sponge was calculated by taking the mean of: (area of counted pixels (mm^2)/area of Matrigel (mm^2)) × 100 of five randomly selected images per sponge. The mean microvessel density was expressed as the absolute number of microvessels counted/area of Matrigel (mm^2). The cross-sectional area occupied by these blood vessels was also simultaneously measured. The vessel maturation index was measured as: ((number of α-SMA-positive vessels/number of CD31-positive vessels) × 100).

SPONGE SINGLE CELL PREPARATION AND FLOW CYTOMETRY ANALYSIS

Single cell suspensions were isolated from sponges and spleen as previously described [Choi et al., 2011]. Briefly, the sponges and corresponding spleens were isolated from C57BL/6 mice, minced and incubated for 60 min at 37°C in an enzyme mixture. Following the blockage of Fc receptors using culture supernatant of 2.4G2 hybridoma, the cells were stained with fluorophore-conjugated antibodies. The stained cells were acquired using LSR Fortessa (Becton Dickinson, Mississauga, Canada) and were analyzed using FlowJo (Tree Star Inc., Ashland, OR). The monoclonal antibodies used in both flow cytometry analysis and FACS were anti-mouse CD45, CD64, CD3, CD19, Ly6G (clone: 1A8), MHCII, CD11c, and corresponding isotype controls were purchased from BioLegend.

ANALYSIS OF PHAGOCYTOSIS

Sponge and splenic $CD45^+CD11c^+MHCII^+$ cells isolated from C57BL/6 mice, were incubated with 0.00134% of 0.50 μm Fluoresbrite® YG Microspheres (Polysciences, Inc., Warrington, PA) for 30 min at 37°C. The cells were then labeled with monoclonal antibodies against CD45, CD11c, MHCII, and CD19 and analyzed by flow cytometry.

MIXED LEUKOCYTE REACTIONS

Sponge and splenic $CD45^+$ cells were FACS (Beckman Coulter MoFlo, Mississauga, Canada) sorted into $CD11c^+MHCII^+$ cell population and $CD11c^-MHCII^-Ly6G^+$ neutrophils from C57BL/6 mice. For proliferative analysis, splenic T-cells were isolated from BALB/c mice by excluding $B220^+$, $F4/80^+$, $CD49b^+$, and $I\text{-}Ab^+$ cells using anti-rat IgG Dynabeads (Invitrogen, Burlington, Canada). These allogenic T-cells were subsequently labeled with carboxyfluorescein diacetate-succinimidyl ester (CFSE) and were combined with isolated stimulator cells (splenic $CD11c^+MHCII^+$ cells, sponge $CD11c^+MHCII^+$ cells, and neutrophils; stimulator: T-cell ratio of 1:10) in microtest wells at 5,000 of stimulator to 50,000 T-cells/well. Four days later, T-cell proliferation was evaluated by CFSE dilution in flow cytometry.

STATISTICAL ANALYSIS

Results are presented as the mean ± SEM and all comparisons were made between each conditions at corresponding days by analysis of variance (ANOVA) followed by a Bonferroni *t*-test. Differences were considered significant at *P*-values less than 0.05.

RESULTS

VEGF AND ANGIOPOIETINS PROMOTE BLOOD VESSEL FORMATION

Previous studies have demonstrated that VEGF and angiopoietins play precise, complementary, and coordinated roles in angiogenesis. In the present study, we wanted to assess the individual pro-angiogenic activities of VEGF and angiopoietins in a novel variant of the sponge/Matrigel angiogenic model. To monitor vascularization and to examine the angiogenic microenvironment in the sponges, we performed histological analysis using Masson's trichrome staining of the sponges at different time points from day 4 to day 21. Sponges

containing PBS followed the time-course of the host foreign body response in terms of cellular infiltration and neovessels formation (Fig. 1A and C). In contrast, sponges loaded with VEGF, Ang1, or Ang2 (200 ng/200 μl) elicited a robust invasion of various cell types into the Matrigel giving rise to a highly vascularized matrix by day 7 (Fig. 1B and D–F).

Based on endothelial cell-specific CD31 IHC detection (Fig. 2A; upper left insert), for a more comprehensive analysis, sponges containing PBS, VEGF, Ang1, or Ang2 showed marginal amount of EC recruitment by day 4 (Fig. 2A). However, in the presence of any one of the tested growth factors, this effect became significant by day 7 and 14 with an increase of ≈3–5 fold as compared to PBS-treated sponges. At day 21, the percentage of CD31 expression in PBS-treated sponges became comparable to VEGF and Ang2 treated sponges, whereas Ang1 continuously maintained its capacity to recruit ECs. These recruited ECs took their neovessel structure (lumen formation) by day 7 in presence of VEGF, Ang1, or Ang2 (Fig. 2B). Once formed by day 7, the microvessel density remained stable,

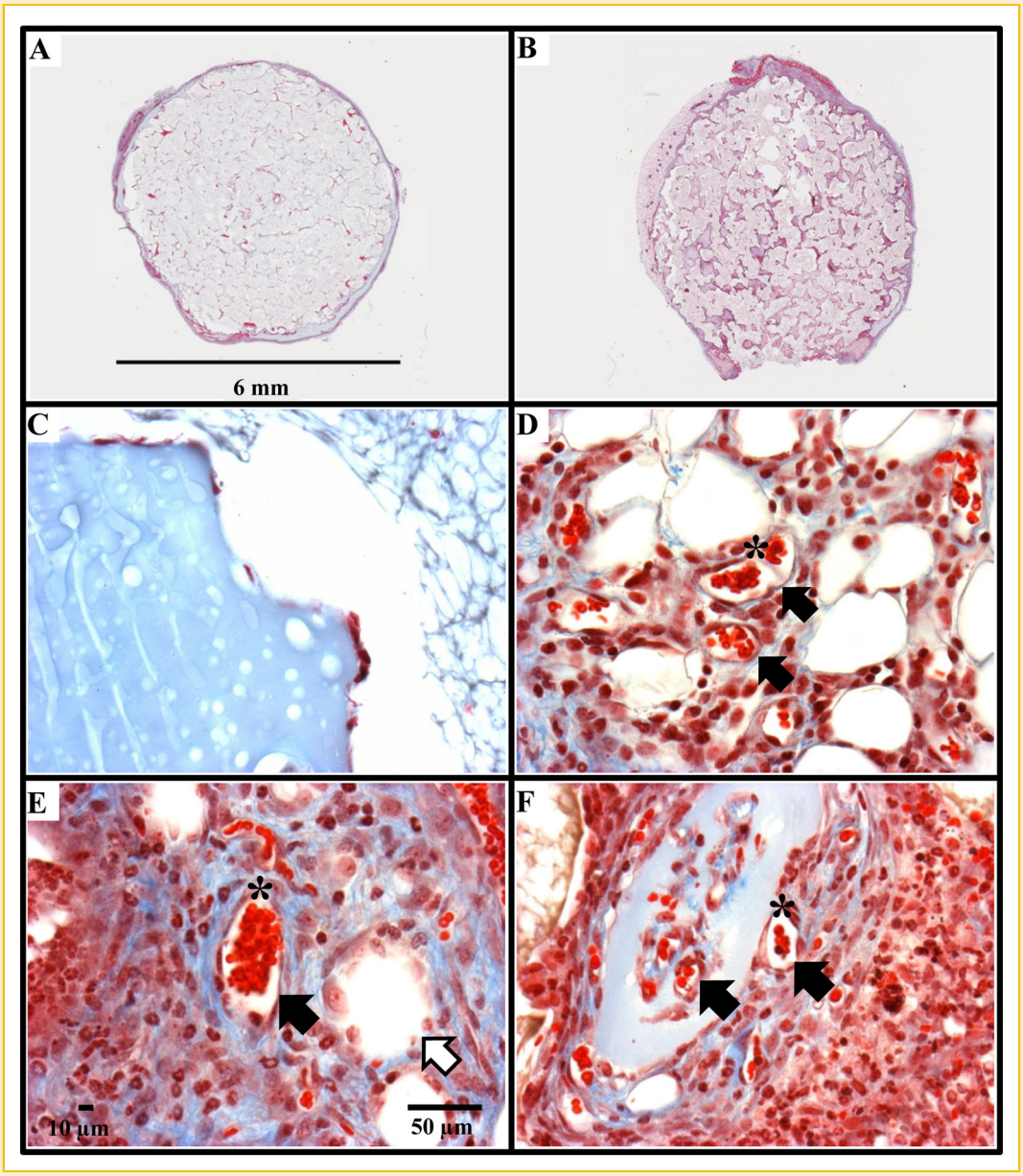


Fig. 1. Pro-angiogenic and inflammatory activities of VEGF and angiopoietins in mice. The images illustrate representative scans (A and B) and representative histological sections (Masson's trichrome staining, 400× magnification) of PVA sponges soaked in growth factor depleted Matrigel containing PBS, VEGF, Ang1, or Ang2 (200 ng/200 μl) harvested at day 7 (C–F). Treatment with VEGF, Ang1, or Ang2 promoted a marked recruitment of numerous inflammatory cells, endothelial cells, and mural cells in the region of Matrigel within the sponges and the formation of neovessels (black arrow) containing circulating red blood cells (star), indicative of functional blood vessels (B and D–F). Neovessel formation from endothelial cells (lumen formation) lacking circulating red blood cells was also observed (white arrow). In contrast, PBS treated sponges showed less cellular accumulation and no blood vessel formation by day 7 (A and C).

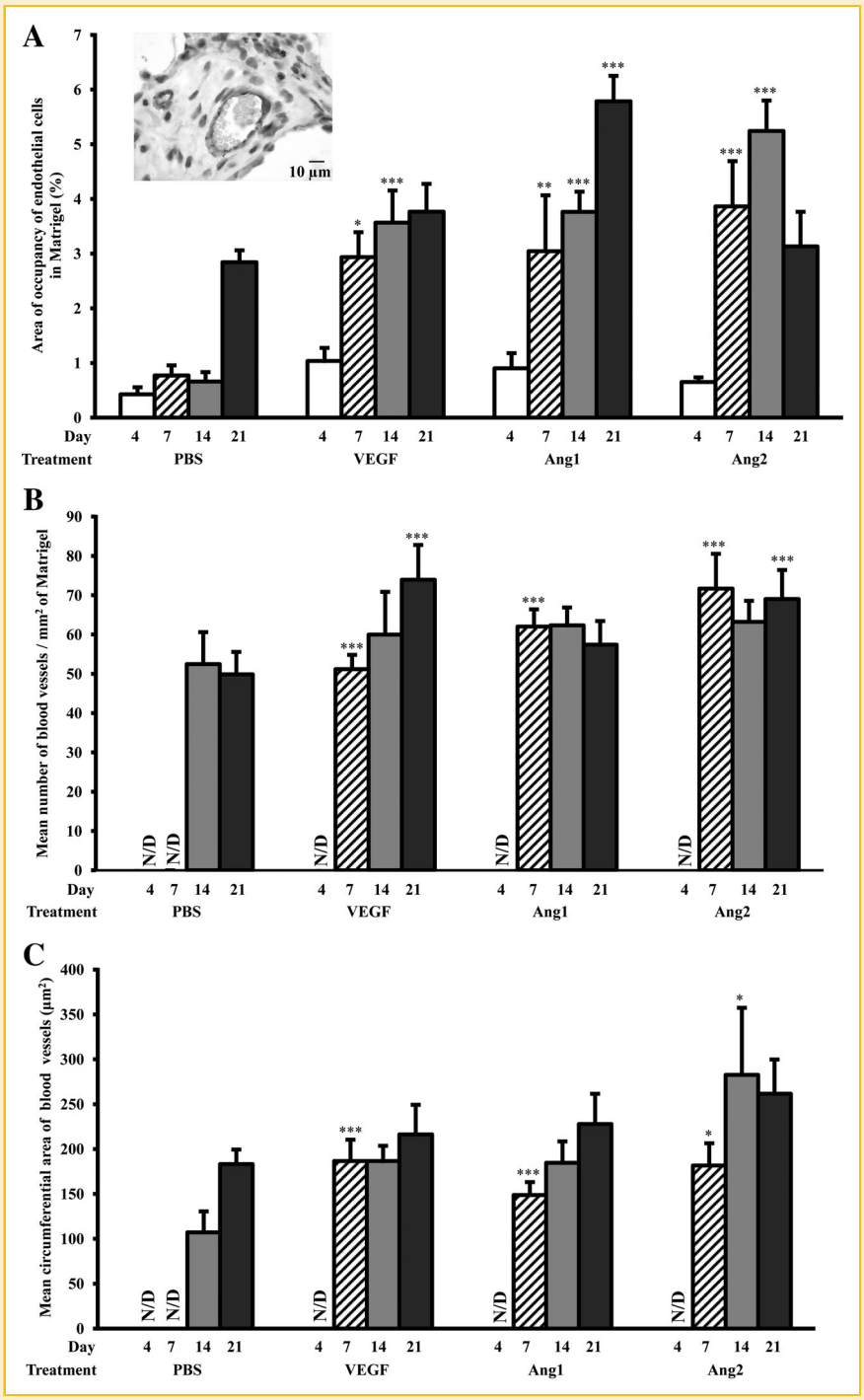


Fig. 2. Effect of VEGF and angiopoietins on angiogenesis in a time-dependent manner. PVA sponges soaked in growth factor depleted Matrigel containing PBS, VEGF, Ang1, or Ang2 (200 ng/200 μl) were removed from the animals at day 4, 7, 14, or 21. Subsequently, IHC staining against endothelial cell specific CD31 protein was performed in order to assess the recruitment of endothelial cells (IHC insert; 1000×; A), microvessel density (B) and the average cross-sectional area occupied by the vessels (C) in the Matrigel. Data are represented as mean ± SEM of 4 to 10 independent experiments per condition. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ as compared to PBS-treatment at corresponding days, N/D: not detectable.

ranging from 50 to 70 vessels/mm^2 of Matrigel. Although delayed in time, the microvessel density under PBS-treatment became comparable (≈50 vessels/mm^2 of Matrigel) to the growth factor-treated sponges by day 14 (Fig. 2B). Finally, in presence of Ang2 the average cross-sectional area occupied by the neovessels formed by day 14 to 21 (>250 μm^2) was greater than the area of vessels formed in presence of VEGF or Ang1 (≈150–225 μm^2). However, under PBS treatment, the primary vessels formed by day 14, were smaller

(≈100 μm^2) but underwent remodeling and nearly doubled by day 21 (Fig. 2C).

VEGF AND ANGIOPOIETINS PROMOTE BLOOD VESSEL MATURATION

Vessel maturation is critical in angiogenesis, as the stability of an induced vasculature is dependent on the mural cell association to prevent vessel regression [Bergers and Song, 2005]. We thus, wanted to elucidate the temporal sequel of VEGF and angiopoietins mediated maturation of neovessels given a pro-inflammatory environment. The recruitment of SMCs was detected based on α-SMA protein expression by day 7 in all tested conditions (Fig. 3A). Yet, treatment with VEGF and angiopoietins individually triggered a more rapid and pronounced recruitment of SMCs, producing a ≈10–12-fold increase as compared to PBS-treated group. By day 14, the number of SMCs detected under growth factor stimulation plateaued, while the venue of SMCs under PBS treatment caught up yet remaining ≈2.5–3-fold lower to what was mediated by VEGF and the angiopoietins. We also observed that by day 21, the area covered by SMCs in presence of VEGF or Ang1 was maintained whereas it partially declined under PBS or Ang2 treatment (Fig.3A).

To assess whether the SMCs remained sparse into the Matrigel and/or associated with neovessels, we performed double IHC staining against CD31 and α-SMA proteins. We observed a common inflection point by day 7 in presence of the different growth factors, favoring the formation of neovessels, the migration of SMCs, and the surrounding of SMCs around the neovessels as compared to PBS-treated sponges (Fig.3B). Sponges harvested at day 4 under all of the tested treatments only supported the recruitment of ECs and not of SMCs (Fig.3A). By day 14 and 21, the maturing blood vessels were covered with multiple layers of SMCs for all conditions (Fig.3B). However, although the number of neovessels surrounded by at least a single layer of SMCs by day 7 was ≈60–70% and reached up to 80% by day 14 or 21 under growth factor treatments, it plateaued to about 40% by day 14 in PBS-treated sponges (Fig.3C).

VEGF AND ANGIOPOIETINS MEDIATED ANGIOGENESIS IS ACCOMPANIED BY INFLAMMATORY CELLS

Inflammatory cells, namely neutrophils and monocytes/macrophages participate in the angiogenic process through the secretion of pro- and anti-inflammatory cytokines by controlling EC activation, migration, and proliferation [El et al., 2000; Lingen, 2001; Voronov et al., 2003]. Using anti-MPO antibody (Fig. 4A; upper right insert), we observed significant recruitment of neutrophils by day 7 in presence of VEGF, which peaked by day 14 covering about ≈2% of total surface area, and then faded away by day 21. In addition, VEGF was more potent as compared to the angiopoietins to promote the recruitment of neutrophils by day 14. Ang2 showed a significant peak (≈1%) in neutrophil recruitment by day 7 and its potency decreased gradually over time. On the other hand, Ang1 showed a mild effect on neutrophil recruitment as compared to PBS-treated

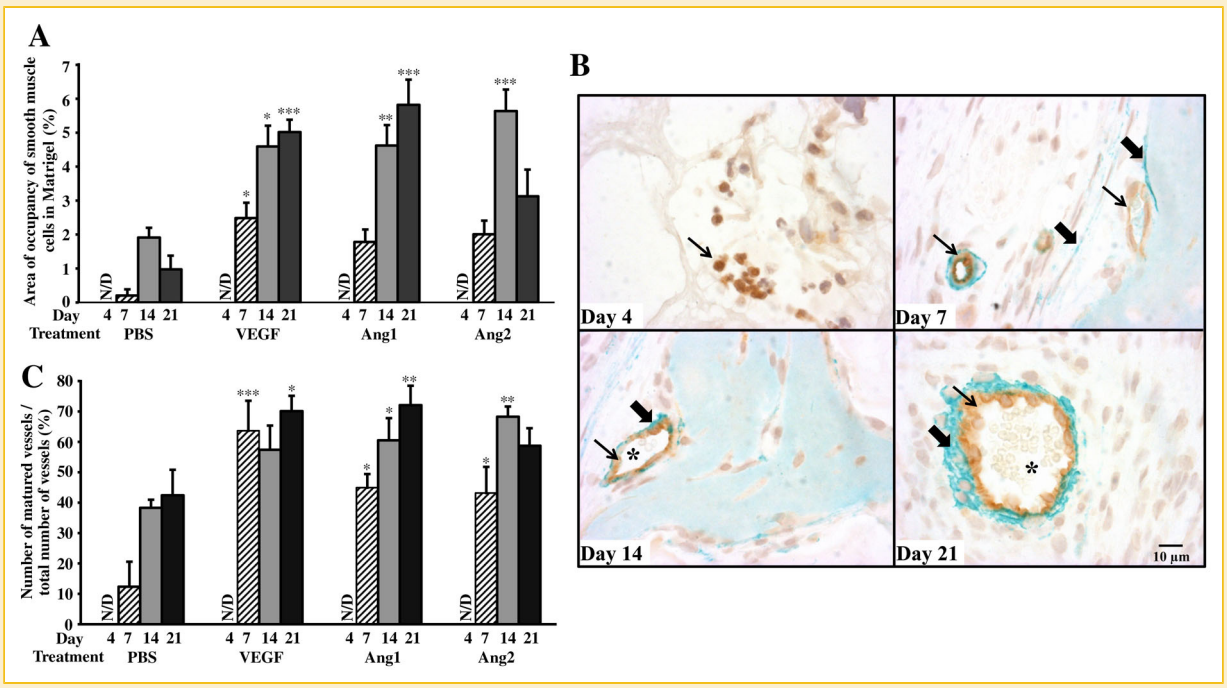


Fig. 3. VEGF and angiopoietins mediated SMC migration and neovascular maturation in the sponges. PVA sponges soaked in growth factor depleted Matrigel containing PBS, VEGF, Ang1, or Ang2 (200 ng/200 μl) were removed from the animals at day 4, 7, 14, or 21. Subsequently, IHC staining against α-SMA was performed in order to assess the venue of SMCs (A). The 4 panels represent immunohistogical snapshots illustrating the temporal evolution of mature blood vessels in the sponges in presence of the studied growth factors (Ang1) at days 4, 7, 14, and 21. Endothelial cells were stained with anti-CD31 (brown staining; thin arrow) and SMCs were stained with anti-α-SMA (turquoise staining; thick arrow) (1000× magnification). The neovessels were not only ensheathed by SMCs, but also contained red blood cells (star) (B). The percentage of mature blood vessels was quantified as the number of neovessels surrounded by SMCs over the total number of blood vessels (C). Data are represented as mean ± SEM of 4 to 10 independent experiments per condition. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ as compared to PBS-treatment at corresponding days, N/D: not detectable.

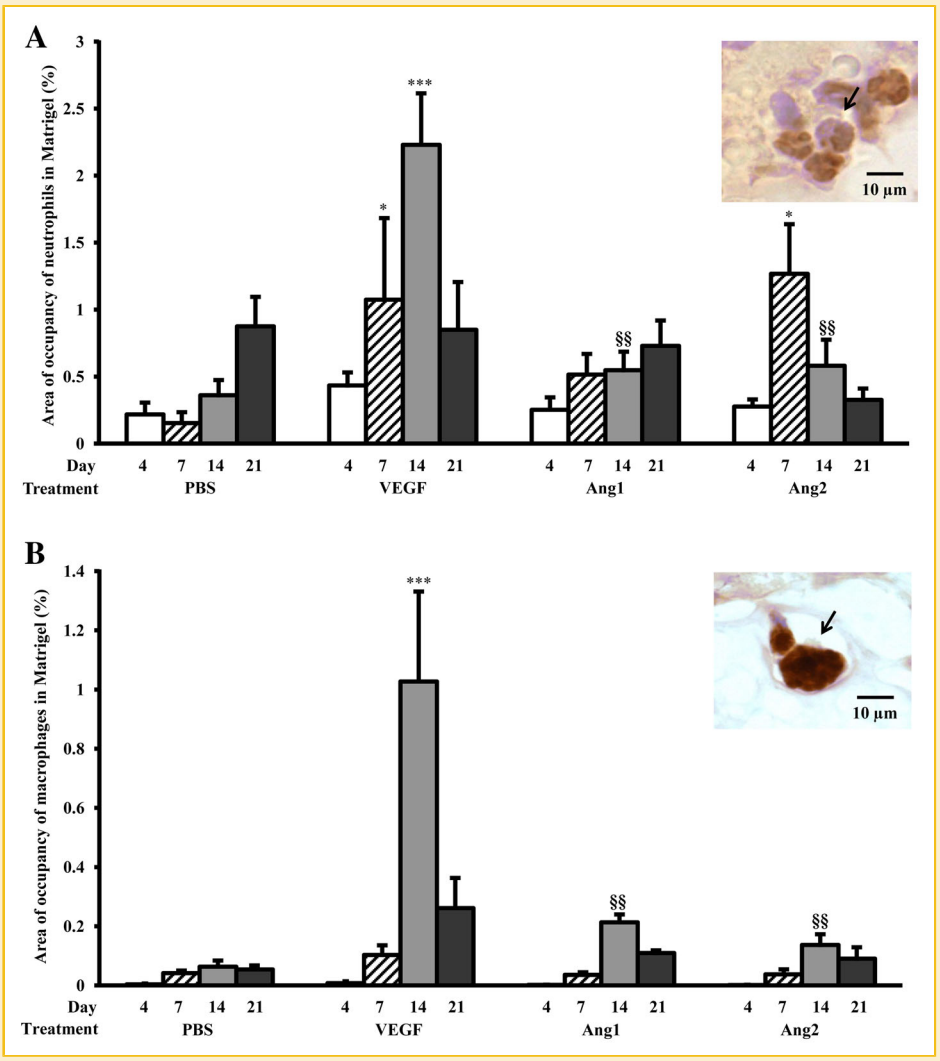


Fig. 4. Identification of VEGF and angiopoietins mediated inflammatory cells influx in the sponges. Neutrophil (A) and macrophage (B) accumulation in the sponge implants were measured as MPO (IHC insert; 1000×; A) and F4/80 (IHC insert; 1000X; B) expression, respectively, in the Matrigel region of the sponges. VEGF, Ang1, and Ang2 mediated inflammatory cells (neutrophil and macrophage) recruitment was temporal-dependent with different potency. Data are represented as mean ± SEM of 4 to 10 independent experiments per condition. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ as compared to PBS-treatment, $^{\S\S}P < 0.01$ as compared to VEGF-treatment at corresponding days, N/D: not detectable.

sponges (Fig. 4A). We looked at the recruitment of monocytes/macrophages based on F4/80 selective protein detection (Fig. 4B; upper right insert). The three growth factors individually tended to have a peak recruitment of macrophages by day 14, which was massive and significant under VEGF treatment (≈1%) as compared to PBS and angiopoietins-treated animals (Fig. 4B).

CHARACTERIZATION OF INFLAMMATORY AND IMMUNE CELLS IN SPONGES BY FACS ANALYSES

To delineate the different leukocyte subsets recruited in the sponges, we utilized multicolor flow cytometry procedure on single cell preparation from sponges. $CD45^{+}$ leukocytes in the sponges were primarily comprised of $CD11c^{+}MHCII^{+}$ cells, $CD11c^{-}MHCII^{-}Ly6G^{+}$ cells (neutrophils), $CD11c^{-}MHCII^{-}CD3^{+}$ cells (T-cells), and $CD11c^{-}MHCII^{+}CD19^{+}$ cells (B-cells) (Fig. 5A). The fate of $CD11c^{+}MHCII^{+}$ cells at this point remained to be investigated. Previous studies demonstrated that dentritic cells (DCs) constitutively express the hematopoietic markers CD45, CD11c, and MHCII in lymphoid tissues such as spleen and lymph nodes. Nonetheless, this marker expression profile on its own is not sufficient to define classical DCs (cDCs) in nonlymphoid tissues. In fact, high and similar levels of CD11c and MHCII expression have been observed in both cDCs and in macrophages [Gautier et al., 2012]. Thus, we performed marker analyses, genetic, and functional studies to specifically identify the $CD11c^{+}MHCII^{+}$ cell population in the sponges as cDCs and/or macrophages. Recently, *Zbtb46* was identified as a selectively expressed transcription factor by cDCs but not by monocytes, macrophages and other lymphoid and myeloid lineages (e.g., neutrophils, T-cells and B-cells) [Satpathy et al., 2012]. Therefore, we harvested sponge cells from $Zbtb46^{+/+}$ (WT) and $Zbtb46^{gfp/+}$ mice

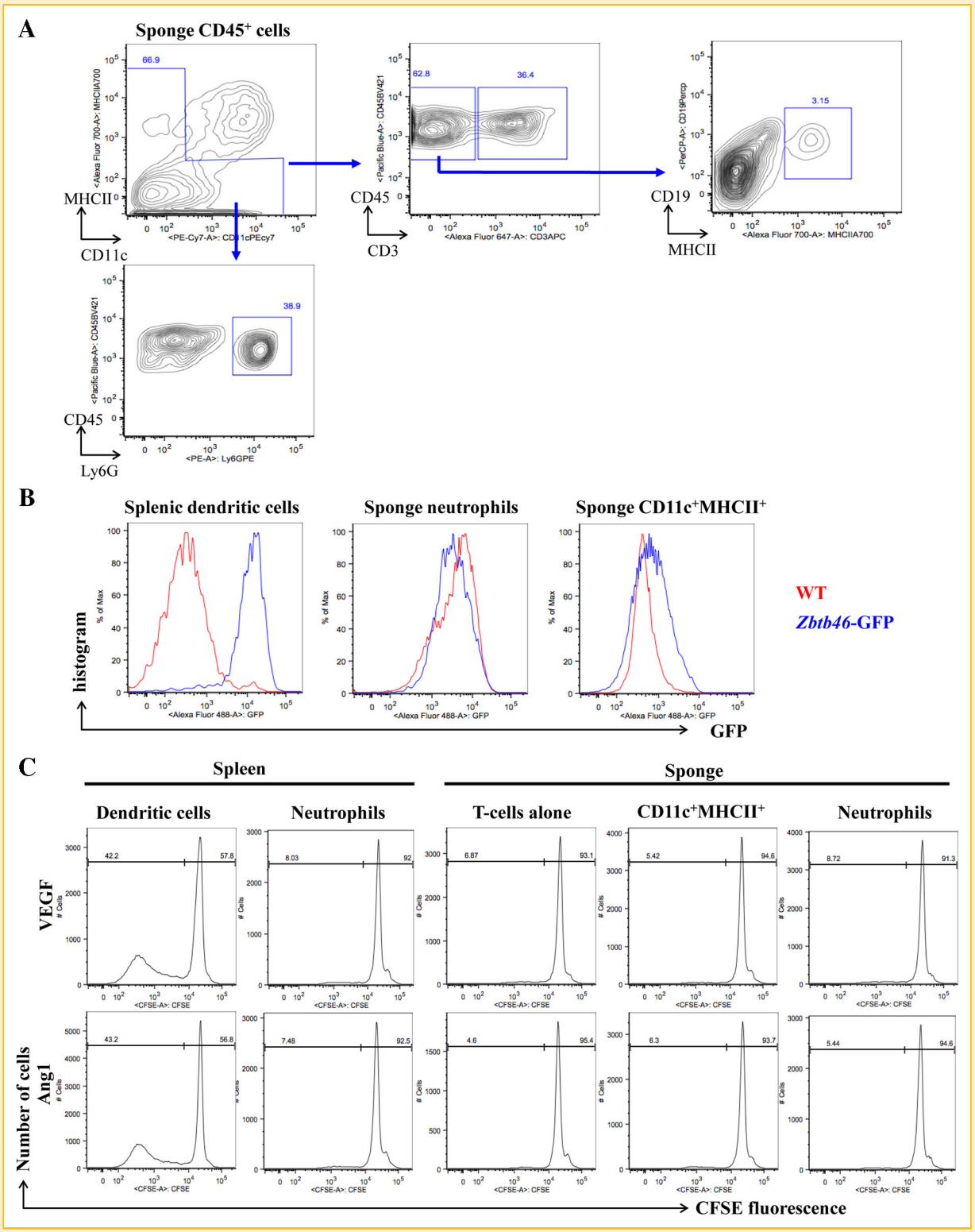


Fig. 5. $CD11c^{+}MHCII^{+}$ cells recruited in the sponges do not possess DC-characteristics. Single cell suspensions from sponges harvested from C57BL/6 mice were examined for surface expression of indicated markers. The data illustrates the expression profile of CD11c and MHCII, CD3 (T-cells), CD19 (B-cells), and Ly6G (neutrophils) within $CD45^{+}$ gated cell population (A). Representative histogram of GFP expression of $CD11c^{+}MHCII^{+}$ cells in spleen ($n = 2$), VEGF-treated sponges and neutrophils ($n = 4$) harvested from *Zbtb46*$^{+/+}$ (WT) and *Zbtb46*$^{gfp/+}$ mice (B). FACS purified $CD11c^{+}MHCII^{+}$ cells isolated from sponges (treated with VEGF or Ang1) and spleens along with neutrophils retrieved from C57BL/6 mice were co-cultured with T-cells purified from BALB/c mice ($CD3^{+}$CFSE-labled T-cells) in MLR. CFSE levels were analyzed four days later. Proliferation of allogenic T-cells results in a reduction of CFSE fluorescence intensity ($n = 4$; C).

and analyzed for GFP expression. As anticipated, $CD45^+$ cell population containing splenic DCs were GFP^+ while neutrophils recruited in the sponges were devoid of GFP expression. In contrast to splenic DCs, $CD11c^+MHCII^+$ cell population isolated from the sponges lacked expression of GFP (Fig. 5B). Next, FACS-sorted $CD11c^+MHCII^+$ cells from sponges and spleens along with neutrophils were tested for their ability to stimulate allogenic T-cells. Only splenic DCs were strong stimulators of T-cell proliferation (Fig. 5C). T-cells alone, neutrophils from sponges and spleen and $CD11c^+MHCII^+$ cells from sponges did not induce allogenic T-cell proliferation. These results confirm that $CD11c^+MHCII^+$ cells in the sponges do not possess DCs functional characteristics.

In contrast, $CD11c^+MHCII^+$ cells from PBS, VEGF, Ang1, and Ang2-treated sponges were positive for F4/80 marker while the neutrophils from the corresponding sponges were negative (Fig. 6A). Although in the past, F4/80 served as a reliable marker of

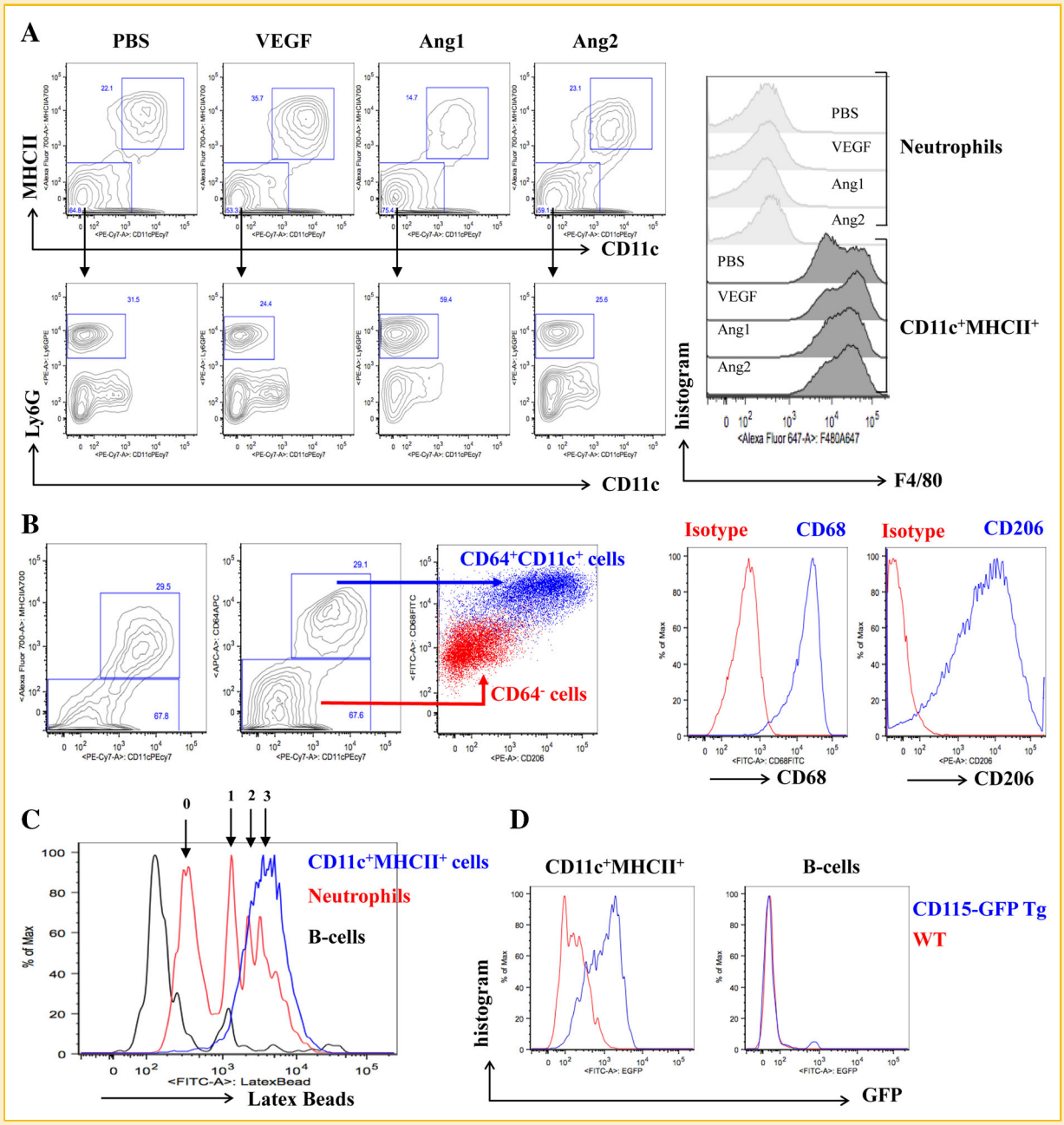


Fig. 6. $CD11c^+MHCII^+$ cells recruited in the sponges are classical and non-classical macrophages. The images illustrate representative histogram (n = 2–4 independent experiments per condition) for F4/80 expression of $CD11c^+MHCII^+$ cells and $CD11c^-MHCII^-$ $Ly6G^+$ neutrophils isolated from sponges pretreated with PBS, VEGF, Ang1, or Ang2 (A). $CD11c^+MHCII^+$ $CD64^+$ cells were stained for the intracellular markers CD68 and CD206 (B). Single cell suspensions isolated from VEGF treated sponge were bathed with 0.5 μm YG microspheres for 30 min at 37°C and the uptake of these microspheres (phagocytosis) were analysed by flow cytometry (C). B-cells, neutrophils, and $CD11c^+MHCII^+$ cells isolated from VEGF treated sponges harvested from *CD115*$^{gfp/+}$ and WT mice were analysed for GFP expression (D).

macrophages, additional analysis of a panel of surface markers is now required to define macrophage population. Recently, the surface marker CD64 expression was identified as a reliable marker of mature tissue macrophages [Gautier et al., 2012]. Interestingly, in our study, we observed that the $CD11c^{+}MHCII^{+}$ cells isolated from the sponges were all $CD64^{+}$. Moreover, they also expressed CD68 and CD206, two additional markers of macrophages [Gautier et al., 2012] (Fig. 6B). To test whether the $CD11c^{+}MHCII^{+}$ cells recruited in the sponges had the phagocytic activity of macrophages, we analyzed their phagocytic capacity by flow cytometry. Neutrophils from the sponges took up 0–2 beads/cell while all sponge $MHCII^{+}CD11c^{+}$ cells were highly phagocytic (>3 beads uptake/cell). B-cells isolated from the sponges served as our negative control and it provided no phagocytic activity (Fig. 6C). To further confirm these isolated cells from the sponges as macrophages, we used transgenic *CD115*$^{gfp/+}$ mice, which express MCSF-1R, a receptor for macrophage-colony stimulating factor [Sasmono et al., 2007]. Indeed, the $CD11c^{+}MHCII^{+}$ cells isolated from the sponges implanted in *CD115*$^{gfp/+}$ mice were GFP positive. However, B-cells (negative control) from the transgenic *CD115*$^{gfp/+}$ and WT mice were GFP negative (Fig. 6D). Taken together, these results demonstrate that the $MHCII^{+}CD11c^{+}$ cells present in the sponges are macrophages and not DCs.

DISCUSSION

Compelling studies have demonstrated the direct participation of neutrophils and monocytes/macrophages in the induction of inflammatory response prior to the initiation of pathological angiogenesis. Indeed, the release of pro-inflammatory cytokines and growth factors provides a suited autocrine/paracrine milieu to fully support blood vessels formation [Schruefer et al., 2005; Aplin et al., 2006; Lin et al., 2006; Gong and Koh, 2010]. As we have previously illustrated the pro-inflammatory activities of VEGF and angiopoietins, we were led to address their capacity to promote inflammatory response associated to in vivo angiogenesis. In the present study, we utilized a novel variant of the murine sponge/Matrigel angiogenic assay to evaluate the sequel of host-derived blood vessel formation and inflammatory cell infiltration into the sponges. Herein, we demonstrate that VEGF, Ang1, and Ang2 individually are highly potent and efficacious in recruiting ECs, SMCs, and inflammatory cells (mainly neutrophils and macrophages, and sparsely T- and B-cells). More importantly, these tested growth factors given individually were not only capable to favor the formation of neovessels but also their maturation as observed by the coordinated ensheating of SMCs around the neovessels and the presence of circulating red blood cells in the vessel lumen. Hence, this study suggests the potential contribution of both inflammatory cells and angiogenic growth factors to fully support blood vessel formation and their maturation.

Recent efforts in clinical trials focus on localized therapy for restoring blood flow in ischemic regions as tissue loss in these patients was localized [Simons et al., 2000]. While growth factor therapy remained a gold standard for the induction of local therapeutic angiogenesis, translating this concept into an effective and safe therapy for patients became a challenge. Presently, biomaterial based approaches is being successfully utilized in animal models to study the capacity of growth factors, cytokines/chemokines, and nonprotein mediators to promote blood vessel formation [Andrade et al., 1997]. One such method is the subcutaneous implantation of PVA sponges in mice, which promotes a robust infiltration of inflammatory cells, providing a pro-inflammatory environment, and giving rise to a highly vascularized sponge matrix. However, due to continuous inflammation, these newly formed vessels were postulated to be fragile, permeable, and dilated with no indication of neovessel maturation (lack of SMCs ensheathing) [Andrade et al., 1997]. A major disadvantage of such matrix implantation is that it induces non-specific inflammatory host response and thus limits to acute studies [Staton et al., 2009]. Later, the Matrigel plug assay became the widely used model for studies involving in vivo testing for angiogenesis, as it provides a natural environment for the formation of neovessels without inducing non-specific immune response [Staton et al., 2009]. Yet, although Matrigel injection containing VEGF in mice successfully promoted the formation of neovessels, the model did not lead to the maturation of the newly formed vessels [Tengood et al., 2010]. As inflammation is an important stimulus for the induction of new vessel growth, we hypothesized that the combination of both these approaches might fulfill the required environment to favor the formation and maturation of neovessels. The classical sponge/Matrigel model, encompassing both the sponge model and the Matrigel assay, requires the subcutaneous injection of Matrigel containing the protein of interest, 20–30 min prior to the surgical introduction of PVA sponges [Akhtar et al., 2002; Norrby, 2006]. This method has been identified to provide variable amount of test compound within the implants and to trigger the fibrotic encapsulation of the sponges [Norrby, 2006]. In our variation of the sponge/Matrigel model, we have soaked PVA sponges into Matrigel containing the tested growth factors prior to the surgical implantation. We observed that our technique was simple, less time consuming, that each sponge implant contained equal volume of the tested growth factors and it did not induce non-specific immune response.

We observed an early onset of EC migration in the sponges within the first 4 days and a significant number of blood vessel formation by day 7 under VEGF or angiopoietin stimulation, thus, challenging the classical role of angiopoietins in angiogenesis. Interestingly, the amount of ECs migrated into the sponges kept increasing up to day 14 or 21, while the number of blood vessels once formed by day 7 remained stable, suggesting that the model itself exerts a restrain on the maximal capacity of blood vessel formation even in presence of free ECs. Our data is in line with previous studies reporting the pro-angiogenic and mitogenic activities of VEGF in various in vivo models including the chick chorioallantoic membrane [Plouet et al., 1989], the rabbit cornea [Phillips et al., 1994], and the primate iris [Tolentino et al., 1996]. However, the capacity of angiopoietins to initiate the angiogenic cascade remains controversial. For instance, while some in vivo reports demonstrated that Ang1 alone is unable to induce angiogenesis but can potentiate VEGF mediated angiogenic response [Asahara et al., 1998; Chae et al., 2000], others showed that Ang1 can promote a robust neovascularization in Matrigel implants [Babaei et al., 2003]. The implication of Ang2 in

angiogenesis is tied with VEGF where it promotes destabilization of pre-existing blood vessels in the absence of VEGF [Holash et al., 1999; Lobov et al., 2002]. Yet, other studies reported that Ang2 alone can induce vascular remodeling and angiogenesis in absence of VEGF [Kim et al., 2000b; Mochizuki et al., 2002]. Our study illustrates that the pro-inflammatory environment itself is sufficient to initiate the angiogenic cascade and the addition of the tested growth factors further allows this effect to be more potent and efficient.

Interestingly, we also observed the venue and the ensheathing of SMCs around neovessels by day 7 in presence of the tested growth factors. Indeed, all three growth factors promoted the maturation of blood vessels with equal potency. Although, our result is consistent with the stabilizing effect of Ang1 on vascular endothelium, it is also in contradiction with the proposed role for VEGF and Ang2 during angiogenesis. In fact, VEGF and angiopoietins are incapable of directly activating SMCs. Yet, they can promote the activation of ECs and support the migration of inflammatory cells (e.g., macrophages and neutrophils) which can promote the release of various growth factors and cytokines (e.g., FGF, VEGF, Ang1, interleukins [IL-1β, IL-8 and -10], and CXCL1) [Gaudry et al., 1997; Noonan et al., 2008; Dinarello, 2009; Neagoe et al., 2009]. Ang2 in particular, has been shown to possess pro-inflammatory characteristics on both ECs and neutrophils [Lemieux et al., 2005; Fiedler and Augustin, 2006; Fiedler et al., 2006; Kim and Koh, 2011]. In addition, neutrophils and macrophages can equally trigger the release of numerous metalloproteinases, neutrophil elastase, and reactive oxygen species (ROS), which can facilitate extracellular matrix degradation, favoring the migration and proliferation of ECs and SMCs (reviewed in [van Hinsbergh et al., 2006]). In addition, the presence of neutrophils and macrophages in the sponges at day 7 during the recruitment of SMCs may initiate a paracrine compensation pathway in order to trigger the maturation event. Interestingly, from the histological sections, we observed that the newly formed vessels in presence of VEGF, Ang1, or Ang2 were "functional" based on the presence red blood cells in the neovessels and that they appeared to be non-leaky. Vascular permeability study must be conducted in order to confirm this later statement. However, as not all the neovessels formed in the sponges are necessarily matured at any given time, it is thus, not possible to confirm the absence of vascular leakiness. Furthermore, we also observed that the blood vessels once formed undergo no or marginal diameter remodeling. Ang1 in the past has been identified to play an important role in the reorganization of EC into tubule-like structures during angiogenesis by stimulating the production of proteases. Plasmin and matrix metalloproteases, examples of such proteases, decrease the EC-substratum interaction allowing the ECs to reshape the vessel lumen [Kim et al., 2000a]. However, in our study, upon the formation of neovessels by day 7 (with growth factors), we did not observe additional remodeling over time. This may be due to the rapid maturation of the newly formed vessels taking place simultaneous to blood vessel formation which may prevent further unrestricted enlargement of the growing vessels [Hoeben et al., 2004]. As for the PBS-treated sponges, the delayed recruitment of SMCs may explain the slight remodeling of the area of occupancy that took place between day 14 and 21. Together, VEGF, Ang1, and Ang2 alone are capable of mediating the maturation process in the presence of a pro-inflammatory environment suggesting that inflammation plays a major role in the angiogenic process.

This notion is further strengthened as observed under various pathological conditions. For instance, suppression of inflammatory response by genetic abnormalities, pathophysiological processes, or pharmacotherapy produce adverse effects in the ability of the host to induce new vessel growth [Jones et al., 1999]; hence inflammation, once considered to be a homeostatic response protecting the body from invading pathogens, is now been shown to function as a critical stimulus for neovessel growth. Neutrophils being the most abundant leukocyte in the circulation have been demonstrated to play important roles during pathological angiogenesis. Although, the exact mechanism through which tumor associated neutrophils mediate or modulate angiogenesis has not been fully elucidated, the importance of neutrophils in tumor angiogenesis has been noted from human biopsies [Van den Steen et al., 2000; Nozawa et al., 2006]. Similarly, increased macrophage infiltration in various types of cancer correlates positively with vascularity, tumor stage and malignancy [Torisu et al., 2000; Chen et al., 2003]. Once again the exact function of the macrophages in the tumor environment remains a nuance.

Likewise, although we did not study the exact roles of inflammatory cells in angiogenesis, we observed the presence of neutrophils, macrophages, and sparsely T- and B-cells, in the tissue section by IHC and/or flow cytometry. Surprisingly, the presence of neutrophils, expected to be one of the first cells recruited at the site of inflammation, was still observed at latter time points raising the question whether we have continuous recruitment of neutrophils in sponges or if they have been differentiated to other cell types. Recently, it was suggested that neutrophils could differentiate into neutrophil-DC hybrids with DC-like properties in the setting of experimentally induced inflammatory lesions in mice [Geng et al., 2013; Matsushima et al., 2013]. DCs are professional antigen presenting cells, which reside in peripheral tissues in an immature state. Upon microbial contact and stimulation by inflammatory cytokines, it possesses a unique ability to induce both primary and secondary T- and B-cell responses. It is now clear that DCs express a wide array of pro- and anti-inflammatory mediators that mediate a significant role in those pathophysiological settings characterized by DC activation and angiogenesis [Sozzani et al., 2007]. Thus, we hypothesized that neutrophils may differentiate into neutrophil-DC hybrids in our system. However, $CD11c^{+}MHCII^{+}$ cells, which we initially thought to be DCs, turned out to be neither DCs nor neutrophil-DC hybrid cells. These cells: (1) did not express DC-lineage transcriptional factor Zbtb46; and (2) did not stimulate allogenic T-cells in MLR assay. Instead, they expressed macrophage specific markers including F4/80, CD68, CD206, and CD115/mCSF1R and were highly phagocytic. Hence, our sponge/Matrigel model, apart from T- and B-cells, contains two major inflammatory cell populations: neutrophils and macrophages.

In summary, our murine sponge/Matrigel model in presence of the pro-angiogenic growth factors (VEGF, Ang1, or Ang2) allowed the formation of new vessels and more importantly, it led to their maturation. Moreover, the recruitment of inflammatory cells in the Matrigel by the provided growth factors further accelerated these processes with greater potency. Thus, such pro-inflammatory/

angiogenic model along with the growth factors may provide a suited autocrine/paracrine environment capable of triggering and supporting the formation and maturation of neovessels, illustrating the necessity of inflammation in the creation of mature blood vessels. Further studies will be needed through selected depletion of neutrophils and monocytes/macrophages to delineate the role of these cells in such angiogenic model.

ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Institutes of Health Research (CIHR; MOP-97943 (MGS) and MOP-125933 (CC)) and from the Heart and Stroke Foundation of Quebec (MGS). Cheolho Cheong is a Chercheur-Boursier Junior of Fonds de Recherche du Québec-Santé.

REFERENCES

Akhtar N, Dickerson EB, Auerbach R. 2002. The sponge/Matrigel angiogenesis assay. Angiogenesis 5:75–80.

Andrade SP, Machado RD, Teixeira AS, Belo AV, Tarso AM, Beraldo WT. 1997. Sponge-induced angiogenesis in mice and the pharmacological reactivity of the neovasculature quantitated by a fluorimetric method. Microvas Res 54:253–261.

Aplin AC, Gelati M, Fogel E, Carnevale E, Nicosia RF. 2006. Angiopoietin-1 and vascular endothelial growth factor induce expression of inflammatory cytokines before angiogenesis. Physiol Genomics 27:20–28.

Asahara T, Chen D, Takahashi T, Fujikawa K, Kearney M, Magner M, Yancopoulos GD, Isner JM. 1998. Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization. Circ Res 83:233–240.

Babaei S, Teichert-Kuliszewska K, Zhang Q, Jones N, Dumont DJ, Stewart DJ. 2003. Angiogenic actions of angiopoietin-1 require endothelium-derived nitric oxide. Am J Pathol 162:1927–1936.

Bergers G, Song S. 2005. The role of pericytes in blood-vessel formation and maintenance. Neuro Oncol 7:452–464.

Carmeliet P, Jain RK. 2011. Molecular mechanisms and clinical applications of angiogenesis. Nature 473:298–307.

Chae JK, Kim I, Lim ST, Chung MJ, Kim WH, Kim HG, Ko JK, Koh GY. 2000. Coadministration of angiopoietin-1 and vascular endothelial growth factor enhances collateral vascularization. Arteriosclerosis 20:2573–2578.

Chen JJ, Yao PL, Yuan A, Hong TM, Shun CT, Kuo ML, Lee YC, Yang PC. 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.

Choi JH, Cheong C, Dandamudi DB, Park CG, Rodriguez A, Mehandru S, Velinzon K, Jung IH, Yoo JY, Oh GT, Steinman RM. 2011. Flt3 signaling-dependent dendritic cells protect against atherosclerosis. Immunity 35:819–831.

Costa C, Incio J, Soares R. 2007. Angiogenesis and chronic inflammation: Cause or consequence. Angiogenesis 10:149–166.

Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD. 1996. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell 87:1161–1169.

de Muinck ED, Simons M. 2004. Re-evaluating therapeutic neovascularization. J Mol Cell Cardiol 36:25–32.

Dinarello CA. 2009. Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol 27:519–550.

Dor Y, Djonov V, Abramovitch R, Itin A, Fishman GI, Carmeliet P, Goelman G, Keshet E. 2002. Conditional switching of VEGF provides new insights into adult neovascularization and pro- angiogenic therapy. EMBO J 21:1939–1947.

Dumas E, Martel C, Neagoe PE, Bonnefoy A, Sirois MG. 2012. Angiopoietin-1 but not angiopoietin-2 promotes neutrophil viability: Role of interleukin-8 and platelet-activating factor. Biochim Biophys Acta 1823:358–367.

El Awad, Kreft B, Wolber B, Hellwig-Burgel EM, Metzen T, Fandrey E, Jelkmann J. 2000. Hypoxia and interleukin-1beta stimulate vascular endothelial growth factor production in human proximal tubular cells. Kidney Int 58:43–50.

Fiedler U, Augustin HG. 2006. Angiopoietins: A link between angiogenesis and inflammation. Trends Immunol 27:552–558.

Fiedler U, Reiss Y, Scharpfenecker M, Grunow V, Koidl S, Thurston G, Gale NW, Witzenrath M, Rosseau S, Suttorp N, Sobke A, Herrmann M, Preissner KT, Vajkoczy P, Augustin HG. 2006. Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nat Med 12:235–239.

Gaudry M, Bregerie O, Andrieu V, El Benna, Pocidalo J, Hakim MA. 1997. Intracellular pool of vascular endothelial growth factor in human neutrophils. Blood 90:4153–4161.

Gautier EL, Shay T, Miller J, Greter M, Jakubzick C, Ivanov S, Helft J, Chow A, Elpek KG, Gordonov S, Mazloom AR, Ma'ayan A, Chua WJ, Hansen TH, Turley SJ, Merad M, Randolph GJ. 2012. Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. Nat Immunol 13:1118–1128.

Geng S, Matsushima H, Okamoto T, Yao Y, Lu R, Page K, Blumenthal RM, Ward NL, Miyazaki T, Takashima A. 2013. Emergence, origin, and function of neutrophil-dendritic cell hybrids in experimentally induced inflammatory lesions in mice. Blood 121:1690–1700.

Gong Y, Koh DR. 2010. Neutrophils promote inflammatory angiogenesis via release of preformed VEGF in an in vivo corneal model. Cell Tissue Res 339:437–448.

Griffioen AW, Molema G. 2000. Angiogenesis: Potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. Pharmacol Rev 52:237–268.

Haddad LE, Sirois MG. 2014. Angiopoietin-1 upregulates de novo expression of IL-1beta and Il1-Ra, and the exclusive release of Il1-Ra from human neutrophils. PloS One 9:e 88980.

Hanahan D, Folkman J. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86:353–364.

Henry TD, Annex BH, McKendall GR, Azrin MA, Lopez JJ, Giordano FJ, Shah PK, Willerson JT, Benza RL, Berman DS, Gibson CM, Bajamonde A, Rundle AC, Fine J, McCluskey ER. 2003. The VIVA trial: Vascular endothelial growth factor in ischemia for vascular angiogenesis. Circulation 107:1359-1365.

Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA. 2004. Vascular endothelial growth factor and angiogenesis. Pharmacol Rev 56:549–580.

Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ. 1999. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science 284:1994–1998.

Jones MK, Wang H, Peskar BM, Levin E, Itani RM, Sarfeh IJ, Tarnawski AS. 1999. Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: Insight into mechanisms and implications for cancer growth and ulcer healing. Nat Med 5:1418–1423.

Kim I, Kim HG, Moon SO, Chae SW, So JN, Koh KN, Ahn BC, Koh GY. 2000a. Angiopoietin-1 induces endothelial cell sprouting through the activation of focal adhesion kinase and plasmin secretion. Circ Res 86:952–959.

Kim I, Kim JH, Moon SO, Kwak HJ, Kim NG, Koh GY. 2000b. Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Oncogene 19:4549–4552.

Kim H, Koh GY. 2011. Ang2, the instigator of inflammation. Blood 118:4767–4768.

Lemieux C, Maliba R, Favier J, Theoret JF, Merhi Y, Sirois MG. 2005. Angiopoietins can directly activate endothelial cells and neutrophils to promote proinflammatory responses. Blood 105:1523–1530.

Liekens S, De Clercq E, Neyts J. 2001. Angiogenesis: Regulators and clinical applications. Biochemical pharmacology 61:253–270.

Lin YJ, Lai MD, Lei HY, Wing LY. 2006. Neutrophils and macrophages promote angiogenesis in the early stage of endometriosis in a mouse model. Endocrinology 147:1278–1286.

Lingen MW. 2001. Role of leukocytes and endothelial cells in the development of angiogenesis in inflammation and wound healing. Arch Pathol Lab Med 125:67–71.

Lobov IB, Brooks PC, Lang RA. 2002. Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo. Proc Natl Acad Sci USA 99:11205–11210.

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD. 1997. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277:55–60.

Maliba R, Brkovic A, Neagoe PE, Villeneuve LR, Sirois MG. 2008. Angiopoietin-mediated endothelial P-selectin translocation: Cell signaling mechanisms. J Leukoc Biol 83:352-360.

Maliba R, Lapointe S, Neagoe PE, Brkovic A, Sirois MG. 2006. Angiopoietins-1 and -2 are both capable of mediating endothelial PAF synthesis: Intracellular signalling pathways. Cellular Signal 18:1947–1957.

Marchand GS, Noiseux N, Tanguay JF, Sirois MG. 2002. Blockade of in vivo VEGF-mediated angiogenesis by antisense gene therapy: Role of Flk-1 and Flt-1 receptors. Am J Physiol Heart Circ Physiol 282:H194–H204.

Matsushima H, Geng S, Lu R, Okamoto T, Yao Y, Mayuzumi N, Kotol PF, Chojnacki BJ, Miyazaki T, Gallo RL, Takashima A. 2013. Neutrophil differentiation into a unique hybrid population exhibiting dual phenotype and functionality of neutrophils and dendritic cells. Blood 121:1677–1689.

Mochizuki Y, Nakamura T, Kanetake H, Kanda S. 2002. Angiopoietin 2 stimulates migration and tube-like structure formation of murine brain capillary endothelial cells through c-Fes and c-Fyn. J Cell Sci 115:175–183.

Neagoe PE, Brkovic A, Hajjar F, Sirois MG. 2009. Expression and release of angiopoietin-1 from human neutrophils: Intracellular mechanisms. Growth Factors 27:335-344.

Neagoe PE, Dumas E, Hajjar F, Sirois MG. 2012. Angiopoietin-1 but not angiopoietin-2 induces IL-8 synthesis and release by human neutrophils. J Cell Physiol 227:3099–3110.

Noonan DM, De Lerma Barbaro, Vannini A, Mortara N, Albini L. 2008. Inflammation, inflammatory cells and angiogenesis: Decisions and indecisions. Cancer Metastasis Rev 27:31-40.

Norrby K. 2006. In vivo models of angiogenesis. J Cell Mol Med 10:588–612.

Nozawa H, Chiu C, Hanahan D. 2006. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. Proc Natl Acad Sci USA 103:12493–12498.

Phillips GD, Stone AM, Jones BD, Schultz JC, Whitehead RA, Knighton DR. 1994. Vascular endothelial growth factor (rhVEGF165) stimulates direct angiogenesis in the rabbit cornea. In vivo 8:961–965.

Plouet J, Schilling J, Gospodarowicz D. 1989. Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells. EMBO J 8:3801–3806.

Qazi BS, Tang K, Qazi A. 2011. Recent advances in underlying pathologies provide insight into interleukin-8 expression-mediated inflammation and angiogenesis. Int J Inflamm 2011:908468.

Sasmono RT, Ehrnsperger A, Cronau SL, Ravasi T, Kandane R, Hickey MJ, Cook AD, Himes SR, Hamilton JA, Hume DA. 2007. Mouse neutrophilic granulocytes express mRNA encoding the macrophage colony-stimulating factor receptor (CSF-1R) as well as many other macrophage-specific transcripts and can transdifferentiate into macrophages in vitro in response to CSF-1. J Leukoc Biol 82:111–123.

Satpathy AT, Kc W, Albring JC, Edelson BT, Kretzer NM, Bhattacharya D, Murphy TL, Murphy KM. 2012. Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. J Exp Med 209:1135–1152.

Schruefer R, Lutze N, Schymeinsky J, Walzog B. 2005. Human neutrophils promote angiogenesis by a paracrine feedforward mechanism involving endothelial interleukin-8. Am J Physiol Heart Circ Physiol 288:H1186–H1192.

Simons M, Bonow RO, Chronos NA, Cohen DJ, Giordano FJ, Hammond HK, Laham RJ, Li W, Pike M, Sellke FW, Stegmann TJ, Udelson JE, Rosengart TK. 2000. Clinical trials in coronary angiogenesis: issues, problems, consensus: An expert panel summary. Circulation 102:E73–E86.

Sozzani S, Rusnati M, Riboldi E, Mitola S, Presta M. 2007. Dendritic cell-endothelial cell cross-talk in angiogenesis. Trends Immunol 28:385–392.

Staton CA, Reed MW, Brown NJ. 2009. A critical analysis of current in vitro and in vivo angiogenesis assays. Int J Exp Pathol 90:195–221.

Stewart DJ, Kutryk MJ, Fitchett D, Freeman M, Camack N, Su Y, Della Siega, Bilodeau A, Burton L, Proulx JR, Radhakrishnan G. 2009. VEGF gene therapy fails to improve perfusion of ischemic myocardium in patients with advanced coronary disease: Results of the NORTHERN trial. Mol Ther 17:1109–1115.

Teichert-Kuliszewska K, Maisonpierre PC, Jones N, Campbell AI, Master Z, Bendeck MP, Alitalo K, Dumont DJ, Yancopoulos GD, Stewart DJ. 2001. Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2. Cardiovas Res 49:659–670.

Tengood JE, Kovach KM, Vescovi PE, Russell AJ, Little SR. 2010. Sequential delivery of vascular endothelial growth factor and sphingosine 1-phosphate for angiogenesis. Biomaterials 31:7805–7812.

Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD. 2000. Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med 6:460–463.

Tolentino MJ, Miller JW, Gragoudas ES, Chatzistefanou K, Ferrara N, Adamis AP. 1996. Vascular endothelial growth factor is sufficient to produce iris neovascularization and neovascular glaucoma in a nonhuman primate. Arch Ophthalmol 114:964–970.

Torisu H, Ono M, Kiryu H, Furue M, Ohmoto Y, Nakayama J, Nishioka Y, Sone S, Kuwano M. 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.

Van den Steen PE, Proost P, Wuyts A, Van Damme J, Opdenakker G. 2000. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. Blood 96:2673–2681.

van Hinsbergh VW, Engelse MA, Quax PH. 2006. Pericellular proteases in angiogenesis and vasculogenesis. Arterioscler Thromb Vasc Biol 26:716–728.

Voronov E, Carmi Y, Apte RN. 2007. Role of IL-1-mediated inflammation in tumor angiogenesis. Adv Exp Med Biol 601:265–270.

Voronov E, Shouval DS, Krelin Y, Cagnano E, Benharroch D, Iwakura Y, Dinarello CA, Apte RN. 2003. IL-1 is required for tumor invasiveness and angiogenesis. Proc Natl Acad Sci USA 100:2645–2650.