Université de Montréal

The inflammatory role of angiogenic growth factors: A neutrophil perspective

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Thèse présentée à la Faculté de médecine en vue de l'obtention du grade de
Ph.D. en pharmacologie

Mars 2015

Université de Montréal

Faculté des études supérieures et postdoctorales

Cette thèse intitulée:

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RÉSUMÉ

De par sa présence dans tous les vaisseaux sanguins, l'endothélium joue un rôle clef dans le processus d'hémostase, tant par sa libération de facteurs anticoagulants que par ses changements protéiques qui permettent à l'organisme de déclencher la réparation tissulaire. La fonction anticoagulante de l'endothélium peut être mise en défaut en cas d'atteinte de son intégrité, entrainant la formation de thrombus, le rejet précoce de greffes ou encore l'induction de l'athérosclérose. L'intégrité de l'endothélium est donc capitale pour la prévention de nombreuses maladies cardiovasculaires.

Chez l'adulte, les cellules endothéliales (CE), normalement quiescentes, sont rapidement activées en cas d'hypoxie ou d'inflammation, leur permettant ainsi d'amorcer le processus angiogénique comme suit: Tout d'abord, l'induction de l'hyperperméabilité vasculaire permet l'extravasation des protéines plasmatiques. Ensuite, la dégradation de la lame basale par des métalloprotéases permet aux CE de se détacher, de proliférer, de migrer et de s'organiser pour former l'ébauche du futur vaisseau. La dernière étape consiste en la maturation du vaisseau, c'est-à-dire son recouvrement par des cellules murales, telles que les cellules musculaires lisses et les péricytes. Ces processus sont régulés par de nombreux facteurs angiogéniques tels que les membres de la famille Notch, du *vascular endothelial growth factor* (VEGF), du *fibroblast growth factor* (FGF), des angiopoïétines, et des *matrix metalloproteases* (MMP). L'angiogenèse pathologique, soit une insuffisance ou un excès de vascularisation, est impliquée dans les blessures chroniques, les accidents cardiovasculaires, les pathologies coronariennes artérielles, les pathologies tumorales, l'arthrite rhumatoïde, la rétinopathie diabétique, l'athérosclérose, le psoriasis et l'asthme. Ces pathologies sont souvent issues d'une dérégulation de l'activité endothéliale, fréquemment observée conjointement à l'expression continue de molécules d'adhésion leucocytaires, à l'augmentation de la perméabilité vasculaire, et aux anomalies de la vasoréactivité. L'activation non-contrôlée de l'endothélium entraîne ainsi une inflammation chronique et la formation de structures vasculaires anarchiques.

Les premiers leucocytes à répondre à l'appel inflammatoire sont les neutrophiles. Equippées d'une panoplie de produits antibactériens puissants mais aussi nocifs pour les tissus qui les entourent, ces cellules polylobées participent à chaque étape du processus inflammatoire, depuis l'induction de l'hyperperméabilité vasculaire jusqu'à la résolution. En effet, grâce à leurs récepteurs, les neutrophiles détectent et interprètent les signaux

biochimiques présents dans la circulation et à la surface de l'endothélium, et libèrent aussi leurs propres médiateurs tels le VEGF, les MMP, et l'interleukine-8 (IL-8), dont les effets sont à la fois paracrines et autocrines. Existent-ils d'autres modulateurs typiques de la fonction endothéliale capables d'influencer le comportement des neutrophiles? En effet, notre laboratoire a démontré que chez l'humain, une stimulation directe aux angiopoïétines incitait les neutrophiles à adhérer aux CE, à migrer, à synthétiser et à relâcher l'IL-8, voire même à vivre plus longtemps. La présence du récepteur des angiopoïétines, Tie2, à la surface des neutrophiles laisse présager que la famille possèderait d'autres fonctions leucocytaires encore non-identifiées. Par ailleurs, dans un modèle classique de l'angiogenèse *in vivo* (matrigel), nous avons observé que sous l'effet du FGF1 et 2, les ébauches des nouveaux vaisseaux étaient parfois accompagnées d'une infiltration de cellules granulocytaires.

Ainsi, en partant de ces observations, l'objectif de nos études (présentées ci-après) était d'approfondir nos connaissances sur la relation entre neutrophiles et facteurs angiogéniques, notamment les FGF et les angiopoïétines. Par tests *in vitro*, nous avons confirmé que les neutrophiles humains exprimaient plusieurs récepteurs du FGF (FGFR1-4) d'une façon hétérogène, et qu'ils migraient vers un gradient des ligands FGF1 et 2. Par ailleurs, nous nous sommes intéressés aux voies de signalisation inflammatoires activées par les ligands FGF1, FGF2, Ang1 et Ang2. Grâce à une stratégie génique ciblant 84 gènes inflammatoires, nous avons identifié plusieurs cibles d'intérêt touchées par Ang1, dont certains membres de la famille de l'IL-1, alors qu'aucun des gènes testés n'avait changé de façon significative sous l'effet des FGF ou d'Ang2. Suite à des cinétiques approfondies, nous avons démontré qu'Ang1 stimulait la transcription de l'ARN messager de l'IL-1β, et augmentait simultanément la quantité de protéine immature (pro-IL-1β; inactive) et clivée (IL-1β « mature »; active). En parallèle, Ang1 augmentait la sécrétion de l'antagoniste naturel de l'IL-1β, l'IL-1RA, sans pour autant stimuler la relâche de l'IL-1β. A l'instar des endotoxines bactériennes dont les effets liés à l'IL-1 dépendaient de la kinase p38, ceux d'Ang1 découlaient presque entièrement des voies de signalisation du p42/44.

Mots clés: angiogenèse, inflammation, neutrophiles, FGF, angiopoïétines, interleukine-1

ABSTRACT

Endothelial cells (ECs) form a monolayer that lines the inside of all blood vessels; thus, as the first barrier that separates blood elements from all things that fall beyond the blood vessel, ECs are strategically placed to play a central role in many essential physiological processes. While it is found mostly in a quiescent state in adult organisms, the endothelium retains a high level of plasticity that allows it to react to stimulus and dynamically control the passage of blood components to and from the bloodstream. For instance, upon detecting an activating angiogenic signal, ECs forgo their quiescence and undergo biochemical and structural changes necessary for the initiation of angiogenesis. Thus, activated ECs down-regulate their own expression of junctional molecules and secrete proteins to digest the extracellular matrix (ECM), thereby giving them the space to proliferate and migrate. Relaxing endothelial junctions also increases permeability, opening up the doorway for leukocyte infiltration. These cells can then modulate angiogenesis via their own set of mediators. Though the instigating stimuli may differ, the biochemical sequence of events that initiates angiogenesis is also common to the inflammatory response. In the latter case, changes in EC biochemistry include the release of chemotactic agents and expression of surface adhesion molecules, increasing the efficiency of leukocyte infiltration, particularly those of the myeloid lineage. Evidently, because angiogenesis and inflammation can be initiated by the same sequence of events, they will inevitably share effector molecules.

Of the recruited leukocytes, neutrophils are generally the first responders at the site of inflammation, contributing mediators that propagate and eventually resolve inflammation. We and other groups have shown that endothelial modulators such as angiogenic growth factors exert a direct action on neutrophil activity independently of the presence of the endothelium. In particular, our laboratory has shown that members of the angiopoietin family and their receptor Tie2 are expressed by neutrophils and are capable of activating neutrophil intracellular signalling pathways that impact their survival, adhesion, migration, and protein production. The ability of angiopoietins to directly engage neutrophils illustrates an intimate link between angiogenesis and inflammation, and provides an explanation for why vascular pathologies are often accompanied by an exacerbated inflammatory response.

In the studies presented herein, we sought to expand our understanding of the relationship between angiogenic growth factors and neutrophil behavior. In a pilot experiment using *in vivo* subcutaneous matrigel plugs, short-term treatment with fibroblast growth factors (FGF) 1 and 2

resulted in significant neovascularization; interestingly, the tissues surrounding the matrigel plug showed an increase in polymorphonuclear cell infiltration. Encouraged by the paucity of information in the literature regarding FGF-neutrophil interaction, we looked at the expression of FGF receptors (FGFRs) on neutrophils from different human donors, as well as the ability of FGFs to induce neutrophil chemotaxis. We demonstrated that the expression of FGFR was strongly dependent on genetic background: Overall, FGFR2 showed the highest incidence as a neutrophil cell-surface receptor, but none of the receptors were universally or uniformly expressed. Despite the genetic factor, neutrophils migrated in response to both FGF1 and FGF2 *in vitro*, suggesting that other neutrophil adaptors may be engaging FGFs.

Given the shared ability of FGFs and angiopoietins (Ang) to induce neutrophil migration, we performed a wide-scale RNA assay to determine which genes were being engaged by the main ligands of both families. While none of FGF1, FGF2 or Ang2 had a strong effect on the 84 inflammatory cytokine genes tested (FGFs - unpublished data, 2011), at least two target genes belonging to the interleukin-1 (IL-1) family were significantly upregulated following Ang1 treatment. Further analysis showed that Ang1 not only stimulates gene transcription, but also translation and processing of the precursor of IL-1β (pro-IL-1β), and both precursor and mature proteins accumulate in the cell simultaneously. Interestingly, although no IL-1β is secreted from neutrophils after Ang1 or endotoxin (LPS) treatment, substantial quantities of the naturally occurring IL-1β antagonist (IL-1RA) are released, thereby tipping the balance in favor of inhibiting IL-1β activity. Finally, the activities of Ang1 on IL-1β and IL-1RA production and/or release are largely mediated by p42/44 MAPK; in contrast, the effects of LPS are driven by recruitment of p38.

Keywords: angiogenesis, inflammation, neutrophils, FGF, angiopoietins, interleukin-1

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Abbreviations

Ang	Angiopoietin
ANGPTL	Angiopoietin-like
BAEC	Bovine aortic endothelial cells
DMSO	Dimethyl sulfoxide
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EPC	Endothelial progenitor cell
Erk	Extracellular signal-regulated kinases
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FHF	Fibroblast growth factor homologous factor
FRS2	Fibroblast growth factor receptor substrate 2
GRO	Growth-related oncogene
HSPG	Heparan sulfate proteoglycan
HUVEC	Human umbilical vein endothelial cell
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor κB
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1RA	Interleukin-1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
iNOS	inducible nitric oxide synthase
IP3	Inositol 1,4,5-triphosphate
IRAK	Interleukin-1 receptor -associated kinase
JNK	c-JUN N-terminal kinase
LPS	Lipopolysaccharide
LTB	Lymphotoxin Beta
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor-κB
NK	Natural killer
NO	Nitric oxide

PAF	Platelet-activating factor
PAI-1	Plasminogen activator inhibitor-1
PBMC	Peripheral blood mononuclear cell
PDGF	Platelet-derived growth factor
PECAM	Platelet endothelial cell adhesion molecule
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol-3-phosphate kinase
PKC	Protein kinase C
PLC	Phospholipase C
PlGF	Placental growth factor
PMA	Phorbol myristate acetate
PR3	Proteinase 3
PSGL-1	P-selectin glycoprotein ligand-1
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SH2	Src homology 2
SIGIRR	Single immunoglobulin Interleukin-1 receptor-related
SMC	Smooth muscle cells
STAT	Signal transducer and activator of transcription
TAM	Tissue-associated macrophages
TEM	Tie2-expressing monocytes
TGF-β	Transforming growth factor-β
Tie	Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains
TIGIRR	Three immunoglobulin Interleukin-1 receptor-related
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumor Necrosis Factor
TRAF	Tumor Necrosis Factor receptor-associated factor
VCAM	Vascular cell adhesion molecule
VE-cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VRAP	Vascular endothelial growth factor receptor -associated protein

To my beloved late grandmother whose unconditional love, spirit, and passion for education have never left me;

To my grandfather whose prayers, support, and belief in me have been unwavering all these years;

To my mother, my confidante, best friend, and unwitting lamentation wall, who can finally find pride in calling me "doctor".

Acknowledgements

Words cannot adequately describe the mixed and bittersweet emotions I feel as I put the finishing touches on this work.

Completing a thesis, along with juggling the challenges of a new career, has been an incredible but rewarding endeavor. Without the insistence of my supervisor, Dr. Martin Sirois, his steadfast support, persistent "gentle" reminders and a fair bit of back-and-forth emailing, this work would not have come to light (I still remember him firmly saying, "no matter where you're going, you *will* finish this thesis"). Although I still don't understand what the number 25 signifies in his self-attributed title of "Caliph 25", Dr. Sirois is undoubtedly the Caliph of mentorship.

The support of my family – particularly my beloved mom, little sister and grandfather – has been instrumental in helping me get to where I am today, and I am incredibly grateful for having such love in my life. I am also thankful for my partner's love and moral support, and for the Hinch family tenderly shaming me into completing this work.

A quick thank you to my current and former colleagues and friends, Elizabeth Dumas, Fadi Hajjar, Lara Bou Khzam, Marie Clavet, Maya Mamarbashi and all the staff at the Montreal Heart Institute for their invaluable scientific insight and help with experiments. Particularly, I owe much laughter and gratitude to Paul-Eduard Neagoe, whose level of geekiness is only surpassed by his giant heart. May he one day discover that Tie2 is expressed ubiquitously after stimulation with Diet Pepsi.

Finally, many thanks to the University of Montreal, the Department of Pharmacology, the Montreal Heart Institute, and all the provincial and federal funding institutions for providing me with the appropriate and necessary tools to conduct all the work mentioned herein.

1.0 INTRODUCTION

1.1 Angiogenesis: Fundamentals

In mammalian organisms, receiving adequate blood supply through a functional network of blood vessels is arguably the most important determinant of organ survival. Because blood perfusion is directly proportional to the amount of oxygen and nutrients a tissue receives, the delivery system must retain a degree of plasticity, such that it can continuously expand or regress in accordance with blood demand. The dependence of the mammalian organism on timely blood vessel development and remodelling is demonstrable very early in life: In humans for instance, during the second week following implantation of the developing zygote, the outer layer of the implanted blastocyst (the chorionic sac) projects finger-like structures called chorionic villi into the endometrial lining of the uterus. These villi release chorionic gonadotropin, a hormone that controls maternal progesterone secretion in the ovaries. In turn, progesterone insures that maternal vessels grow and extend from the uterus towards the villi. Failure to adequately develop and maintain these vascular connections is unforgiving; indeed, in the case of faulty vascular structure formation in the decidua, spontaneous abortion – and thus loss of embryo – is the most likely outcome.

1.1.1 Terminology

There are two distinct mechanisms for the formation of vascular structures in vertebrates. While *vasculogenesis* describes the migration and assembly of mesoderm-derived endothelial precursor cells (the angioblasts) and their organization into primitive networks, *angiogenesis* refers to the formation of new blood vessels (neovascularization) from pre-existing structures, through the proliferation and rearrangement of local vessel wall elements (endothelial and mural cells). Both processes play a critical role in embryo development and are often intertwined throughout gestation. In humans, vasculogenesis is evident in the fourth week of zygotic development; a fetal circulatory loop is established *de novo*, with the emergence of a beating heart and the major embryonic vessels – the dorsal aorta and an intra- and extra-embryonic (blood islands in the yolk sac) vascular plexus (1). By the end of the fourth week, embryonic blood vessels extend from the fetus, likely through vasculogenesis (rather than protrusion of embryonic vessels into the placenta

by angiogenesis), through the umbilical cord and into the chorionic villi, where they meet maternal blood vessels that have developed, through angiogenesis, out of the *decidua basalis* of the endometrium (review (2)) (**Figure 1**, p.2). The zone where embryonic and maternal vessels meet constitutes the placenta, where nutrients and waste are exchanged via diffusion. If the fetal or maternal portions of the placenta are deficient, i.e. if the placental relationship is not successfully established, fetal growth can be severely impaired.

Because of its role in establishing the initial vascular scaffold, vasculogenesis was perceived as strictly an embryonic process. However, recent evidence suggests that bone marrow-derived committed (i.e. lineage restricted) progenitor cells, often termed endothelial progenitor cells (EPCs), which give rise to endothelial progeny are present in the adult (4) and retain their vasculogenic potential (5). Thus, neovascularization in the adult may not only involve "classical" angiogenic processes (i.e. proliferation/rearrangement of local vessel wall elements) as was previously thought, but also the work of circulating EPCs.

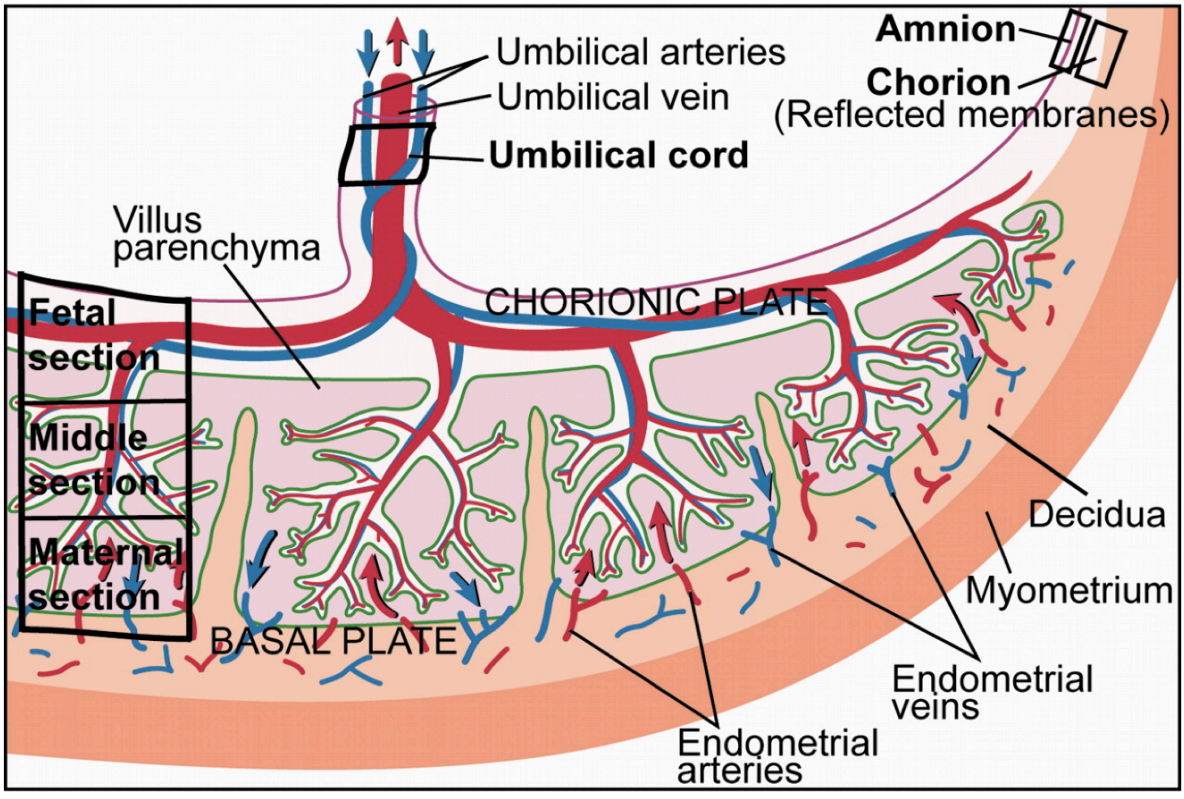

Figure 1: Schematic of human term placenta.

The extensive development and maintenance of new blood vessels in the fetal chorionic plate and the maternal decidua basalis is essential to life. **Reproduced with permission from PNAS: Sood *et al*, 2006 (3).**

While the distinction between vasculogenesis and angiogenesis remains ill-defined and must be reconsidered as new discoveries in the field of vascular biology emerge (review (6)), for the purpose of this study, we define angiogenesis as the formation of new blood vessels of *any* size (encompassing arterio and venogenesis) and using *any* mechanism (including endothelial cell rearrangement and sheer stress) from a pre-existing structure. Under this definition, we distinguish two main mechanisms of angiogenesis thought to occur in virtually all tissues and organs, referred to as sprouting and splitting (intussusceptive) angiogenesis. This section will outline the sequential steps of vessel branching under normal physiological conditions, before delving into the molecular players involved.

1.1.2 Basic vessel structure and quiescence

Vessel lumens are lined by a single layer of endothelial cells (ECs) that are tightly connected by junctional molecules such as vascular endothelial cadherins (VE-cadherin) and claudins. The monolayer of ECs is ensheathed in a "sleeve" made of basement membrane and covered by mural cells (vascular smooth muscle cells and pericytes). In a quiescent state, ECs and pericytes produce elements of the basement membrane and maintain it. Furthermore, pericytes suppress EC proliferation and secrete pro-survival signals such as angiopoietin-1 (Ang1), fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs) and Notch to help protect the endothelium from insult.

1.1.3 Sprouting angiogenesis

This type of angiogenesis is characterized by the selection of a "sprout", or an EC guide that directs the re-arrangement of surrounding ECs. When a quiescent vessel detects an angiogenic signal (hypoxic conditions, tumor-induced, tissue injury etc.), ECs and surrounding support cells release vessel-destabilizing factors such as Ang2 and VEGF that concomitantly loosen up the endothelium at the spot where the sprout is to form (**Figure 2**, p.5): Ang2 promotes the detachment of pericytes and proteolytic cleavage of the basement membrane by matrix metalloproteases (MMPs). In parallel, VEGF relaxes endothelial junctions, increasing vascular permeability and allowing for extravasation of plasma elements (such as fibrinogen and fibronectin); these elements then lay down a provisional extracellular matrix (ECM) scaffold towards which ECs will migrate. Proteases in the surrounding milieu degrade ECM components,

thereby liberating trapped angiogenic factors such as VEGF and FGF; this step establishes new signalling gradients that will serve to stimulate and guide the sprout. To prevent endothelial migration en masse towards the angiogenic signal, one endothelial cell - the tip cell - is chosen over all the others to lead the sprout, a selection that is made through a complex and timely interplay between VEGF, neuropilin and Notch signals. The remaining cells assume an auxiliary role as stalk cells that proliferate and form the stalk and the lumen of the new vessel, driven by FGFs, Notch and placental growth factor (PlGF) among others. Before it can be functional, the vessel must undergo a maturation step and the destabilizing signals have to be removed: MMPs are inhibited by tissue inhibitors of metalloproteases (TIMPs) and plasminogen activator inhibitor-1 (PAI-1), allowing for the deposition of a basement membrane again; at the same time, stabilizing signals such as platelet-derived growth factor B (PDGF-B), Ang1, transforming growth factor-β (TGF-β), ephrin-B2 and Notch stimulate pericyte recruitment and adhesion, as well as tightening of endothelial junctions to allow for optimal perfusion in the new vessel (review (7)).

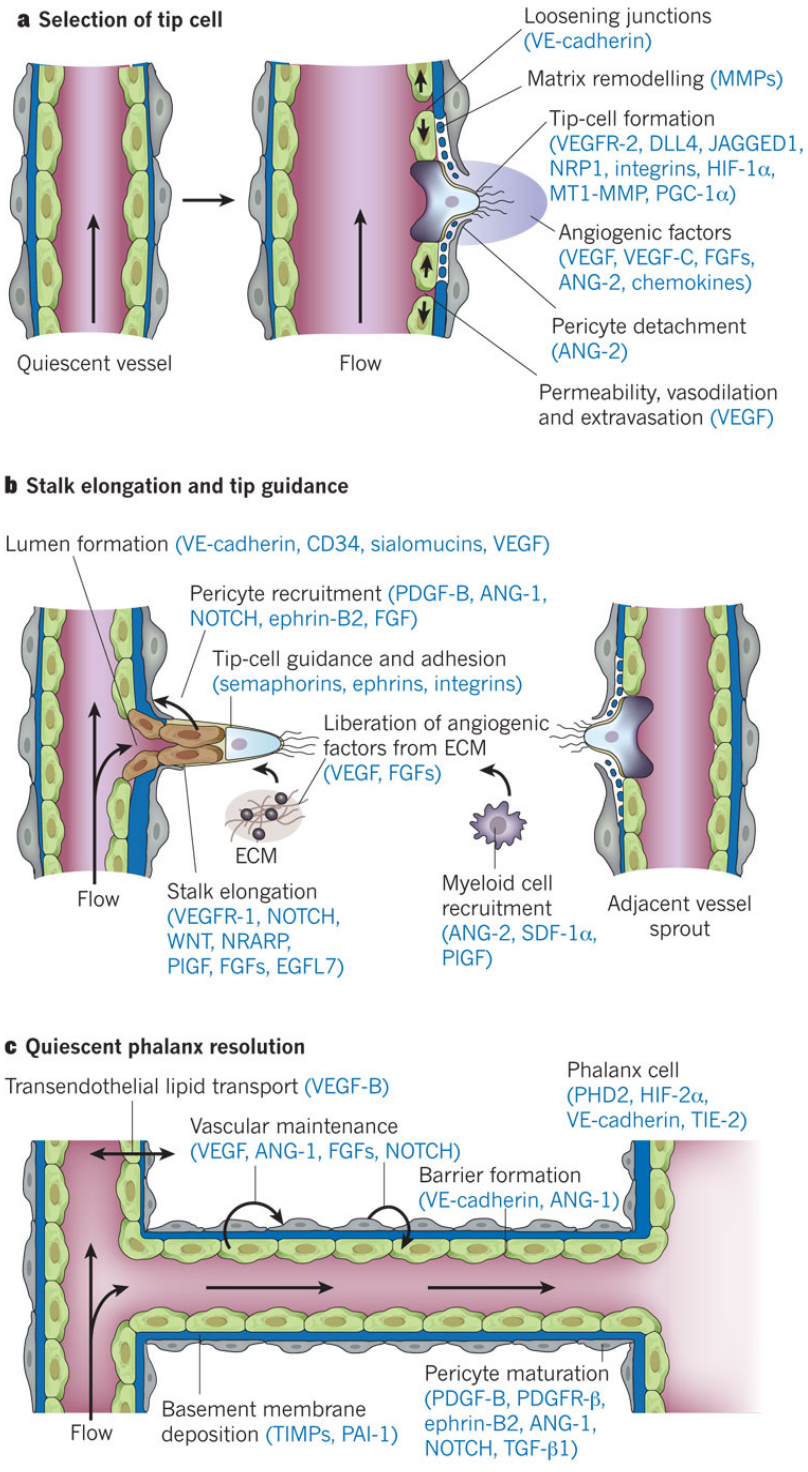

Figure 2: Molecular basis of sprouting angiogenesis.

a) Following stimulus, the quiescent vessel dilates and an endothelial cell (EC) guide, the "tip cell", is selected (DLL4; JAGGED1) to ensure branch formation. Tip-cell formation requires degradation of the basement membrane (BM), pericyte detachment and loosening of EC junctions. Increased permeability permits extravasation of plasma proteins (ex: fibrinogen and fibronectin) to deposit a provisional matrix layer, and proteases remodel pre-existing interstitial matrix, all enabling EC migration. For simplicity, only the BM between ECs and pericytes is depicted, but in reality, pericytes and ECs are both embedded in BM. **b)** Tip cells navigate in response to guidance signals (ex: semaphorins and ephrins) and adhere to the ECM (via integrins) to migrate. Stalk cells proliferate, elongate and form a lumen, and sprouts fuse to establish a perfused neovessel. Proliferating stalk cells attract pericytes and deposit BMs to become stabilized. Recruited myeloid cells (ex: subsets of macrophages, monocytes) can produce pro-angiogenic factors or proteolytically liberate factors from the ECM. **c**) After fusion of neighbouring branches allowing perfusion, the neovessel resumes quiescence by promoting a phalanx phenotype, re-establishment of junctions, deposition of BM, maturation of pericytes and production of vascular maintenance signals. Other factors promote transendothelial lipid transport.

Reproduced with permission from Macmillan Publishers Ltd: Carmeliet *et al*, 2011 (7).

1.1.4 Splitting angiogenesis

In intussusceptive angiogenesis, also called *splitting angiogenesis,* the vessel wall extends into the lumen, forming transluminal tissue "pillars" that subsequently fuse and divide a single vessel into two (8-10). This type of angiogenesis mostly creates new capillaries where capillaries have already been formed, either by sprouting angiogenesis or vasculogenesis. It is also of great importance in "pruning" existing vessels, i.e. in remodeling the branching patterns of vascular beds (11, 12). The advantage of this mechanism is that blood vessels are formed more rapidly and in a metabolically more efficient manner, since there is no need for immediate EC proliferation, basement membrane degradation, or invasion of the surrounding tissue (review (13)). While the driving factors for intussusception remain poorly understood, altering blood flow by clamping leads to an increase in the number of tissue pillars and to immediate changes in vessel branching, suggesting that blood flow and blood pressure are driving intussusceptive forces (14). Furthermore, factors that mediate sprouting angiogenesis are also likely implicated. Indeed, Ang1 is a good example: in knockout mice for the ligand or for its receptor Tie2, similar vascular remodeling defects are observed, whereby the vasculature remains primitive in appearance and fails to undergo adequate branching (**Figure 3**, p.7) (15, 16). Importantly, in the Ang1 knockout study, the authors note that the defects in vascular morphology are not due to a lack of ECs, as the number of cells is comparable between $Ang1^{-/-}$ and control mice (15). Instead, the study points out that vessels of $Ang1^{-/-}$ mice lack both "periendothelial cell" (i.e. pericytes) coverage and "tissue folds" (i.e. tissue pillars), which the authors propose are "responsible for vessel branching". Finally, Thurston *et al* have also shown that in mice, overexpression of Ang1 alone or co-expression of Ang1 and VEGF leads to the presence of abundant "small holes" in the capillary plexus (17), which is suggestive of increased intussusception.

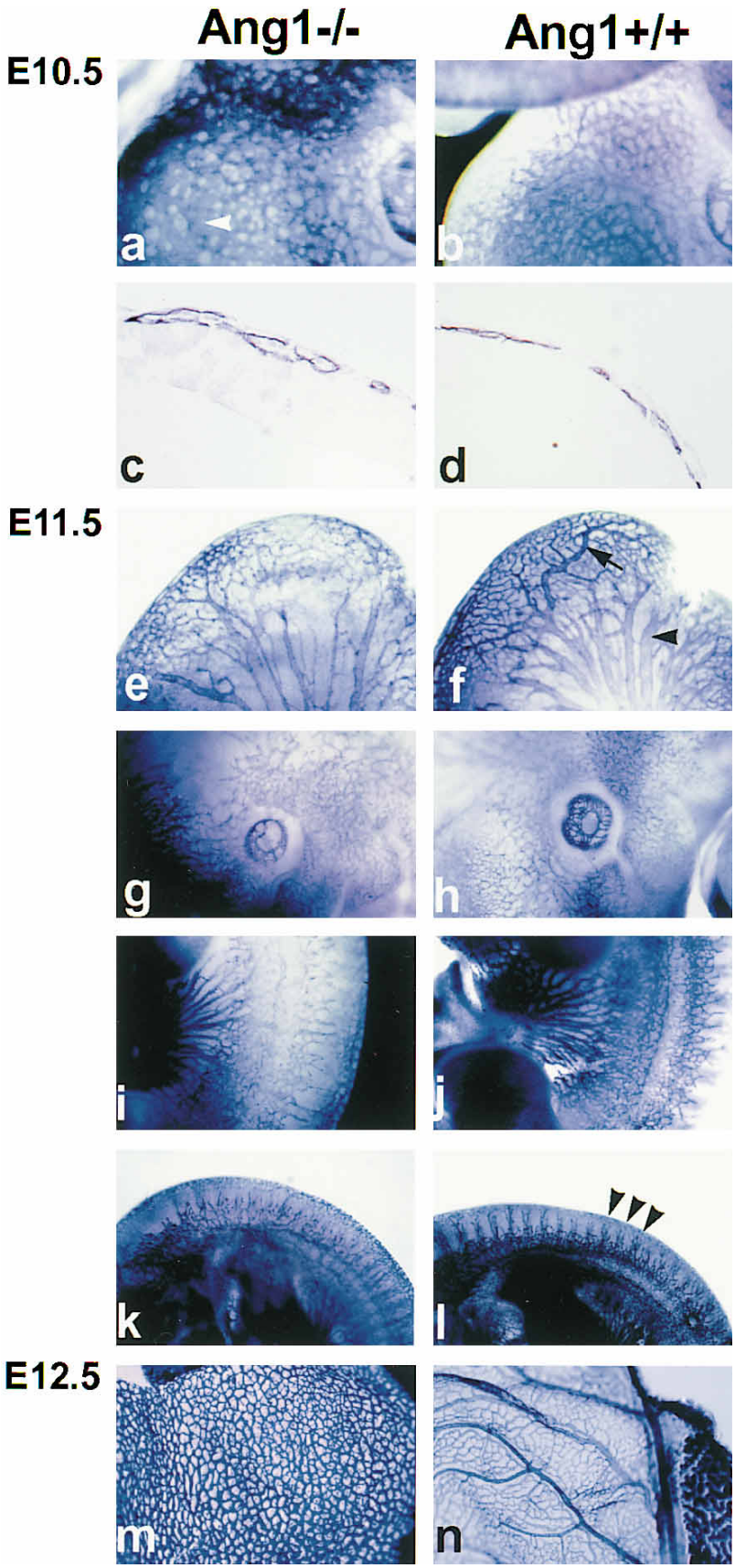

Figure 3: Vascular defects in Ang1-deficient embryos.

Whole-mount PECAM staining in the forebrain (A and B), head region (E and F), eye (G and H), umbilical and primitive gut (I and J), intersomitic region (K and L), and yolk sac (M and N) of Ang1$^{-/-}$ embryos.

The dilated appearance of vessels in the forebrain is confirmed by sections (C and D). Arrowhead in (A) indicates syncitial vessels in contrast to the finer vascular network seen in (B); arrowhead in (F) indicates large vessels whose counterparts are much smaller in size in Ang1$^{-/-}$ embryos (E); arrow in (F) indicates highly branched and meandering intermediate-size vessels typically not seen in Ang1$^{-/-}$ embryos (E); arrowheads in (L) indicate intersomitic vessels that have regressed in Ang1$^{-/-}$ embryos of this age (K).

Perhaps the most striking example of a remodeling deficit is seen in the yolk sac vasculature (M and N), which is similar to deficits seen in Tie2-deficient embryos (16); in both cases, it appears as if remodeling of the initially homogeneous capillary network to form both large and small vessels does not occur.

1.2 The vascular endothelial growth factor (VEGF) family

As the name suggests, the vascular endothelial growth factor (VEGF) family is extensively involved in regulating vascular development. With five growth factors (VEGF-A-D; placental growth factor (PlGF)), three receptors (VEGFR1-3), and various splicing products with properties distinct from those of their originators, the VEGF family plays a fundamental role in the initiation of fetal vasculogenesis and adult angiogenesis. The importance of this family is evidenced by the exhaustive list of genetic studies targeting its members, ranging from complete gene deletions to smaller modifications, and generally resulting in major vascular impairments or embryonic lethality (**Table I**, p.14). In addition to possessing unique features, VEGFs may also have overlapping characteristics and functions with angiogenic factors from other families; thus, the VEGF family is a good starting point for understanding the molecular mechanisms of angiogenesis.

1.2.1 VEGF members

VEGF-A. VEGF-A (sometimes referred to simply as "VEGF") was first discovered from its capacity to induce rapid vascular permeability (accordingly, it was called "vascular permeability factor") (18, 19), with 50,000 times more potency than histamine (20). VEGF-A is critical for embryonic vascular development. Indeed, both complete and partial deletions of VEGF-A in mice (*Vegfa*$^{-/-}$ and *Vegfa*$^{+/-}$, respectively) lead to embryonic lethality, with severe impairments in the initial assembly of the vasculature (21, 22). VEGF-A is also critical for organ vascular development during embryogenesis, and as an impressive body of literature shows, is essential in post-embryonic angiogenesis.

VEGF-A exerts a range of functions on different cell types in physiological and pathological conditions, including leukocytes, neurons, epithelial cells and tumors. In particular, the ligand directly and potently modulates EC behavior, promoting survival, differentiation, proliferation and migration (23). Overexpression of VEGF-A (splicing variant $VEGF_{164}$; see below) in mouse ear, heart, and skin promotes vessel (capillaries and lymph vessels) proliferation and enlargement, but also results in a leaky vasculature, myocardial thinning and the skin shows a persistent and chronic inflammatory response (24) from increased fluid accumulation in tissues and infiltration of leukocytes. These observations suggest that while VEGF-A is necessary for the

initiation of both the embryonic capillary plexus and adult angiogenesis, it requires additional assistance from other mediators to achieve vessel maturation, pruning, and homeostasis, steps that are essential for the establishment of a fully functional vascular system (17, 25).

VEGF-A has four main isoforms derived from alternative splicing of exons 6-7 and that have different affinities for heparan sulfate proteoglycans (HSPGs) and ECM components (26). Based on their number of amino acids after signal sequence cleavage, the isoforms are labelled $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$[1] (27, 28). The higher the amino acid count, the greater the affinity to HSPGs: Indeed, $VEGF_{165}$ has intermediate properties in terms of heparin affinity (29), while $VEGF_{189}$ and $VEGF_{206}$ are almost completely bound to the cell surface or the ECM (30). In contrast, $VEGF_{121}$ lacks heparin-binding properties and is freely diffusible (30).

The importance of HSPG affinity was demonstrated in several murine genetic manipulations where only one VEGF isoform is expressed. In mice exclusively expressing $VEGF_{120}$ ($Vegf^{120/120}$), angiogenesis defects are most noticeable in the heart (where $VEGF_{120}$ is normally only 5% of total VEGF-A), with lower myocardial capillary density and impaired vessel branching into the innermost tissues of the heart (the endomyocardium), compared to normal VEGF-expressing controls (31). Retinal angiogenesis in $Vegf^{120/120}$ mice is also severely impaired, with defects in venous development and profound impairments in arterial growth (32). Interestingly, $Vegf^{120/120}$ mice show lowered Tie2, Ang1, VE-cadherin and VEGF-C levels. Mice expressing only $VEGF_{188}$ ($Vegf^{188/188}$) exhibit skeletal defects, decreased vascularization surrounding joint cartilage (epiphyseal vascularization), decreased retinal capillary pruning and while venular outgrowth is normal, arterioles are significantly underdeveloped[2] (33, 34). These results indicate that $VEGF_{120}$ or $VEGF_{188}$ alone are insufficient for normal angiogenesis. In contrast, $Vegf^{164/164}$ mice develop normally, are healthy and have normal retinal angiogenesis (34).

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[1] Mouse VEGF-A isoforms have one less amino acid than their human counterparts (thus, $VEGF_{120}$, $VEGF_{164}$, $VEGF_{188}$, and $VEGF_{205}$).

[2] Although VEGF isoform-specific signalling cannot be excluded, an argument in support of VEGF diffusion properties is provided by the observation that mice expressing either $VEGF_{164}$ or $VEGF_{120}$, in addition to $VEGF_{188}$ ($Vegf^{164/188}$ and $Vegf^{120/188}$ mice, respectively), show no evidence of defects in epiphyseal vascularization (Maes *et al*, 2004).

Other VEGF members. VEGF-B, VEGF-C, VEGF-D and PlGF have temporal and spatial restrictions on their functions. VEGF-B has limited angiogenic activity in certain tissues such as the heart, and mitotic activity on neuronal tissue; loss of VEGF-B does not affect vascular development (35). As for VEGF-C and VEGF-D, their involvement in embryonic vascular development appears to be species-dependent: In xenopus tadpoles and zebrafish, both isoforms affect embryonic blood vessel formation (36, 37). In contrast, knockout mice for VEGF-C, VEGF-D, or both at the same time (*Vegfc*$^{-/-}$; *Vegfd*$^{-/-}$), exhibit normal embryonic vascular development (38-40). That being said, the absence of VEGF-C is lethal to mice later in embryogenesis (E15.5), as the lymphatic system fails to develop and *Vegfc*$^{-/-}$ mice die before birth due to fluid accumulation in tissues (39, 40). *Vegfd* knockout mice have only a subtle defect in lymphatic phenotype, involving a decrease in the abundance of lymphatic vessels in the lungs, suggesting that VEGF-D is functionally redundant with VEGF-C in the stimulation of developmental lymphangiogenesis (41). Interestingly, though VEGFR3 mediates the effect of both ligands, *Vegfc*$^{-/-}$ / *Vegfd*$^{-/-}$ double-knockout mice do not phenocopy the severe cardiovascular defects observed in *Vegfr3*$^{-/-}$ embryos, suggesting the possible existence of a third VEGFR3 ligand or a ligand-independent mechanism regulating blood vessel formation.

Finally, PlGF appears to be dispensable for vascular development, and its role is restricted to pathological conditions (42). Binding of PlGF to VEGFR1 modulates the angiogenic response to VEGF, but not to FGF2; this is possibly achieved through displacing VEGF from VEGFR1 and making it more available to bind and activate VEGFR2.

1.2.2 VEGF Receptors (VEGFRs)

Nomenclature. As receptor tyrosine kinases (RTK), VEGFRs belong to the same superfamily as PDGF and FGF receptors. The structure of the three VEGFRs (VEGFR1-3) is similar to other RTKs, with an extracellular portion comprised of Immunoglobulin-like domains, a single trans-membrane domain, a tyrosine kinase domain and a C-terminal tail. In humans, additional receptor variants are generated by alternative splicing or proteolytic cleavage, giving rise to secreted forms of VEGFR1-2 (sVEGFR1-2), and a C-terminal truncated VEGFR3 (23).

Properties. VEGFRs have different binding properties depending on the ligand; once bound, they are capable of forming both homodimers and heterodimers (23). The dimerization drives

autophosphorylation of the receptors at various intracellular tyrosine residues and the subsequent recruitment of a broad array of second messengers. Inactivation of the receptors occurs through dephosphorylation of tyrosine residues by phosphatases, and likely by internalization and degradation by either the proteasome or the lysosomal pathways. The fate of activated VEGFRs, especially after internalization, remains to be elucidated.

Activity of VEGFR1. VEGFR1 binds to VEGF-A, VEGF-B and PlGF. *Vegfr1* null mice perish in utero at day E8.5-9, and exhibit an overgrowth of ECs. An interesting feature of VEGFR1 is that its level of tyrosine phosphorylation in response to VEGF-A is so low that it can only be readily detected in transfected models overexpressing VEGFR1. It would even appear that the tyrosine kinase (TK) domain of VEGFR1 is not necessary to its activity, as the selective deletion of the TK domain (ligand-binding and anchoring domains intact) (*Vegfr*1(TK)$^{-/-}$) does not seem to alter development or angiogenesis in mice (43). Interestingly, removal of both the TK and the transmembrane domains of VEGFR1 results in early embryonic lethality for 50% of the mice, owing to vascular malformations, while the remaining 50% develop normally (44). Finally, while the TK domain is dispensable for vascular development, *Vegfr*1(TK)$^{-/-}$ mice exhibit major defects in macrophage/monocyte recruitment in response to VEGF-A and PlGF (43), implying that in these cells, ligand-bound VEGFR1 has the capability of transducing downstream signals. In vascular biology, because signal transduction through VEGFR1 is apparently not necessary, VEGFR1 is postulated to act as a "trap" for VEGF, and may thus alter VEGFR2 activity by influencing VEGF availability[3].

Activity of VEGFR2. On the other hand, the role of VEGFR2 in vascular development is more pronounced (**Table I**, p.14). VEGFR2-deficient mice (*Vegfr2*$^{-/-}$) die early in utero; however, what is particularly interesting about these embryos is that they do not even develop a primitive vascular network, as blood island formation is impaired and vasculogenesis halted. These mice are also severely deficient in their hematopoietic progenitor count.

In mammals, VEGFR2 naturally binds a single ligand, VEGF-A. After proteolytic processing, human VEGF-C and -D can also bind to VEGFR2, but this happens with lower

[3] Activity of VEGFR1/VEGFR2 heterodimers compared to homodimers remains to be elucidated.

affinity than binding to VEGFR3 (23). Unlike VEGFR1, phosphorylation of stimulated VEGFR2 is readily detectable in intact cell models, and a single mutation in a specific tyrosine phosphorylation site (Tyr1173 in mice; Tyr1175 in humans) leads to a similar phenotype to *Vegfr2*$^{-/-}$, i.e. where vasculogenesis and hematopoiesis are severely impaired (45). This receptor is responsible for mediating the majority of VEGF-A's effects on the vascular system and particularly on ECs, including stimulating permeability, EC proliferation, migration, and sprouting (23). After receptor dimerization and autophosphorylation, several downstream signalling transducers can be recruited, involving Akt, p38 mitogen-activated protein kinase (MAPK), Src, focal adhesion kinase (FAK)/paxilin, Ras, protein kinase C (PKC), p42/44 MAPK and phospholipase C (PLC)-γ among others. However, only a limited group of src homology 2 (SH2)-domain-containing molecules has been shown to bind directly to the phosphor-tyrosine (pTyr) residues of VEGFR2. These include PLC-γ and adaptor proteins Shb and Sck, all three of which bind pTyr1175 mentioned above, and VEGFR-associated protein (VRAP) that binds pTyr591 (pTyr949 in mice). Meanwhile, activation of PI3K and Src occurs indirectly via adaptor proteins Shb and likely VRAP, respectively (46).

Activity of VEGFR3. This receptor binds to VEGF-C and D, and is important for lymphangiogenesis and lymph EC development and function. During early embryonic development, VEGFR3 is necessary for the formation of the blood vasculature, and is widely expressed on ECs of blood vessels; progressively, expression becomes restricted, fisrt to the venous endothelium before lymphatic vessels emerge and then to the endothelium of lymphatic vessels (41, 47, 48).

Vegfr3$^{-/-}$ knockout mice die early in utero (at E9.5-10.5, before the emergence of the lymphatic system), due to defective remodeling and maturation of blood vessels into larger vessels (49). The involvement of VEGFR3 in lymphangiogenesis is derived from studies using conditional knockouts and demonstrating that declining levels of functional receptor in the embryo do not impact development of the vasculature; rather, they impair lymphatic vessel development and function: the less VEGFR3, the more severe the impact on lymphangiogenesis. Finally, VEGFR3 activity is modulated by co-receptors such as neuropilin-2 (expressed in venous and lymphatic vessels) and by heterodimerizing with VEGFR2 in lymphatic ECs, resulting in a modified pattern of VEGFR3 phosphorylation and differential downstream

signalling (50).

Co-receptors: Neuropilins. Neuropilins are transmembrane glycoproteins involved in axonal guidance in vertebrates during the development of the nervous system. They have relatively small cytoplasmic domains with no catalytic activity, and are receptors for a class of axonal guidance proteins known as the semaphorins. Neuropilins are also co-receptors to VEGFs. Just like HSPGs, neuropilins impact the stability of the VEGF-VEGFR signalling complex at the cell-surface. For instance, the human VEGF-A gene consists of nine alternatively spliced exons that regulate interactions with HSPGs and neuropilins. Thus, $VEGF_{121}$ lacks the HSPG- and neuropilin-binding domains while $VEGFA_{189}$, includes both and is therefore retained in the vicinity of the cell-surface.

Neuropilins are expressed in several cell types. In particular, Neuropilin-1 is present in arteries, whereas the related neuropilin-2 is expressed in venous and lymphatic vessels. Although neuropilins lack an intrinsic catalytic domain, upon binding to semaphorins, they are capable of relaying signals from the cell membrane through association with the plexin family of transmembrane proteins (51). It is currently unknown whether VEGF binding to neuropilins could lead to a similar engagement of plexins, or whether neuropilins could transduce VEGF signalling through alternate mechanisms independently of VEGFRs.

Table I: VEGF and Receptor function by gene targeting

Genotype	Phenotype of mouse
***Vegfa*$^{+/-}$**	Lethal at embryonic day E11-12, defective vascular development
***Vegfa*$^{-/-}$**	Generated by aggregation of embryonic stem cells with tetraploid embryos, more severe defects in vascular development than heterozygote, embryonic lethal E9.5-10.5.
***Vegfa*$^{120/120}$**	50% die shortly after birth owing to bleeding in multiple organs and the remaining mice die before postnatal day 14 owing to cardiac failure. Impaired myocardial angiogenesis, ischemic cardiomyopathy, skeletal defects, and defects in vascular outgrowth and patterning in the retina.
***Vegfa*$^{164/164}$**	Viable, healthy.
***Vegfa*$^{188/188}$**	Impaired retinal arterial development, dwarfism, defective epiphyseal vascularization, impaired development of growth plates and secondary ossification centers, knee-joint dysplasia.
***Vegfb*$^{-/-}$**	Reduced heart size, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia.
Human VEGF-C overexpression	Hyperplasia of lymphatic vessels.
***Vegfc*$^{-/-}$**	Prenatal death owing to edema, lack of lymphatic vessels.
***Vegfc*$^{+/-}$**	Cutaneous lymphatic hypoplasia, lymphedema.
***Vegfd*$^{-/-}$**	Normal development, slight reduction of lymphatic vessels adjacent to lung bronchiole.
***Plgf*$^{-/-}$**	Impaired angiogenesis during ischemia, inflammation, wound healing and cancer.
***Vegfr1*$^{-/-}$**	Embryonic lethal E8.5-9.0, increased hemangioblast commitment, vascular disorganization owing to endothelial-cell overgrowth.
***Vegfr1(TK)*$^{-/-}$**	Normal development, VEGF-induced macrophage migration suppressed, decreased tumor angiogenesis.
***Vegfr1(TM-TK)*$^{-/-}$**	50% of mice die during embryonic development, owing to vascular defects.
***Vegfr2*$^{-/-}$**	Embryonic lethal E8.5-9.5, defective blood-island formation and vasculogenesis.
***Vegfr3*$^{-/-}$**	Embryonic lethal before formation of lymphatics owing to cardiovascular failure. Embryos show vascular remodeling defects and pericardial fluid accumulation.
Human VEGFR3 overexpression	Inhibition of fetal lymphangiogenesis, regression of lymphatic vessels, lymphedema.
***Neuropilin-1*$^{-/-}$**	Embryonic lethal, defective neural patterning, vascular regression.
***Neuropilin-1* overexpression**	Cardiovascular defects, heart malformation, excess blood-vessel formation, dilated blood vessels, hemorrhage, anomalies in nervous system and limbs.
***Neuropilin-2*$^{-/-}$**	40% show perinatal death close to birth. Survivors are smaller than littermates. Defects in neuronal patterning, severe reduction of small lymphatic vessels and capillaries.
***Neuropilin-1* and *2* double KO**	Embryonic lethal E8.5, defective vascular development.

Adapted with permission from Macmillan Publishers Ltd: Olsson *et al*, 2006 (23).

1.3 Beyond VEGF: The fibroblast growth factor (FGF) family

A substantial body of evidence exists outlining the importance of the fibroblast growth factor (FGF) family in controlling multiple developmental processes throughout embryogenesis, from mesoderm patterning to major organogenesis (**Table II**, p.26). FGFs also wield multiple effects in the adult organism, with a significant contribution to physiological processes such as angiogenesis and wound healing. On the flipside, because FGF signalling affects multiple cell types and regulates tissue survival, growth, migration and neovascularization – processes that are essential for tumor development –, the family is an ideal target for cooption by cancer cells. Indeed, numerous studies in humans and murine models have confirmed the oncogenic potential of FGFs: For instance, *Fgf19* transgenic mice overexpressing FGF19 in skeletal muscle develop liver tumors (52); additionally, studies have reported that gain-of-function mutations or single-nucleotide polymorphisms in FGF receptors (FGFRs) in both mice and humans are associated with multiple cancers types ((53-56) among others; and review (57)). In particular, there is strong correlation between FGFR2 anomalies and breast (58) and gastric cancers (59).

1.3.1 FGF members

Nomenclature. All FGFs are small (20-30 kDa) secreted glycoproteins, with similar core regions made of 120-130 amino acids arranged in 12 antiparallel β-strands (β1-12) and flanked by amino (N) and carboxy (C) terminals. Most sequence differences that confer specific FGF biological properties are found within the N and C tails. Based on nomenclature, the FGF family comprises 23 members, of which only 18 are FGFR ligands. Indeed, four of the 23 members (FGF11-14) lack several key FGFR-binding residues and thus do not bind or activate FGFRs, despite a remarkably high sequence homology with other members of the FGF family and a high affinity for heparin (60). For that reason, they are generally not considered canonical FGFs and are often referred to as FGF homologous factors 1-4 (FHF1-FHF4)[4]. Finally, FGF15 is only found in mice and is the ortholog of human FGF19.

[4] Additionally, the carboxyl terminus of FHFs folds against the rest of the ligand in a way that blocks many FGFR-binding residues from interacting with FGFRs.

The 18 FGF members are divided into six subfamilies based on sequence homology and phylogeny (**Figure 4**, p.16), grouped as follows:

1. FGF1 and FGF2;
2. FGF4, FGF5 and FGF6;
3. FGF3, FGF7, FGF10 and FGF22;
4. FGF8, FGF17 and FGF18;
5. FGF9, FGF16 and FGF20;
6. FGF19, FGF21 and FGF23.

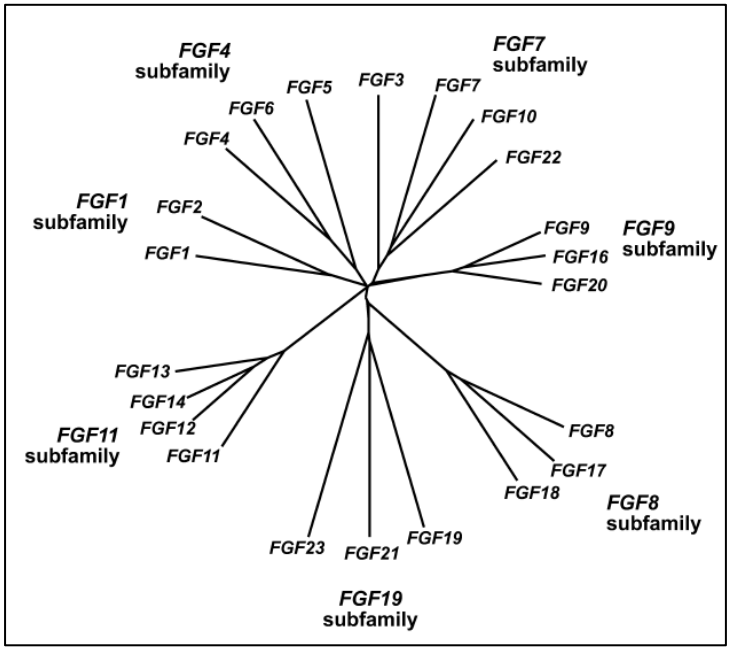

Figure 4: Human FGF subfamily distribution according to phylogeny.

Twenty-two FGF*s* are illustrated, including FGF11-14. Branch lengths are proportional to the evolutionary distance between each gene. FGF19 is a human ortholog of mouse FGF15. FGF11/FHF1 subfamily is shown for reference. **From Itoh *et al*, 2007 (61).**

Binding to HSPGs. Similarly to the VEGF family, varying affinities of FGFs for HSPGs impacts both the bioavailability and the function of the FGF ligands. The binding site for HSPGs is located within the FGF core, at β1- β2 as well as in parts of the region spanning β10 and β12. Directed mutagenesis of the HSPG-binding sites of two FGF members belonging to the same subfamily, FGF7 and FGF10, converted FGF10 into FGF7 with respect to diffusion characteristics and morphogenetic activity. The study suggests that differences in the affinity to HSPGs not only defines whether an FGF ligand acts in an endocrine or paracrine manner, but also affects the biological characteristics of FGFs within the same subfamily (62).

The first five subfamilies of FGFs have a distinctive heparin-binding site that forms a contiguous and positively charged surface. In contrast, the HSPG-binding site of the FGF19 subfamily contains ridges formed by the β1- β2 and β10-β12 that sterically hinder binding of HSPGs to the FGF core (this allows them to diffuse freely; see below). Finally, HSPGs were shown to simultaneously bind the ligand and the receptor, each heparin oligosaccharide interacting with one ligand and both receptor monomers within the dimer (63). This trivalent interaction strengthens both ligand-receptor and receptor-receptor protein contacts.

Endocrine ligands. Because of a high binding affinity to HSPGs, members of the first five FGF subfamilies are retained in the ECM or in close proximity to the cell surface where they exert their effects; thus, they function as paracrine ligands. On the other hand, the FGF19 subfamily was recently shown to act in an endocrine manner (64), enabled by poor binding to HSPGs and an ensuing capacity to diffuse from the production site into the circulation (on the flipside, the low affinity for HSPGs also reduces FGF-FGFR binding strength, stability and duration). Interestingly, the endocrine function of this subfamily is dependent on the presence of klotho proteins (α or β) in target tissues, which increase ligand-binding affinity by binding both endocrine FGFs and their corresponding receptors ((65); (review (66)).

1.3.2 FGF Receptors (FGFRs)

FGFs carry out their biological functions through four highly conserved heparin-binding tyrosine kinase receptors (FGFR1-4) and their multiple isoforms. Upon ligand binding, FGFRs dimerize, and a conformational shift allows for transphosphorylation of the intracellular kinase domains (the activation loop) of each monomer, as well as the phosphorylation of tyrosine (Tyr) residues in the C tail, the kinase insert and the juxtamembrane domain of the receptor itself. Receptor pTyr residues function as docking sites for adaptor proteins, including FGFR substrate 2 (FRS2) – a protein largely specific to FGFRs – and PLCγ, that activate Ras, MAP Kinases (p42/44, p38 etc.), PKC, Akt, Jun and signal transducer and activator of transcription (STAT) signalling, among others (66) (**Figure 5**, p.18).

Structure. Mammalian FGFR genes encode receptors consisting of three extracellular immunoglobulin-like domains (Ig I-III / D1-3), a single-pass transmembrane (TM) domain, and a cytoplasmic split tyrosine kinase (TK) domain (**Figure 5**, p.18). A feature of the FGFRs is a serine-rich sequence in the linker region between Ig-I and Ig-II, called the acid box (A). FGFs interact with both Ig-II and Ig-III domains of an FGFR monomer, with the Ig-III interactions primarily responsible for ligand-binding specificity; meanwhile, heparin binds to the first half of Ig-II (68, 69).

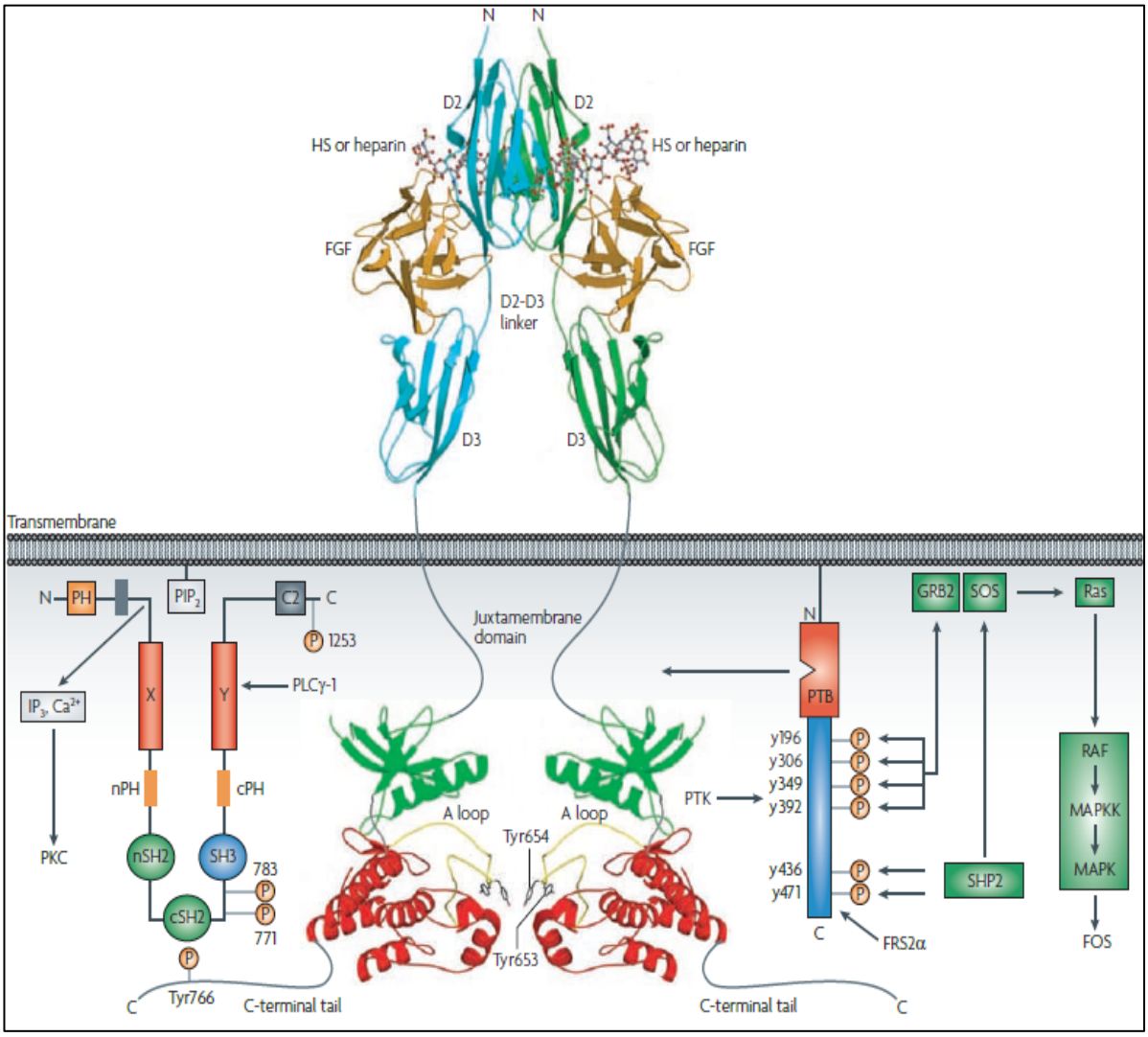

Figure 5: FGF2-FGFR1c-heparin crystal structure and signalling targets.

Structure of a dimerized FGFR bound to FGF (orange) and Heparin (HS). Structurally unresolved regions are shown as grey lines. N- and C-terminal lobes of the kinase domain are in green and red, respectively. The two major targets, PLCγ and FRS2α, are shown. HS, heparan sulphate; IP3, inositol-1,4,5-trisphosphate; PH, pleckstrin homology domain; PIP2, phosphatidylinositol-4,5- bisphosphate; PKC, protein kinase C; PTB, phosphotyrosine binding domain; PTK, protein tyrosine kinase; SH, Src homology domain. **Reproduced with permission from Macmillan Publishers Ltd: Beenken *et al*, 2009 (66, 67).**

Several FGFR isoforms exist (**Figure 6**, p.19): In normal tissues, FGFRs express all three Ig domains and are referred to as FGFR-α. Exon skipping completely removes the Ig-I domain and/or the acid box, giving rise to a "short" FGFR isoform called FGFR-β that has different ligand affinities (70). This alternative splicing event results in higher ligand and heparin affinity (69, 71), and is tied to tumour transformation into a malignant and invasive phenotype,

suggesting that Ig-I normally plays an autoinhibitory role in FGFR signalling. Additional splicing events in the second half of the Ig-III domain of FGFR1-3, specifically in exons 8 and 9, generate two other isoforms called IIIb/ "b" (where exon 9 is skipped) and IIIc/ "c" (where exon 8 is skipped). This brings the total to seven receptors with unique ligand-binding specificity and tissue localizations. For instance, FGFRb isoforms are generally expressed in epithelial cells, whereas FGFRc isoforms are present in mesenchymal cells. As for differential ligand binding capabilities, this is reflected in the ability of FGF2 to only activate IIIc forms of FGFRs (72); meanwhile, distinct residues within the N-terminal of FGF1 enables it to bind and activate all receptors and their splice variants, warranting its designation as "universal ligand" (73, 74).

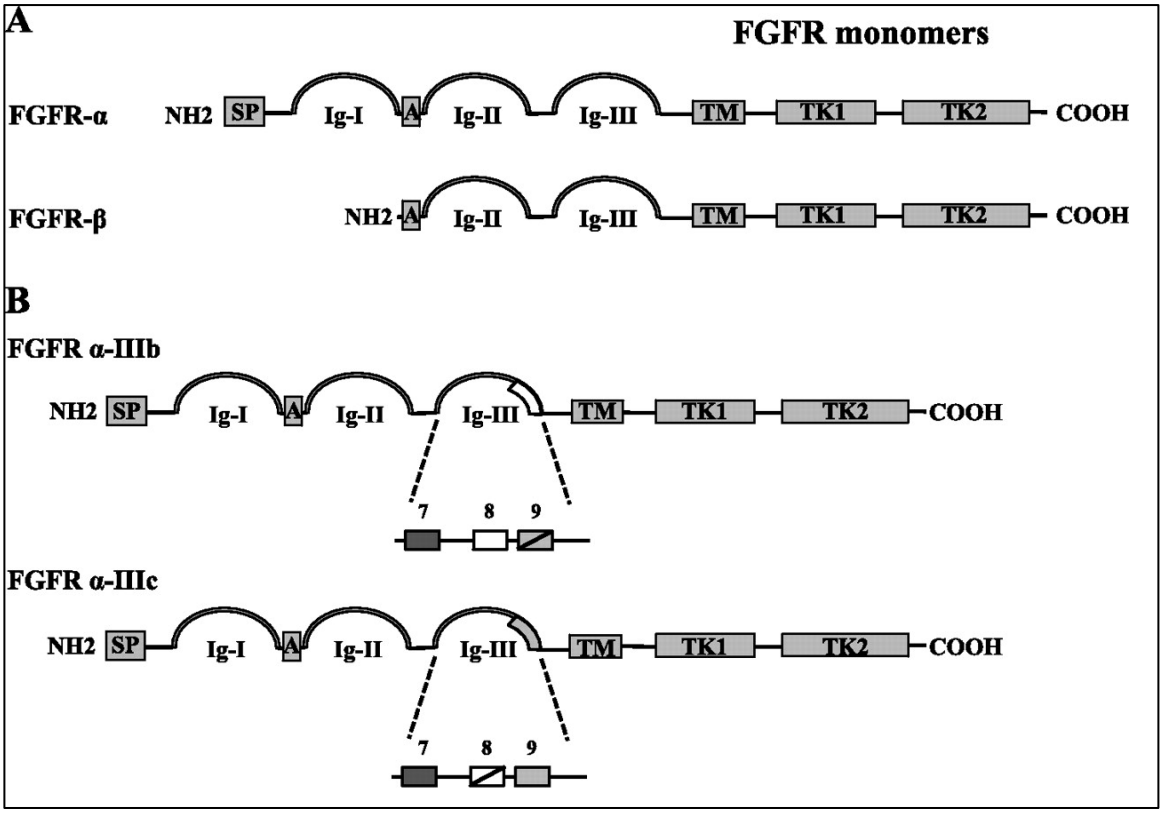

Figure 6: mRNA structure of FGFR monomers.

FGFRs are composed of up to three extracellular Ig-like domains that determine their binding properties to members of the FGF family. **A)** FGFRs can be expressed either as a long form (FGFR-α) containing all three Ig domains or as a shorter form lacking Ig-I (FGFR-β). **B)** Alternative splicing exons 8–9 of the Ig-III domain of FGFR-α or β generates isoforms referred to as "IIIb" (spliced exon 9) or "IIIc" (spliced exon 8) that exert different FGF-binding preferences. SP: Signal peptide; A: Acid box; TM: Transmembrane domain; TK: Tyrosine Kinase domain. **From Haddad *et al*, 2011 (77)**.

A fifth receptor, FGFR5/FGFRL1, has 50% sequence homology with FGFRs and can bind certain FGFs and heparin with high affinity; however, this receptor lacks the hallmark intracellular split kinase domain. In cultured cell models, FGFR5 is found mostly in soluble form containing all three Ig domains. Interestingly, cleavage of FGFR5 in both human and murine models occurrs at similar regions, if not the same sites. FGFR5 is thought to act as an FGF decoy receptor, sequestering FGFs and limiting their ability to activate signal-transducing FGFRs. This is supported by the observation that introducing FGFR5 mRNA in *Xenopus* embryos (75) results in a phenotype that mimics the overexpression of a truncated dominant-negative FGFR1 (76).

Mechanism of dimerization. There are two proposed models for FGFR dimerization. The prevailing model is referred to as "symmetrical" (vs. the "asymmetric"[5] model (78)), and is derived from the crytal structures of FGF2-FGFR1c, FGF1-FGFR2c and FGF10-FGFR2b. This model postulates that when FGFRs dimerize, they form a positively charged canyon where heparin can bind. Each FGF ligand is bivalent and each receptor is trivalent with respect to protein-protein interactions (63) (**Figure 5**, p.18 & **Figure 7**, p.22): One FGF ligand binds between the Ig-II (D2) and Ig-III (D3) regions of one FGFR, forming a 1-1 FGF-FGFR monomeric complex. That same ligand has a distinct secondary interaction site with the Ig-II domain on the adjacent FGF-FGFR monomer, thereby promoting the formation of a 2-2 FGF-FGFR dimer. Indeed, mutations in the FGF10 secondary site that do not impact FGF10 tertiary structure, binding to receptor monomer, or to heparin, still decrease FGF10 activation of FGFR2b due to impaired dimerization (63). Furthermore, the crystal structure of the FGF10-FGFR2b dimer complex shows that direct FGFR2b-FGFR2b protein contacts (a hydrophobic interaction and a hydrogen bond at Ala172 and Ser220, respectively) occur at the bottom of the Ig-II domains (**Figure 7**, p.22). Replacing Ala172 with Phe results in increased hydrophobic interactions and lessens the need for heparin for dimerization. Accordingly, this more stable FGFR2 dimer leads to a gain-of-function (63). The orientation of the Ig-III domain is governed by a proline residue (Pro253) in the linker region between Ig-II and Ig-III; in the symmetric model, this proline adopts a *trans* conformation (63, 79).

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[5] In the "asymmetric" model, FGF-FGFR halves are held together only by heparin (Pellegrini *et al*, 2000). The biological relevance of this model was refuted in (Ibrahimi *et al*, 2005).

In the crystal structure of 2-2 FGF2-FGFR1c, two heparin molecules are bound in the canyon (**Figure 5**, p.18 & **Figure 7**, p.22). Each heparin oligosaccharide interacts with one ligand and both receptors in the dimer, thus promoting FGFR dimerization by augmenting FGF-FGFR binding at primary and secondary interaction sites, as well as by stabilizing receptor-receptor contacts. In the absence of heparin, a series of sulfate ions bind into the canyon and have been proposed to mimic the sulfate moieties of heparin.

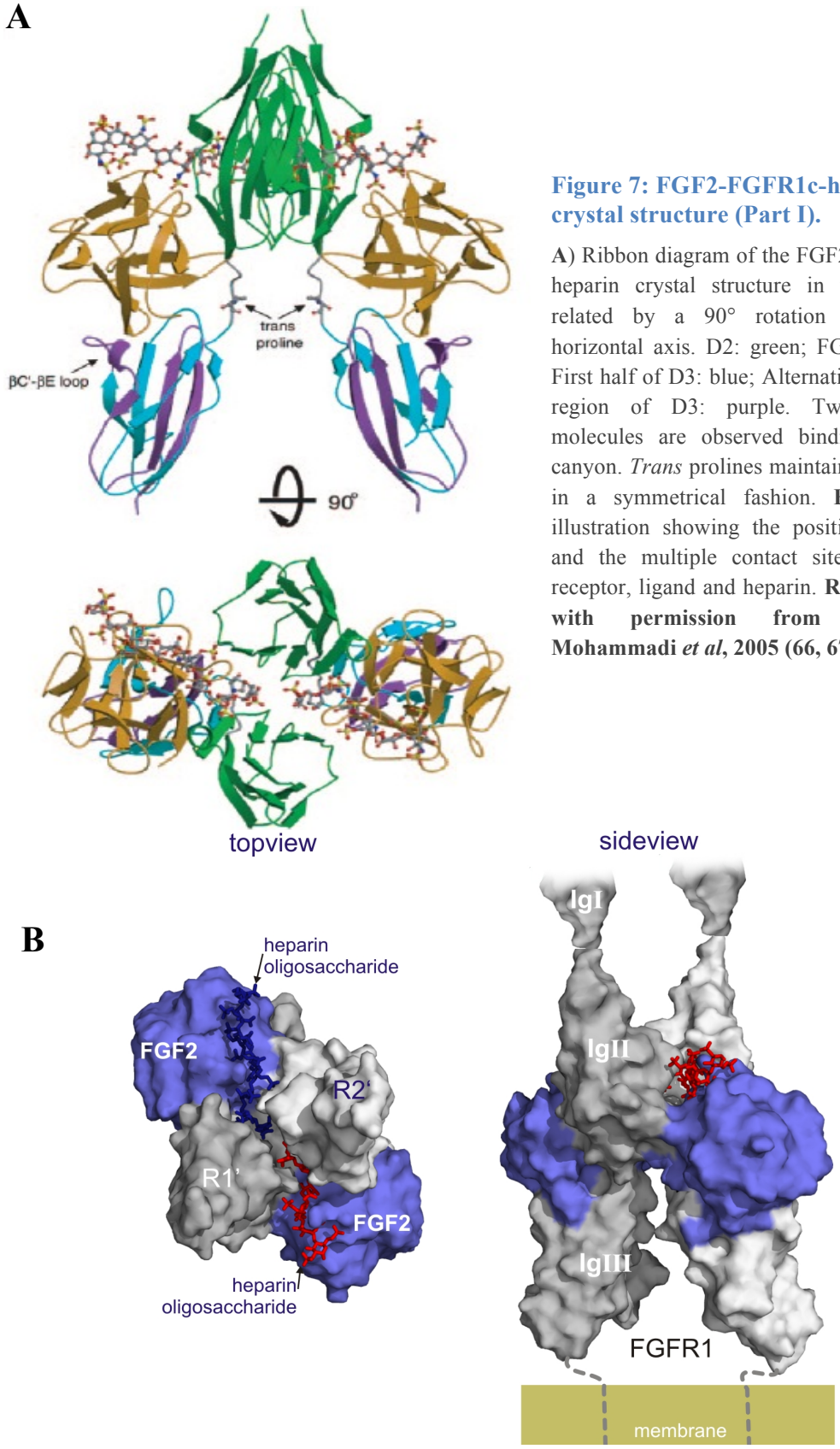

Figure 7: FGF2-FGFR1c-heparin crystal structure (Part I).

A) Ribbon diagram of the FGF2-FGFR1c-heparin crystal structure in two views related by a 90° rotation about the horizontal axis. D2: green; FGF: orange; First half of D3: blue; Alternative splicing region of D3: purple. Two heparin molecules are observed binding in the canyon. *Trans* prolines maintain the dimer in a symmetrical fashion. **B)** Surface illustration showing the positive canyon and the multiple contact sites between receptor, ligand and heparin. **Reproduced with permission from Elsevier: Mohammadi *et al*, 2005 (66, 67).**

1.3.3 FGF1 subfamily signalling

Targeted gene disruption studies have provided much insight into the function of FGFs (**Table II**, p.26). While a complete review of the physiological and pathological functions of each and every member of the FGF family would be beyond the scope of this document, attention will be given to two prototypical members of the family, FGF1 and FGF2.

As knockout mice for FGF1, FGF2, or both at the same time are viable and fertile and *Fgf1*$^{-/-}$ mice even appear to be completely normal, the physiological roles of FGF1 and FGF2 are still unclear.

FGF2 is a very potent inducer of angiogenesis, and its expression is potentiated by hypoxia[6] (80) and hemodynamic stress. In an *in vitro* collagen gel assay, FGF2 was shown to be twice as potent as VEGF in stimulating EC organization into capillary-like structures (81). *In vivo,* exogenous FGF2 stimulates proliferation of ECs (82), smooth muscle cells (83), macrophages and fibroblasts, thereby promoting the development of large vessels with adventitia. FGF2 also promotes migration of ECs, has anti-apoptotic properties (84), regulates EC production of proteases such as plasminogen activator and MMPs (85), has vasodilation capabilities through the release of nitric oxide (86) and promotes cell differentiation and maturation (87, 88).

FGF2 was also shown to improve myocardial circulation post-infarct by increasing vessel density in several animal models including rabbits, dogs and pigs (89-91). Because promoting revascularization has obvious therapeutic applications, the potential of FGF2 in coronary and peripheral vascular diseases in humans became of interest and was explored in several clinical trials: In a Phase I unblinded and uncontrolled trial with patients with severe ischemic heart disease, a single bolus of FGF2 was shown to be safe (92, 93) and reduced the size of ischemic regions in the myocardium, improved treadmill performance, reduced the frequency of angina and increased myocardial perfusion (92, 94). However, in the Phase III FGF Initiating RevaScularization Trial (FIRST) (a multicenter, randomized, double-blind, placebo-controlled trial) a single intracoronary infusion of FGF2 did not improve exercise tolerance or myocardial

[6] As described by Conte *et al*: "Hypoxia is a major pathophysiological trigger of angiogenesis. In solid tumours, the angiogenic switch responsible for tumor development is induced by hypoxia. In cardiovascular diseases, ischemia corresponds to a shortage of the blood supply, resulting in tissue damage because of the lack of oxygen and nutrients."

perfusion despite a trend, early in the study, towards symptomatic improvement (95). Using a different approach with a more direct FGF2 delivery system, the long-term benefits of FGF2 administration were tested in a separate Phase I double-blind randomized and placebo-controlled study. This method, deemed safe (96), required open-chest delivery and introduced heparin beads containing adsorbed FGF2 implanted over the ischemic myocardium of patients. The early promising results found in this trial were maintained over the course of 3 years of follow-up (97), making it one of the few examples of a sustainable long-term positive response to FGF2 (review (66)).

The potential benefits of FGF1 and FGF2 in peripheral revascularization were also tested. In separate Phase I and/or II studies, patients suffering from peripheral artery diseases (critical limb ischemia or claudication) received a naked plasmid DNA encoding FGF1 or an intra-arterial FGF2 injection, and both delivery systems were deemed safe and efficacious (98, 99). Improvement compared to placebo was observed in both cases, as FGF1 treatment lead to significantly reduced pain and risk of major amputation (100), while FGF2 improved calf blood-flow (99). However, in larger phase III trials (TAMARIS (101) and TRAFFIC (102), respectively), neither FGF1 nor FGF2 demonstrated any sustainable significant benefits.

Other possible physiological roles for the FGF1 subfamily include inflammation: One or both of these ligands can be up-regulated in the pericardial fluid of patients with cardiac ischemia (103), as well as in inflammatory disorders such as asthma[7] (104) (review (105)), bowel syndrome, Crohn's disease, ulcerative colitis (106) and rheumatoid arthritis (107). Diseased glomeruli from patients with Proliferative Lupus Nephritis, an inflammatory disorder that results in renal damage, show an increase in infiltrating macrophages and T lymphocytes expressing FGF1 and FGFR1, which are also actively synthesized during the development of pulmonary fibrosis (108). FGF2 also modulates leukocyte infiltration, a hallmark of inflammation, by enhancing the expression of endothelial adhesion molecules ICAM-1/2 (109, 110) and E-selectin (111), and leukocyte chemoattractants such as monocyte chemoattractant protein-1 (MCP-1) (112-114). Several reports have suggested that FGF1 and FGF2 are secreted by, and may act as

[7]"Asthma is caused by an inappropriate inflammatory response toward usually innocuous inhaled substances, and is characterized by recurrent but normally reversible respiratory symptoms. These episodic symptoms occur as a result of airway obstruction, secondary to inflammation and excessive airway narrowing that develop following each exposure to the offending substance" (Bosse *et al*, 2008).

immunoregulators of, infiltrating neutrophils, monocytes, macrophages and T lymphocytes, often in tandem with powerful inflammatory cytokines (108, 112, 115, 116). Indeed, FGF2 enhances the effect of TNF-α (109, 113) and IFN-γ (113) on monocytes, T cells, and neutrophils recruitment to inflamed dermal sites, and increases respiratory burst induction and H_2O_2 production by neutrophils (110, 113). Interestingly, a recent study showed that FGF2 and IL-1 precursors may share a common secretory pathway; the inflammatory enzyme caspase-1, the main culprit in the activation of IL-1β, has the ability to regulate FGF2 secretion, particularly in stress situations such as during wound healing (117), providing a direct link between the inflammasome complex and FGF2 function.

Based on the observations that VEGF overexpression results in leaky, immature, and unstable vessels, while FGF alone is unable to sustain vascularization, it is evident that a single growth factor family is not sufficient to sustain functional neovascularization. Along similar lines, targeting one family exclusively may not provide the desired therapeutic benefit.

Table II: FGF and Receptor function by gene targeting

Member	Phenotype of KO mouse	Physiological role
FGF1	Viable (118); Normal	Relevance not established Role in angiogenesis; adipogenesis
FGF2	Viable; Mild vascular defects characterized by decreased vascular smooth muscle contractility, delayed wound healing hematopoietic deficiencies, low blood pressure and thrombocytosis (119)	Relevance not established Role in angiogenesis; vascular tone; inflammation (asthma) Mitogen for articular chondrocytes (120)
FGF1 + FGF2	Viable; Same phenotype as $FGF2^{-/-}$ mice (118)	-
FGF3	Viable; Inner ear agenesis in humans	Inner ear development
FGF4	Embryonic lethal (**E4.5**); Defects in trophoblastic proliferation	Cardiac valve leaflet formation; Limb development
FGF5	Viable; Abnormal hair growth	Hair growth cycle regulation
FGF6	Viable; Defective muscle regeneration	Myogenesis
FGF7	Viable; Matted hair; reduced nephron branching in kidney	Branching morphogenesis
FGF8	Embryonic lethal (**E8**)	Brain, eye, ear and limb development
FGF9	Postnatal death (**PD0**); Gender reversal Lung hypoplasia	Gonadal development Organogenesis
FGF10	**PD0**; Failed limb and lung development	Branching morphogenesis
FGF16	Strain-dependent survival (121) (viable: (122); lethal (E11.5): (123))	Unclear; effects on heart development, cardiomyocyte proliferation (122, 123)
FGF17	Viable (124); Abnormal brain development	Late cerebellar development (124)
FGF18	**PD0**; Delayed long-bone ossification	Bone development Chondrocyte and cartilage development (120)
FGF19	Embryonic lethal (**E13.5 – PD21**); Increased bile acid pool	Bile acid homeostasis Lipolysis Gall bladder filling
FGF20	Viable; Deafness (125)	Inner ear development; differentiation of cells in the lateral cochlear compartment (outer hair and supporting cells) within the organ of Corti (125)
FGF21	Viable; Late postnatal weight gain (week 14) (126)	Fasting response Glucose homeostasis Lipolysis and lipogenesis
FGF22	Viable, fertile, no obvious abnormalities (127)	Unknown
FGF23	Lethal at postnatal week 4 -13; Hyperphosphatemia Hypoglycemia Immature sexual organs	Phosphate homeostasis Vitamin D homeostasis
FGFR1	Embryonic lethal, soon after implantation (128)	Limb initiation, development, cell survival, autopod formation and digit patterning (129)

FGFR2	Embryonic lethal, soon after implantation (130)	Adrenal cortex development Bone development, osteoblast differentiation, cranial ossification(131)
FGFR3	Viable; Skeletal defects, severe kyphosis ("hunchback"), softening of bones (osteomalacia) at 4 months Deafness (132, 133)	Development and maintenance of growth plate cartilage (134)
FGFR4	Viable; Features of metabolic syndrome, hyperlipidemia, glucose intolerance, and insulin resistance, in addition to hypercholesterolemia	Lipid and glucose homeostasis (135) Bile acid homeostasis, gall bladder filling (136)
FGFR5/ FGFRL1	Normal embryonic development until term; but then **PD0** (137) from respiratory defects (hypoplastic diaphragm); Skeletal alterations, craniofacial dysplasia Heart valve defects Embryonic Anemia Failure to develop functional metanephric kidneys (138)	Inhibition of FGF signalling; Myogenesis (diaphragm muscle) Heart, bone, kidney development

Where no reference is indicated, adapted with permission from Macmillan Publishers Ltd: Beenken *et al.*, 2009 (66).

1.4 The Angiopoietin (Ang) family

In a healthy adult organism, most vessels are found in a stable quiescent state that they only relinquish in the presence of an activating signal. Under an angiogenic stimulus for instance, activated ECs alter the biochemical balance surrounding them; thus, they modify their own expression of junctional proteins such that intracellular connections are loosened, and they secrete destabilizing agents that degrade the basement membrane and promote the dissociation of mural support cells. After the new vessel is established, activating signals must be removed to ensure a return to EC quiescence and homeostasis. This step is called the resolution phase (139) (see **1.1 Angiogenesis: Fundamentals**). The loss of endothelium quiescence is a hallmark of another physiological process, inflammation (see **1.5 Inflammation**). The sequence of events here is similar to the angiogenic cascade in that it involves biochemical changes, shedding of EC tight junctions and induction of permeability. Additionally, depending on their activation status, ECs can alter their cell-surface adhesion molecules, enabling them to capture leukocytes from the circulation and to assist in leukocyte transmigration to sites of injury.

Thus, the endothelium must retain a high level of plasticity to be able to dynamically respond to physiological stimuli. Evidently, control of the endothelial quiescence-activation switch is an attractive target for developing tumors. As well, deregulation of endothelial quiescence is prevalent in a range of inflammatory diseases and vasculopathies, highlighting the need for a robust control mechanism that regulates the timing and duration of endothelial activation. Based on a body of evidence from the last two decades, the angiopoietin-Tie system has emerged as a strong candidate for controlling this switch (reviewed in (140)).

1.4.1 The family

Early transgenic mice studies, and multiple publications since then, have revealed two striking observations regarding the angiopoietin family: First, it is largely specific to the vascular system, being mainly expressed by or acting on cell types that are directly involved in neovascularization. Second, the loss of any of its members early in development is generally lethal (**Table III**, p.45).

A distinction is made between the angiopoietin (Ang) and the very similar angiopoietin-like (ANGPTL) families based on the ability of the former and inability of the latter to bind and

activate Tie receptors. In humans, there are three ligands for the Tie receptors, termed the angiopoietins (Ang1, 2 and 4). In mice, there are also three Ang proteins (Ang1-3), with Ang3 commonly considered the ortholog of human Ang4 despite having diverging sequences, functions and tissue distributions (141). An effort to identify more ligands through sequence homology lead to the discovery of seven proteins, called angiopoietin-like (ANGPTL1-7), that are structurally related to Ang but are not "classical" angiopoietins in that they do not bind Tie receptors (141) (**Figure 8**, p.29). No specific receptors for the ANGPTL family have been identified thus far.

The angiopoietin family has both agonist and antagonist members within its ranks, best illustrated by the relationship between Ang1 and Ang2 in the context of Tie2 activation. In effect, Ang1 is considered a "traditional" agonist of Tie2, inducing receptor phosphorylation (143, 144), Tie2-mediated recruitment of downstream signalling effector molecules (145-147) and promotion of various biological outcomes (discussed later), most of which can be competitively antagonized by Ang2 (148). The latter ligand, however, is far from being a simple antagonist, as it has been shown that in certain vascular beds and with some cell types, Ang2 can actually activate Tie2 in a similar fashion to Ang1 (145, 149-152). Finally, Ang3 and Ang4 appear to be agonists for the ECs originating from their respective species only (153); however, studies on the distinct role and activities of either ligand are limited.

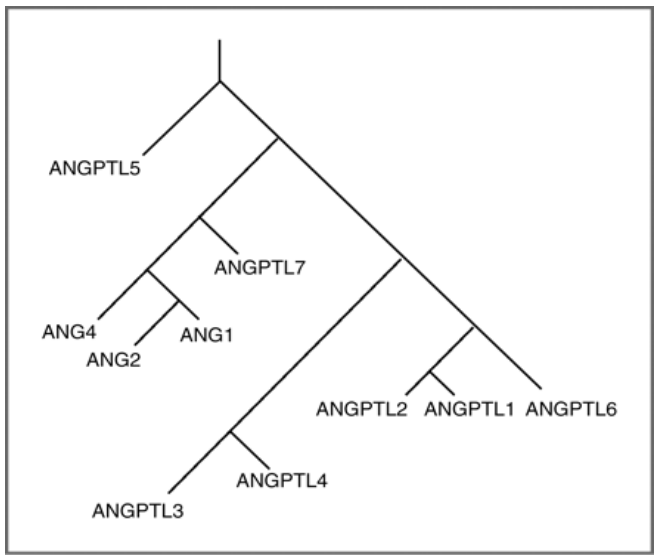

Figure 8: Phylogenetic tree of Ang and ANGPTL families in humans.

Both families of proteins share structural similarities. However, ANGPTLs do not bind or activate Tie. ANGPTLs are commonly considered to be orphan ligands. **Reproduced with permission from AACR: Tan *et al*, 2012 (142).**

1.4.2 Tie receptors

1.4.2.1 Properties

The acronym "Tie" stands for tyrosine kinase with immunoglobulin (Ig) and epidermal growth factor (EGF) homology domains. The Tie receptors were originally identified as orphan

receptors, following a search in the 1990's to identify protein tyrosine kinases expressed by ECs. A few years later, Ang1 was discovered as an activating ligand for Tie2, followed by the rest of the angiopoietins. To date, Tie1 still remains an orphan receptor without any specific ligands.

Nomenclature. Going from the N-terminal to the cell surface (**Figure 9**, p.31), the extracellular portion of Tie receptors consists of two Ig-like domains, three EGF-like domains, another Ig-like domain and three fibronectin type III-like repeats. The transmembrane domain is followed by a catalytic C-terminal tyrosine kinase domain. Tie1 and Tie2 have an overall similar protein structure, with about 76 and 30% homology in their intracellular and extracellular domains, respectively. Binding of angiopoietins to Tie2 occurs at the second extracellular Ig-like domain of the receptor; interestingly, unlike VEGFRs and FGFRs, binding of Tie2 to its ligands does not lead to any significant conformational change compared to the unbound receptor (154).

At the cell surface, Tie2 can be found in an uncomplexed form or heterodimerized with Tie1, the Tie1-Tie2 heterodimers being kept together via electrostatic interactions between the extracellular portions (ectodomains) of the receptors (155). Upon ligand binding, Tie2-Tie1 heterodimers generally dissociate, allowing for Tie2 homodimer clustering (155), Tie2 autophosphorylation and initiation of signalling. Studies have shown that Tie1 phosphorylation in Tie2-Tie1 heterodimers can also occur in response to Ang1, but this happens in *trans* and is dependent on the presence of a functional Tie2 (156, 157).

Expression. As a testament to their importance in vascular biology, and similarly to the VEGFRs, Tie receptors are highly specific to vascular endothelium: Both Tie receptors are mostly expressed in vascular ECs during embryogenesis (158, 159), and while the expression pattern is more restricted in adult tissues, both receptors continue to be predominantly found at the level of the endothelium (160). Indeed, Tie2 is homogenously expressed in the endothelium of quiescent and angiogenically active arteries, veins, and capillaries of all adult tissues, and Tie1 expression is significantly restricted to vascular bifurcations and branching points (161). The significance of this patterning remains to be clarified. Interestingly, Tie2 is also expressed on various innate immune cells, notably of the myeloid lineage (145, 151, 162, 163), suggesting a possible role for the Ang-Tie family in leukocyte-driven angiogenesis and inflammation.

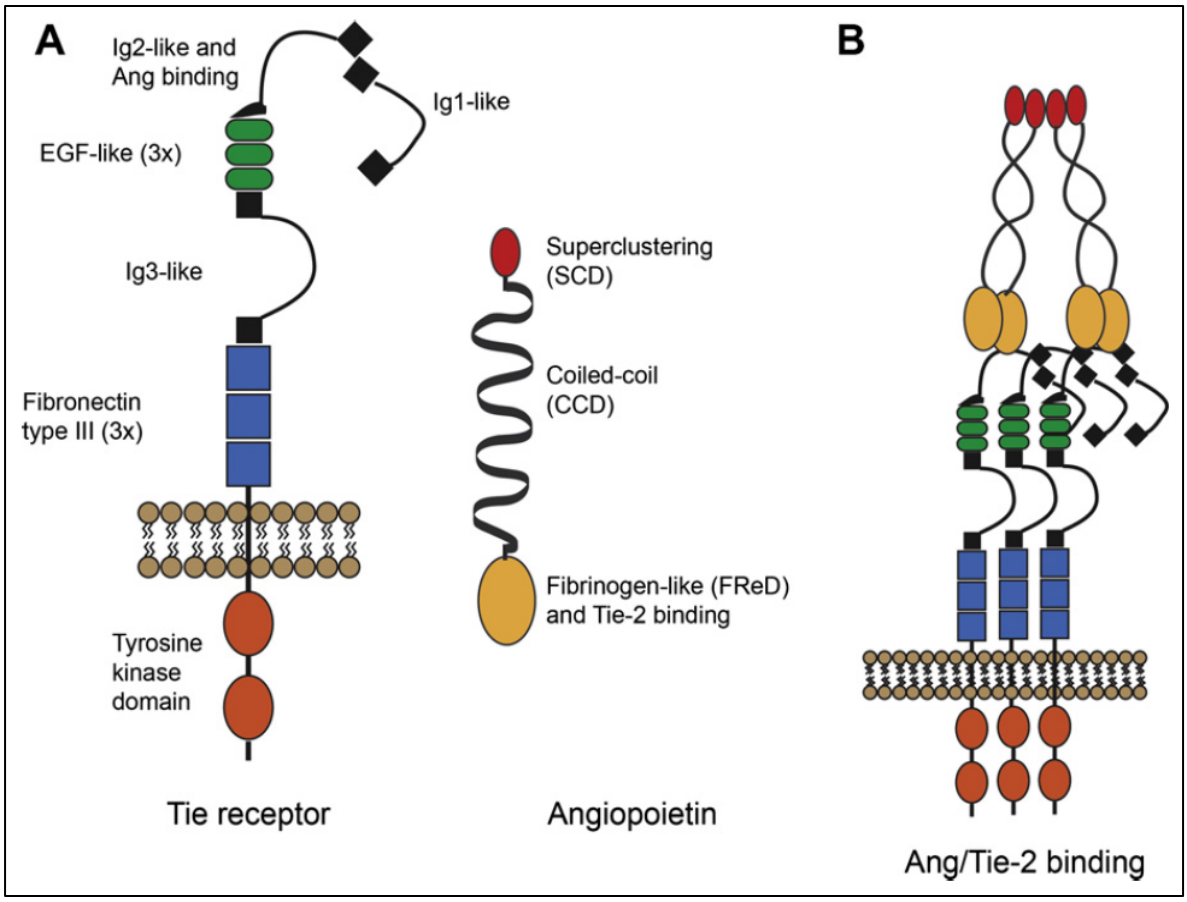

Figure 9: The molecular structure of Tie receptors and Ang ligands.

A) The N-terminal extracellular region of Tie receptors consists of two Ig-like domains, three epidermal growth factor (EGF)-like domains flanked by another Ig-like domain, followed by three fibronectin type III domains. Ang bind to the Ig-2 domain. The C-terminal contains a split tyrosine kinase domain. The binding of Ang to Tie-2 is mediated by a fibrinogen-like domain (FReD). **B)** Ang bind to Tie-2 receptor as multimers. Clustering of receptor molecules brings their kinase domains into close proximity allowing phosphorylation of each other in *trans* and resulting into receptor activation and the initiation of downstream signal transduction. **Reproduced with permission from Elsevier: Fagiani *et al.*, 2013 (140).**

Ligand binding. A close comparison of the Ang1-Tie2 and Ang2-Tie2 crystal structures recently revealed that both Ang1 and Ang2 bind to Tie2 in a very similar manner (**Figure 10**, p.32), underlining the fact that altered ligand presentation cannot account for the distinct biological activities of the two ligands. Rather, ligand function appears to be controlled by a small stretch of residues close to the Tie2-ligand binding interface (located within the β6-β7 loop of Ang1 and Ang2) and near the area where Tie2 and Tie1 interact (154), suggesting a possible role for Tie1 in controlling Ang signalling. In support of this theory, although Tie1 does not bind angiopoietins directly, its heterodimerization with Tie2 hinders the access of Ang1 – but not of Ang2 – to Tie2

(164), and reduces both basal (155) and Ang1-driven Tie2 phosphorylation (and therefore activity) (165).

Signal transduction. Binding of Ang1 to Tie2 stimulates a number of intracellular signalling pathways, including PI3K/Akt, eNOS, MAPKs and Dok-R/Nck/Pak pathways (144, 166). Additionally, activated Tie2 recruits ABIN-2, a suppressor of NFκB activity with anti-apoptotic activities on ECs (147, 167). Ang1 preferentially binds uncomplexed Tie2: Upon Ang1 (but not Ang2) binding, Tie2-Tie1 heterodimers dissociate allowing for Tie2 clustering (155), auto-phosphorylation at five tyrosine residues within the Tie2 intracellular kinase domain that serve as docking sites for SH2-containing signalling effectors (144), and initiation of signalling (144, 168). On the other hand, it seems that Ang2 has no particular preference in binding Tie2 in either homo or heterodimers, as the ligand's weak activation of Tie2 and its ability to antagonize Ang1 are not affected by the presence of Tie1 (164). Thus, since Ang1 binds and activates Tie2 preferentially in the absence of Tie1, the stabilizing actions of Ang1 on the endothelium are diminished with higher levels of Tie1. In support of this, the ratio of Tie1 to Tie2 increases at

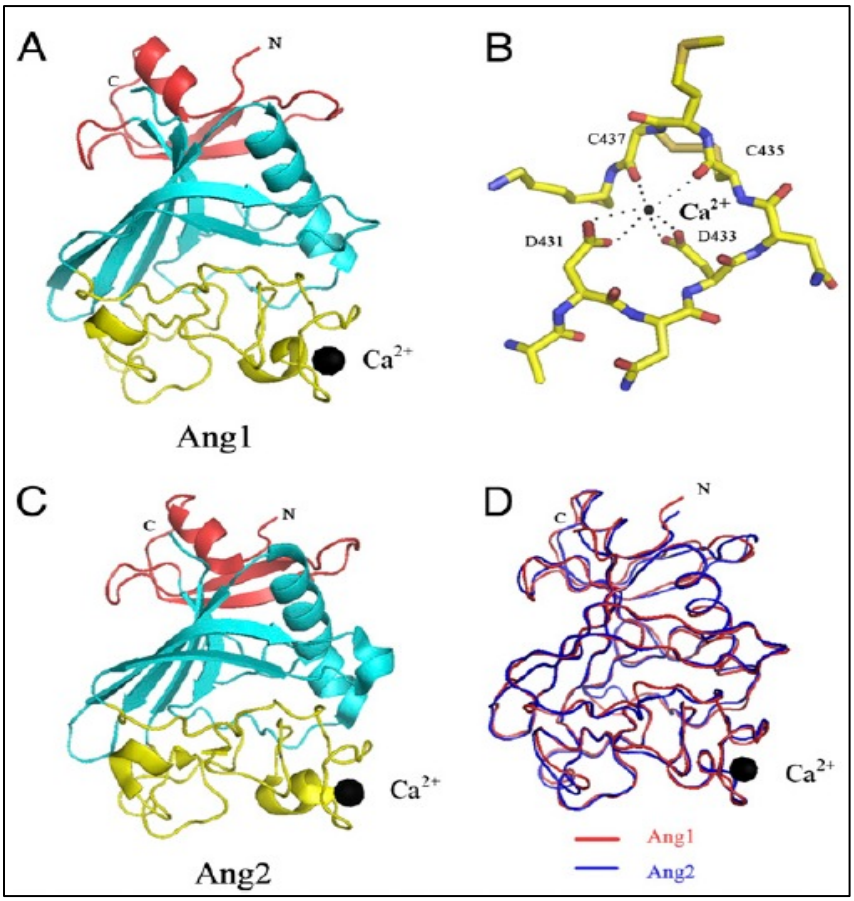

Figure 10: Crystal structures of Ang-Receptor binding domain (RBD).

(A) The refined model of Ang1-RBD with the individual subdomains shown in different colors (A domain, red; B domain, cyan; P domain, yellow). The black sphere represents the bound calcium atom. (B) Close-up view of the Ca2+-binding site. (C) Structure of the previously determined Ang2-RBD colored as in A. (D) Structural alignment in coil representation of the Ang1-RBD (shown in red) and the Ang2-RBD (shown in blue). The superimposition shows the high similarities in the tertiary structures of both ligands. **Reproduced with permission from PNAS: Yu *et al*, 2013 (154).**

sites of active neovascularization during embryonic development (169) as well as in hypoxic conditions or upon VEGF stimulation in the adult (170).

Ectodomain cleavage. The ratios of Ang1:Ang2 and Tie1:Tie2 are important determinants of the state of activation of the endothelium. There is an additional layer of control of Ang-Tie activity, found at the structural level of the receptors themselves. Once exposed at the cell-surface, both Tie1 and Tie2 can undergo proteolytic cleavage that removes the ectodomain of the receptors, targeting the truncated receptors for internalization and proteasomal degradation. As mentioned above (***Ligand Binding***), loss of Tie1 ectodomain would increase Ang1 signalling. In contrast, loss of Tie2 ectodomain would suppress Ang1 signalling by two routes, first through the loss of the ligand-binding domain and second, through the sequestration of Ang1 by the released fragment.

Ectodomain cleavage of Tie1 generates a 45 kDa cell-associated fragment of the receptor, comprising of transmembrane and intracellular domains (171). Similarly, Tie2 ectodomain cleavage results in the formation of a 55 kDa cell-associated fragment (171), and in the appearance of a soluble fragment approximately 75–85 kDa in size (172). Though the resulting truncated receptors are similar in size, Tie1 and Tie2 ectodomain sheddings can significantly differ in their temporal dynamics: For instance, while VEGF and PMA induce Tie1 cleavage within minutes, it takes several hours before any sign of Tie2 cleavage is detected (171). The direct physiological impact of this is the acute enhancement of Ang1 signaling through Tie2. Thus, Tie1 cleavage provides the system with a rapid way to adapt to new angiogenic requirements and to acute changes in the biochemical environment.

1.4.2.2 Tie-mediated biological activities

Mutagenesis studies have underlined that Tie expression is critical in the later stages of embryonic vascular development (**Table III**, p.45). Null embryos for one or both receptors show that neither receptor is required for early vasculogenesis, as angioblast differentiation and formation of the primitive plexus do occur; however, in Tie1 null embryos, vessels lose their integrity, leading to widespread edema, hemorrhage and finally to death between E13.5 and E14.5 (173). Tie2 null embryos have an even more severe phenotype, as embryonic death starts earlier at E9.5 to E12.5 (16, 174). Though they advance through the early steps of cardiovascular

development, Tie2 null embryos exhibit a poorly organized capillary plexus with impaired vessel branching, fewer ECs and an important lack of supporting mural cells, as well as impairments in hematopoiesis and development of the endocardium. Finally, double knockouts for both Tie1 and Tie2 are similar in phenotype to Tie2-null embryos, but some defects are more severe and the onset of fatality is even earlier (occurring at E9.5- 10.5) (175). Thus, while both receptors appear dispensable to the early assembly of the vasculature or to defining hematopoietic lineages in the embryo, they are essential at later stages of embryonic angiogenesis (176), for heart development, and for post-natal hematopoiesis (177).

1.4.3 Tie2 ligands

1.4.3.1 Properties

Nomenclature. Angiopoietins (Ang) are secreted glycoproteins, with a signal peptide, an N-terminal superclustering domain (SCD), a central coiled-coil domain (CCD), a short 20-residue linker sequence followed by a C-terminal fibrinogen-related domain (FreD) containing three sub-domains, A, B and P. The P-domain mediates interaction of Ang to Tie2, and to other FReD-containing proteins (such as fibrinogen) (**Figure 9**, p.31). The genes coding human Ang1 and Ang2 are found on chromosome 8, giving rise to proteins containing 498 and 496 amino acid residues, respectively, with 60% sequence homology. Monomeric Ang proteins have a molecular weight of approximately 64-70 kDa in non-reducing conditions (and ~ 57 kDa in reducing conditions) (178). However, all family members exist primarily as oligomers assembled through the coiled-coil domain of the proteins, forming trimers, tetramers, and pentamers. Moreover, in solution, half of Ang1 and less than 10% of Ang2 exist as higher order multimers (several oligomers together) brought together by the N-terminal superclustering motifs. Oligomerization is essential for Ang1 function, as the ligand needs to be in a tetrameric form at the very least to bind and activate Tie2 (179, 180). Finally, directed mutagenesis demonstrated that three critical residues (residues 463-465 in Ang1 and 461-463 in Ang2) within the angiopoietin β6-β7 loop in the fibrinogen domain appear to be necessary to confer ligand biological activity. Swapping these residues in Ang2 for those of Ang1 allows the mutant Ang2 to disrupt the pre-existent Tie1/Tie2 complex on the cell surface and phosphorylate Akt in a comparable manner to native Ang1. Similarly, replacing the three residues in Ang1 with those of Ang2 leads to the loss of Ang1 agonistic properties (154).

Expression pattern. Ang1 is produced and secreted primarily by perivascular cells, with a very low expression in ECs (cell lysates taken from sub-confluent HUVECs contained over 8-fold more Ang2 than Ang1). In contrast, Ang2 is constitutively expressed by ECs where it is kept stored in specific storage granules, known as Weibel–Palade bodies (WPBs)[8], along with other presynthesized molecules such as P-selectin, IL-8 and endothelin-1 (181). In response to multiple endothelial modulators such as thrombin, histamine and serotonin, ECs release the content of WPBs within seconds to minutes, suggesting that this is the first step in the switch from an endothelial quiescent state to an active one. Interestingly, leukocytes also express differential levels of Ang1 and Ang2: circulating non-activated monocytes contain comparable high levels of both Ang1 and Ang2 and appear to be the only peripheral blood mononuclear cells to express and readily secrete angiopoietins (182). Meanwhile, neutrophils express and store Ang1 but not Ang2, and the physiological conditions necessary for the release of Ang1 remain unknown (183).

1.4.3.2 Cellular effects and signalling of Ang1

Murine studies with angiopoietin-null embryos have shown that, similarly to the receptors, Ang ligands are not required for the initial formation of the vasculature during embryogenesis, but are instead essential for vessel maturation, stability and remodeling.

Ang1-deficient mice die between E11.5-12.5, with a comparable phenotype to Tie2 deficiency: although the vasculature has formed, it displays decreased complexity with dilated vessels, diminished branching, and reduced numbers of small vessels. Additionally, blood vessels have fewer ECs, and exhibit poor association of ECs with basement membrane and with perivascular cells, suggesting a possible role for Ang1 in promoting endothelial proliferation or survival[9] and vessel integrity. In a mouse model overexpressing Ang1 in skin, vessel diameter enlargement and an increase in EC numbers and coverage by pericytes provide additional evidence that Ang1 is involved in EC proliferation, survival and vessel maturation (184). In parallel, in support of its role in maintaining vessel integrity/quiescence, Ang1 counters the hyperpermeability observed following VEGF administration: While overexpressing VEGF-A

[8] The primary constituent of WPBs is von-Willebrand factor (vWF), which exists under multiple processed forms.
[9] It is possible that there are fewer ECs because they die from poor attachment to ECM components, rather than from a lack of pro-survival/pro-proliferation stimulus in the absence of Ang1.

alone in mice results in leaky hyperpermeable vessels that sustain a chronic inflammatory response, co-overexpression with Ang1 gives rise to vessels that are stable and resistant to leakage (17).

Aside from the above-noted genetic manipulations, an exhaustive review of the literature for the specific cellular functions of Ang1 reveals a high level of context-dependency, largely influenced by the intrinsic properties of both the cellular model[10] and the angiopoietins themselves. The most significant effects generally attributed to Ang1 are highlighted below.

Apoptosis. The most consistently reported cellular effect of Ang1 is its ability to inhibit apoptosis under a range of conditions and in a variety of cell models, the majority of which is of endothelial lineage (albeit of various origins). The ligand suppresses apoptosis in human umbilical vein ECs (HUVECs) (146, 147, 185), bovine aortic and microvascular ECs (186), bovine mesenteric vein and arterial ECs (152), porcine retinal ECs (187), porcine pulmonary arterial ECs (185), mouse cortical neurons[11] (188), mouse skeletal and cardiac myocytes (188), and in isolated human neutrophils in which Ang1 reduces both apoptosis and necrosis (189). The anti-apoptotic effect of Ang1 on ECs appears to ultimately involve PI3K pathway (146, 147, 186). In neutrophils, the intracellular mechanisms that mediate the survival effects of Ang1 remain to be elucidated; however, because IL-8 production appears to be necessary (189), it is possible that p42/44 – which mediates IL-8 production in neutrophils (190) – participates in this process.

It is unclear to what extent Tie2 is involved in Ang1's pro-survival effects. Some groups did not test the requirement for Tie2 *directly* via blocking antibodies, but concluded it is involved because of an increase in the phosphorylation of downstream signalling effectors after stimulation with Ang1 (187). Others have shown that a soluble Tie2-Fc fusion can block the effects of Ang1 on HUVEC survival, at a 2-μg/mL concentration corresponding to a 5-fold Molar excess[12] over Ang1 concentration (146). In neutrophils, despite lower expression of Tie2 than in HUVECs, it takes significantly more blocking antibodies (up to 20 μg/mL) to inhibit only one component of Ang1-induced pro-survival effects, as the blockade impacts neutrophil necrosis but

[10] This includes the sensitivity to angiopoietins, the overall properties of the vascular bed from which the cell model originates, the experimental conditions, and finally, interpretational bias.
[11] Ang1 protects neurons from apoptotic but not from necrotic stress.
[12] The reported K_D value of Ang1 for Tie2 is about 3 nM - calculated as 173 ng/mL when the molecular weight of Ang1 is 57.7 kDa (Hori *et al*, 2004).

not apoptosis (189). The ability of Ang1 to promote survival of mouse cortical neurons (188) just as well as cardiac myocytes (188) also casts doubt as to the involvement of Tie2: while mice neurons express Tie2, myocytes do not, leaving the possibility that Ang1-induced myocyte survival may be mediated by other molecules – possibly by integrins (see **1.4.6 Integrins**) (188, 191).

Proliferation. Ang1 has been reported to have no (143, 192), weak (152) or significant impact on EC growth (193). The varying response to Ang1 may be due to different EC types. For instance, arterial, venous or lymphatic bovine ECs (bmVECs, bmAECs, and bmLECs, respectively) from the same mesenteric vascular bed respond very differently to an *in vitro* administration of human Ang1[13], whereby there is no proliferative response in bmVECs, a weak response in bmAECs, and a slightly higher response in bmLECs compared to bmAECs (152). In all three EC cell types, Ang1 only weakly increases Tie2 and Erk1/2 phosphorylation (albeit at a higher phosphorylation rate in bmLECs), which might explain why the proliferative response is so weak.

Migration. As previously covered (see **1.1 Angiogenesis: Fundamentals**), cell motility is a fundamental part of neovascularization that occurs at various stages of the process, and impacts multiple cell types (7). Briefly, at the level of the endothelium, EC migration encompasses three components: First, a single EC is selected to migrate ahead of the pack to produce a sprout, which is essential for guiding and coordinating the movement of the remaining ECs (sprouting). Stalk ECs then move and re-arrange to form the stem of the new vessel (tube-like arrangement). Finally, additional migration of ECs occurs to prune the new vessel structure (remodelling). Cell migration also describes the movement and recruitment of perivascular cells to the new vessel during the maturation step. Thus, because cellular motility is multifaceted, the ability of Ang1 to promote it will undoubtedly be cell model-dependent.

Although early studies showed that Ang1 had no effect on EC motility (143, 194), most subsequent reports indicate otherwise. *In vitro*, Ang1 stimulates migration of HUVECs (192) and all three bovine mesenteric EC types (bmVEC, bmAEC and bmLEC) (152), and promotes EC

[13] The authors make the assumption that bovine and human Tie2 would both be activated in a similar fashion by human angiopoietins, based on 95% receptor sequence homology and conserved residues at the Ang-binding site.

tubule-like structure formation on collagen/matrigel matrices (195-197). Besides altering EC cytoskeletal properties, Ang1 likely facilitates EC motility by stimulating the secretion of proteinases, such as MMP3 and MMP9 precursors, and inhibiting the secretion of tissue inhibitor of metalloproteinase (TIMP)-2 (197), all of which contribute to the degradation of the basement membrane. Finally, Ang1 actively contributes to the maturation process by promoting the recruitment and migration of pericytes to the newly-formed vessel, demonstrable both *in vitro* with the matrigel invasion assay (196), and *in vivo* in a mouse hindlimb ischemia model (195).

Finally, in addition to promoting endothelial migration, Ang1 can promote motility of other non-endothelial cell types. The involvement of Tie2 in this regard does not appear mandatory. In effect, Ang1 induces the migration of Tie2-positive rat aortic smooth muscle cells and rat dorsal ganglia (thereby promoting neurite outgrowth) (198, 199), Tie2-negative fibroblasts (200), and Tie2-positive (151, 201) and negative (163) phagocytic leukocytes, including neutrophils (145), eosinophils (162), monocytes and macrophages (163).

1.4.3.3 Cellular effects and signalling of Ang2

Unlike their Ang1 and Tie2 null counterparts, Ang2 null mice all survive *in utero* to full term, and even appear normal at birth. However, postnatal, the severity of the *Ang2*$^{-/-}$ genotype increases depending on the genetic background of the mice (**Table III**, p.45): While the vast majority of 129/J mice die in the first 14 days after birth as a consequence of severe lymphatic dysfunction (from chylous ascites[14] and edema) (149), C57BL/6 mice develop only transient postnatal chylous ascites and their postnatal mortality is less than 10% (202). The 129/J *Ang2*$^{-/-}$ model was useful in showing that Ang2 is dispensable for embryonic vascular development but is required for post-natal remodelling, especially noticeable in the retina and the lymphatic system. Indeed, major defects in retinal arborisation of these mice after birth are caused by the retina's failure to initiate vascular regression (149). Moreover, as per the vasculature, Ang2 is not requisite for the establishment of the embryonic lymphatic system but is required for proper lymphatic patterning and normal functioning shortly after birth.

[14] Chylous ascites is a disorder that arises when lymphatic drainage is impaired, resulting in the accumulation of chyle, a milky-like fluid rich in lymph and triglycerides, in the peritoneal cavity.

In the mid-1990s, after the discovery of Ang1, Maisonpierre *et al* used homology screening and identified a second Tie2 ligand, Ang2; this important study ultimately resulted in labeling the ligand as a natural antagonist of Ang1 (148). First, using both human and bovine endothelial-derived cell lines, the group observed that Ang2 fails to induce Tie2 phosphorylation, even at supra-physiological[15] concentrations (1250 ng/mL) ("supra" in relation to basal circulating levels of Ang2, found here (203, 204)). Instead, Ang2 competitively blocks Ang1-driven phosphorylation of Tie2 when used at a concentration of 4 to 8-fold molar excess. In contrast, in a non-EC cell model using NIH 3T3 fibroblasts with ectopically expressed Tie2, Ang2 is equivalent to Ang1 in inducing Tie2 phosphorylation. These results prompted two possible explanations: Ang2 is a general agonist, but ECs contain signalling molecules that can discriminate between the angiopoietins and selectively prevent Ang2 from activating Tie2. Alternatively, Ang2 is an antagonist in an EC context, but not in other cell types.

The group of Maisonpierre *et al* also looked at the temporal expression patterns of Ang1 and Ang2, observing that Ang1 appears earlier than Ang2, at E9-11, and is found most prominently in the heart myocardium surrounding the endocardium (143). Later in development, circa E12.5-13, Ang1 becomes much more widely distributed, especially in mesenchymal tissues surrounding the vasculature (143). In contrast, Ang2 is not expressed in mice in developing heart or around any other vascular beds at early embryonic stages E9-10.5 (148). Later in development, at E12.5, both Ang2 and Ang1 are found associated with the embryonic vasculature, with Ang1 showing wide diffusion in mesenchyme tissues surrounding vessels, and Ang2 expression being punctate and restricted to cells in the lumen of vessels (143, 148). On the other hand, in the adult rat, Ang2 is localized to sites of active vascular remodeling; for instance, in the female reproductive tract (ovary), Ang2 is either strongly co-expressed with VEGF leading the vessel sprout, or present alone at the site of vessel regression (in atretic follicles) (148). Meanwhile, Ang1 expression appears to follow vessel formation, suggesting that Ang1 plays a later role than VEGF. This expression pattern led to the proposal of a model in which Ang2, by antagonizing Ang1, destabilizes the endothelium and prepares it for the robust pro-angiogenic action of VEGF.

[15] Physiological concentrations of circulating Ang2 are not often mentioned or measured in clinical experiments. Based on two studies from the literature, in healthy volunteers (n=6-29), baseline ("control") blood levels of Ang2 were circa 0.5 ± 0.20 ng/mL and those of Ang1 were circa 67 ± 20.7 ng/mL. These levels were significantly higher in critically ill patients, with Ang2 levels increasing over 40-fold in the cohort afflicted with septic shock (Kumpers *et al*, 2008; Kumpers *et al*, 2009).

In accordance, in the absence of another pro-angiogenic stimulus, Ang2 alone leads to vessel regression (148, 205).

Further to the above, and keeping in mind the time at which Ang1 is expressed in embryos, Maisonpierre *et al* injected an Ang2-containing vector with Tie2 transcriptional elements (this construct would direct Ang2 into all vascular structures where Tie2 is normally expressed) into developing mice embryos with the expectation that, if Ang2 were an antagonist, such a treatment would phenocopy Tie2 and Ang1 knockout mice. In effect, these transgenic Ang2-overexpressing embryos die *in utero* at E9.5-10.5, even earlier than either $Ang1^{-/-}$ or $Tie2^{-/-}$ embryos (**Table III**, p.45). Sections and analyses performed at E9 revealed several defects in the vasculature – including vessel discontinuities, poor association of ECs to the basement membrane and collapse of the endocardial lining of the heart – that are highly reminiscent of defects observed in Ang1/Tie2-deficient mice. However, the phenotype of these Ang2 transgenic mice is more severe, especially in the extent of vascular discontinuities, possibly due to the timing and spread of Ang2 expression (and how that relates to Tie2/Ang1 expression), the cellular context or varying VEGF and/or FGF gradients in the developing vasculature.

1.4.3.4 Ang2 and the endothelium

While the role of Ang1 as an EC agonist is not contested, there is little consensus for the true function of Ang2 in endothelial biology. The general belief is that Ang2 does not exert a direct stimulatory role on ECs, but instead simply antagonizes Ang1, thereby annulling Tie2 activity and its associated quiescence-promoting effects and allowing the endothelium to be more responsive to activating stimuli. This dogma generally holds in vascular EC biology, but fails in the context of the endothelium of the lymphatic system (149, 152): In effect, in 129/J mice lacking Ang2, replacing the gene locus of Ang2 with that of the agonist Ang1 (thereby generating $Ang2^{Ang1/Ang1}$ mice) rescues the lymphatic defects, but not the angiogenic ones, suggesting that Ang1 and Ang2 can interchangeably act as agonists in lymphatic tissue (149).

Though the above suggests some functional redundancy with Ang1, Ang2 can drive distinct cellular events that could be regarded as agonistic or antagonistic, depending on the context. Lymphatic system aside, in peripheral vascular biology, Ang2 competitively antagonizes binding of Ang1 to Tie2 in *in vitro* binding studies (206) and functional assays (164, 207), as well as *in vivo* (208). Meanwhile, many conflicting reports – sometimes even when performed in

the same cell models – have reported that Ang2 exerts no (148, 152, 209), weak (152, 206), or strong (206, 210-212) agonistic activity, assessed by looking at Tie2 phosphorylation and certain changes in EC behavior under various conditions of Ang2 exposure. As outlined below, there are multiple parameters that can alter EC response to Ang2, compounding the difficulty of determining its true function.

Ang2 origin. There is evidence that the origin of Ang2, whether it is produced endogenously or is administered as a recombinant exogenous product, has a significant impact on the activities of the ligand. Indeed, it is reported that at physiological concentrations, exogenous Ang2 does not lead to Tie2 phosphorylation or to anti-apoptotic effects (209). In contrast, the endogenous form of the ligand seems to stimulate both. By eliminating the endogenous production of Ang2 in HUVECs via siRNA, basal phosphorylation of Tie2 and the downstream effector protein Akt is reduced, implying that naturally produced Ang2 is responsible for phosphorylating and activating both proteins. Targeting endogenous Ang2 via siRNA also leads to an increase in caspase-3 and caspase-9 activity, proteins that generally stimulate apoptotic activity, implying that endogenous Ang2 can work as a pro-survival factor (206). Interestingly, when FOXO1 gene is overexpressed in both HUVECs and bovine lung microvascular ECs, it promotes the secretion of relatively low doses of Ang2 (circa 30 ng/mL); however, this quantity of Ang2 is sufficient and responsible for phosphorylating Tie2 and Akt and for promoting EC survival, as anti-Ang2 antibody treatment blocks all three effects (210).

Effect of dosage. The concentration of Ang2 appears to play a role in determing the extent of its agonistic properties, which may skew interpretrations as to the ligand's "true" function. Indeed, a high concentration of exogenous Ang2 (800 ng/mL) (209) can mirror the effects of a low dose of Ang1 (200 ng/mL) (146) with regards to Tie2/PI3K phosphorylation and the induction of endothelial survival. Meanwhile, lower doses of Ang2 (50 - 400 ng/mL) are either ineffective (209) or have weak activity (206).

Time is of the essence. In addition to concentration, the duration of exposure to Ang2 is at times a determinant of activity. For instance, despite high concentrations of the ligand, it takes a prolonged exposure of bovine mesenteric venous and arterial cells to Ang2 (800 ng/mL) to attain

detectable Tie2 phosphorylation, though detection remains weak (152). This is in contrast to an equivalent dose of Ang1, whereby a short-term exposure is sufficient to promote not only Tie2 and Erk1/2 phosphorylation, but also cell survival and migration. In a human EC model (HUVECs), a brief exposure to Ang2 also fails to phosphorylate Tie2; however, extending the duration of exposure to Ang2 leads to an increase in Tie2 phosphorylation and extensive capillary-like tube formation in a 3D fibrin matrix, in a similar fashion to Ang1 and VEGF (212).

EC origin. Inter- and intra- species differences and the intrinsic properties of ECs (vascular bed, availability of downstream signalling effectors) generate differential responses to Ang2, similarly to their behaviour with Ang1. For instance, in humans, Ang2 promotes marked endothelial progenitor cell (EPCs) migration but fails to induce migration of HUVECs (213) or microvascular ECs (HMECs) (192), suggesting that terminally-differentiated ECs respond differently to Ang2 than do their progenitors. Species differences are evident even when using a high concentration of Ang2: In mouse brain capillary ECs, a markedly high dose of Ang2 (2 μg/mL) leads to phosphorylation of Tie2 and cellular migration (211). In HUVECs, Ang2 (800 ng/mL) also promotes Tie2 and Akt phosphorylation (209). In contrast, the ligand is only weakly capable of phosphorylating Tie2 in bovine mesenteric venous and arterial cells, and fails to promote survival, migration (152, 192) or Erk1/2 phosphorylation in the bovine cell lines (152). Similarly, while Ang2 promotes a rapid and sustained synthesis of platelet-activating factor (PAF) in BAECs, it fails to do so in HUVECs (214). Finally, highlighting the impact of different vascular beds from the same species on EC responsiveness, Ang2 fails to phosphorylate Tie2 or p42/44 in mesenteric venous and arterial cells lines, but is highly effective at inducing Tie2, p42/44, p38 and Akt phosphorylation in BAECs (214).

In vivo. As illustrated above, the *in vitro* component of Ang2 strongly suggests context-dependent agonistic/antagonistic activities. There is some evidence *in vivo* to suggest that Ang2 adopts the role of an agonist in vascular biology: For instance, an intravenous injection of Ang2 results in Tie2 and Akt phosphorylation in extracts from the heart taken at 15-30 minutes post-treatment, albeit with slightly less potency than an Ang1 injection (210). Although it is possible that these effects are due to an unidentified secondary mediator released in response to Ang2, the

fact that Tie2 and Akt phosphorylation occurs so rapidly makes a good argument for direct Ang2–Tie2 interaction.

There is also indication that the timing of Ang2 expression may alter the physiological response to the ligand: In a study where Ang2 is overexpressed in the retina of transgenic mice, the expectation was that the excess of Ang2 alone would lead to capillary regression in the developing eye. Instead, these mice actually display a 46% increase in vascular density of the capillary network at day 10 after birth and a reduction in pericyte coverage, compared to wild type littermates (215). Similarly, in rats, a transcorneal injection of Ang2 (10 ng) at 5 days after birth (before the normal onset of pupillary membrane regression) produces a rapid dose-dependent increase in the diameter of capillaries, an effect that involves direct changes in EC morphology rather than pericyte recruitment (216). In stark contrast**,** Ang2 alone fails to promote neovascularization in an *in vivo* model of angiogenesis (the mouse corneal micropocket assay) (217) or to induce EC junction re-arrangement (218).

Collaboration with VEGF. Several groups have reported that VEGFs and the angiopoietins, particularly VEGF and Ang2, play synergistic and complementary roles in vascular development. Based on spatio-temporal expression patterns of angiogenic factors in the adult rat, Maisonpierre *et al* postulated that the ability of Ang2 to antagonize Ang1-Tie2 signalling destabilizes the endothelium and sensitizes it to the sprout-inducing action of VEGF, in the absence of which vessels recede. Thus, Ang2 can promote two seemingly opposing vascular activities, but the determination of which path to take – growth or regression – is decided by the biochemical environment, notably by potent angiogenic factors such as VEGF. In support of this, in aged corpora lutei and in follicular atresia[16], two physiological states in which surrounding vessels naturally undergo extensive regression, Ang2 mRNA is uniformly present in large amounts while VEGF mRNA is scarce (148). The impact of the Ang2/VEGF collaboration on vessel development has been demonstrated more directly in rodent models of ocular vascularization: In the mouse corneal micropocket assay, Ang2 strongly potentiates the action of VEGF on neovascularization (217). Perhaps more convincingly, in the absence of endogenous Ang2,

[16] Condition in which large vesicular follicles fail to ovulate and the surrounding vessels in the theca interna recede as the follicle regresses.

knockout mice (*Ang2*$^{-/-}$) are unable to initiate ocular (hyaloid) vessel regression and instead develop major vascular defects in the eye shortly after birth (149). On the other hand, in the absence of endogenous VEGF, Ang2 was shown to promote EC death and vascular regression (216).

The collaboration between VEGFs and angiopoietins appears to extend to pathological conditions. It has been previously shown (219) that a subset of tumors develops the ability to hijack host vessels to support its growth, thereby forming initially well-vascularized masses. Perhaps as a host defence mechanism, the coopted vasculature does not immediately undergo angiogenesis to support the tumor but instead undergoes substantial regression, resulting in a secondarily avascular tumor and massive tumor cell loss. Ultimately, however, the remaining tumor is rescued by robust angiogenesis at the tumor margin. The pattern of VEGF and Ang2 expression as the tumor cycles between regression and angiogenesis is highly indicative of synchronized activity between the two growth factors. In effect, in several models of rat and human brain tumors (glioblastoma), there is a marked induction of Ang2 expression in coopted vessels (but not in surrounding normal tissues) before VEGF expression is induced, which is thought to mark these vessels for regression (219). As blood supply becomes inadequate for the growing tumor and forces it into a hypoxic state, a significant induction of VEGF expression is observed in conjunction with Ang2, coinciding with robust angiogenesis in the tumor periphery (205, 219). Thus, in tumors, Ang2 and VEGF appear to reprise the roles played during vascular remodeling, making co-blockade an appealing target for anti-cancer therapies (7).

Table III: Angiopoietin and Receptor function by gene targeting

Family member	Phenotype of KO mouse	Physiological role
Ang1$^{-/-}$	Embryonic lethal [E.11.5 – 12.5] (15) Severe heart and vascular defects, poorly formed vascular network, defects in branching. Poor association of ECs with basement membrane and perivascular cells, especially in the endocardium.	Vessel remodeling, maturation: increases coverage by perivascular cells, anti-permeability action EC survival and proliferation (17)
Ang1 overexpression (dermis)	Dramatic increase in diameter of dermal vessels, but not in number (140).	
Ang2$^{-/-}$	Strain-dependent lethality: - 129/J: Normal at birth, 90% lethality by PD14 (149) - C57Bl/6: normal at birth, less than 10% lethality into adulthood (C57Bl/6) (202). 129/J: Major defects in post-natal retinal vessel remodeling; in lymphatic vasculature, profound lack of central lacteals (capillaries that absorb dietary fats) in intestinal villi, poor association of lymphatic perivascular cells to ECs. Impaired inflammatory response (C57Bl/6) (202, 218).	Remodeling of blood and lymphatic vessels Sensitizes the endothelium (in angiogenesis and inflammation) to action of endothelial modulators. In the absence of VEGF, causes vessel regression (148). Tie2 agonist in the lymphatic system and Tie2 antagonist in the retinal vasculature (149)
Ang2 overexpression	Embryonic lethal [E9.5-10.5] “Moth-like” discontinuous vasculature Poor association of endothelial cells in the heart with basement membrane; endocardial lining collapse (148).	
Tie1$^{-/-}$	Embryonic lethal [E13.5-14.5] Loss of vessel integrity, widespread edema and hemorrhage (173). Lymphatic vascular abnormalities (220).	Support for later stages of angiogenesis and EC proliferation (176). Negative regulation of Ang1 activity (164). Lymphatic vessel development (220).
Tie2$^{-/-}$	Embryonic lethal [E9.5-12.5] Vasculature does not develop beyond capillary plexus Impaired vessel remodeling, hematopoiesis and heart development (174).	Blood and lymphatic vascular maintenance, remodeling
Tie1 and Tie2 double KO	Embryonic lethal [E9.5- 10.5] Similar but more severe defects than Tie2-/- (175).	Adult angiogenesis Formation of microvasculature during late organogenesis

1.4.4 Angiopoietins in inflammation

Because angiopoietins are involved in dictating endothelial activation, the family will almost inevitably impact other physiological processes where endothelium participation is required. While the process of inflammation is discussed in greater detail in **1.5 Inflammation**, it can be easily understood how Ang1, through its ability to promote homeostasis and inhibit permeability and vascular leakage, would have an indirect role in inhibiting inflammation (thus functioning as an "anti-inflammatory" agent). In parallel, because it can antagonize Ang1 function and enable the action of factors such as VEGF, Ang2 may exhibit certain pro-inflammatory activities. However, just as their agonist/antagonist relationship is neither black nor white, the role of angiopoietins in inflammation paints a far more complex picture, one that can be independent from the ligands' endothelial activities. Indeed, angiopoietins have been shown to directly activate leukocytes in the absence of the endothelium or of any other tissues ((151), among others). A literature review of the role of Ang-Tie2 in inflammation showcases the polyvalence of this family, leaving many unanswered questions, and strongly cautioning the reader against committing the angiopoietins to a pro- or anti-inflammatory classification.

1.4.4.1 Inflammatory action of Ang1

Endothelial tight junctions, which are composed of small trans-membrane proteins such as occludins, claudins, and junctional adhesion molecules (JAMs), are an important determinant of vessel permeability. For instance, blood-brain-barrier (BBB) capillaries are composed of ECs that are particularly tight – more so than anywhere else in the peripheral vasculature – to prevent the majority of blood elements from reaching the brain. Conversely, postcapillary venules express lower levels of tight junctions and are therefore more sensitive to permeability factors and leukocyte transmigration (221).

Anti-inflammatory. Ang1 was shown to influence the organization of EC junctions and in doing so, can counteract some elements of EC function that are conducive to the initiation and development of an inflammatory process: For instance, *in vitro*, Ang1 prevents the action of VEGF, thrombin, bradykinin and histamine (140) on increasing permeability of ECs from the peripheral vascular system, and the ligand directly enhances mRNA expression of tight junctions (occludins) in brain capillary ECs (180). In mouse skin *in vivo*, co-overexpression of VEGF and

Ang1 shows an additive effect on angiogenesis but unlike VEGF overexpression alone, the combination results in leakage-resistant vessels with little inflammation (17). In other mouse models, Ang1 downregulates pulmonary inflammation by limiting granulocyte infiltration and Ang2 and cytokine production in murine lungs subjected to ventilator-induced injuries (222), and also attenuates inflammation in LPS-induced injuries (223). Interestingly, in apparent contrast with the protective effects of Ang1 in LPS-challenged animals, Ang1 does not prevent alveolar-capillary permeability, pulmonary edema (i.e. vascular leakage) or impaired gas exchange in the ventilation model (222). Going on the assumption that LPS-induced plasma leakage primarily involves ECs while mechanical stretch destabilizes both alveolar-epithelial and capillary-endothelial barriers, the authors propose that the reason for this discrepancy is that Ang1 treatment only modulates endothelial inflammation.

Additionally, Ang1 inhibits the early steps of the cascade that leads to leukocyte infiltration in inflamed tissues, as follows: First, Ang1 limits the ability of inflammatory agents to increase leukocyte attraction; for instance, Ang1 inhibits the ability of thrombin to stimulate EC release of potent leukocyte chemoattractant IL-8 (224). Ang1 also reduces the capability of leukocytes to adhere to the endothelium activated by VEGF, TNF-α and thrombin, by altering the expression of endothelial E-selectin and adhesion molecules that belong to the immunoglobulin family (ICAM-1 and VCAM-1), proteins that respectively ensure the rolling and firm adhesion of leukocytes onto the endothelium (225, 226). Thus, by sealing the vasculature and attenuating the expression of endothelial surface adhesion molecules that recruit inflammatory leukocytes, Ang1 exerts a protective anti-inflammatory effect.

Pro-inflammatory. On the other hand, Ang1 exerts certain pro-inflammatory activities on ECs, which in some cases appear to directly contradict the observations noted above: In cultured bovine aortic ECs (BAECs) but not in HUVECs, Ang1 promotes endothelial synthesis of PAF (214), a phospholipid that acts as trigger and amplifier of inflammatory and thrombotic cascades (227). Additionally, *in vitro*, Ang1 increases the translocation to the surface of ECs of P-selectin, a protein that mediates the rolling of leukocytes onto the endothelium; in accordance, neutrophil adhesion onto EC monolayers is thus increased (151). Ang1 even appears to relax endothelial tight junctions in the airway capillaries of mice (228), thereby facilitating the process of leukocyte transmigration. Not only that, but in this same model, overexpression of Ang1, acting

via Tie2, induces a phenotype change in capillaries that renders them more amenable to leukocyte transmigration: in effect, Ang1 leads to remodeling of these capillaries into a postcapillary venule phenotype[17] expressing ICAM-1, P-selectin and EphB4, which are typical lineage markers of venous ECs (228). These studies underline the ability of Ang1 to exert differential and at-times opposing activities depending on the choice of experimental parameters.

1.4.4.2 Inflammatory action of Ang2

Unlike the 129/J *Ang2*$^{-/-}$ model, the high survival rate of C57BL/6 *Ang2*$^{-/-}$ mice allowed for a more exhaustive look at the impact of Ang2 deficiency on specific tissues. Ang2 appears to control the responsiveness of the endothelium to acute inflammatory stimuli, but shows no direct stimulatory effect by itself**.** Looking at vascular permeability *in vivo*, Ang2 null mice (*Ang2*$^{-/-}$) show a significantly attenuated response to the hyperpermeability-inducing action of histamine, bradykinin and VEGF (218). Additionally, these mice exhibit a decreased inflammatory response to infection compared to normal littermates, with significantly less neutrophil infiltration in the peritoneal cavity in the first hours of exposure to thioglycollate or *S. aureus*. However, in a long-term (36 h.) lung infection experiment with *Streptococcus pneumonia*, wild type and Ang2-deficient mice show no difference in their ability to mount an inflammatory reaction (202). These observations are indirect evidence that Ang2 can transiently modulate endothelial junctional properties.

Beyond EC junctions, there is conflicting information about the role of the ligand in modulating expression of EC adhesion molecules, and thus, of leukocyte adherence and/or transmigration. In acute conditions, Ang2 increases EC surface expression of P-selectin and neutrophil capture by EC monolayers (151). However, in *Ang2*$^{-/-}$ mice, rolling of leukocytes in response to TNF-α is normal, but then firm adhesion is impaired (202), suggesting that Ang2 may only affect Ig-type adhesion molecules. On the other hand, while Ang2 alone increases neutrophil adhesion onto ECs *in vitro* (151), it can only promote monocyte adhesion in the presence of sub-saturating concentrations of TNF-α (202). Taken together, these observations suggest that Ang2

[17] As outlined in **1.4.4.1 Inflammatory action of Ang1**, postcapillary venules are the preferred site for leukocytes to transmigrate because they express fewer junctional proteins.

may modulate a leukocyte-specific subset of adhesion molecules, but it is more likely that it plays a permissive role that prepares the endothelium for the action of other inflammatory cytokines.

1.4.5 Angiopoietins and myeloid biology

Recent studies in transgenic mice demonstrated that the majority of adult blood cells derive from a *Tie2*$^{+}$ hematopoietic precursor (229)**,** and Tie2 continues to be expressed downstream in multiple lineages and by the majority of the progeny, including by myeloid progenitors (229). The presence of functional Ang-Tie signalling mechanisms in myeloid cells and the corresponding biological significance are explored in more detail below.

1.4.5.1 Neutrophils

When our laboratory first discovered that Ang1 could stimulate P-selectin translocation to the endothelial cell-surface, we wondered whether the resulting increase in this adhesion molecule would have a direct impact on leukocyte capture and adhesion. In effect, when freshly isolated and untreated human neutrophils were added *in vitro* to EC monolayers stimulated with as low as picomolar concentrations of Ang1 or Ang2, they adhered more efficiently than to untreated control ECs. Interestingly, when both ligands were co-administered, we observed a supplementary increase in neutrophil adhesion without any additional P-selectin translocation. This observation raised two plausible explanations: First, that angiopoietins could impact the expression of other unidentified EC adhesion molecules, thereby assisting P-selectin in capturing and anchoring neutrophils onto the EC surface. Alternatively, that angiopoietins could directly interact with neutrophils to induce their "adhesive state", for example by promoting the expression of neutrophil-specific adhesion molecules[18]. While we did not discount the first possibility given the well-documented effects of Ang1 on the endothelium, we explored the latter hypothesis and discovered that the angiopoietin signal-transducing receptor, Tie2, was expressed on the neutrophil cell-surface (151). This was confirmed shortly thereafter by an independent group (230).

[18] Leukocyte-specific adhesion molecules and the process of leukocyte recruitment, rolling and adhesion are coverered in greater detail in **1.4.6 Integrins** and **1.5.3 Leukocyte recruitment**.

Interestingly, Ang-Tie signalling exhibits certain distinguishing properties in neutrophils vs. ECs: First, Tie1 is not expressed on neutrophils (151), indicating that the mechanisms for negatively regulating angiopoietin activity may be different than in ECs. Second, the agonist-antagonist relationship between the two ligands is even less clear than in ECs. In fact, Ang1 and Ang2 exhibit a fair degree of overlap in function with human neutrophils tested *in vitro*: Both ligands are equipotent in sensitizing neutrophils and increasing their migration towards an IL-8 gradient, their adhesion onto EC monolayers, and their synthesis of PAF. Finally, the two ligands are both capable of recruiting the PI3K pathway and promoting Akt phosphorylation in neutrophils (151). That being said, certain neutrophil responses *in vitro* are unique to Ang1; for instance, the ligand extends neutrophil lifespan (189) and induces IL-8 *de novo* synthesis and secretion, whereas Ang2 fails to fulfill either of those tasks (190). Whether the exclusive Ang1 activities result in pro- or anti-inflammatory responses (an increase in neutrophil viability means a delay in neutrophil degranulation and/or a slow consistent release of pro-inflammatory mediator content; IL-8 is an inflammatory cytokine) or even pro-angiogenic effects (neutrophils release large amounts of MMPs that degrade EC basement membrane and IL-8 is a pro-angiogenic factor) will likely be dependent on surrounding biochemical gradients and the state of responsiveness of the endothelium.

Finally, a particularly interesting observation is that in all our neutrophil experiments, the addition of Ang2 to an equivalent dose of Ang1 does not antagonize the action of the latter ligand, as would have been expected from a competitive, and seemingly equipotent, antagonist. This further suggests differential Ang-Tie signalling properties in myeloid biology compared to ECs.

1.4.5.2 Eosinophils

These granulocytes constitute the bulk of cell infiltrates during infectious diseases, and are important mediators during allergic reactions and in the pathogenesis of asthma. Similarly to neutrophils, eosinophil migration into sites of inflammation/infection is under tight cytokine control, generally driven by CXCL type of cytokines.

Only one study has looked at the impact of angiopoietin/Tie2 signalling on eosinophil behavior, demonstrating the presence of a functional Tie2 receptor on the cell-surface of isolated human circulating eosinophils (162). Of the two angiopoietins, only an Ang1 gradient was shown

to promote *in vitro* eosinophil chemotaxis; this migration towards Ang1 was impaired by the addition of blocking-Tie2 antibodies but also by incubation with Ang2, suggesting that Tie2 mediates Ang1-induced chemotaxis, and that Ang2 retains its antagonistic action in this context. Finally, chemotaxis towards Ang1 was mediated by PI3K, partially by Src, with no contribution from PKC. The study did not look at the expression of Tie1, or the specific contributions of other signalling pathways such as Erk1/2 or p38 MAPK.

1.4.5.3 Monocytes/Macrophages

The general understanding of monocyte biology is that these myeloid cells can be subdivided into two main groups: A short-lived "inflammatory" subset constituting the majority of monocytes and that homes to inflamed tissues, and a longer-living "resident" subset, that shows a preference for non-inflamed environments (231, 232). The progeny of monocytes mirrors this subdivision, with cells differentiating into pro-inflammatory M1 (classically activated) or tissue repair/immunosuppressive M2 (alternatively activated) macrophages, a process referred to as macrophage polarization. M1/M2 phenotypic and functional characteristics are plastic, and are determined by the biochemical environment available to mononuclear cells during differentiation.

Monocytes contain high levels of both Ang1 and Ang2, but appear to selectively secrete only Ang1 upon contact with confluent ECs *in vitro* (182). This action induces EC survival-related signalling and suggests a possible role for Ang-Tie and monocyte-EC interaction in re-establishing homeostasis in inflammatory or ischemic conditions.

Investigation into the expression of Tie2 in human peripheral blood (PB) identified a small subset of Tie2-expressing monocytes (TEMs), comprised within the less abundant "resident" population, constituting 2-7% of PB mononuclear cells (PBMCs) in healthy donors (233). The Tie2 receptor on TEMs is functional, as Ang2 exerts an agonistic chemotactic effect on human TEMs *in vitro* in a dose and Tie2-dependent manner, but has no such effect on inflammatory monocytes (233). The impact of Ang1 in this context was not tested. In accordance, in transgenic mice overexpressing Ang2 in ECs only, prolonged expression of Ang2 (> 6 months) leads to a marked increase in leukocyte infiltration into several organs. The majority of the leukocyte infiltrate is made up of cells of the myeloid lineage, particularly of monocytes. Interestingly, the frequency of Tie2-positive cells is not increased in these transgenic mice during inflammation (peritonitis) (234).

Remarkably, TEMs preferentially home to tumors in both mice (235) and humans (233), compared to a near absence in non-neoplastic tissues[19]. The response to Ang2 *in vitro* provides a possible homing mechanism for tumors to selectively recruit TEMs.

TEMs appear to be endowed with a marked pro-angiogenic capability, providing a possible explanation for their preponderance in tumors: Using a gene targeting strategy that selectively eliminates TEMs, De Palma *et al* showed that tumor growth in mice was stunted; conversely, in a tumor transplantation model, injection of TEMs lead to an increase in tumor neovascularization (233, 235). These observations prompted the authors to suggest that TEMs represent a circulating reservoir of cells *committed* to a proangiogenic function. However, Ang-Tie2 signalling in the progeny of monocytes does not seem to be particularly pro-angiogenic. First, there is no relationship between Tie expression and macrophage classification induced *in vitro*, as both M1 and M2 subtypes expressed Tie2 and Tie1; rather, Tie expression was dependent on the polarizing cytokine, as it was observed to occur to various levels following both M1 (IFN-γ; GM-CSF)- and M2 (M-CSF; IL-10; IL-4)-inducing cytokine stimulation (236).

Neither angiopoietin is capable of changing Tie expression levels or of driving macrophage polarization, indicating that Ang-Tie signalling alone is insufficient to drive an expression feedback loop or to promote differentiation. Furthermore, Ang1 and Ang2 alone are weak inducers of macrophage gene expression, independently of macrophage polarization conditions. Indeed, treatment with Ang1 and Ang2 alone had a marginal effect on the expression of 84 angiogenic genes (angiogenic growth factors and inhibitors) in both M1 and M2 macrophages.

Similarly to mRNA, macrophage cytokine protein production shows no particular trend with Ang stimulation but is more a function of the polarizing conditions: For instance, in M2 macrophages polarized by IL-10, both Ang1 and Ang2 induce a trend towards inhibiting anti-angiogenic thrombospondin (TSP)-2, fail at inducing IL-6, and only Ang1 is capable of promoting IL-10 production. In M1 macrophages polarized by interferon (IFN)-γ, Ang1 only promotes IL-6 (but not TSP-2 or IL-10 production) whereas Ang2 has no effect. Finally, both

[19] Tumor samples show a marked increase in TEM presence compared to surrounding normal tissues. However, in the blood, there seems to be no difference in number of circulating TEMs vs. healthy controls: Limited data from 7 cancer patients indicates that the incidence of TEMs in the blood of these patients (mean: 4.9% ± 3.0% of PBMCs) is not significantly different than in healthy donors (mean: 3.3% ± 1.5%) (Venneri *et al*, 2007).

angiopoietins cooperate with TNF-α in a highly overlapping manner to induce protein production of pro-inflammatory cytokines and chemokines, including IL-8, CCL-3, CCL-7, and IL-12B independently of macrophage polarization conditions (236). Taken together, these results indicate that Ang-Tie signalling in monocytes/macrophages is not restricted to cells with an angiogenic phenotype.

1.4.6 Integrins

Properties. Integrins are heterodimeric transmembrane cell adhesion proteins that are a crucial, yet often overlooked, component of cell signalling. They are ubiquitously expressed at the cell-surface and function as "receptors" for elements of the ECM such as collagens, fibronectin, fibrinogen and laminins, attaching the cell's cytoskeleton to the matrix. Integrins also have "counter-receptors" on other cells that facilitate cell-cell rather than cell-matrix interactions (review (237)).

Control of ECM-integrin signalling is bidirectional (238): matrix elements binding to integrin can activate intracellular signalling pathways that communicate the character and status of the bound matrix (outside-in stimulation). Alternatively, the cell can enhance or inhibit its own integrin activity, likely via phosphorylation, thereby controlling its cell-cell and cell-matrix interactions (inside-out stimulation). For instance, weak binding of a T lymphocyte to an antigen triggers intracellular signalling inside the T cell that activates its β2 integrins, consequently promoting stronger adhesion. Return of integrins to an inactive state allows the T cell to disengage (239).

Although they have no intrinsic kinase activity, integrins can activate both distinct downstream signalling mechanisms and influence signalling from conventional growth factor receptors. For instance, in cultured ECs, integrin adhesion to fibronectin potentiates the effect of growth factor on MAP kinase signalling (240). In addition, many types of cell cultures will not grow or proliferate in response to extracellular growth factors in the absence of integrin binding to ECM elements (241).

An integrin molecule is composed of two non-covalently associated transmembrane glycoprotein subunits called α and β. In humans, there are 9 types of β and 24 types of α in addition to a number of splicing variants, generating a wide range of human integrin heterodimers. One cell can express several types of integrin. For instance, endothelial cells

express at least 7 different types of integrins ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$) at varying times (242). The same integrin molecule can have different ligand-binding properties depending on the cell type where it is expressed, suggesting intrinsic cell-specific factors that interact with integrins and differentiate between ligands.

While β_1 subunits are found on almost all vertebrate cells ($\alpha_5\beta_1$ binds fibronectin; $\alpha_6\beta_1$ is a laminin), the β_2 (CD18) subunits are expressed exclusively on the surface of lymphoid and myeloid blood cells (243, 244), where they mainly mediate cell-cell interactions via binding to Ig-type adhesion proteins/counter-receptors. A genetic defect wherein white blood cells lack the entire family of β_2 subunits (*leukocyte adhesion deficiency*) exists in humans who suffer repeated bacterial infections. Finally, the β_3 integrins are found on a variety of cells, including platelets; a genetic deficiency in β_3 integrins leads to excessive bleeding (*Glanzmann's disease*).

Binding to growth factors. While other angiogenic growth factors including $VEGF_{165}$ and FGF2 are capable of regulating integrin expression on endothelial cells (245-247), the relationship between integrins and Ang-Tie signalling is not fully resolved. Ang1 and Ang2 do not contain an RGD motif or any known integrin-binding site, and to the best of our knowledge, no crystal structure of either angiopoietins or Tie2 has been resolved showing binding to integrin moieties. However, there is a body of evidence in the literature that suggests collaboration between Ang/Tie and integrins (notably the β_1, β_3 and β_5 integrin heterodimers). For instance, Tie2 and $\alpha_5\beta_1$ interact constitutively: when lysates from untreated ECs cultured on native ECM *in vitro* are immunoprecipitated with different integrins (of the β_1 and β_3 variety), Tie2 only co-immunoprecipitates with $\alpha_5\beta_1$ (whereas VEGFR2 is not detected in $\alpha_5\beta_1$ immunoprecipitates). As well, Tie2 appears to co-localize with $\alpha_5\beta_1$ in unstimulated ECs *in vivo* (248).

Interestingly, treatment with angiopoietins can alter the ability of Tie2 to complex with different types of integrins: Ang1 appears to engage $\alpha_5\beta_1$ in EC migration assays, and $\alpha_5\beta_1$, $\alpha_2\beta_1$ and $\alpha_v\beta_3$ in adhesion assays *in vitro,* in the presence of Tie2 (248) or independently of the receptor (191, 200, 249). In the chick chorioallantoic membrane (CAM) assay[20] (250, 251),

[20] The chick embryo CAM is an extraembryonic membrane, mediating gas and nutrient exchanges until hatching. Since the CAM has a dense capillary network, it has been commonly used as *in vivo* model to assess the angiogenic potential of mediators and various cell preparations, including tumor specimens (Ribatti *et al*, 2008). Briefly (methodology reviewed in Deryugina *et al*, 2008), fertilized chicken eggs are incubated at 37°C as soon as

blockade of either of $\alpha_5\beta_1$ or $\alpha_v\beta_3$ inhibits Ang1-induced angiogenesis, with $\alpha_5\beta_1$ blockade showing the greatest inhibitory effect (248). Finally, while monomeric Ang1 binds Tie2 very weakly and fails to phosphorylate it, it binds $\alpha_5\beta_1$ with a similarly low affinity (249) that is nevertheless capable of activating integrin-linked kinase (ILK) (178), a major regulator of β_1- and β_3-generated signalling (252). This activation is independent of Tie2 (178).

There is also evidence that Ang2 engages similar integrins as Ang1 upon binding to ECs (200, 253), with certain unique properties in activating integrin-mediated signalling. In HUVECs in vitro, of the two angiopoietins, only Ang2 stimulation induces complex formation between Tie2 and $\alpha_v\beta_3$ integrin and recruits focal adhesion kinase (FAK); subsequently, Ang2 stimulation leads to integrin internalization and ubiquitinylation**,** targeting it for lysosomal degradation (253). Additionally, under shear, Ang2 engages β_2-integrins in human monocytes to promote their adhesion to ICAM-1 and VCAM-1, whereas Ang1 shows no such effect; this Ang2 function is completely abrogated by anti-β_2 treatment and only partially by anti- α_L (CD11a) and α_M (CD11b), implying that other α subunits may contribute to this effect (234).

The above suggests that integrins can mediate certain angiopoietin activities, at times independently of Tie2, providing an attractive explanation for why the blockade of Tie2 does not always abolish angiopoietin signalling. Indeed, in a rat neuronal cell line that does not express Tie2, Ang1 promotes cell survival and neurite outgrowth in a Tie2-independent, β_1-integrin-dependent manner (254). In human neutrophils, blockade of Tie2 inhibits only some aspects of the *in vitro* functions of angiopoietins, but not all: For instance, the receptor is necessary for the chemotaxis of neutrophils towards a gradient of Ang1 or Ang2 (145), as well as for adhesion driven by the combination of Ang1 and Ang2, but not when either ligand is used alone (151). Additionally, Tie2 blockade abrogates Ang1's anti-necrotic effects, but has little influence over its anti-apoptotic properties (189). While the contribution of integrins has not been evaluated, given their abundance at the surface of neutrophils and the ability of angiopoietins to recruit them to elicit variable responses in other cell models, it is possible that they could be involved in mediating some of the aforementioned activities. However, the relative affinities between

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embryogenesis begins. Angiogenic materials are generally introduced over the CAM after day 7-10 of embryonic growth, either in-shell through a window cut in the egg shell, or *ex ovo* on the CAM of embryos grown in sterilized weigh boats. Angiogenesis occurs after 72-96 hours, at which point evaluation of the extent of vascularization can be made.

angiopoietins/Tie receptors and integrins, the role and contribution of the individual integrin subunits to signalling in different cellular contexts, and the nature of the interaction of integrins with monomeric vs. multimeric angiopoietins at both the endothelial and neutrophil cell-surface remain to be elucidated.

1.5 Inflammation

Inflammation is a physiological response to cellular injury, and may be initiated by physical (trauma, burn etc.) or chemical (toxins) agents, infections (microbial, viral and/or the immunological response to them), or necrotic tissue. Regardless of the initiating agent, the inflammatory process involves a multitude of cell types working closely together on either side of the endothelium, in a predictable sequence of cellular and biochemical events. These are: coagulation, vasodilation, increase in blood flow and vascular permeability, fluid accumulation, leukocyte recruitment, formation of granulation tissue and finally, tissue repair.

Phenotypically, inflammation classically manifests itself as redness, heat, swelling and pain. In reality, these symptoms represent a complex interplay of vascular, cellular, and neurological events that aim at removing infectious agents, re-establishing hemostasis and ultimately at restoring vessel integrity. The inflammatory orchestra is composed of platelets, neutrophils, mast cells, monocytes, endothelial cells and fibroblasts. The conductor is an intricate network of temporally and spatially regulated expression of cytokines. An inflammatory response is meant to be transient ("acute"); thus, the activities of its key regulators must be tightly controlled to avoid excessive tissue damage and spillover to normal tissue. Failure to downgrade the response and to dissolve the instigating signals turns the inflammatory process into a chronic pathological condition (255). The sequential steps of inflammation are covered in more details below.

1.5.1 Initiation

Coagulation. When tissue trauma or microbial invasion cause damage to the endothelial lining of a blood vessel, contact of the blood with cells that are not usually exposed to blood (the sub-endothelial surface) leads to a rapid activation and aggregation of platelets at the site of injury, first to plug off the leak and contain blood loss, then to support complex coagulation cascades (molecules on the surface of platelets are required for many of the reactions) and ultimately, to restore the integrity of the vessel (256). With the support of activated platelets, exposure of sub-endothelial tissue elements such as collagen, vWF and tissue factor (TF) to plasma components (Factor VII) activates coagulation cascades that lead to deposition of fibrinogen and maturation into fibrin strands, and establishing a scaffold that strengthens the platelet clot and traps platelets

and leukocytes. Platelets that are trapped in this fibrin 3-D scaffold also release leukocyte chemoattractants, permeability factors and growth factors that precipitate the inflammatory response, but also support the process of wound healing (255, 257).

1.5.2 Propagation

Vascular response. Disturbances in the cellular environment surrounding the site of injury causes all neighbouring tissues, including endothelial and connective tissues, leukocytes, platelets and mast cells, to release products. In particular, engagement of the coagulation process stimulates local ECs to secrete pro-inflammatory cytokines, such as TNF-α and IL-1, which further activate endothelial and vascular support cells in a paracrine manner (255). The action of secreted permeability factors (NO, VEGF, serotonin, PAF, histamine, leukotrienes, etc.) causes changes in endothelial cytoskeletal and junctional molecules such that cells can contract, increasing permeability and enabling outpouring of protein-rich fluid/plasma into the inflamed tissue. The motion of fluid brings along serum proteins, including components of the complement system and antibodies, necessary for the elimination of infectious agents.

1.5.3 Leukocyte recruitment

In addition to allowing plasma movement, ECs facilitate the localization, capturing and infiltration of circulating blood leukocytes into the injured tissue by displaying specific subsets of adhesion molecules that they normally would not express. ECs also secrete a special type of mediators that recruit leukocytes, called "chemoattractants". Tissue infiltration by circulating leukocytes is a three-step process involving rolling on the endothelium, firm adhesion, and transmigration across the endothelial barrier (258) (**Figure 11**, p.61)**.** Four superfamilies of adhesion molecules are involved in mediating these events: (1) integrins, (2) immunoglobulin-like proteins known as intercellular adhesion molecule (ICAM) 1 and 2, and vascular cell adhesion molecule (VCAM), (3) the selectins (L-, P- and E- selectin) and (4) mucin-like selectin ligands (PSGL-1, CD34, gly-CAM etc.). L-selectin is constitutively and exclusively expressed on leukocytes and binds to certain sulfated sialomucins that may be attached to several different endothelial plasma membrane proteins or proteoglycans (including CD34, MadCAM-1, and gly-CAM-1). On the other hand, E-selectin is exclusive for endothelial cells (ECs). P-selectin is found in the membrane of secretory storage granules in platelets (i.e., α granules) and endothelial

cells (i.e., Weible-Palade bodies). In general, stable microvessels do not express the arrangement of ligands necessary for recognition by L-selectin, nor do they show basal expression of E-selectin. However, when activated by TNF-α and IL-1, ECs synthesize and display E-selectin, endothelial ligands for L-selectin, and P-selectin translocation occurs to the EC surface (review (259)**)**.

At the same time, chemotactic signals are released and sensed by circulating leukocytes that begin their migration towards the site of injury. As cells circulating in the blood are subject to blood pressure and shear, their velocity must be reduced before they can be effectively captured by the endothelium. This is where the selectins and their counter-receptors come in, ensuring the tethering and rolling of leukocytes on the endothelial surface. Once at the site of the highest chemoattractant gradient, leukocytes cease their rolling and adhere more strongly by engaging integrins (inside-out/outside-in activation, see **1.4.6 Integrins**) and binding to endothelial receptors (ICAMs). Subsequently, integrins collaborate with ICAMs and platelet endothelial cell adhesion molecule (PECAM)-1 to facilitate the process of leukocyte transmigration (diapedesis) across the endothelial monolayer.

Chemotaxis. The initial step of leukocyte recruitment involves directing the leukocyte to where it is needed, and guiding it along the blood vessel to the specific site of injury where it eventually needs to cross. Chemoattractants are the "homing" molecules for leukocytes that express the appropriate counter-receptor. When a sufficient chemoattractant gradient is detected, leukocytes migrate towards it in a process called "chemotaxis". Numerous molecules have the potential to attract leukocytes, including transforming growth factor β (TGFβ), N-formyl-methionyl-leucyl-phenylalanine (fMLP), leucotriene B_4 (LTB_4) and the complement fragment C5a, which are capable of attracting and activating multiple leukocyte types (260-262).

Chemokines. The recent identification and characterization of a large family of related chemoattractant proteins, called "chemokines"[21], has shed additional light on the process of

[21] A distinction is sometimes made between the terms "cytokine" and "chemokine". While both are generally small proteins, a cytokine's role is to modify intracellular pathways and signalling, impacting cellular response. On the other hand, a chemokine primarily functions as a chemoattractant for specific leukocytes. Some cytokines, such as IL-8, are also chemokines.

chemotaxis. Chemokines are a large superfamily of highly homologous small peptides, comprising at least 46 ligands that bind to 18 functional G-protein-coupled receptors and two decoy or scavenger receptors (264). Thus, multiple chemokine ligands can bind to the same receptor. The two main chemokine superfamilies are named according to the arrangement of the first two cysteine residues near the amino terminus: In the CC family, the two cysteines are adjacent, whereas in the CXC family there is one amino acid between them. Adding an L or an R at the end of the name indicates that the chemokine is a ligand (CCL/CXCL) or a receptor (CCR/CXCR), respectively. Members of the CXC family, particularly CXCL8 (IL-8), typically recruit neutrophils, whereas the CC chemokines such as CCL2 (monocyte chemotactic peptide (MCP)-1), CCL5 (regulated on activation normal T cell expressed and secreted; RANTES) and CCL3 / CCL4 (macrophage inflammatory peptides (MIP)-1α and -1β), preferentially attract mononuclear cells (255).

Chemotactic hierarchy. As leukocytes travel through the vast vasculature and encounter different chemotactic signals from various microenvironments, it is unclear how they prioritize in terms of which signal to respond to. For instance, during a bacterial infection, multiple chemoattractants can be released from the bacteria themselves (LPS, formylated proteins) but also from nearby activated macrophages and endothelial cells (IL-1β, IL-8, PAF, TNF-α). These multiple sources of chemoattractants result in a complex milieu of opposing chemoattractant gradients, and therefore leukocytes must have the ability to selectively ignore certain chemotactic cues and "prioritize" those that will guide them to the site of infection. There is evidence that human and mouse neutrophils respond to such guidance signals in a "hierarchical manner"[22] (265), preferring to migrate towards formylated peptides and activated complement fragments (C5a) over "intermediate" chemokines such as IL-8 (266-268). Recently, chemotactic hierarchy was demonstrated to occur in vivo: in a model of necrotic foci, the gradient of CXCL2/MIP-2 was consistently observed to abruptly end approximately 100 to 150 μm proximal to the border of necrotic tissue, and yet neutrophils still migrated directly into the area of cell death. Thus, neutrophils were rapidly migrating away from high concentrations of CXCR2 ligands and

[22] By necessity, in the setting of multiple chemoattractants, the neutrophils must prioritize, favouring end target chemoattractants (e.g., formylated peptides and C5a) emanating from the site of infection over intermediary endogenous ones (e.g., IL-8 and LTB_4) encountered en route to sites of infection. (Heit *et al*, 2002)

towards the necrotic centre, suggesting that a "necrotactic" stimulus (perhaps a formyl-peptide receptor ligand) was hierarchically overriding CXCR2 signalling (269, 270).

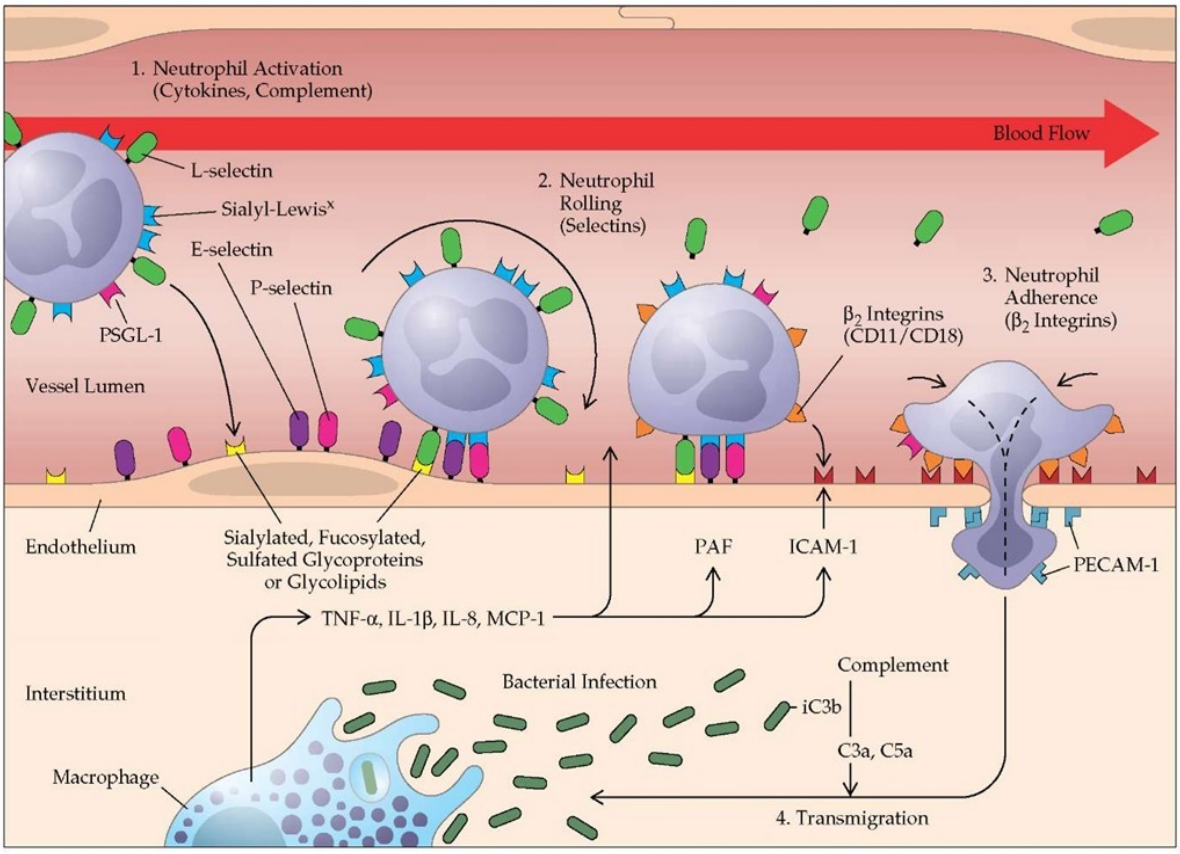

Figure 11: Sequential engagement of adhesion molecules in sepsis.

There are three types of selectin molecules: L-selectin (CD62L), E-selectin (CD62E), and P-selectin (CD62P). L-Selectin is constitutive and binds to certain sulfated or sialylated endothelial proteins or proteoglycans. E-selectin is inducible and binds carbohydrate ligands related to sialylated LewisX or LewisA moieties on leukocytes. P-selectin is stored in endothelial storage vesicles and binds to glycans and sulphated sialomucins displayed on leukocyte P-selectin glycoprotein ligand-1 (PSGL-1). Under the activity of inflammatory mediators, ECs display E-selectin, P-selectin, and the arrangement of ligands necessary for binding L-selectin, as well as the integrin receptors (ICAM). Leukocytes attach weakly and roll over the endothelium via selectins, then adhere more firmly via integrins. Transmigration is mediated via PECAM-1 and integrin activity. **Reproduced with kind permission from Springer Science and Business Media: Opal *et al*, 2008 (263); text adapted from Pober *et al*, 2001 (259).**

Rolling/adhesion characteristics. Once rolling leukocytes have encountered and responded to a sufficiently high chemotactic gradient, they begin to exit the vasculature and migrate into the adjacent tissue. This transendothelial migration (TEM) is enabled by engagement of selectins and then of integrins (**Figure 11**, p.61). Thus, the expression pattern of adhesion molecules is an

important determinant of leukocyte emigration and is controlled by both intrinsic and extrinsic factors. For instance, circulating leukocytes preferentially migrate in post-capillary venules, even in conditions where shear stress is made equivalent to arterioles/venules and where blood flow is reversed, suggesting the existence of specific cell-surface characteristics. In accordance, P-selectin and VCAM-1 expression are most abundant on post-capillary venules compared to large vessels, arterioles or capillaries[23]. Additionally, extrinsic factors control adhesion molecule expression: for example, microvessels constitutively express CD34 protein, but L-selectin ligands are usually not present; they can be synthesized and displayed following TNF-α or IL-1 treatment (review (259)).

Diapedesis. The mode of leukocyte crossing the endothelial barrier (TEM) has been a question of much debate for over four decades (271), with the reigning theory being that leukocytes traverse through gaps between adjacent cells (**Figure 11**, p.61), usually where cell junctions are found ("paracellular") (272). However, an early morphological study of leukocyte infiltration revealed that a significant amount of leukocytes seemed to transmigrate without disrupting the EC junctions; rather they migrated through an endothelial cell ("transcellular") (273). Due to the technical challenges of capturing transcellular migration experimental settings, and precipitated by the discovery of junctional proteins and observations that blockade of junctional constituents resulted in inhibition of leukocyte migration, the theory of "transcellular" migration fell out of favour for many years (274-276). Recently, advances in electron microscopy and computer remodelling showed that leukocytes, specifically neutrophils, could migrate through a transcellular pore, passing cleanly through the cytoplasm of the EC in proximity to but at a site distinct from EC junctions (260, 277, 278). However, it is entirely possible that different vascular beds and/or inflammatory conditions may alter the method by which leukocytes migrate.

Phagocytosis. Once recruited to the inflammatory foci, the major role of leukocytes is the rapid recognition and elimination of pathogens and cell debris that may have penetrated the epithelial barriers, normally the first level of host defense. The first wave of leukocytes, the neutrophils,

[23] Postcapillary venules also express less junctional molecules thereby facilitating the action of permeability factors and allowing greater access for transmigrating leukocytes (see **1.4.4 Angiopoietins in inflammation)**.

will begin microbial elimination by phagocytosis (279), where microbial organisms are engulfed and destroyed via neutrophil-derived reactive oxygen species, proteolytic enzymes, and antimicrobials from cytoplasmic granules (259, 261). As the neutrophils are clearing the area, neighboring tissue resident macrophages and circulating monocytes converge to the site. Monocytes are recruited and infiltrate to inflamed foci via similar mechanisms to neutrophils (262); upon their arrival in the tissue, they mature into macrophages and rapidly engage in the clearance of pathogens, tissue debris and dead cells, including apoptotic neutrophils (280). Phagocytosis is dependent on leukocytes recognizing the pathogen or the antigen it presents; there are two main mechanisms to achieve this, either directly via pattern-recognition receptors, or via complement/opsonins (review (279). The latter mechanism is better characterized (281): Opsonins bind to bacterial antigens and enable them to be recognized by leukocyte receptors that trigger phagocytosis; more specifically, opsonins can be two kinds, antibodies (IgG) or complement fragments that enable antigen recognition by Fc or C3 receptors, respectively. FcγR-mediated phagocytosis of IgG-opsonized particles relies on the formation of pseudopod extensions for engulfment, whereas C3 receptor-mediated phagocytosis does not (281). Phagocyting leukocytes contain cytoplasmic "compartments"[24] that store microbicidal products and enzymes; once a pathogen is engulfed, these compartments fuse with the phagocytic vacuole and release their product into it. Macrophages not only phagocytose pathogens and dead cells, but they participate in coordinating the adaptive immune response through antigen presentation.

1.5.4 Resolution

Clearance. The clearance of the offending stimuli, cellular debris and recruited cells from the inflammatory site marks the beginning of the final stage of inflammation, called the resolution phase (282). Resolution is an active process that alters the cytokine and cellular environment in favor of an anti-inflammatory phenotype. Mediators such as glucocorticoids or cytokines such as IL-4, TGFβ and IL-1RA dampen the activity and release of additional inflammatory mediators. IL-4 inhibits further recruitment of neutrophils via suppression of neutrophil IL-8 production and induces differentiation of T lymphocytes into T-cell helper type 2 (TH2) lymphocytes, which secrete further macrophage inhibitory cytokines, primarily IL-10 and IL-13 (255).

[24] These compartments are the granules and the lysosomes, found in neutrophils and macrophages, respectively.

Granulocyte apoptosis is an essential component of resolution, as it effectively prevents further pathogen movement (270, 283). Apoptotic neutrophils are ingested by tissue resident and recruited macrophages, a process that presents multiple benefits: Besides providing a second insurance that the pathogen is destroyed, this process prevents apoptotic neutrophils from progressing to a necrotic state and the potential for bystander tissue damage. Additionally, macrophages that uptake an apoptotic neutrophil switch to a tissue repair and anti-inflammatory phenotype (called "M2"), thereby preventing further macrophage activation (284).

Granulation tissue. As mentioned earlier, clot formation is an immediate response that provides a quick and temporary remedy for blood leakage. The final solution requires repairing the tissue, generating a "granulation" tissue that includes inflammatory cells, fibroblasts, myofibroblasts and matrix tissue, in addition to growing vessels. Components of the initial clot and surrounding leukocytes are essential in this process: Along with platelets trapped in the fibrin structure, M2 macrophages release angiogenic growth and regulatory factors including platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), VEGF and TGFβ, which coordinate several cellular events necessary for the reparative phase. Non-apoptotic neutrophils also have the potential to contribute to tissue repair, as they store VEGF (270, 285) and Ang1 (183) but the conditions for their release *in vivo* and in inflammatory settings are still unknown. Thus, neighboring fibroblasts are recruited by FGF, PDGF and TGFβ signals and they upregulate their surface expression of integrins in the presence of inflammatory mediators, enabling them to recognize, bind to, and migrate over the fibrin components of the initial clot. These fibroblasts proliferate (process called "fibroplasia") and lay down a provisional matrix consisting mainly of collagen. By the end of the first week, fibroblasts are the main cells in the wound (286). Later this fibroblast-derived provisional matrix is replaced with an ECM that more closely resembles that found in non-injured tissue. Simultaneously with fibroplasia, angiogenic signals promote endothelial cell (EC) migration, proliferation, and formation of new vessel structures. Similarly to fibroblasts, ECs are drawn into the wound area by fibronectin, migrate in response to inflammatory mediators and chemotactic agents, and grow under the stimulation of angiogenic growth factors and hypoxia. As capillary loops appear, they have the appearance of small granules, giving the tissue its name of granulation tissue (287, 288).

Healing. The final step in wound healing involves tissue remodeling, during which the granulation tissue is gradually replaced and reshaped by a less vascular and less cellular scar tissue. Excess ECM and cellular components are degraded and/or removed, a process that may take months or years after granulation tissue is resolved. Unfortunately, while remodeled tissue is functional, it is generally not as good as the original.

1.5.5 Inflammatory myeloid leukocytes

1.5.5.1 Neutrophils

The arrival of neutrophils at the site of injury symbolizes the early stages of an inflammatory response. Neutrophils are usually the first responders among immune cells at the site of vessel damage, often arriving within minutes of trauma. These phagocytic cells, often termed "inflammatory leukocytes", account for 50-70% of the white blood cell count in the adult human (vs. up to 30% in mice). Thus, based on their propensity alone, appreciating the immune component of inflammation requires a comprehensive understanding of the neutrophil and its functions. Because the previously described processes of leukocyte recruitment and chemotaxis were largely drawn from observations of neutrophil biological behavior, they will not be addressed again here. Instead, this section will focus on key characteristics of neutrophil biology, laying the groundwork for the two novel studies presented in this document.

Neutrophils are equipped with an impressive arsenal of biological weaponry that enables them to effectively destroy most[25] invading pathogens they encounter. Some of these weapons are pre-stored pools of antimicrobials, but neutrophils exiting the bone marrow retain their ability to transcribe and translate additional proteins as needed, and in response to environmental cues. Together with eosinophils and basophils, neutrophils form the granulocyte family of white blood cells, a family whose hallmark is a lobulated nucleus and the presence of "granules", which are unique storage structures for the variety of mediators and antimicrobial products.

Lifespan. Neutrophils were traditionally seen as short-lived cells, with lifespans <24 hours and an estimated half-life of 8 hours (289); however, a study (that is often overlooked) challenged

[25] Some pathogens have developed mechanisms to resist neutrophil phagocytosis. See text in ***Lean, mean, killing machines.***

this concept through a novel *in vivo* labeling technique that showed that circulating human neutrophils actually live significantly longer, with an average lifespan of 5.4 days and a half-life of 3.8 days. Interestingly, the same technique used in mice showed that the average half-life of circulating mouse neutrophils was 12.5 hours, corresponding to an expected lifespan of 0.75 days (290). This revised *in vivo* survival rate is substantially different from that of isolated neutrophils used in *ex vivo* manipulations: Indeed, after isolation from peripheral blood of healthy human volunteers, 90% of unstimulated neutrophils have undergone apoptosis by 24 hours (189). These differences have important implications for studies focusing on elucidating neutrophil biology and their role in diseased states. First, the data highlights that murine and human neutrophil half-lives are very different, warranting caution when extrapolating data to humans. Second, the stark difference in lifespan begs the question as to the real relevance of *ex vivo* studies. Finally, the novel concept that neutrophils have relatively long estimated circulatory lives suggests a more complex involvement in immunity and inflammation.

Granules. Neutrophils develop continuously out of the bone marrow under the action of granulocyte colony stimulating factor (G-CSF) (291), and their entrance into circulation is tightly controlled. As granulocyte precursors (myeloblasts) mature to neutrophils, they synthesize proteins that are sorted into different granules. Granules are classified into three types based on the cargo: azurophilic (primary) granules, which contain myeloperoxidase (MPO), specific (secondary) granules which contain lactoferrin, and gelatinase (tertiary) granules which contain MMP9 (aka Gelatinase B). Primary and secondary granules can be further subdivided according to their particular content. Granules are produced continuously throughout a neutrophil's lifespan and their content depends on the transcriptional cues inside and outside the neutrophil. Thus, as the neutrophil matures and intracellular conditions change, so do granular loads, resulting in multiple granule species with overlapping cargoes. Neutrophils also carry secretory vesicles that are formed during the final stages of neutrophil maturation by endocytosis, thereby incorporating cytoplasmic elements. The existence of multiple types of granules can be explained by the fact that some of the proteins cannot co-exist in the innate form: for example, neutrophil elastase digests neutrophil gelatinase-associated lipocalin (NGAL)-164 (261). Additionally, granules have different propensity for mobilization, likely to correspond with the different stages in neutrophil activation. Secretory vesicles are the first and easiest to mobilize; upon appropriate activation, they rapidly translocate to the cytoplasmic membrane where they fuse with the cell-surface and

expose adhesion proteins such as β2 integrins (292). As activation progresses, neutrophils mobilize gelatinase and specific granules, and their azurophilic granules are the last to be deployed.

Lean, mean, killing machines. Neutrophils eliminate their targets through a multitude of mechanisms (detailed review in (282)) (**Figure 12**, p.68). Besides direct phagocytosis in which pathogens are encapsulated and degraded (via reactive oxygen species[26] or granule fusing to the phagosome), neutrophils can "degranulate", i.e. the granules fuse to the plasma membrane and release their antimicrobial content to the extracellular milieu. Neutrophils can also eject neutrophil extracellular traps (NETs) made of DNA and to which are attached histones, proteins and granular enzymes: Briefly, (a) azurophilic and specific granules can fuse to either the cytoplasmic membrane, releasing their products to the outside, or to the phagosome, dumping their antimicrobials directly into the engulfed pathogen. When these granules fuse, they also release components of the NADPH oxidase machinery that assemble on the cytoplasmic or the phagosomal membrane and initiate the reactive oxygen species (ROS) cascade, reducing molecular oxygen to superoxide. Many downstream reactions can occur following superoxide, as it is a powerful antimicrobial. (b) As for phagocytosis, it is the major mechanism to remove dead cells and pathogens (279). After engulfment, the nascent phagosome requires substantial maturation before it becomes effectively lethal to the pathogen. Phagosome maturation in neutrophils involves fusing of granules to the phagosomal surface and emptying of antimicrobial content. Simultaneously, NADPH oxidase assembly on the phagosomal membrane leads to ROS production. (c) Neutrophils can also undergo NETosis, an active form of cell death that leads to release of decondensed chromatin that traps microbes and exposes them to high gradients of granular and cytoplasmic products. The process is not well understood, but the ROS pathway appears to be required.

Some pathogens have developed strategies to survive inside the hostile neutrophil phagosomal environment (summarized in (282)): For instance, *Staphylococcus aureus* expresses a polysaccharide capsule that has antiphagocytic properties. *Helicobacter pylori* and *Francisella tularensis* can disrupt one or more stages of the NADPH oxidase cascade, from targeting of the

[26] Neutrophil production of reactive oxygen species is a process called "oxidative burst".

NADPH oxidase to the phagosome to preventing the triggering of the oxidative burst and inhibiting ROS production. Finally, other pathogens, such as *Salmonella typhimurium* and *Streptococcus pyogenes*, can efficiently block granule fusion with the phagosome.

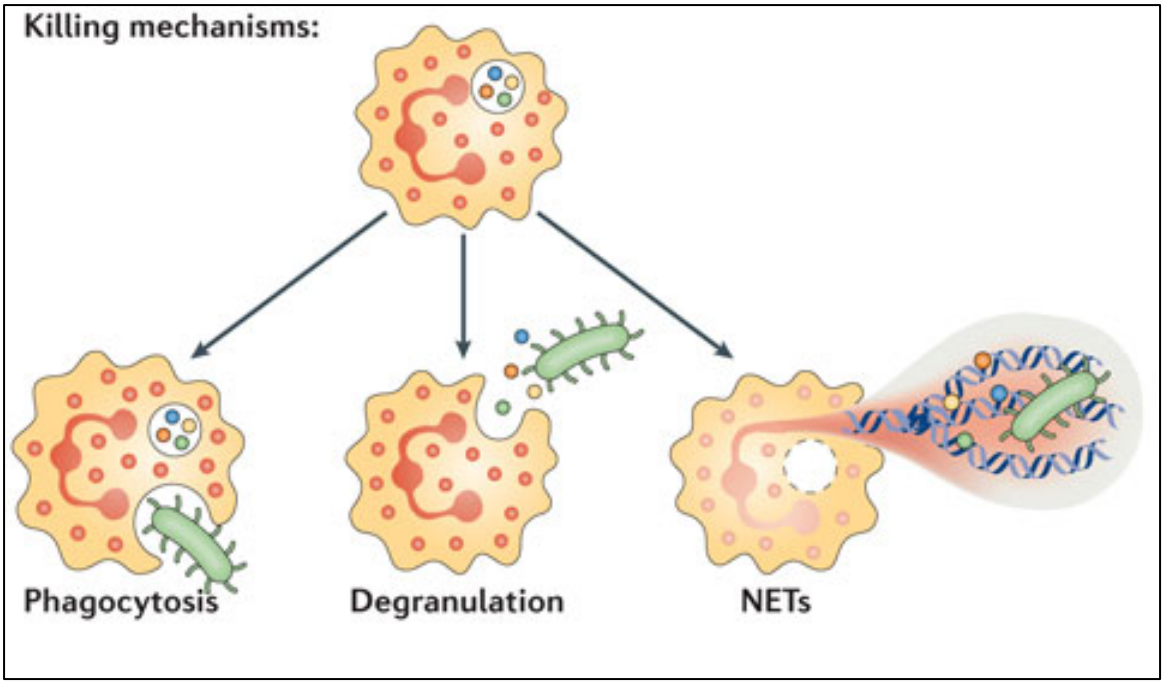

Figure 12: Neutrophil killing mechanisms.

Neutrophil can destroy pathogens by ingestion or targeting outside the cell. All three methods involve deployment of granular content. **Reproduced with permission from Macmillan Publishers Ltd: Kolaczkowska *et al*, 2013 (261).**

Cytokine production. Neutrophils are an impressive source of cytokines, conferring them with the ability to intrinsically modulate their own activity and that of neighbouring cells. Ex vivo, and under a range of conditions, neutrophils have been shown to produce proteases, reactive oxygen species (26, 27), and granular content. Compared with other inflammatory leukocytes (such as mononuclear cells), neutrophils typically produce lower amounts of cytokines per cell, but they are so abundant at inflammatory sites that their contribution to total cytokine levels cannot be dismissed. Interestingly, there is little consensus (293) with regards to the list of mediators that neutrophils can produce, as these tend to vary with experimental conditions (population purity, detection method, species, type of activation inducers etc.). Thanks to advances in detection methods, this question has recently been revisited. The cytokines released by neutrophils are often synthesized *de novo*, but they also contain pre-formed pools of mediators that they can

secrete even in the absence of stimulation. When activated, neutrophils undergo a transcriptional burst that results in the synthesis and/or secretion of signalling molecules at various levels, including secretion of IL-8, IL-1 receptor antagonist (IL-1RA), TNF-α, MIP-1β, MIP-1α (CCL3), growth-related oncogene (GRO)-α, and macrophage colony-stimulating factor (M-CSF) (293-295)). On the other hand, neutrophils did not secrete any IL-1β, IL-6, IL-10, IL-12 and GM-CSF. Although these studies are performed at single time-points and do not measure intracellular cytokine production, their importance is in their revelation that neutrophils can contribute both pro- and anti-inflammatory mediators.

1.5.5.2 Monocytes/Macrophages

The interaction of neutrophils with other immune cells is quite complex. At least between neutrophils and their mononucleated blood relatives, the cross talk appears to be circular. Monocytes and tissue resident macrophages are generally the first cells to detect danger signals and the cytokines they produce in response serve to summon large numbers of neutrophils to the inflammatory site. Neutrophils then recruit monocytes via classical monocyte chemoattractants, such as CC chemokines CCL2, CCL3, CCL19, and CCL20, and via granule mediators, including LL-37, azurocidin (HBP/CAP37), and CG (reviewed in (282)). Monocyte recruitment is also facilitated by the changes to the endothelium used to accommodate neutrophil recruitment, such as upregulation of endothelial adhesion factors, increase of transendothelial permeability and enhancement of production of chemoattractants by other cell types.

In addition to monocytes, nearby tissue monocyte-derived macrophages, which are distributed ubiquitously, converge on the scene. Newly recruited monocytes also mature into macrophages once in the inflammatory tissue, and quickly begin clearance of damaged tissues, microorganisms and dying neutrophils. Thus, neutrophil-mononucleated cell cooperation is extremely important in the resolution of inflammation and initiation of tissue repair: Once neutrophils have executed their antimicrobial agenda, they die via a spontaneous apoptosis program that preserves the integrity of their membrane and prevents the leaking of noxious agents. Phagocytosis of apoptotic neutrophils also reprograms macrophages to adopt an anti-inflammatory phenotype: Indeed, ingestion of apoptotic cells by macrophages drives the production of the anti-inflammatory cytokines tumor growth factor (TGF)-β and IL-10 (296), which as we have previously seen (see **1.4 Angiopoietins**), drives macrophage polarization to an

immune-suppressive and tissue repair phenotype (M2). Thus, the benefits of apoptotic neutrophils are three-fold: First, it acts as insurance that cytotoxic microbes are eliminated from the system in a controlled manner, even if the neutrophil attached to them is dead. Second, it ensures that neutrophil content is kept from damaging surrounding tissues. Finally, apoptotic neutrophils stimulate macrophages into a pro-resolution phenotype, restricting further propagation of the inflammatory response.

1.5.6 Remarks

The inflammatory process involves an intricate network of cells and mediators that must work under tight control and regulation to eliminate microbes with as little damage to the host as possible. As the first line of immune defense against invaders, neutrophils have developed capabilities that make them the pre-eminent pathogen exterminators of the system. In addition to this important role, neutrophils also network with many other cells involved in the inflammatory response, including vascular and immune cells, thereby helping in the propagation and eventually in the resolution of inflammation. It is therefore not surprising that deregulation of neutrophil function can have harmful consequences, leading to inflammatory and auto-immune diseases (reviewed in (282)). Many challenges remain in understanding neutrophil function, especially because much of our current knowledge is based on observations in mice when there are definite species differences that cannot be ignored.

As we have seen thus far, human neutrophils express typically-angiogenic growth factor receptors, VEGFR-1 (297) and Tie2 (151). What is the significance of this, and how does a neutrophil roaming the bloodstream avoid being accidentally activated and launching an inflammatory cascade at sites of active angiogenesis? Is there specialization among neutrophils, much like monocytes? Could neutrophils undergo a switch from a default inflammatory cell to an angiogenic phenotype, like macrophage polarization into M2? How does the neutrophil phenotype change in diseased conditions? There is strong evidence that cytokine production is time and stimulus-dependent. What are a neutrophil's cytokine and growth factor production capability and the conditions under which different cytokines are deployed? Because inflammation sees multiple cytokine networks that overlap in time, how exactly do neutrophils "prioritize"? Answering these questions is key to understanding neutrophil function in physiological and diseased conditions.

2.0 RESEARCH THEMES

At the onset of inflammation, neutrophils are the first inflammatory cells to respond to the site of infection. Sequential molecular and cellular events allow for neutrophils to exit the bloodstream via the endothelial barrier, infiltrate the tissue and neutralize the infection. Once the threat is effectively eliminated, neutrophils actively participate in dampening the inflammatory response, either by modifying the cytokine environment in favor of resolution and tissue repair, or by undergoing spontaneous apoptosis. All of these events require active participation by the endothelium and the final endgame is to re-establish homeostasis. Additionally, before vessel integrity and blood flow are adequately rehabilitated, the new cellular infiltrates and surrounding tissues are going to be in a hypoxic state. The system remedies this problem by forging new blood vessel routes via angiogenesis, which increases blood flow, oxygen and nutrient supply to immune cells, thereby favoring additional immune cell activation and recruitment. Thus, angiogenesis and inflammation *can* and *do* occur conjointly. Because they overlap, these two processes will inevitably share cellular players that exert a level of flexibility allowing them to adapt their function depending on the cytokine environment available to them. This is perhaps best exemplified by the M2 macrophage, with its ability to switch from an M1 (inflammatory) to an M2 (tissue repair) phenotype when inflammation is almost over, or the Tie2-expressing monocytes (TEMs) that can promote angiogenesis in favorable environments (tumors). Evidence suggests that neutrophils have the potential to behave in the same manner as their mononucleated cousins: In effect, neutrophils express receptors for tumor necrosis factor (TNF)-α (17), Interleukin (IL)-8 (6), vascular endothelial growth factor (VEGF) (9, 89), platelet activating factor (PAF) (78) and angiopoietins (53). Thus, the purpose of both studies presented herein was to characterize the ability of angiogenic factors to modulate neutrophil biological responses or in other words, the changes in neutrophil biochemistry in the presence of angiogenic modulators.

Article #1 – FGFs and neutrophils

Cells of hematopoietic origin were shown to express functional FGFRs (2), and infiltrating macrophages and T lymphocytes from diseased glomeruli of patients with proliferative lupus nephritis exhibit FGFR1 at their surface. Given that our group and others have previously shown that neutrophils express functional receptors of at least two families of potent angiogenic factors

(VEGFR-1 and Tie2), we wished to determine if human neutrophils expressed FGF receptors by biochemical and cytological analyses, and if so, to determine the functionality of the identified receptor(s). We also addressed the ability of FGF1 and FGF2 to induce neutrophil chemotaxis *in vitro*.

Article #2 – Angiopoietins and neutrophils

Despite their purported stabilization/destabilization antagonism, we showed that both Ang1 and Ang2 had an equal ability to induce a pro-inflammatory response from endothelial cells (ECs) *in vitro*, promoting P-selectin translocation to the cell-surface and PAF synthesis. The existence of a functional Tie2 on the surface of neutrophils suggests that angiopoietins may exert modulatory functions on inflammatory leukocytes as well. Indeed, we previously showed that Ang1 and Ang2 promoted chemotaxis of human neutrophils *in vitro*, and primed neutrophils to migrate towards an IL-8 gradient. More recently, we demonstrated that Ang1 increased human neutrophil lifespan *ex vivo*, and induced *de novo* synthesis and release of IL-8. Although pro-inflammatory in nature, the significance of these events *in vivo* is currently unknown. The purpose of this study was to elucidate the ability of angiopoietins to activate human neutrophils at a biochemical level, especially given the propensity of these cells to transcribe and translate mediators under different stimuli. Targets of angiopoietin stimulation in human neutrophils would then be further characterized.

2.1 Article #1

Haddad, L. E., L. B. Khzam, F. Hajjar, Y. Merhi, and M. G. Sirois. 2011. Characterization of FGF receptor expression in human neutrophils and their contribution to chemotaxis. *American journal of physiology Cell physiology* 301: C1036-1045.

Characterization of FGF receptors expression on human neutrophils and their contribution to chemotaxis

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Running title: FGFR expression on human neutrophils

Key Words: • neutrophil • FGF • inflammation • FGFR • cell migration

Figures: 6, Tables: 1.

Contract grant sponsor: Canadian Institutes of Health Research (CIHR)

Contract grant number: MOP-97943

Contract grant sponsor: Heart and Stroke Foundation, Quebec

2.1.1 Abstract

Several members of the fibroblast growth factor (FGF) family are potent endothelial cell (EC) mitogens and angiogenic factors, and their activities can be mediated by four tyrosine kinase receptors (FGFR1–4). In addition, FGFs can induce the release of inflammatory mediators by ECs and the expression of adhesion molecules at their surface, thereby favoring the recruitment and transvascular migration of inflammatory cells such as neutrophils. Neither the expression nor the biological activities that could be mediated by FGFRs have been investigated in human neutrophils. By biochemical and cytological analyses, we observed that purified circulating human neutrophils from healthy individuals expressed varying levels of FGFRs in their cytosol and at their cytoplasmic membrane. FGFR2 was identified as the sole cell surface receptor, with FGFR1 and -4 localizing in the cytosol and FGFR3 being undetectable. We assessed the capacity of FGF1 and FGF2 to induce neutrophil chemotaxis in a modified Boyden microchamber and observed that they increase neutrophil transmigration at 10−10 and 10−9 M and by 1.77- and 2.34-fold, respectively, as compared with PBS-treated cells. Treatment with a selective anti-FGFR2 antibody reduced FGF1-mediated chemotaxis by 75% and abrogated the effect of FGF2, while the blockade of FGFR1 and -4 partially inhibited (15–40%) FGF-chemotactic activities. In summary, our data are the first to report the expression of FGF receptors in human neutrophils, with FGF1 and FGF2 promoting neutrophil chemotaxis mainly through FGFR2 activation.

Key Words: neutrophil • FGF • inflammation• FGFR • cell migration

2.1.2 Introduction

Inflammation is an essential component of host defense against harmful stimuli, involving a complex interplay between vascular tissues and blood leukocytes. Leukocyte recruitment to sites of inflammation occurs via a series of molecular and cellular events, beginning with the tethering and rolling of leukocytes on the endothelium lining blood vessels, followed by firm arrest, diapedesis, and finally leading to extravasation into the vascular wall (Muller 2003). These cellular responses are accompanied by changes in the expression of effector molecules such as surface adhesion proteins, receptors and mediators secreted by the endothelium and recognized by neutrophils (Springer 1994). Therefore, the efficacy of neutrophils in reaching infected tissues is dependent on recognizing and responding to alterations of endothelial functions. Interestingly, many of the mediators that neutrophils respond to are also extensively involved in angiogenesis, a phenomenon that leads to the formation of new blood vessels from pre-existing vasculature. Factors such as vascular endothelial growth factor (VEGF), angiopoietins (Ang1, Ang2), interleukins (IL)-1, -6 and -8, all of which are extensively involved at different stages of angiogenesis, have been shown to modulate neutrophil survival, degranulation, respiratory burst, adhesion and chemotaxis (Coxon, Rieu et al. 1996, Rollin, Lemieux et al. 2004, Maliba, Lapointe et al. 2006, Brkovic, Pelletier et al. 2007, Neagoe, Brkovic et al. 2009).

Another set of potent angiogenic modulators, the family of fibroblast growth factors (FGFs), is also involved in the inflammatory process, but the contribution of specific FGFs to different stages of inflammation remains to be elucidated. Evidence of the involvement of FGFs in inflammation comes from observations that the two most studied members of the family, FGF1 and FGF2, are up-regulated in inflammatory disorders such as bowel syndrome, Crohn's disease, ulcerative colitis (Kanazawa, Tsunoda et al. 2001) and rheumatoid arthritis (Byrd, Zhao et al. 1996). Other reports have suggested that FGF1 and FGF2 are secreted by and may act as immunoregulators of infiltrating neutrophils, monocytes, macrophages and T lymphocytes, often in tandem with powerful inflammatory cytokines (Barrios, Pardo et al. 1997, Byrd, Ballard et al. 1999, Ohsaka, Takagi et al. 2001). The notion that FGF2 alters neutrophil behavior secondary to the activation of endothelial cells (ECs) rather than through direct interaction is supported by evidence that FGF2 enhances EC surface-expression of adhesion molecules ICAM-1/2 (Zittermann and Issekutz 2006), E-selectin (Paludan 2000) and monocyte chemoattractant protein-1 (MCP-1) (Takagi, Takahashi et al. 2000, Ohsaka, Takagi et al. 2001, Yamashita,

Yonemitsu et al. 2002) prior to neutrophil rolling and adhesion. Although one study showed that FGF2 primes neutrophil respiratory burst and increases their surface expression of integrins CD11b/CD18 (Takagi, Takahashi et al. 2000), the effects of direct FGF stimulation, especially those of FGF1, on neutrophil biological activities remain largely unexplored.

In general, the effects of FGFs, of which there are 23 members, are mediated by binding to four high-affinity tyrosine kinase receptors (FGFR1-4) and their splice variants (Jaye, Schlessinger et al. 1992). The diversity in FGF signalling is due, in part, to different FGF/FGFR combinations. Additionally, alternative splicing in the FGFR immunoglobulin (Ig)-like domains generates additional receptor isoforms with novel ligand affinities. Finally, effector cells will usually express different heparan-sulfates at their surface, which are responsible for stabilizing FGF/FGFR complexes and enhancing FGFR downstream signalling (Vainikka, Partanen et al. 1992, Cotton, O'Bryan et al. 2008).

Although it has been shown that cells of hematopoietic origin express functional FGFRs (Allouche, Bayard et al. 1995), no information has been reported regarding the receptors that mediate the effects of FGF1 and FGF2 on neutrophils. Therefore, the aim of the following study was to determine whether neutrophils expressed FGFRs and if so, to characterize the contribution of the identified FGFRs to the effects of FGF1 and FGF2 on neutrophil chemotaxis.

2.1.3 Materials and Methods

2.1.3.1 Neutrophil purification

Venous blood was obtained from healthy donors free from medication for at least 10 days prior to the experiments and in accordance with the guidelines of the Montreal Heart Institute's ethical committee. Neutrophils were isolated using Ficoll-Hypaque gradient, as described previously (Theoret, Bienvenu et al. 2001, Rollin, Lemieux et al. 2004, Neagoe, Brkovic et al. 2009), and resuspended in RPMI medium (Lonza, Allendale, NJ, USA) supplemented with 25 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and 1% penicillin/ streptomycin. Ninety-eight (98) percent of the isolated cells were polymorphonuclear cells, as determined with a Coulter counter, and viability was found to be greater than 98%, as assessed by Trypan blue dye exclusion assay.

2.1.3.2 FGF receptor identification by RT-qPCR analyses

Total RNAs were obtained from freshly isolated human neutrophils (10^7 cells) by using the RNeasy extraction kit (Qiagen, Mississauga, ON, Canada). Total RNAs (100 ng) was reverse transcribed using random hexamers and the Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Burlington, ON, Canada) as described by the manufacturer. Reactions were performed on a MX3500P (Stratagene, La Jolla, CA) using 10 ng of cDNAs, Syber Green (Invitrogen) and 300 nM of the primers (table below) for each receptor. cDNAs were submitted to 45 cycles of amplification (temperatures for annealing: 60°C; dissociation: 55°C) and gave single peaks for each product.

Reverse transcriptase polymerase chain reaction (RT-qPCR) products were purified on a 2% acrylamide gel, quantified using QIAquick Gel Extraction Kit (Qiagen) and sequenced (Genome Quebec Innovation Centre, McGill University, Montreal, QC, Canada). The concentration of the purified products was measured by nanodrop and eluted amplicons used in another set of RT-qPCR reactions as serial dilutions to generate standard curves for each set of oligonucleotides. The number of copy was calculated using the following formula: number of copies = (amount (ng) * 6.022x1023) / (length (bp) * $1x10^9$ * 650 (g/mole of bp). Standard curves of cDNA copies were generated by RT-PCR and used to determine the number of mRNA copies for each receptor.

Receptor	Forward oligonucleotide	Reverse oligonucleotide
FGFR1 (all variants)	CTACAAGGTCCGTTATGCCAC	TGCTACCCAGGGCCACTGTTTTG
FGFR2 (all variants)	AAGCCCAAGGAGGCGGTCAC	CAGGAACACGGTTAATGTCA
IIIb	GATAAATAGTTCCAATGCAGAAGTGCT	TGCCCTATATAATTGGAGACCTTACA
IIIc	AGATTGAGGTTCTCTATATTCGGAATG	CTGTCAACCATGCAGAGTGAAAG
α	CTCTCAACCAGAAGTGTACGTGGCTGC	GACATTCACCATGAAGYACCAAG
β (same reverse)	CCACATTAGAGCCAGAAGGAGCACC	GCTGGTTTCGTACCTTGTAGCCTCC
	CCACATTAGAGCCAGAAGATGCCA	
FGFR3 (all variants)	TACTGTGCCACTTCAGTGTGC	ATCCGCTCGGGCCGTGTCCAGTAA
FGFR4 (all variants)	CCAGCGCATGGAGAAGAAAC	ACCACGCTCTCCATCACGAGAC

2.1.3.3. Immunohistochemistry (IHC)

Freshly isolated human neutrophils (1.2 x 10^8 cells) were centrifuged and the pellet was fixed in 10% PBS-buffered formalin overnight, dehydrated in a graded series of ethanol solutions and xylene, and embedded in paraffin. Six (6) µm–thick sections were rehydrated, blocked in 10% normal goat serum (NGS), and incubated overnight with rabbit polyclonal anti–human FGFR1-4 IgG or normal rabbit IgG (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Following a 30-minute incubation with the secondary antibody (goat anti-rabbit IgG, 1% NGS), sections were rinsed 3 times in Tris buffer, and incubated in Vectastain ABC alkaline phosphatase solution (Vector laboratories, Burlington, ON, Canada) and revealed with the Vector black alkaline phosphatase substrate solution for 30 minutes each as recommended by the manufacturer. Slices were rinsed, immerged in methyl green (10 minutes, 60°C), dehydrated, mounted with resinous medium, and examined with a light microscope under 40x and 100x magnification. No cross-reactivity was observed between FGFR1-4 antibodies.

2.1.3.4. Confocal microscopy

Neutrophils were isolated as aforementioned, led to adhere on glass coverslips precoated with poly-L-lysine (BD BioCoat; Becton Dickinson, Mississauga, ON, Canada) or 1 hour and fixed with a 2% paraformaldehyde solution. Nonspecific binding of primary antibodies was prevented by preincubating fixed neutrophils with 10% serum from the species used to raise secondary antibodies. Neutrophils were exposed to mouse monoclonal anti-human FGFR1-4 IgG (R&D Systems, Minneapolis, MN) and to a secondary goat anti-mouse antibody coupled to the Cy3-fluorochrome (Invitrogen). No cross-reactivity was observed between FGFR1-4 antibodies. Neutrophil preparations were mounted using DABCO (1,4-diazabicyclo-2-2-2-octane)/glycerol (1:1) solution and glass coverslip. Stained neutrophils were observed by confocal microscopy

(Zeiss Axiovert 100 M microscope equipped with a 63x/1.4 Plan-Apochromat oil objective lens; Zeiss, Oberkochen, Germany; and adapted with an LSM 510 confocal system). Images were recorded with LSM 510 software (Zeiss) and exported in tagged-image file format (TIFF).

2.1.3.5 Flow Cytometry (FACS)

Neutrophils (10^7 cells/mL) were rinsed and resuspended in RPMI, and mouse IgG (150 µg/mL) was added for 30 minutes to prevent nonspecific binding via FcRs. Neutrophils were centrifuged, rinsed, resuspended in PBS-BSA (10^6 cells/mL, 1% BSA), and incubated with PE-Conjugated mouse monoclonal anti-human FGFR1-4 IgG (25 or 50 µg/mL, R&D Systems), or with control PE-conjugated mouse monoclonal IgG1 (50 µg/mL, R&D Systems) for 30 minutes at room temperature. Cells were rinsed and fixed with 2% paraformaldehyde. Flow cytometric analysis (10^5 events) was performed using a FACScan (Becton Dickinson, San Jose, CA, USA).

2.1.3.6 Neutrophil migration

In vitro chemotactic assays were performed in a 48-well modified Boyden chamber apparatus (Neuro Probe Inc., Gaithersburg, MD) as described (Lemieux, Maliba et al. 2005, Brkovic, Pelletier et al. 2007). Briefly, The bottom wells were loaded with RPMI containing PBS, IL-8 (25 nM), FGF1 (10^{-14} - 10^{-8} M) or FGF2 (10^{-14} - 10^{-8} M) to a final volume of 27 µl. The top wells were loaded with neutrophils (10^6 cells/mL; 50 µl from a RPMI suspension). The two sections (top and bottom wells) were separated by a porous 3-µm polycarbonate membrane filter. The modified Boyden chamber apparatus was incubated at 37°C for 1 hour in a humidified incubator in the presence of 5% CO2. At the end of the incubation period, the upper part of the modified Boyden chamber (upper wells) was removed, and the upper side of the membrane was wiped carefully with the rubber scraper provided by the manufacturer. The polycarbonate membrane was fixed in methanol, colored with the Kwik-Diff staining solution kit (Thermo Shandon, Pittsburgh, PA), mounted on a glass slide, and examined with a light microscope under 40X magnification. The number of cells in five random fields was counted, and the results were expressed as relative neutrophil migration (number of cells from tested group/number of cells from corresponding control vehicles).

In another set of experiments, neutrophils were pre-treated with 0.1 µg/mL of blocking anti-FGFR1, FGFR2 or FGFR4 antibodies (R&D Systems, Minneapolis, MN) or with an isotype-

matched control IgG for 30 minutes prior to a 1-hour migration towards PBS, IL-8 (25 nM), FGF1 (10^{-9} M) or FGF2 (10^{-9} M). In the latter experiment, maximal effect was set to 100% and corresponds to the number of IgG-pretreated neutrophils migrating towards FGF1 or FGF2. The migration of neutrophils pretreated with FGFR antibodies was expressed as a percentage of the maximum FGF-induced response in absence of FGFR antibodies.

2.1.3.7 Statistical analysis

Data are presented as mean ± SEM. Statistical comparisons were made by student t-test or by a one-way analysis of variance (ANOVA), followed by Tukey post-hoc where applicable using GraphPad Prism (Mac version 5.0b). Differences were considered significant at P values less than .05.

2.1.4 Results

2.1.4.1 Neutrophil expression of FGFR mRNA

Using neutrophils isolated from venous blood of 22 healthy volunteers, we determined the mRNA expression of FGF receptors (1 to 4; FGFR1-4), as described in materials and methods. Neutrophils expressed an average of 4.72 x 10^5 copies of total FGFR mRNA, irrespective of the subtypes. FGFR2 mRNA had the highest level of expression (3.10 ± 0.67 x10^5 mRNA copies) followed by FGFR4 (1.07 ± 0.24 x10^5 copies) and FGFR1 (0.6 ± 0.12 x10^5 copies), whereas FGFR3 mRNA level was undetectable (Figure 1A). As the levels of total FGFR mRNA copies varied between individuals, we represented the distribution of each of FGFR1-4 mRNA per donor. We arbitrarily separated the donors into low (< 4 x10^5 copies; 11/22 donors) and high expressers (>4 x10^5 copies; 11/22 donors) (Figure 1B); regardless of which group the donors belonged to, FGFR2 represented the highest proportion of total FGFR mRNA, corresponding to 51.0 ± 9.5 and 67.9 ± 5.1% of the total FGFR mRNA for low and high expressers (Figure 1C).

2.1.4.2 Neutrophil expression and localization of FGFR proteins

Independently of the above mRNA analyses, neutrophils from 16 different donors were examined for FGFR1-4 protein expression and localization by conventional immunohistochemistry (IHC). Due to variations in staining intensities and the number of cells that were marked between different donors, we distinguished four staining patterns (Figure 2A-E) to which we attributed qualitative scores: score 0 (background staining; Figure 2A), score 1 (faint but detectable staining in some cells; Figure 2E), score 2 (high staining in some cells; Figure 2B) or score 3 (high staining in most cells; Figure 2C). The staining scores for each donor, along with the means of the scores for comparative purposes, are presented in Table 1. FGFR1 staining showed high staining on a small subset of the neutrophils for most donors (Figure 2B). The majority of donors scored high for FGFR2, with a much larger fraction of neutrophils intensely stained (Figure 2C). FGFR3 and FGFR4 expression fell between scores 0 and 1 as most donors showed either very faint staining in a minor population of neutrophils or no detection at all (Figure 2D and E). We also observed differences in the localization of the staining, especially between FGFR2 and FGFR1 (and to a lesser extent FGFR4), as shown by the boxed in magnified images in Figure 2. When viewed at 100X magnification, neutrophils stained for FGFR2 showed a distinct darker coloration at the cytoplasmic periphery, unlike FGFR1 or FGFR4, which showed a rather diffuse

intracellular staining pattern. These observations suggest that FGFR2 is the only neutrophil cell-surface receptor, whereas FGFR1 and FGFR4 are cytosol-bound.

Data obtained from flow cytometry analyses reflected the same variability in expression levels of FGFR1-4 from one donor to another. By confocal microscopy, we determined whether the receptors were localized intracellularly or at the cell-surface. We observed that FGFR1 and FGFR4 staining was peri-nuclear and diffused across the cytosol of the neutrophils, whereas FGFR2 staining was mainly confined to a thin strip in close proximity to the cell surface. Once again, FGFR3 detection was similar to the non-specific IgG background (Figure 3).

2.1.4.3 Characterization of FGFR2

FGF receptors share the same basic protein structure, which is characterized by up to three extracellular immunoglobulin (Ig) domains (denoted by Ig-I, -II, or -III). These Ig domains define the affinity and responsiveness of FGFRs for different FGF ligands (Miki, Bottaro et al. 1992, Hanneken 2001, Cotton, O'Bryan et al. 2008). In normal tissues, FGFRs express all three Ig domains and are referred to as 'FGFR-α' (Figure 4A). The loss of Ig-I, gives rise to 'short' forms of FGFRs called 'FGFR-β', correlating with transformation to a malignant phenotype and invasiveness (Yamaguchi, Saya et al. 1994, Karajannis, Vincent et al. 2006). FGFR-β forms are thus found in cancerous cell lines, having a thousand-fold higher affinity to FGF1 but not for FGF2. The complexity of the FGFR family is increased by alternative splicing in exons 8-10 of Ig-III domain, generating FGFR-IIIb and FGFR-IIIc isoforms (Figure 4B). These splicing events confer additional ligand binding properties to FGFRs (Turner and Grose 2010).

Because we identified FGFR2 as the only cell-surface receptor, we further characterized the expression of its different isoforms. We looked at the FGFR2 mRNA transcript, first to establish whether all three Ig domains were coded, and second to determine which of exons IIIb or IIIc in Ig-III were spliced. As per our previous experiments, we performed RT-qPCR analyses and determined that FGFR2α is nearly the exclusive isoform (Figure 4C). We then quantified the mRNA of both FGFR2α-IIIb and FGFR2α-IIIc subtypes and observed that in neutrophils, FGFR2 mRNA was primarily FGFR2α-IIIc.

2.1.4.4 In vitro FGF1 and FGF2 - mediated chemotaxis

Previous studies reported that FGF2 modulates the recruitment of polymorphonuclear cells, monocytes, and T cells in vivo (Zittermann and Issekutz 2006, Zittermann and Issekutz 2006). However, the possibility that FGFs could be direct stimulators of leukocyte recruitment was not addressed. To this end, we used a modified Boyden microchamber model and observed that both FGF1 and FGF2 were capable of mediating neutrophil chemotaxis at picomolar concentrations. In addition, treatment with FGF1 or FGF2 (10^{-14} - 10^{-8} M) induced a bell-shape activity on the number of migrating neutrophils as compared to control PBS-treated cells. The maximal effect was achieved at 10^{-10} M for FGF1 and 10^{-9} M for FGF2, corresponding to 1.77 and 2.34 fold increase over PBS-induced migration, respectively. The positive control IL-8 (25 nM) increased migration by 2.72 (± 0.22) folds compared to control PBS-treated neutrophils (Figure 5A).

In another set of experiments, we assessed the contribution of FGFR1, FGFR2 and FGFR4 to FGF-mediated chemotaxis. Neutrophils were pre-treated with blocking monoclonal antibodies against FGF receptors (FGFR1, -2 and -4) or with a control isotype-matched IgG (0.1 μg/mL) for 30 minutes prior to stimulation with PBS, IL-8 (25 nM), FGF1 or FGF2 (10^{-9} M). We observed that the effects of FGF1 on neutrophil chemotaxis were suppressed by 75.2% with the selective anti-FGFR2 antibody and partially reduced with the anti-FGFR1 or anti-FGFR4 by 39.6 and 31.9% respectively. FGF2-induced neutrophil migration was almost completely abrogated by treatment with the anti-FGFR2 antibody (96% reduction), whereas anti-FGFR1 and FGFR4 antibodies reduced migration by 28.9% and 14.6% respectively (Figure 5B). In contrast, basal and IL-8-induced neutrophil migration was unaffected in presence of anti-FGFR antibodies (data not shown).

2.1.5 Discussion

In the current study, we demonstrate that circulating neutrophils isolated from the blood of healthy individuals express varying levels of FGFR1, -2 and -4 mRNA, with FGFR2 mRNA showing the most consistent and highest level of expression. At the protein level, donors showed a heterogeneous expression of FGF receptors. We identified FGFR2 as the most expressed and unique neutrophil cell-surface receptor, whereas FGFR1 and FGFR4 appeared to be cytosol-bound. Neither the mRNA nor the protein for FGFR3 was significantly detectable. In addition, we observed that FGF1 and FGF2 were capable of stimulating neutrophil migration under in vitro conditions, mainly through FGFR2 activation.

FGF1 and FGF2 are potent heparin-binding pro-angiogenic growth factors that exert their biological functions through the activation of high affinity tyrosine kinase receptors (FGFRs), heparan-sulfate proteoglycans (HSPGs) and integrins expressed on the surface of effector cells (Forsten-Williams, Chua et al. 2005). Although the presence of FGFRs on human neutrophils has not been reported, these leukocytes from a biological standpoint seem naturally well equipped to respond to FGF stimulation. Firstly, neutrophils express integrins as well as HSPGs, accessory co-receptors that facilitate dimerisation of tyrosine kinase receptors and amplify the signalling triggered by HSPG-binding growth factors such as FGFs. Secondly, neutrophils present at their surface the receptors for at least two other families of angiogenic growth factors with similar properties to FGFs. Indeed, neutrophils were shown to express VEGFR-1 and Tie2 receptors, which are namely responsible for VEGF and angiopoietins (Ang)-mediated neutrophil chemotaxis, respectively (Ancelin, Chollet-Martin et al. 2004, Lemieux, Maliba et al. 2005).

2.1.5.1 Detection and localization of FGF receptors on human neutrophils

The initial objective of our study was to determine whether neutrophils expressed FGF receptors. Quantitative RT-PCR analyses showed a trend among individuals to express more mRNA for FGFR2 than any of the other FGF receptor subtypes. FGFR1 and FGFR4 mRNA were also detectable with a generally lower number of copies, whereas FGFR3 was minimally or not detected. Owing to the fact that the presence of the mRNA does not always coincide with the expression of its corresponding protein, we examined FGFR protein expression by immunostaining and confocal microscopy. FGFR2 was detected on a large subset of neutrophils for many of the donors, with a staining pattern to the cell-surface membrane. FGFR1 and FGFR4

showed a less intense but uniformly diffused staining across the cytoplasm of a small fraction of neutrophils, suggesting that if these receptors were indeed cytoplasmic, they were not confined to specific subcellular fractions or to vesicles. FGFR3 protein detection was absent to very low, accordingly to its corresponding mRNA levels.

2.1.5.2 Characterization of FGFR2

The specificity of the FGF-neutrophil interaction is determined, not only on the basis of which FGFR is expressed at the cell surface, but also by the subtype and isoform of the surface-receptor. Most mammalian FGFRs exist as the FGFR-α isoform, which consists of three extracellular immunoglobulin-like domains (Ig I-III). The lack of Ig-I, which gives rise to short forms of FGFR called 'β', leads to a thousand-fold increase in FGF1 responsiveness and an important reduction in FGF2 affinity. FGFs interact with the Ig-II and Ig-III domains, with the Ig-III interactions primarily responsible for ligand-binding specificity. Alternative splicing of exons 8 and 9 in the Ig-III domain gives rise to 'IIIb' and 'IIIc' variants for FGFR1, -2 and -3, thus generating seven possible FGFR subtypes at the cell surface that bind a specific subset of FGFs. FGF1 is capable of activating all seven FGFRs. In contrast, FGF2 only binds the IIIc forms of FGFRs (Dionne, Crumley et al. 1990, Dell and Williams 1992, Jaye, Schlessinger et al. 1992, Cheon, LaRochelle et al. 1994, Ibrahimi, Zhang et al. 2004, Ibrahimi, Zhang et al. 2004, Mohammadi, Olsen et al. 2005, Cotton, O'Bryan et al. 2008). Using qPCR analysis, we confirmed that nearly all the FGFR2 mRNA from human neutrophils comprises all three Ig domains, coding for the α-isoform. Furthermore, the majority of the FGFR2 mRNA contains exon 9 in the second part of Ig-III domain, which would thereby generate IIIc isoforms. The presence of FGFR2α-IIIc isoform on the cell surface of human neutrophils would allow them to interact with FGF1, FGF2, FGF-4, and FGF-6 but not with FGF-3, FGF-7 or FGF10 (Bottaro, Rubin et al. 1990, Dell and Williams 1992, Miki, Bottaro et al. 1992). Given the role of FGFs in angiogenesis, this finding could potentially have important physiological implications; indeed, in cases such as following a cerebral stroke or cardiac ischemia, where an increase in angiogenesis is desired to improve reperfusion while maintaining a minimal influx of inflammatory cells, the choice of FGF ligand could become critical.

Our data regarding FGFR1-4 protein expression and localization are in agreement with previous studies reporting the presence of soluble cytosolic forms for FGF receptors in blood and

different cell types (Root and Shipley 2000, Hanneken 2001). The function of the cytoplasmic FGFRs can only be speculative at the moment, but we did consider the possibility that FGF2 could induce the translocation of its own receptors from the cytosol of the neutrophils to the cell surface. However, when neutrophils were treated with FGF2 for up to 24 hours, FGFR1-4 detection was not significantly increased, as measured by flow cytometry (data not shown). Nevertheless, this observation does not exclude the possibility that FGFR1-4 translocation could be induced by other more potent inflammatory mediators such as IL-8, TNF-α or bacterial lipopolysacharide (LPS), all of which can promote protein translocation to the surface of neutrophils (Csernok, Ernst et al. 1994, Rollin, Lemieux et al. 2004). It is possible that the soluble forms of FGFR1 and -4 are proteolytically cleaved by matrix metalloproteases (MMPs) inside the cell before they reach the cytoplasmic membrane, especially since neutrophils synthesize, store and release large amounts of MMP-2 (Cuadrado, Ortega et al. 2008) and MMP-9 (Sopata and Dancewicz 1974, Hibbs, Hasty et al. 1985). This proteolysis can occur in different locations within the cell, especially since MMPs have been shown to cleave substrates in nuclear, mitochondrial, cytoplasmic and vesicular compartments, including the intracellular cytoskeletal matrix (Cauwe and Opdenakker). Furthermore, the presence of FGF2 in the cytoplasm of neutrophils has been reported (Pazgal, Zimra et al. 2002), which raises the possibility that FGF2 could have undetermined intracellular functions and could be sequestered by soluble FGFR proteins. Further experimentation will be required before a functional role for these cytoplasmic receptors can be ascertained.

2.1.5.3 FGF1 and FGF2 - mediated chemotaxis

Numerous angiogenic factors have been shown to modulate leukocyte behavior under inflammatory condition. For instance, VEGF has been reported to promote several chronic inflammatory disorders (Dvorak, Detmar et al. 1995, Lee 2005, Tammela, Enholm et al. 2005, Roy, Bhardwaj et al. 2006, Yla-Herttuala, Rissanen et al. 2007, Mac Gabhann and Popel 2008, Scaldaferri, Vetrano et al. 2009). It has been shown that FGF2 can modulate the interaction of leukocytes with ECs in vitro (Byrd, Zhao et al. 1996, Wempe, Lindner et al. 1997, Zittermann and Issekutz 2006), secondary to the stimulation of adhesion molecule expression on ECs. We addressed the capacity of FGF1 and FGF2 to directly modulate neutrophil chemotaxis in an EC-free environment by using a modified boyden microchamber model. We observed that both FGF1

and FGF2 were capable of stimulating neutrophil recruitment, albeit with different potencies. These effects were almost completely abrogated with blocking anti-FGFR2 antibodies, and only partially blocked by anti-FGFR1 and FGFR4 antibodies, suggesting that most of the chemotactic activities of FGFs are mediated by FGFR2 activation.

In summary, our results demonstrate for the first time the exclusive expression of FGFR2 at the surface of human neutrophils. The predominance of the FGFR2α-IIIc isoform suggests that neutrophils respond to only a specific subset of FGF ligands. Accordingly, we observed that FGF1 and FGF2, both of which bind strongly to FGFR2α-IIIc, act as chemotactic agents for the recruitment of neutrophils in vitro, mainly through direct interaction and activation of FGFR2. Consequently, this study delineates a key inflammatory role for FGF1 and FGF2 and supports the possibility of additional functions for FGF/FGFR complex in modulating polymorphonuclear leukocyte pro-inflammatory activities.

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2.1.6 Acknowledgements

This work was supported by grants from the Canadian institutes of health research (CIHR; MOP-97943) and from the Heart and Stroke Foundation of Quebec. Dr. Sirois was recipient of a senior scientist scholarship from the Fonds de la recherche en santé du Québec (FRSQ). We would like to thank Marie-Elaine Clavet-Lanthier and Dominique Lauzier for their technical assistance and expertise in histology, as well as Maya Mamarbashi for her assistance in designing PCR oligonucleotides sequences and RT-qPCR experiments. Finally, we would like to thank all the volunteers that have agreed to generously donate blood.

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2.1.8 Figure Legends

Figure 1: ***Fibroblast growth factor (FGF) receptor (FGFR) 1–4 mRNA expression in human neutrophils.*** Circulating neutrophils isolated from 22 healthy individuals were profiled for FGFR1–4 mRNA expression. A: number of FGFR1–4 mRNA copies as well as means ± SE are presented. Donors are the same across columns, with each symbol per column corresponding to a single donor. B: distribution of FGFR1–4 mRNA copies per donor is illustrated. Each column represents a single donor, and the peak of the columns corresponds to the total number of FGFR copies. Donors were divided into low ($<4 \times 10^5$ total FGFR mRNA copies) and high ($>4 \times 10^5$ total FGFR mRNA copies) expressers. C: distribution of each FGFR1–4 mRNA illustrated in B is expressed as a percentage of total FGFR mRNA per individual. Dotted lines indicate the average FGFR2 percentage observed in low or high expressers.

Figure 2: ***Expression and localization of FGFR1–4 proteins by immunohistochemistry (IHC).*** Human neutrophils mounted in paraffin were stained with specific anti-human FGFR1–4 IgG antibodies. Representative IHC staining images for each receptor are illustrated as follows: control IgG (*A*), FGFR1 (*B*), FGFR2 (*C*), FGFR3 (*D*), and FGFR4 (*E*). The magnified (×100) boxes in the images were chosen from their corresponding fields (black arrows) and show the distinct localization patterns for each receptor.

Figure 3: ***Localization of FGFR1–4 by confocal microscopy***. Human neutrophils were stained with specific anti-human FGFR1–4 antibodies and a secondary Cy3-coupled antibody. Labeled neutrophils were viewed under confocal microscope to assess the protein localization of FGFR1–4. Columns, *left* to *right*, correspond to images of neutrophils taken from random fields: phase contrast showing membrane integrity, specific FGFR staining (secondary antibody coupled to Cy3 dye), and nuclear counterstaining (To-pro). The three-dimensional reconstructions of confocal Z-stack images (*last column*) correspond to neutrophils chosen at random from the larger associated fields.

Figure 4: ***FGFR2 subtype and isoform expression in human neutrophils.*** FGFRs are composed of up to three extracellular Ig-like domains that determine their binding properties to members of the FGF family. *A*: FGFRs can be expressed either as a long form (FGFR-α) containing all three Ig domains or as a shorter form lacking Ig-I (FGFR-β). *B*: alternative splicing in exons 8–9 of the Ig-III domain generates isoforms referred to as “IIIb” (spliced exon 9) or “IIIc” (spliced exon 8)

that exert different FGF-binding preferences. *C*: mRNA was isolated from the neutrophils of at least 7 healthy donors and assessed for FGFR2α and -β subtypes as well as IIIb-IIIc isoforms by quantitative (q)PCR analyses. The number of mRNA copies and means ± SE are shown, with each symbol per column representing a single donor.

Figure 5: ***Effect of FGF1 and FGF2 on neutrophil chemotaxis.*** *A*: untreated human neutrophils were set to migrate in a modified Boyden microchamber toward PBS, IL-8 (25 nM, positive control), or increasing concentrations of FGF1 or FGF2 (10^{-14} to 10^{-8} M). Cells from 5 random fields were counted, and migration is expressed relative to control-PBS. *B*: neutrophils were pretreated with 0.1 μg/mL of specific blocking antibodies for FGFR1, -2, or -4 or an isotype-matched IgG and then set to migrate toward PBS, IL-8, FGF1, or FGF2 (10^{-9} M). Migration of IgG-treated neutrophils toward FGF1 or FGF2 was set as the maximal effect (100%), and the migration of FGFR-pretreated neutrophils toward FGF1 or FGF2 is expressed as a percentage of the maximal effect. Results are presented as means ± SE for at least six independent experiments. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 as compared with control-PBS (*A*) or FGF1/FGF2 (*B*).

Table 1: Immunohistochemistry scores for FGFR1-4 expression

Neutrophils from 16 donors were assessed for the presence and localization of FGFR1-4 proteins by immunohistochemistry. Qualitative scores were attributed per individual for each receptor as follows: IgG background staining (*score 0*), faint but detectable staining in some cells (*score 1*), high staining in some cells (*score 2*), and high staining in most cells (*score 3*). Means ± SE for each receptor are shown in the bottom row.

Donors	FGFR1	FGFR2	FGFR3	FGFR4
1	2	3	1	2
2	3	1	0	1
3	1	3	0	0
4	0	0	0	0
5	1	3	0	2
6	3	3	1	2
7	1	2	0	1
8	0	2	0	1
9	2	2	0	0
10	2	2	0	1
11	3	2	0	1
12	1	2	0	1
13	0	2	1	0
14	1	2	1	0
15	1	1	1	0
16	2	2	1	1
Mean	1.43	2	0.37	0.81
SEM	0.36	0.50	0.09	0.20

Figure 1: FGFR1–4 mRNA expression in human neutrophils

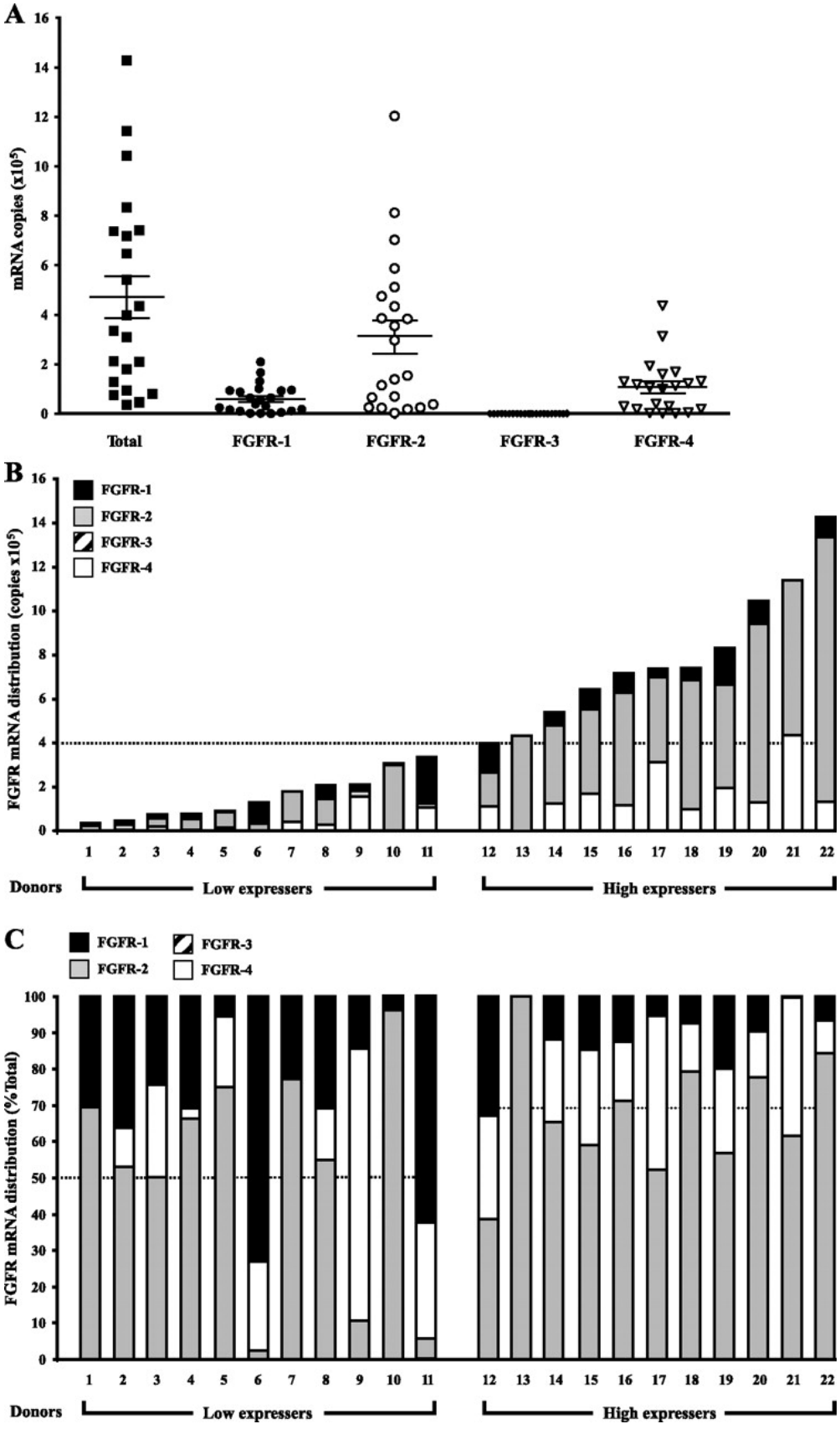


Figure 2: FGFR1–4 proteins by immunohistochemistry (IHC)

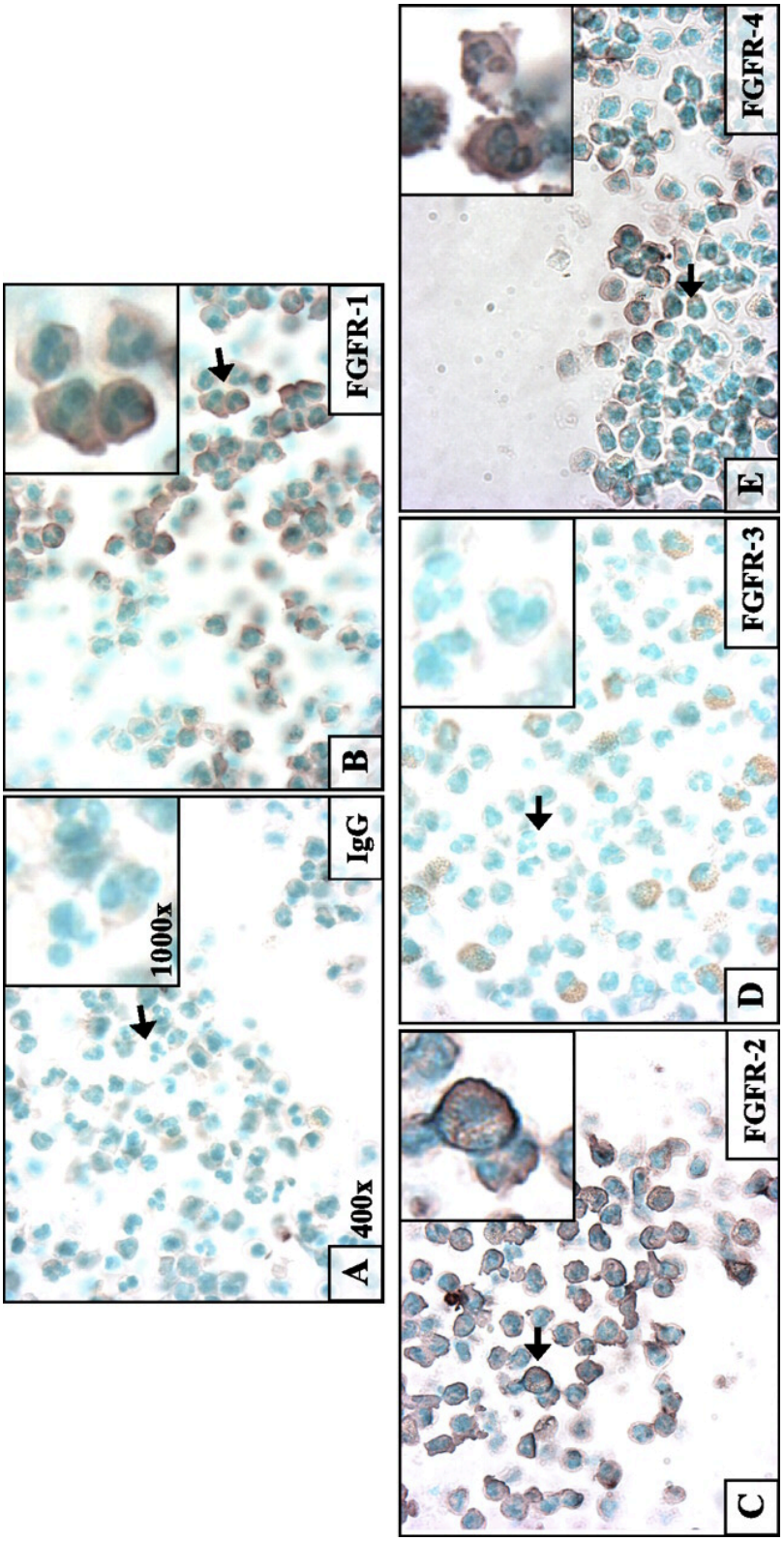


Figure 3: FGFR1–4 by confocal microscopy

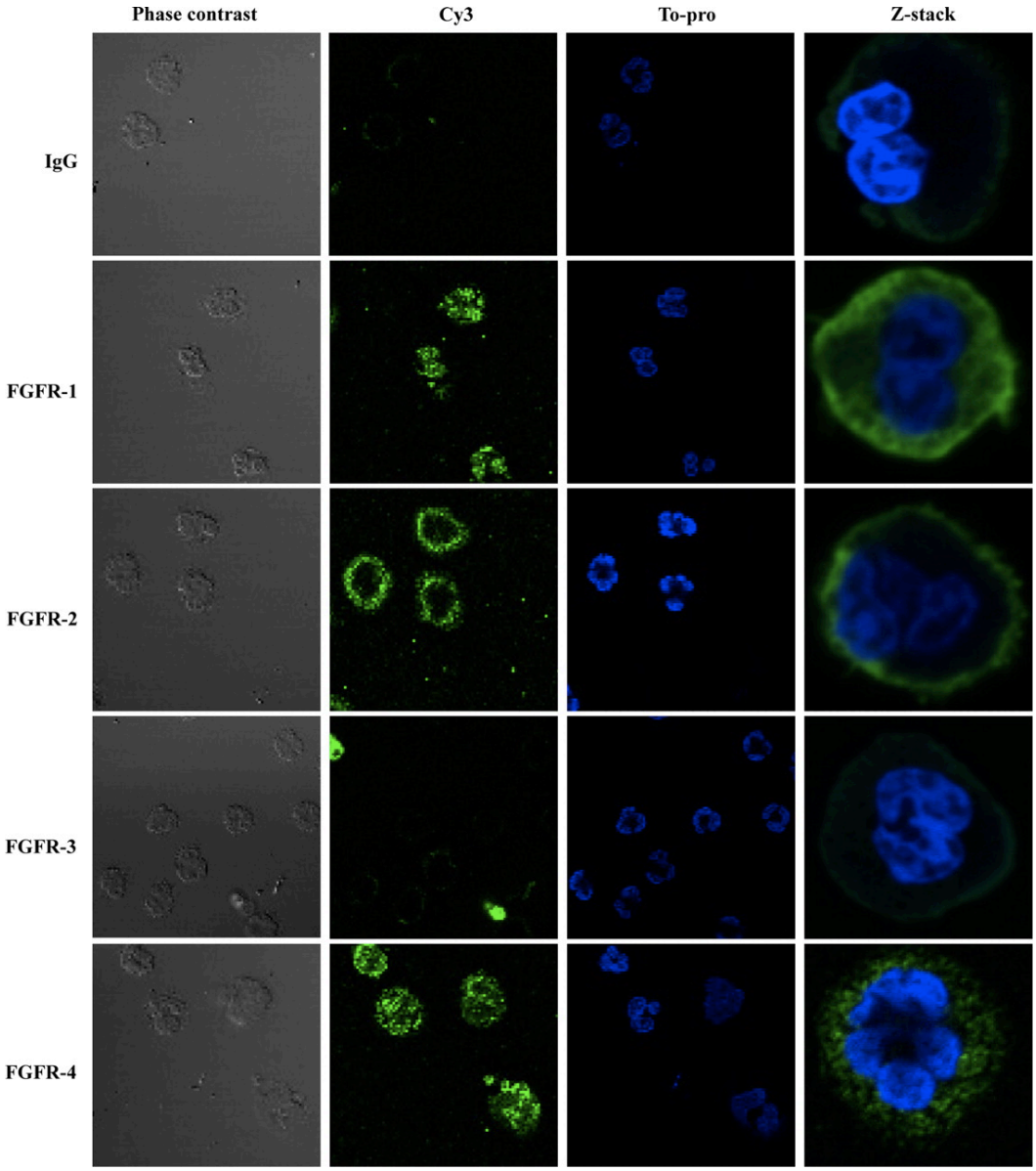


Figure 4: FGFR2 subtype and isoform expression

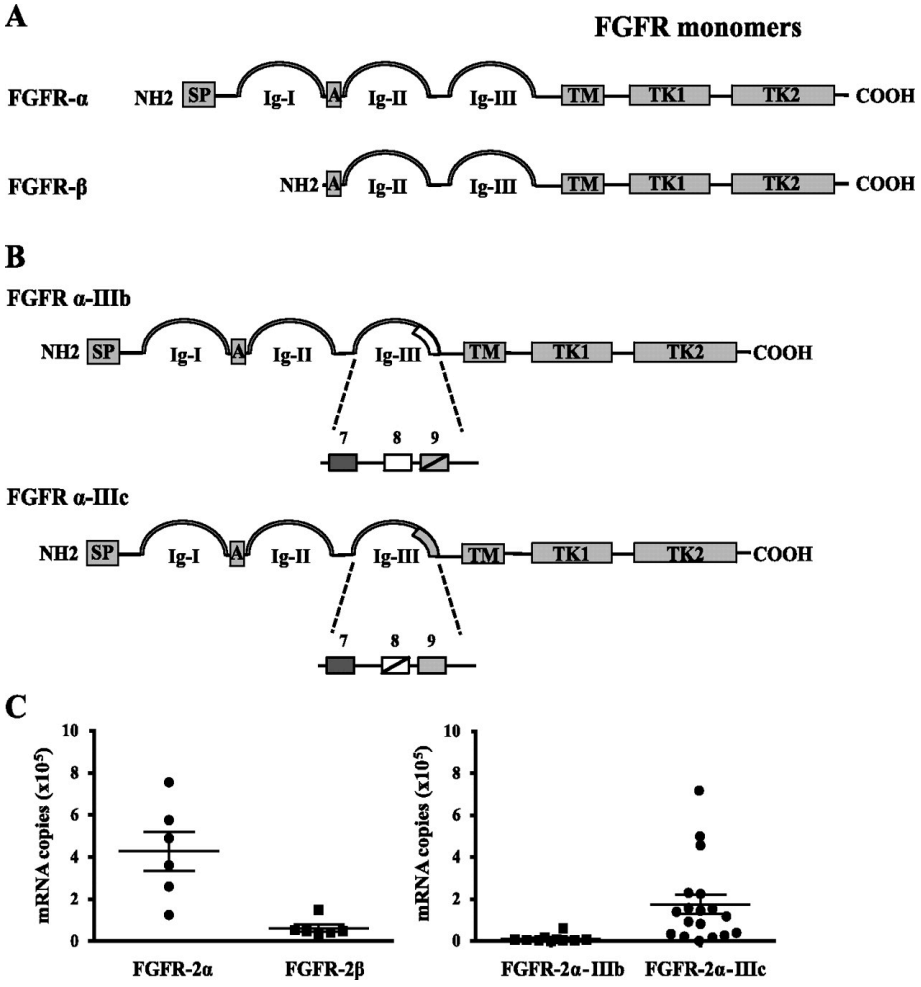
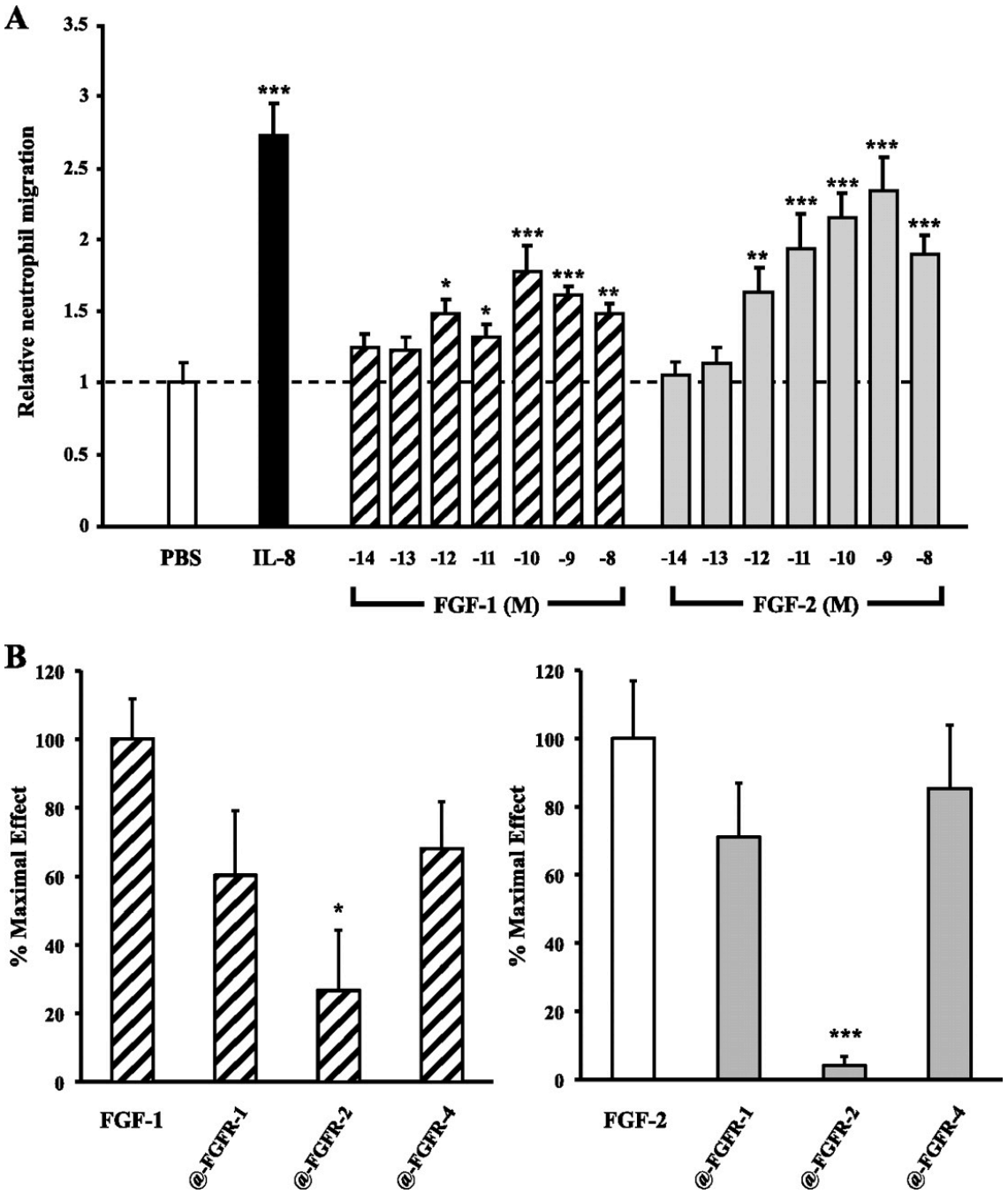


Figure 5: Chemotactic effect of FGF1 and FGF2



2.2 Article #2

Haddad, L.E. and M.G. Sirois, 2014. Angiopoietin-1 upregulates de novo expression of IL-1β and Il1-Ra, and the exclusive release of Il1-Ra from human neutrophils. *PLoS One*, 2014. **9**(2): p. e88980.

ANGIOPOIETIN-1 UPREGULATES DE NOVO EXPRESSION OF IL-1β AND IL1-RA, AND THE EXCLUSIVE RELEASE OF IL1-RA FROM HUMAN NEUTROPHILS

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Running title: Ang1 upregulates IL-1 production in neutrophils
Key Words: Angiopoietins • Ang1 • Ang2 • neutrophil • IL-1 • inflammation • cytokines • MAPK
Figures: 7, Tables: 3.
Contract grant sponsor: Canadian Institutes of Health Research (CIHR)
Contract grant number: MOP-97943
Contract grant sponsor: Heart and Stroke Foundation of Québec

2.2.1 Abstract

The expression of the angiopoietin (Ang) receptor, Tie2, on both endothelial and inflammatory cells supports the idea that Ang signalling may play a fundamental role in initiating and maintaining the inflammatory response. We have previously shown that Ang1 and/or Ang2 alter the innate immune response by enhancing human neutrophil survival, chemotaxis and production of inflammatory cytokine interleukin-8 (IL-8) *in vitro*. Thus, we hypothesized that Ang1 and Ang2 could modulate other inflammatory signals in neutrophils, a possibility we explored through a gene-based assay looking at changes in the mRNA expression of 84 inflammatory cytokines and their receptors. We observed that Ang1 (10^{-8} M), but not Ang2, increased mRNA expression of prominent pro-inflammatory cytokine IL-1β and its natural antagonist IL-1RA, by up to 32.6- and 10.0-fold respectively, compared to PBS-control. The effects of Ang1 extended to the proteins, as Ang1 increased intracellular levels of precursor and mature IL-1β, and extracellular levels of IL-1RA proteins, by up to 4.2-, 5.0- and 4.4-fold respectively, compared to PBS-control. Interestingly, Ang1 failed at inducing IL-1β protein release or at increasing intracellular IL-1RA, but the ratio of IL-1RA to mature IL-1β remained above 100-fold molar excess inside and outside the cells. The above-noted effects of Ang1 were mediated by MAP kinases, whereby inhibiting MEK1/2 lead to up to 70% effect reduction, whereas the blockade of p38MAPK activity doubled Ang1's effect. These findings suggest that Ang1 selectively alters the balance of neutrophil-derived inflammatory cytokines, favoring the blockade of IL-1 activity, a consideration for future therapies of inflammatory diseases.

2.2.2 Introduction

Inflammation is characterized by a sequence of events that involve activation of the endothelium, release of endothelial mediators, vascular remodeling to allow for increased permeability and blood flow, and leukocyte – especially neutrophil – recruitment and infiltration into inflamed tissues. Because acute inflammation and angiogenesis can be triggered by the same molecular events, it is not surprising that most molecules that alter permeability, such as vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)-α and nitric oxide (NO), are potent pro-angiogenic factors (review; (Arroyo and Iruela-Arispe 2010)).

Angiopoietins (Ang) are a family of angiogenic growth factors that play a major role in modulating vascular integrity and maturation. While the expression of the Ang receptor Tie2 on both endothelial and inflammatory cells (Feistritzer, Mosheimer et al. 2004, Lemieux, Maliba et al. 2005, Ahmad, Cudmore et al. 2010) suggests a potential involvement in inflammation, a literature review of the specific contributions of the primary family members, Ang1 and Ang2, reveals a dichotomy of pro- and anti-inflammatory properties that is often influenced by the presence of other inflammatory mediators. From an anti-inflammatory perspective, Ang1 counteracts some components of the activity of pro-inflammatory factors on endothelial cells (ECs), inhibiting increases in EC permeability induced by VEGF, thrombin, bradykinin and histamine in vitro (Gamble, Drew et al. 2000, Pizurki, Zhou et al. 2003, Oubaha and Gratton 2009). Additionally, Ang1 downregulates the release of chemokine IL-8 by ECs (Pizurki, Zhou et al. 2003), and inhibits adherence and transmigration of neutrophils across EC monolayers stimulated with VEGF, TNF-α and thrombin (Gamble, Drew et al. 2000, Kim, Oh et al. 2002, Pizurki, Zhou et al. 2003), likely through altering the expression of endothelial E-selectin and intracellular/vascular cell adhesion molecules (ICAM-1/VCAM-1) (Gamble, Drew et al. 2000, Kim, Moon et al. 2001). In mouse skin in vivo, co-overexpression of VEGF and Ang1 shows an additive effect on angiogenesis but results in leakage-resistant vessels with little inflammation (Thurston, Rudge et al. 2000). In stark contrast, Ang1 exerts certain pro-inflammatory activities: Ang1 increases endothelial P-selectin translocation, a protein that mediates the rolling of leukocytes onto the endothelium under inflammatory conditions(Maliba, Brkovic et al. 2008). Ang1 alone also has the ability to directly impact leukocyte behavior, stimulating neutrophil IL-8 synthesis and release(Neagoe, Dumas et al. 2011), and acting in a Tie2-dependent manner to recruit neutrophils and eosinophils, to increase neutrophil lifespan, and to promote neutrophil

adhesion onto extracellular matrix (Dumas, Martel et al. , Feistritzer, Mosheimer et al. 2004, Lemieux, Maliba et al. 2005, Sturn, Feistritzer et al. 2005, Brkovic, Pelletier et al. 2007). The contribution of Ang2 to acute inflammation is even less defined, with some evidence of pro-inflammatory properties such as enhancing TNF-α-dependent adhesion of leukocytes to EC monolayers, as well as TNF-α-induced expression of ICAM-1 and VCAM-1 (Fiedler, Reiss et al. 2006). Ang2 alone also promotes a transient endothelial P-selectin translocation and its effects on neutrophil adhesion and chemoattraction are Tie2-dependent and similar to those of Ang1 (Lemieux, Maliba et al. 2005, Sturn, Feistritzer et al. 2005); however, unlike Ang1, Ang2 fails to promote neutrophil IL-8 synthesis and/or release, to increase neutrophil survival, or to counteract the effects of Ang1 on the aforementioned processes (Dumas, Martel et al. , Neagoe, Dumas et al.). Thus, the distinct contributions of Ang1 and Ang2 to acute inflammation remain to be clearly delineated.

Neutrophils are generally the first responders at sites of inflammation. They contribute substantially to inflammation through their ability to produce proteases, reactive oxygen species (Cassatella 1995, Ely, Seeds et al. 1995), and to a lesser extent, cytokines including interleukin (IL)-6, TNF-α and IL-1 receptor antagonist (IL-1RA) (Kuhns, Young et al. 1998, Lapinet, Scapini et al. 2000, Jablonska, Kiluk et al. 2001, Xing and Remick 2003, Riedemann, Guo et al. 2004). Building on our recent findings that Ang1 promotes significant IL-8 production in human neutrophils in vitro in a time-dependent manner (Neagoe, Dumas et al.), we broadened our investigation to 84 other pro-inflammatory cytokines and their receptors, and looked at changes in their mRNA expression following angiopoietins stimulation. The first part of this study identified three related targets, all belonging to the IL-1 family of inflammatory cytokines, IL-1α, IL-1β, and IL-1RA, as well as a number of other potential interests unrelated to the IL-1 family. The second part of this study focused on identifying the kinetics and mechanisms that mediate the effects of Ang1 on IL-1 family members in neutrophils.

2.2.3 Materials and Methods

2.2.3.1 Neutrophil purification

The study was conducted in accordance with the Declaration of Helsinki and approved by the Montreal Heart Institute's ethical committee (Montreal, QC, Canada; ethics No. ICM #01-406). All of the subjects provided written informed consent to the experimental protocol before participating in the study. Venous blood was obtained from healthy donors free from medication for at least 10 days prior to the experiments. Venous blood was obtained by drawing 100 mL (4 x 25 mL) of blood using a 21G needle into 30 mL syringes prefilled with 5 mL of Anticoagulant Citrate Dextrose Solution USP (ACD) Formula A (Baxter Healthcare; Deerfield, IL). The blood was then transferred into 4 x 50 mL tubes and spun for 15 min at 200g at room temperature. Following the centrifugation, the platelet rich plasma (PRP) was removed from the top layer and 20 mL of a 4% Dextran solution (138 mM NaCl, 5 mM KCl, 0.34 mM Na2HPO4, 0.4 mM KH2PO4, 4.2 mM NaHCO3, 5.6 mM Glucose, 10 mM HEPES, 12.9 mM Sodium Citrate and 250 mM Dextran; pH 7.4) was added per tube. The tubes were gently mixed and red blood cells were left to sediment for 45 minutes at room temperature. The upper layer containing the white blood cells was collected and gently deposed on a 12.5 mL layer of Ficoll-Paque Plus (GE Healthcare; Baie d'Urfé, QC, Canada) in 50 mL tubes and spun for 28 minutes at 400g and at room temperature (Theoret, Bienvenu et al. 2001, Rollin, Lemieux et al. 2004, Neagoe, Brkovic et al. 2009). Following this centrifugation, the monocytes and lymphocytes were separated from the neutrophils by Ficoll gradient. The reminiscent red blood cells and neutrophils were found in the pellet. In order to eliminate the red blood cells from the neutrophils, we used a water lysis procedure by which we added 20 mL of distilled water over the neutrophils and red blood cells pellet and mix gently for 20 seconds, followed by the quick addition of 20 mL of HBSS 2X solution while continuing mixing, for a final concentration of HBSS 1X (pH 7.4). Neutrophils were then spun for 10 minutes at 200g and at room temperature. The pellet was then resuspended in RPMI 1640 medium with Corning Glutagro (Mediatech, Manassas, VA) supplemented with 25 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) and 1% penicillin/streptomycin. Contamination of isolated neutrophil suspension with peripheral blood mononuclear cells was less than 0.1% as determined by morphological analysis and flow cytometry, and viability was found to be greater than 98%, as assessed by Trypan blue dye exclusion assay.

2.2.3.2 RNA studies

Two RT-qPCR -based techniques were used. The first of these is a gene-based screening method; more specifically, real time quantitative polymerase chain reaction (RT-qPCR) arrays were used to identify targets of angiopoietins stimulation in inflammation. The second method was used to confirm array results and to expand mRNA expression kinetics. Recombinant human Ang1 and Ang2 were obtained from R&D Systems (Minneapolis, MN) and bacterial lipopolysaccharide (LPS) from Sigma-Aldrich (St Louis, MO).

RT-qPCR array analyses: Neutrophils (10^7 cells/mL; 1 mL) from at least three independent donors were treated with PBS, Ang1 (10^{-8} M) or Ang2 (10^{-8} M) for 90 minutes prior to DNAse treatment and total RNA extraction with the RNeasy extraction kit (Qiagen, Mississauga, ON, Canada). RNA samples were evaluated for integrity using a Bioanalyzer 2000 system (Genome Quebec Innovation Centre, McGill University, Montréal, QC, Canada); when all three samples (PBS, Ang1 and Ang2) from the same donor showed an mRNA integrity above 8.5, they were selected for use in arrays. RNA integrity between selected samples differed by less than 0.5. Following isolation, 2 μg of RNA were processed with RT2 First Strand Kit (SA Biosciences, Frederick, MD) according to manufacturer's instructions. Quantitative PCR analyses of chemokines and receptors were assessed with the Chemokines & Receptors PCR Array (SA Biosciences), RT2 SYBR® Green qPCR master mix (SA Biosciences) and a Stratagene Mx3500p qPCR System (Stratagene, La Jolla, CA). PCR array data were analyzed by the RT2 Profiler PCR Array Data Analysis program, available through SA Biosciences' web portal and based on the ΔΔCt method with four different housekeeping genes. Data were normalized to 4 housekeeping genes (B2M, HPRT1, RPL13A and GAPDH) and represented in a volcano plot of fold change in expression of each gene (compared to PBS-control) against its p-value.

RT-qPCR kinetics: Total RNAs (100 ng) from PBS, LPS (1 μg/mL), Ang1 (10^{-9} and 10^{-8} M) or Ang2 (10^{-9} and 10^{-8} M)-treated neutrophils were extracted as mentioned above and reverse transcribed using random hexamers and the Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Burlington, ON, Canada) according to manufacturer's instructions. Reactions were carried out on a MX3500P (Stratagene) using 10 ng of cDNAs, Syber Green (Invitrogen) and 300 nM of specific primers as follows (5' to 3'): • IL-1α forward (Fwd)

TGACCTGGAGGCCATCGCCAA; reverse (Rev) GCAGCAGCCGTGAGGTACTGA, • IL-1β Fwd ACGCTCCGGGACTCACAGCA; Rev TGAGGCCCAAGGCCACAGGT, • IL-1RA Fwd GATGTGGTACCCATTGAGCCTCATGC; Rev ACTGGTGGTGGGGCCACTGT. cDNAs were submitted to 40 cycles of amplification (temperatures for annealing: 60°C; dissociation: 55°C) and gave single peaks for each product. RT-qPCR products were purified on a 2% acrylamide gel, quantified using QIAquick Gel Extraction Kit (Qiagen) and sequenced. Gene expression was normalized using β-microglobulin as the housekeeping gene and results were expressed relative to calibrator T_0 (gene expression at time of isolation) or to the control-PBS at each time point.

In another set of experiments, neutrophils were pretreated with inhibitors of p38 MAP kinase (SB203580; 10 μM), MEK1/2 (U0126; 20 μM), Akt (Triciribine; 5 μM), DMSO (vehicle) or PBS for 30 minutes prior to a 1-hour stimulation with PBS, LPS (1 μg/mL), Ang1 (10^{-10} - 10^{-8} M) or Ang2 (10^{-10} - 10^{-8} M). Total RNAs were then extracted and submitted to RT-qPCR analyses as aforementioned.

2.2.3.3 Quantification of cytokines by ELISA

Purified neutrophils (10^7 cells/mL; 1 mL) were incubated in RPMI and treated with PBS, LPS (1 μg/mL) Ang1 (10^{-10} - 10^{-8} M) or Ang2 (10^{-10} - 10^{-8} M) for up to 24 hours at 37°C and 5% CO2. Upon the incubation period, neutrophils were centrifuged at 900 g for 6 minutes and supernatants collected and stored at -80°C. The centrifuged cells were then lysed in ice-cold 1% Triton-RPMI solution containing a cocktail of protease inhibitors. The complete kinetics of synthesis and release of IL-1α, IL-1β and IL-1RA as well as those for pro-IL-1β were evaluated from cell-lysates and supernatants respectively, using Quantikine (pro-IL-1β; R&D Systems) or Duoset ELISA development kits (IL-1α, 1β, 1RA; R&D Systems) and in accordance with manufacturer's instructions.

In another set of experiments, neutrophils were pretreated with DMSO-soluble inhibitors of p38 MAPK (SB203580; 1 and 10 μM), MEK1/2 (U0126; 2 and 20 μM), Akt (Triciribine; 1 and 5 μM), DMSO or PBS for 30 minutes prior to a 2-hour stimulation with PBS, LPS, Ang1 or Ang2. Final DMSO concentration in reaction volumes did not exceed 0.2%. Upon agonist stimulation, supernatants and lysates were collected and the concentrations of cytokines assessed by ELISA.

2.2.3.4 IL-1β maturation

IL-1β is synthesized in the cytoplasm as a 31-kDa precursor pro-protein (pro-IL-1β) that is cleaved to its mature 17-kDa form by IL-1β-converting enzyme (ICE; also known as caspase-1). Neutrophils (10^7 cells/mL; 1mL) treated with PBS, LPS (1 μg/mL), Ang1 (10^{-10} - 10^{-8} M) or Ang2 (10^{-10} - 10^{-8} M) for up to 6 hours were assessed for caspase-1 activity using the Caspase-1 Fluorometric Assay (R&D Systems). Upon each incubation period, neutrophils were centrifuged at 900 g for 6 minutes and the supernatants were gently discarded. Cells were then lysed with 250 μl of lysis buffer (provided by the manufacturer) and protein concentrations were determined with the BCA protein assay. The enzymatic reaction for caspase-1 activity was carried out in a 96-well flat bottom microplate using 50 μl (150 μg) of total protein and the caspase-1 fluorogenic substrate WEHD-AFC. The plates were incubated at 37°C for 2 hours and read on a fluorescent microplate reader (excitation 400 nm, emission 505 nm).

In previous studies, Perregaux et al. reported that the potassium ionophore, nigericin is capable of inducing efficient cleavage and release of newly synthesized IL-1β from LPS-treated macrophages (Perregaux, Barberia et al. 1992, Perregaux and Gabel 1994). We tested this hypothesis in 4 sets of neutrophils (10^7 cells/mL; 1 mL), each set treated with PBS, LPS (1 μg/mL), Ang1 (10^{-8} M) or Ang2 (10^{-8} M), for 2 hours at 37°C and 5% CO2 to induce maximal accumulation of pro-IL-1β. Upon the first incubation period, neutrophils were centrifuged at 900 g for 6 minutes. Lysates and supernatants were immediately collected from one set; for the remaining three sets, supernatants were carefully removed and replaced with RPMI containing vehicle (DMSO + ethanol), Cycloheximide (CHX; 10 μg/mL; + ethanol) to halt new protein synthesis, or CHX and the potassium-proton ionophore nigericin (N; 20 μM) for a further 45 minutes treatment (37°C and 5% CO2). Upon this second incubation period, the three sets were centrifuged at 900 g for 6 minutes, and supernatants and lysates were collected and assayed for pro-IL-1β and IL-1β concentrations by ELISA, as previously described. CHX was dissolved in DMSO to a final DMSO concentration that did not exceed 0.1%. Nigericin was dissolved in ethanol to a final ethanol concentration that did not exceed 0.05%.

2.2.3.5 Statistical analyses

Results are presented as the mean ± SEM of independent experiments performed on the neutrophils of at least three independent donors. Comparisons were made by one-way analysis of variance (ANOVA) followed by a Dunnett or tukey post-hoc test where applicable, using GraphPad Prism (Mac version). Differences were considered significant at p values <0.05.

2.2.4 Results

2.2.4.1 Expression of inflammatory cytokines and their receptors

We have recently reported that Ang1 (10^{-8} M) promotes the synthesis and release of the inflammatory cytokine IL-8 from neutrophils within 60 minutes and peaking within 2 hours of stimulation, whereas Ang2 has no such effect (Neagoe, Dumas et al.). Extending these observations, we assessed the potential of angiopoietins to modulate the expression of 84 inflammatory cytokines in neutrophils. Neutrophils were treated with PBS (control vehicle), Ang1 or Ang2 (10^{-8} M; 90 minutes), and total mRNAs were extracted for RT-qPCR array analyses. For follow-up experiments, we selected genes with a nominal P-value <0.05 and a change in expression level ≥ 4-fold (Figure 1A and B).

Based on the above criteria, most genes that were assayed did not fluctuate significantly following treatment with either angiopoietins (see Tables 1 and 2 for a list of all genes and their p-value). However, Ang1, but not Ang2, significantly upregulated the expression of 3 genes belonging to the IL-1 family (Figure 1A): IL-1α (49.65-fold increase; $p<0.001$), IL-1β (17.23-fold increase; $p<0.01$) and the endogenous antagonist IL-1RA (8.85-fold increase; $p<0.01$) as compared to PBS-treated cells. Neither other members of the IL-1 family nor the biologically active receptor IL-1R1 varied significantly under our experimental conditions (Table 1).

Under less stringent statistical parameters ($p\approx0.05$ and gene modulation ≥ 2-fold change), qPCR arrays identified three potentially interesting targets of Ang1 treatment: IL-8/CXCL8, Lymphotoxin Beta (LTB) and C-C chemokine receptor type 1 (CCR1) (Table 1 and Figure 1A). In parallel, Ang2 showed a tendency to up-regulate IL-8 receptor B (IL-8RB)/CXCR2 (Table 2 and Figure 1B). The significance of these potential targets will be covered briefly in the discussion.

2.2.4.2 Effect of angiopoietins on the mRNA expression of IL-1α, IL-1β and IL-1RA

Given the strong response of neutrophils in up-regulating IL-1 expression, and the importance of the latter family in initiating and modulating the inflammatory response, we sought to confirm and expand on the above using custom primers for IL-1α, IL-1β and IL-RA. Kinetics were performed by treating neutrophils with PBS, Ang1 (10^{-10} - 10^{-8} M), Ang2 (10^{-10} - 10^{-8} M) or LPS (1 μg/mL; positive control), for up to 6 hours before mRNA extraction. Given that lower concentrations of angiopoietins (10^{-10} and 10^{-9} M) had no significant effect on mRNA expression

compared to PBS-control, only the highest concentration of the angiopoietins (10^{-8} M) is represented in the graphs throughout the study. Additionally, because maximal Ang2 (10^{-8} M) had no significant effect compared to PBS-control, only Ang1 (10^{-8} M) is discussed below.

IL-1β mRNA was abundantly expressed in neutrophils, with a cycle threshold (Ct)< 25 at the time of isolation (T_0), and basal (PBS) IL-1β mRNA did not significantly change over time. Treatment with Ang1 (10^{-8} M) induced a rapid increase in IL-1β mRNA expression within an hour of stimulation, with 32.6-fold increase compared to PBS-treated cells, after which expression progressively returned to basal values (Figure 2A). Levels of IL-1RA mRNA were also abundant in neutrophils, with a Ct< 25 at T_0. Similarly to the potent positive control LPS, Ang1 promoted a significant increase in IL-1RA mRNA expression as early as 1 hour after stimulation, with 3.3-fold expression increase over PBS-control and reaching up to 9.8-fold at 6 hours (Figure 2B). Finally, regardless of treatment, IL-1α mRNA levels were hardly detectable in neutrophils (Table 3), with a Ct> 42 at T_0. Subsequent basal and treated Ct values remained above 35 throughout the 6-hour time-period indicating that IL-1α mRNA is barely, if at all, expressed in neutrophils.

2.2.4.3 Kinetics of protein synthesis and release

Building on the mRNA kinetics studies, we assessed basal expression and de novo protein synthesis and release for all three IL-1 family members following angiopoietin treatment. Kinetics studies were extended to a 24-hour period; at each time point, using the same lysates and/or supernatants, the concentrations of IL-1α, IL-1β or IL-1RA protein were simultaneously evaluated by ELISA. For the same reasons as per the mRNA section, only the highest concentration of Ang1 (10^{-8} M) is discussed below.

Intracellular levels of IL-1β in neutrophils (10^7 cells/mL) were almost undetectable at T_0 (Figure 3A). Basal IL-1β protein levels in PBS-treated neutrophils fluctuated over time, starting with 2.1 pg/mL at 30 minutes, reaching a peak of 37.7 pg/mL at 6 hours and declining to 4.7 pg/mL at 24 hours. Ang1 (10^{-8} M) treatment lead to a steady increase in IL-1β synthesis throughout the first 6 hours of stimulation, going from 16.4 pg/mL at 1 hour, up to 68.1 pg/mL at 6 hours, and then stabilizing between 12 and 24 hours at a value below 22 pg/mL.

Several studies performed on macrophages and monocytes in vitro reported that LPS and other mediators are capable of promoting IL-1β protein synthesis but fail to induce IL-1β release

(Arend, Smith et al. 1991, Perregaux, Barberia et al. 1992, Perregaux and Gabel 1994, Ferrari, Pizzirani et al. 2006, Arend, Palmer et al. 2008). Our current study shows that circulating human neutrophils behave in much of the same manner; indeed, under all conditions tested, no IL-1β was detected in the supernatants (Table 3), suggesting that the decreases in intracellular IL-1β levels over time were not due to its release.

Unlike IL-1β, intracellular levels of IL-1RA were substantial at T_0, with detection at 11 350 pg/mL (Figure 3B). We observed a short drop in intracellular IL-1RA in the first 30 minutes of stimulation, with levels fluctuating between 7 130 – 7 800 pg/mL regardless of treatment. For the remainder of the time-course, variations in intracellular IL-1RA levels were not statistically significant between treatments (Figure 3B), averaging between 7500 pg/mL to up to 16 400 pg/mL. In another stark contrast to IL-1β, we observed that neutrophils constitutively release IL-1RA: At T_0, we detected 629.1 pg/mL of extracellular IL-1RA in the supernatants (Figure 3C). Detection of extracellular IL-1RA under basal conditions continued throughout the entire time-course and corresponded to about 2-10% of total IL-1RA cellular content. Similarly to LPS, Ang1 promoted a statistically significant increase in IL-1RA release as early as 2 hours following stimulation, with 844.6 pg/mL IL-1RA released (vs. 468.2 pg/mL for PBS), after which detection values climbed to a peak of 1 379.3 pg/mL at 4 hours (vs. 498.8 pg/mL for PBS) (Figure 3C). Finally, IL-1α protein was not detected in neutrophil cell lysates or in their corresponding supernatants at T_0 or throughout the time-course under basal conditions or angiopoietin stimulation (Table 3).

2.2.4.4 Is IL-1β a product of de novo synthesis or maturation?

IL-1β is synthesized in the cytoplasm as an inactive 31-kDa-precursor protein (pro-IL-1β) before being cleaved to its mature 17-kDa form (Netea, Simon et al. 2010). Thus, we looked at the modulation of precursor pro-IL-1β levels in human neutrophils, and performed an initial assessment of the possible mechanisms governing pro-IL-1β cleavage. For the same reasons as per the mature protein, only the highest concentration of Ang1 (10^{-8} M) is discussed below.

De novo synthesis: As per the mature protein, intracellular levels of pro-IL-1β in human neutrophils were almost undetectable at T_0 (Figure 4). We observed an increase in pro-IL-1β levels under basal (PBS) conditions, reaching as much as 35.1 pg/mL at 2 hours, but subsequently declining to less than 10.0 pg/mL at 24 hours. Treatment with Ang1 (10^{-8} M)

promoted a substantial increase in pro-IL-1β synthesis starting at 1 hour, with detection reaching a peak of 147.6 pg/mL at 2 hours, and then declining to 16.0 pg/mL at 24 hours, compared to PBS. Irrespective of the treatment, we did not detect pro-IL-1β proteins in the supernatant of neutrophils (Table 3), consistent with reports that pro-IL-1β is not released from cells (Arend, Smith et al. 1991, Hogquist, Unanue et al. 1991, Chin and Kostura 1993, Arend, Palmer et al. 2008, Netea, Simon et al. 2010).

Maturation of pro-IL-1β has been attributed primarily to the activation of caspase-1; however, separate studies reported that the processing of IL-1β might actually occur via a caspase-1-independent mechanism, through enzymes such as serine proteases Cathepsin G, Neutrophil Elastase, and Proteinase-3 (Mayer-Barber, Barber et al. , Young, Thompson et al. 2004, Greten, Arkan et al. 2007, Guma, Ronacher et al. 2009). Using a fluorometric method, we assessed the activity of caspase-1 in human neutrophils treated with PBS, angiopoietins and LPS (1 μg/mL) for up to 6 hours. We did not detect any basal caspase-1 activity beyond threshold, and little to no changes following agonist stimulation (data not shown).

2.2.4.5 Induction of pro-IL-1β maturation and IL-1β release

Studies have reported that the mechanisms leading to the maturation and effective release of IL-1β depend on the subset of leukocytes being investigated. While monocytes readily release IL-1β under LPS treatment (Perregaux, Laliberte et al. 1996, Solle, Labasi et al. 2001), macrophages require a depletion of intracellular potassium induced by ionophores such as nigericin before efficient IL-1β maturation and subsequent release (Perregaux, Barberia et al. 1992, Perregaux and Gabel 1994). Because the mechanisms governing IL-1β maturation and release have never been reported in neutrophils, and given that even LPS failed at promoting IL-1β release, we tested the requirement for a secondary stimulus to drive neutrophil processing of pro-IL-1β and release of the mature protein.

Neutrophils were divided into four sets (Figure 5; Sets 1-4) and were treated with agonists for 2 hours, a time when most of the new pro-IL-1β has already accumulated under Ang1 (refer to Figure 4). Upon this first incubation period, supernatants and cell lysates from Set 1 were collected. For Sets 2-4, supernatants were replaced with new media containing vehicles (DMSO + ethanol) or nigericin (N; in ethanol) for an additional 45 minutes as described in Materials and

Methods. In order to eliminate the contribution of de novo synthesis to possible changes in levels of mature IL-1β (i.e. to confirm that any new IL-1β is a result of processing of the accumulated pro-IL-1β), a protein translation inhibitor, cycloheximide (CHX; in DMSO) was added during this step. To preserve sample comparability, supernatants from Sets 2-4 were supplemented with either DMSO or ethanol, as required.

Data from Sets 1 (Figure 5A and B) were used to establish the baseline of protein kinetics, and were comparable to what we had observed in our previous experiments for all the conditions tested. For pro-IL-1β (Figure 5A), neither the addition of vehicles (Set 2, left panel) nor CHX alone (Set 3, left panel) affected the synthesis of pro-IL-1β. Upon addition of nigericin (Set 4, left panel), we observed a near complete loss of detection of intracellular pro-IL-1β, in comparison to Set 3. This loss was not due to the release of pro-IL-1β, since the pro-protein in its native form was not detected in any of the corresponding extracellular fractions (Figure 5A, right panel).

Based on these observations, we hypothesized that nigericin may have indeed induced the processing of pro-IL-1β into IL-1β, as was reported to happen in macrophages (Perregaux, Barberia et al. 1992, Perregaux and Gabel 1994). However, the concomitant evaluation of IL-1β levels indicated that this was not the case (Figure 5B): While the addition of nigericin (Set 4, left panel) almost completely depleted intracellular IL-1β content compared to neutrophils from Set 3, most of the IL-1β was recovered in the extracellular fraction of nigericin-treated neutrophils (Set 4, right panel). In fact, the amount of IL-1β recovered extracellularly from nigericin-treated neutrophils (Set 4, right panel) nearly matched what had accumulated inside the cells prior to nigericin treatment (Set 3, left panel). Thus, potassium depletion did not promote maturation of pro-IL-1β into IL-1β in human neutrophils, but only the selective exteriorization of IL-1β.

2.2.4.6 Intracellular mechanisms of IL-1 family synthesis and release

Previous studies reported that the biological activities of angiopoietins can be mediated by PI-3K/Akt, p38 MAPK, and p42/44 MAPK pathways as a function of the cellular activities being solicited (Kim, Kim et al. 2000, Kim, Kim et al. 2000, Harfouche, Gratton et al. 2003, Brkovic, Pelletier et al. 2007, Maliba, Brkovic et al. 2008). Thus, we wanted to delineate the signalling pathway(s) involved in mediating the effects of the angiopoietins on synthesis and/or secretion of IL-1 family members in human neutrophils. Neutrophils were pretreated with inhibitors of

p42/44 MAPK Kinase - MEK1/2 - (U0126; (U0)), p38 MAPK (SB203580; (SB)) or Akt (Triciribine; (T)) for 30 minutes prior to agonist challenge, as previously described (Neagoe, Dumas et al. , Cuenda, Rouse et al. 1995, Favata, Horiuchi et al. 1998, Sun, Ramnath et al. 2009). Inhibitor-pretreated neutrophils were then compared to their vehicle (DMSO; D) counterparts.

mRNA changes: Because most of the inducible IL-1 mRNA was synthesized within the first hour of Ang1 treatment, we looked at the effects of the inhibitors on mRNA expression after 1 hour of agonist stimulation. Ang2 (10^{-10} – 10^{-8} M) and lower concentrations of Ang1 (10^{-10} – 10^{-9} M) yielded similar results as PBS-control under all conditions tested; thus, only the highest concentration of Ang1 (10^{-8} M) is discussed below.

Basal levels of IL-1β mRNA were not affected by either pretreatment with DMSO (vehicle) or with inhibitors (data not shown). Addition of the p38 MAPK inhibitor (SB) significantly increased the effect of Ang1 (10^{-8} M) on IL-1β mRNA expression, from 22.4-(Ang1-D) to 44.8-fold (Ang1-SB) expression (Figure 6A). MEK1/2 inhibition (U0) had the opposing effect, leading to a decrease from 22.4- to 7.8-fold (Ang1-U0) expression, corresponding to a 68% inhibition of Ang1 activity. The Akt inhibitor (T) had no significant effect on the activities of Ang1. Interestingly, none of the inhibitors significantly impacted the effects of LPS on IL-1β mRNA expression (Figure 6A).

As for IL-1RA mRNA expression, only MEK1/2 blockade had a mild, but statistically insignificant, decreasing effect on Ang1 treatment (Figure 6B). On the other hand, the blockade of p38 MAPK activity significantly reduced the effect of LPS, from 5.9- (LPS-D) to 2.8-fold (LPS-SB) expression, corresponding to a 63% inhibition. The blockade of MEK1/2 or Akt pathways had no significant effects on the activities of LPS (Figure 6B).

Protein changes: The immediate impact of the aforementioned mRNA changes on the corresponding protein levels was assessed at 2 hours of agonist stimulation, coinciding with the time at which protein synthesis rate was also at its maximum. For the same reasons as per the mRNA experiments, only the highest concentration of Ang1 (10^{-8} M) is discussed below.

Basal (PBS) protein levels were not affected by the addition of DMSO or any of the inhibitors (Figure 7A-D). While p38 MAPK inhibition significantly increased Ang1-induced pro-IL-1β synthesis by 79%, from 38.5 pg/mL (Ang1-D) to 64.3 pg/mL (Ang1-SB), blockade of MEK1/2

lead to a 60% inhibition, with pro-IL-1β protein levels decreasing from 38.5 pg/mL (Ang1-D) to 19.2 pg/mL (Ang1-U0) (Figure 7A). As for LPS, blockade of p38 MAPK lead to an important 80% inhibition of pro-IL-1β protein expression, as levels dropped from 262.1 pg/mL (LPS-D) to 55.9 pg/mL (LPS-SB), despite similar treatment having no effect at the mRNA level. Blockade of MEK1/2 or Akt had no effect on LPS-driven pro-IL-1β levels (Figure 7A).

The inhibition pattern for mature IL-1β mimicked that of pro-IL-1β for both Ang1 and LPS (Figure 7B). Blockade of p38 MAPK increased Ang1-driven IL-1β protein levels by 86%, as levels jumped from 17.3 pg/mL (Ang1-D) to 29.9 pg/mL (Ang1-SB). Blockade of MEK1/2 lead to a 68% inhibition, as IL-1β levels decreased from 17.3 pg/mL (Ang1-D) to 7.1 pg/mL (Ang1-SB). In the case of LPS, as per the precursor protein, mature IL-1β levels were deeply affected by the blockade of p38 MAPK, witnessing a 77% effect inhibition as levels dropped from 219.2 pg/mL (LPS-D) to 52.0 pg/mL (LPS-SB). Surprisingly, the blockade of MEK1/2 activity had a partial but significant effect on IL-1β protein, corresponding to 24% inhibition, as levels went from 219.2 pg/mL (LPS-D) to 166.9 pg/mL (LPS-U0). For both Ang1 and LPS, the Akt pathway did not modulate IL-1β levels significantly. It should be noted that no pro- or mature IL-1β proteins were detected in the extracellular fraction, regardless of treatment (data not shown).

Intracellular IL-1RA protein levels were maintained between 13-18 ng/mL across treatments, with only a very slight increase and decrease in Ang1 and LPS-driven levels, respectively, following p38 MAPK inhibition (Figure 7C). The lack of effect on the intracellular stores of IL-1RA protein following p38 MAPK blockade is noteworthy in the case of LPS, especially given the 63% drop in the corresponding mRNA; this implies that the cell holds IL-1RA mRNA in large excesses, and actually utilizes less than 40% of the total mRNA quantity it produces to convert into protein. However, the impact of inhibitors was immediately noticeable at the level of the release of IL-1RA (Figure 7D), suggesting that the cell prioritizes having a constant pool of intracellular IL-1RA and will modify the amount released in response to different conditions. First, under Ang1, the dynamics of p38 MAPK-MEK1/2 mediation differed from those of IL-1β, in that only the blockade of MEK1/2 had a significant impact, equivalent to 65% inhibition, on extracellular IL-1RA levels, as levels dropped from 963.4 pg/mL (Ang1-D) to 542.5 pg/mL (Ang1-U0). Meanwhile, the blockade of p38 MAPK had no important impact on Ang1-mediated IL-1RA release (Ang1-SB), in line with the apparent lack in p38 MAPK

contribution at the mRNA and the intracellular protein levels. For LPS, both the blockade of p38 MAPK and MEK1/2 exerted a negative effect on IL-1RA release: inhibition of p38 MAPK lead to a marked 75% inhibition, with levels dropping from 1 471.9 pg/mL (LPS-D) to 749.3 pg/mL (LPS-SB). Furthermore, blockade of MEK1/2 resulted in a marked 41% inhibition, with levels decreasing to 952.4 pg/mL (LPS-U0). Finally, for both Ang1 and LPS, the Akt pathway did not modulate IL-1RA release significantly.

2.2.5 Discussion

Vessel destabilization, increase in permeability and leukocyte infiltration are hallmarks of both inflammation and angiogenesis. Under normal physiological conditions, these processes undergo a natural resolution or removal of inciting signals, a critical step in preventing disorganized vascular network formation and a sustained inflammatory reaction. During the resolution step, changes in the microenvironment through local mediators produced lead to an active "push-back" of infiltrating neutrophils, and serve to limit the activity of destabilizers such as VEGF, nitric oxide (NO) and Ang2 while increasing stabilizing elements such as Ang1 (resolution reviewed in (Fagiani and Christofori 2013);(Serhan, Brain et al. 2007)). A previous study suggested that neutrophils might actually contribute to the resolution of inflammation based on their ability to produce endogenous anti-inflammatory mediators but little pro-inflammatory cytokines (Xing and Remick 2003). The present study supports those findings, as we show that during a 24-hour lifespan, neutrophils constitutively release endogenous anti-inflammatory mediator IL-1RA from a pool of stored protein that is continuously replenished, but no IL-1 agonists are produced or secreted. While we observed that Ang1 and LPS "prime" neutrophils to synthesize IL-1β de novo, in the absence of other signals, both precursor and mature IL-1β stores are retained within the intracellular compartment and are degraded over time. Additionally, the intracellular spikes in IL-1β levels were accompanied by parallel increases in the release of IL-1RA. Thus, we propose that neutrophils from healthy individuals naturally and intrinsically curtail the activity of IL-1 agonists, and "put the brakes" on the propagation of IL-1 mediated inflammation.

Of the 11 members of the IL-1 family of ligands, IL-1α and IL-1β are two major agonists with a demonstrated role in inflammation, angiogenesis, and hematopoiesis (Dinarello 2009). Both agonists bind to and activate IL-1 Receptor Type 1 (IL-1R1), and their activity is competitively antagonized by the endogenous IL-1RA. IL-1α and IL-1β are synthesized as precursor proteins; however, while IL-1α is active in both the precursor and the mature form upon release, IL-1β requires cleavage for activation and subsequent secretion. The importance of tight control over IL-1 production/processing is underlined by a number of serious inflammatory diseases, termed "autoinflammatory" (reviewed in (Dinarello 2011)), that are closely correlated with deregulation in bioactive IL-1β secretion, and where the use of recombinant IL-1RA (anakinra) has clear therapeutic benefits. Details on the processing of IL-1β and its release are still unclear, but several groups have pointed to mechanistic cell-dependent differences. In

monocytes, the IL-1β processing enzyme, caspase-1, is constitutively active, and mature IL-1β is released in large quantities (1 500 pg/mL) upon stimulation with LPS (Perregaux, Laliberte et al. 1996, Solle, Labasi et al. 2001) at a rate that is less than 20% of the total precursor pool (Dinarello 2011). For macrophages, an LPS challenge is insufficient; a second intracellular potassium (K+)-depleting stimulus is required to trigger the assembly of a complex called the inflammasome, the subsequent activation of caspase-1, and the processing and release of IL-1β (Perregaux, Laliberte et al. 1996, Brough, Le Feuvre et al. 2002, Dinarello 2009, Netea, Nold-Petry et al. 2009). Based on our current data, we show that processing of pro-IL-1β in neutrophils is neither contingent on caspase-1 activation, nor on K+ depletion. Strictly speaking, K+ emptying did lead to mature IL-1β being detected outside the cells; however, these levels were the result of a simple externalization of already-accumulated mature IL-1β, with no active role per se for K+ depletion in the maturation step. These results provide evidence that IL-1β maturation in human neutrophils is distinct from the release process, and could be mediated by a mechanism other than caspase-1, as suggested by Greten et al. (Greten, Arkan et al. 2007), or K+ efflux. The contribution of other components of the inflammasome to IL-1β processing in neutrophils, however improbable, should be considered and further explored.

2.2.5.1 Intracellular mechanisms

Several studies have suggested that p38 MAPK regulates the synthesis and release of cytokines by many types of blood cells. For example, inhibition of the p38 MAPK pathway in monocytes, macrophages and neutrophils blocked LPS-induced protein transcription (including that of IL-1β), translation and subsequent cytokine release (Lee, Laydon et al. 1994, Lee and Young 1996, Baldassare, Bi et al. 1999, Carter, Monick et al. 1999, Alvarez, Fuxman Bass et al. 2006).

In general, the p38 MAPK pathway responds weakly to growth signals and is preferentially recruited by pro-inflammatory cytokines, whereas p42/44 MAPKs have been shown to be strongly activated by growth factors and growth-promoting hormones. Such is the case for Ang1 in mediating IL-8 de novo synthesis in neutrophils, a process that occurs through a p42/44 MAPK-dependent mechanism, and independently of p38 MAPK or Akt activity (56). Along the same lines, the present study suggests that p42/44 MAPK mediates most of the effects of Ang1 on IL-1 production in neutrophils. However, IL-1RA regulation appears to be less stringent than that of IL-1β: while antagonist de novo synthesis is not affected by any inhibition,

and the release of IL-1RA is mostly regulated by a single signal transduction pathway (p42/44 MAPK), control of agonist production is two-fold involving not only p42/44 MAPK, but also p38 MAPK that exerts a negative regulatory role on the entirety of the IL-1β de novo synthesis process. The negative regulation exerted by p38 MAPK over the IL-1β synthesis process is likely a second insurance that IL-1β production remains tightly controlled when one of the two kinase pathways is unavailable, such as in the presence of a stronger pro-inflammatory signal.

A look into the downstream signalling governing the effects of LPS on IL-1 highlights differences that could be attributed to the potency of the inflammatory signal. According to our data, none of the studied pathways played a role in LPS-mediated IL-1β transcription, which is especially surprising for p38 MAPK given its similar role in macrophage cell lines (Baldassare, Bi et al. 1999). However, p38 MAPK impacted both IL-1β translation and processing, as the precursor and the mature proteins were significantly down-regulated with p38 MAPK blockade. LPS also recruited p42/44 MAPK for IL-1β maturation, and both kinase pathways had a significant contribution to IL-1RA release. Thus, in the context of neutrophil IL-1 production, the recruitment of downstream signalling effectors is stimulus-dependent. Finally, because neutrophils maintained their constitutive synthesis of IL-1RA at the same level despite the inhibitors, it is likely that other signal transduction effectors mediate this process.

2.2.5.2 Other potential targets of angiopoietin stimulation

qPCR arrays identified four additional potential targets of angiopoietin stimulation whose genetic changes could be rendered significant with more exhaustive kinetics studies: IL-8, CCR1 and Lymphotoxin B (LTB) for Ang1, and IL-8RB for Ang2. For instance, providing validation that the above targets might be significant is a recent finding that, when stimulated with Ang1 for 2 or more hours, neutrophils increased their IL-8 de novo synthesis (Neagoe, Dumas et al.), an effect that did not extend to Ang2. Lu P et al. demonstrated that IL-1α and IL-1β induced the production of a CCR1 ligand, CCL3, from human hepatomas (Lu, Nakamoto et al. 2003); while neither CCL3 nor any of the other CCR1 ligands (CCL4/MIP-1β and CCL5/RANTES) were affected by Ang1 treatment, it is possible that increases in IL-1β could drive CCR1 expression, increasing neutrophil responsiveness to surrounding tissue-derived corresponding ligands. As for Ang2, we have previously shown that it has similar agonistic capacity to Ang1 in mediating PAF synthesis, CD11b/CD18 activation and chemotaxis in neutrophils (Lemieux, Maliba et al. 2005,

Brkovic, Pelletier et al. 2007), but this is the first time we report that Ang2 may modulate protein transcription. While the cross-talk between angiopoietins and the aforementioned proteins remains to be elucidated, the involvement of CCL3/CCR1 and IL-8RB in neutrophil migration could offer additional insight into the mechanisms governing differences in Ang1 and Ang2-driven neutrophil chemotaxis.

In conclusion, the identification of several inflammatory targets of angiopoietin stimulation provides further evidence of the implication of angiopoietins in acute inflammation. We showed that Ang1, a blocker of vessel permeability, induces transcription, translation and maturation of one pro-inflammatory IL-1 agonist, IL-1β. Perhaps to counter the damaging activities of IL-1β in the presence of a potential release signal, or perhaps to initiate resolution or to push back any additional neutrophil infiltration, neutrophils upregulate their release of IL-1RA in response to both Ang1 and the more potent pro-inflammatory signal LPS, as was observed under TNF-α treatment (Langereis, Oudijk et al. 2011). These initial observations shed light on the complex interplay of inflammatory cells and mediators at the final stages of angiogenesis and acute inflammation, and provide a possible role for Ang1 in attenuating IL-1–related pathologies.

2.2.6 Acknowledgements

This work was supported by grants from the Canadian institutes of health research (CIHR; MOP-97943) and from the Heart and Stroke Foundation of Quebec. We would like to thank Mr. Fadi Hajjar and Mrs. Maya Mamarbashi for their invaluable insight in all matters pertaining to RT-qPCR experiments, as well as the volunteers that have agreed to generously donate blood.

2.2.7 References

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2.2.8 Figure Legends

Figure 1: ***Expression of inflammatory cytokines and their receptors in neutrophils.*** Circulating neutrophils isolated from 6 different donors were treated with PBS, Ang1 (10^{-8} M) (A) or Ang2 (10^{-8} M) (B) for 90 minutes prior to RNA isolation. Data are expressed in a Volcano plot, as fold change in gene expression (x-axis) compared to PBS-treated cells; values outside the dotted vertical lines indicate significant fold increases (positive values) or fold decreases (negative values). Values below the dashed horizontal line ($p<0.05$) underline statistical significance (y-axis). Each circle corresponds to the fold-expression of a single gene.

Figure 2: ***Kinetics of mRNA expression of IL-1β and IL-1RA.*** Primers were designed to quantify changes in the mRNA levels of IL-1β (A) and IL-1RA (B), following treatment with PBS, angiopoietins (10^{-10} - 10^{-8} M) or LPS (1 μg/mL), for up to 6 hours. For each time point, basal (PBS) mRNA expression is set to unitary value, and the data are presented as fold change compared to its corresponding PBS. For angiopoietins, only values resulting from treatment with the highest concentration (10^{-8} M) are shown. Data are represented as the means ± SEM of at least three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. PSB-control (Dunnett's test).

Figure 3: ***Kinetics of IL-1β and IL-1RA protein expression.*** Neutrophils were treated with PBS, angiopoietins (10^{-10} - 10^{-8} M) or LPS (1 μg/mL), for up to 24 hours. Intracellular IL-1β (A), IL-1RA (B) and extracellular IL-1RA (C) were quantified by ELISA. For angiopoietins, only values resulting from treatment with the highest concentration (10^{-8} M) are shown. No IL-1β was detected extracellularly. Data are represented as the means ± SEM of at least three independent experiments. *$p<0.05$, **$p<0.01$, *** $p<0.001$ vs. PSB-control (Dunnett's test).

Figure 4: ***Kinetics of pro-IL-1β protein expression.*** Neutrophils were treated with PBS, angiopoietins (10^{-10} - 10^{-8} M) or LPS (1 μg/mL), for up to 24 hours. Only intracellular levels of pro-IL-1β were detectable, as no pro-IL-1β was detected in the supernatants at any time points and under any of the conditions tested. For angiopoietins, only values resulting from treatment with the highest concentration (10^{-8} M) are shown. Data are represented as the means ± SEM of at least three independent experiments. *$p< 0.05$, **$p< 0.01$, *** $p< 0.001$ vs. PSB-control (Dunnett's test)

Figure 5: ***Effect of potassium depletion on IL-1β release.*** Neutrophils were treated with PBS, angiopoietins (10^{-10} - 10^{-8} M) or LPS (1 μg/mL), for two hours, followed by an additional 45-minute treatment with potassium ionophore nigericin, CHX, or appropriate vehicles. Changes in intracellular (left panels) and extracellular (right panels) levels of pro-IL-1β (A) and IL-1β (B) before and after ionophore addition were quantified by ELISA. CHX: Cycloheximide. N: Nigericin. Vehicles: DMSO, ethanol. Data are represented as the means ± SEM of at least three independent experiments. *p< 0.05, **p< 0.01, *** p< 0.001 vs. PSB-control (Dunnett's test).

Figure 6: ***Effect of downstream signalling inhibitors on IL-1β and IL-1RA mRNA expression***. Neutrophils were pretreated with inhibitors of Akt (Triciribine; 5 μM), p38 MAPK (SB203580; 10 μM), and p42/44 MAPKK (U0126; 20 μM), vehicle-DMSO (0.2%) or PBS for 30 minutes prior to a 1-hour agonist challenge. Total mRNA was used in RT-qPCR for assessment of mRNA expression of IL-1β (A) and IL-1RA (B). Data are presented as mean ± SEM of at least three independent experiments. *p< 0.05, **p< 0.01, *** p< 0.001 vs. PSB-control within each set (Dunnett's test); §p< 0.05, §§p< 0.01 vs. corresponding agonist-DMSO (Tukey test).

Figure 7: ***Effect of downstream signalling inhibitors on IL-1β and IL-1RA protein synthesis and release.*** Neutrophils were pretreated with inhibitors of Akt (Triciribine; 5 μM), p38 MAPK (SB203580; 10 μM), and p42/44 MAPKK (U0126; 20 μM), vehicle-DMSO (0.2%) or PBS for 30 minutes prior to a 2-hour agonist challenge. Concentrations of intracellular pro-IL-1β (A), IL-1β (B), IL-1RA (C) and released IL-1RA (D) were quantified by ELISA. Data are represented as mean ± SEM of at least three independent experiments. *p< 0.05, **p< 0.01, *** p< 0.001 vs. PSB-control within each set (Dunnett's test); §p< 0.05, §§p< 0.01 and §§§p< 0.001 vs. corresponding agonist-DMSO (Tukey test).

Table 1: List of inflammatory mediators and changes with Ang1.

Human neutrophils from at least 3 different individuals were treated with PBS, Ang1 or Ang2 (Table 2) at 10^{-8} M for 90 minutes. RT-qPCR array analyses were performed to assess expression change of 84 genes involved in the inflammatory response. Each gene from angiopoietin-treated neutrophils was compared to PBS-treated neutrophils and the data expressed as fold change. Negative and positive values denote a decrease and increase in mRNA expression, respectively. Differences were considered significant at Fold ≥ 4 and $p<0.05$. House keeping genes are denoted by an asterisk (*). Members of the IL-1 family that satisfied both requirements were considered significantly upregulated by Ang1, and are shaded in grey.

Genes	Fold	P	Genes	Fold	P	Genes	Fold	P	Genes	Fold	P
ABCF1	2.58	0.36	CCL4	1.12	0.31	CXCL14	-1.16	0.16	IL1F9	-1.18	0.05
BCL6	1.83	0.37	CCL5	-1.29	0.26	CXCL2	1.51	0.60	IL-1R1	2.04	0.56
C3	-1.26	0.56	CCL7	-1.78	0.53	CXCL3	1.51	0.44	IL-1RA	8.85	0.001
C4A	-1.04	0.54	CCL8	-1.16	0.13	CXCL5	-1.22	0.77	IL22	-1.32	0.68
C5	-1.16	0.32	CCR1	3.33	0.04	CXCL6	1.73	0.43	IL5	-1.16	0.75
CCL1	-1.16	0.41	CCR2	-1.47	0.29	CXCL9	-1.16	0.90	IL5RA	-1.34	0.97
CCL11	-3.05	0.66	CCR3	4.19	0.56	CARD18	-1.16	0.32	IL8	2.11	0.04
CCL13	-2.47	0.56	CCR4	-1.16	0.54	IFNA2	1.11	0.22	IL8RA	1.23	0.89
CCL15	-1.16	0.56	CCR5	1.02	0.16	IL10	-1.30	0.56	IL8RB	2.72	0.23
CCL16	-1.16	0.84	CCR6	-1.28	0.81	IL10RA	1.19	0.56	IL9	-1.30	0.96
CCL17	-1.09	0.50	CCR7	1.01	0.50	IL10RB	4.05	0.56	IL9R	-1.19	0.42
CCL18	-1.16	0.82	CCR8	-1.43	0.30	IL13	-2.05	0.42	LTA	-1.42	0.46
CCL19	1.73	0.36	CCR9	-1.16	0.49	IL13RA1	1.29	0.40	LTB	1.95	0.000
CCL2	2.59	0.34	CEBPB	1.07	0.31	IL17C	-2.33	0.35	LTB4R	1.17	0.44
CCL20	3.13	0.48	CRP	-1.32	0.27	IL-1α	49.65	0.000	MIF	-1.07	0.23
CCL21	-2.48	0.49	CX3CR1	1.33	0.94	IL-1β	17.24	0.004	SCYE1	-1.01	0.87
CCL23	1.67	0.00	CXCL1	2.01	0.96	IL1F10	-1.16	0.96	SPP1	1.39	0.88
CCL24	-1.27	0.02	CXCL10	-1.09	0.99	IL1F5	-1.14	0.96	TNF	1.19	0.04
CCL25	-1.17	0.56	CXCL11	-1.16	0.53	IL1F6	-1.16	0.96	CD40LG	-1.16	0.63
CCL26	-1.99	0.57	CXCL12	-1.98	0.90	IL1F7	1.58	0.64	TOLLIP	1.82	0.74
CCL3	-1.10	0.56	CXCL13	-1.67	0.56	IL1F8	-1.76	0.96	XCR1	-1.50	0.43
B2M*	1.24	0.56	HPRT1*	1.07	0.47	RPL13A*	-1.34	0.40	GAPDH*	1.01	0.04

Table 2: List of inflammatory mediators and changes with Ang2.

As per Table 1, each gene from Ang2-treated neutrophils was compared to its counterpart from PBS-treated neutrophils and the data expressed as fold-change. Although genes such as CCL7, CCL11, CCL13, CCL24 and IL10RB showed substantial fold-change differences between Ang2 and PBS, statistical significance denoted by the p-value was far from 0.05. Because of its fold-regulation and a p-value close to 0.05, IL-8RB is a promising target.

Genes	Fold	P	Genes	Fold	P	Genes	Fold	P	Genes	Fold	P
ABCF1	1.48	0.39	CCL4	2.19	0.21	CXCL14	-1.63	0.44	IL1F9	-1.45	0.73
BCL6	1.23	0.64	CCL5	1.04	0.76	CXCL2	3.39	0.34	IL-1R1	1.10	0.68
C3	1.73	0.39	CCL7	-4.43	0.13	CXCL3	1.09	0.91	IL-1RA	1.16	0.95
C4A	-1.09	0.75	CCL8	-1.34	0.62	CXCL5	-3.15	0.34	IL22	-3.17	0.27
C5	-1.16	0.64	CCR1	-1.13	0.64	CXCL6	1.71	0.29	IL5	-1.54	0.40
CCL1	-1.56	0.45	CCR2	1.65	0.43	CXCL9	1.51	0.45	IL5RA	-2.30	0.55
CCL11	-8.00	0.17	CCR3	3.52	0.22	CARD18	-1.47	0.62	IL8	3.21	0.11
CCL13	-12.29	0.24	CCR4	-1.02	0.91	IFNA2	1.06	0.64	IL8RA	2.21	0.46
CCL15	-1.56	0.45	CCR5	-1.31	0.42	IL10	-2.64	0.26	IL8RB	13.48	0.07
CCL16	-1.68	0.45	CCR6	-1.05	0.75	IL10RA	1.12	0.90	IL9	-1.68	0.29
CCL17	-1.24	0.30	CCR7	2.39	0.18	IL10RB	5.03	0.17	IL9R	-1.96	0.12
CCL18	-1.79	0.44	CCR8	-1.80	0.43	IL13	-3.76	0.30	LTA	-1.90	0.27
CCL19	-1.40	0.86	CCR9	-1.22	0.53	IL13RA1	1.70	0.17	LTB	-1.74	0.39
CCL2	-1.99	0.38	CEBPB	-1.51	0.45	IL17C	-2.46	0.64	LTB4R	1.82	0.30
CCL20	1.91	0.40	CRP	-1.91	0.67	IL-1α	3.63	0.32	MIF	-2.01	0.75
CCL21	-2.29	0.35	CX3CR1	-1.23	0.75	IL-1β	2.44	0.31	SCYE1	1.07	0.73
CCL23	1.03	0.94	CXCL1	-1.84	0.64	IL1F10	-1.83	0.44	SPP1	1.55	0.41
CCL24	-4.98	0.17	CXCL10	-1.03	0.96	IL1F5	-1.61	0.23	TNF	-2.69	0.49
CCL25	-1.12	0.83	CXCL11	-1.51	0.44	IL1F6	-1.83	0.44	CD40LG	-1.02	0.60
CCL26	-3.40	0.48	CXCL12	-3.68	0.36	IL1F7	-1.97	0.56	TOLLIP	1.57	0.39
CCL3	1.03	0.86	CXCL13	-3.13	0.45	IL1F8	-1.89	0.29	XCR1	-2.64	0.46
B2M*	1.05	0.74	HPRT1*	-1.22	0.94	RPL13A*	-1.34	0.93	GAPDH*	1.56	0.21

Table 3: IL-1α, β and RA mRNA and protein changes with Ang1.

T_0 represents content at the time of isolation and reflects the state of circulating human neutrophils in healthy individuals; intracellular and extracellular content are assessed after stimulation with Angiopoietin-1 (Ang1); S: stimulated; N.D.: not detectable. Unstimulated neutrophils do not express or store pools of IL-1α mRNA, and that is not altered by the addition of Ang1. Neutrophils hold large pools of IL-1β mRNA, but Ang1 signal is required for translation. Finally, neutrophils constitutively express and release IL-1RA.

Genes	mRNA	Protein		
		T_0	Intracellular (S)	Extracellular (S)
IL-1α	N.D.	N.D	N.D	N.D
IL-1β	+	N.D	+	N.D
Pro-IL-1β	+	N.D	+	N.D
IL-1RA	+	+	+	+

Figure 1: Changes in expression of inflammatory cytokines after Ang

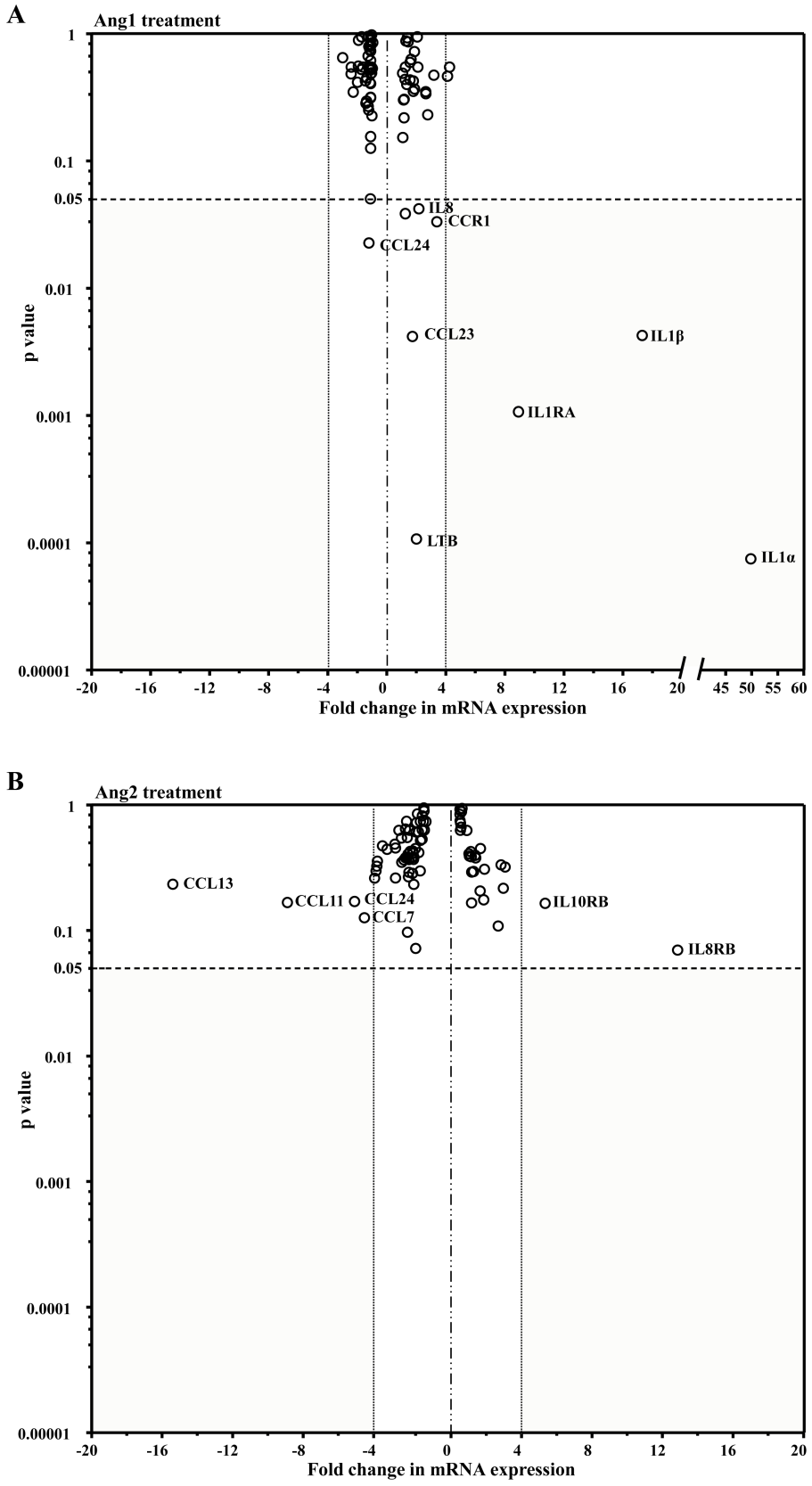


Figure 2: Kinetics of mRNA expression of IL-1β and IL-1RA

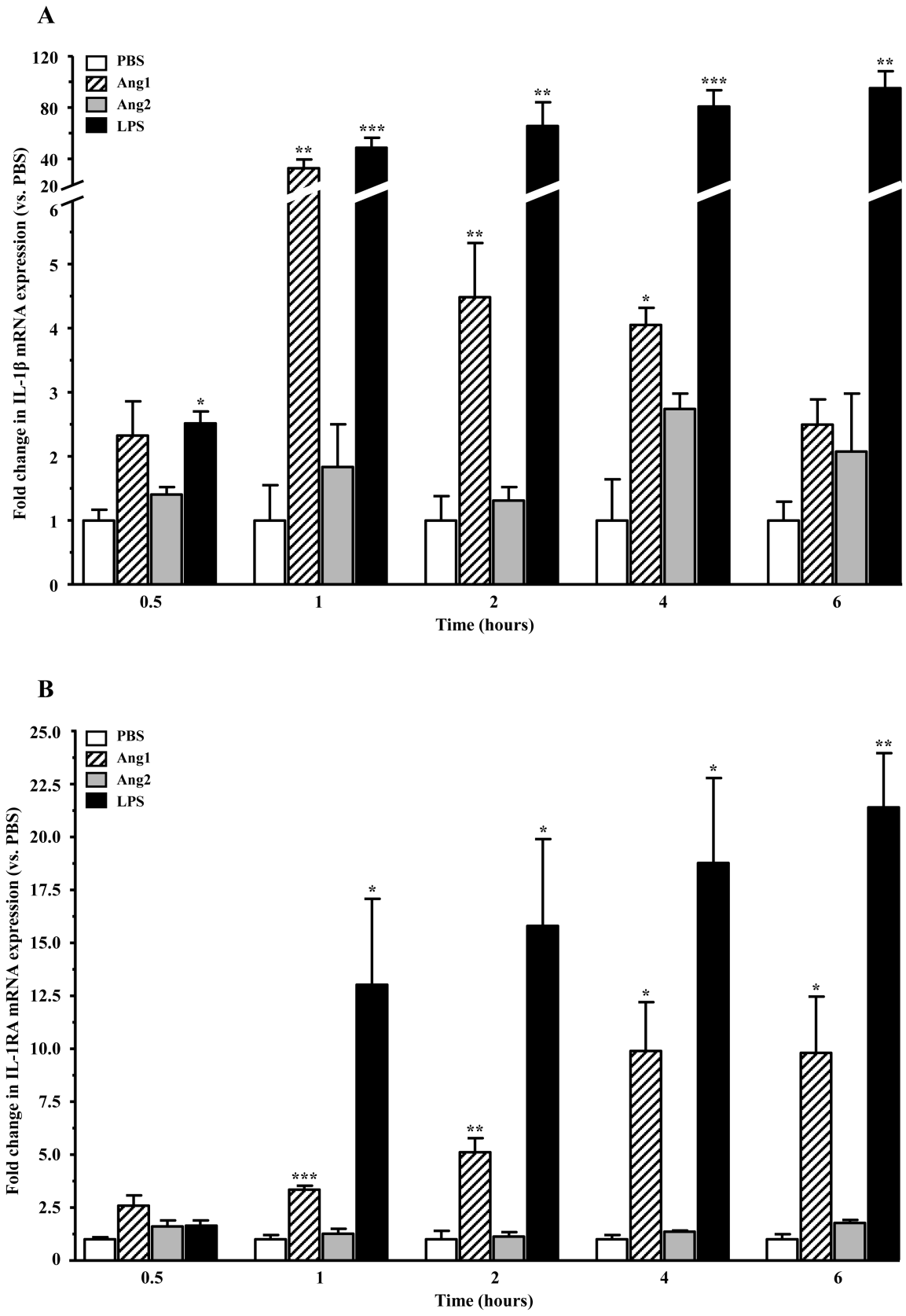


Figure 3 (A): Intracellular IL-1β protein expression

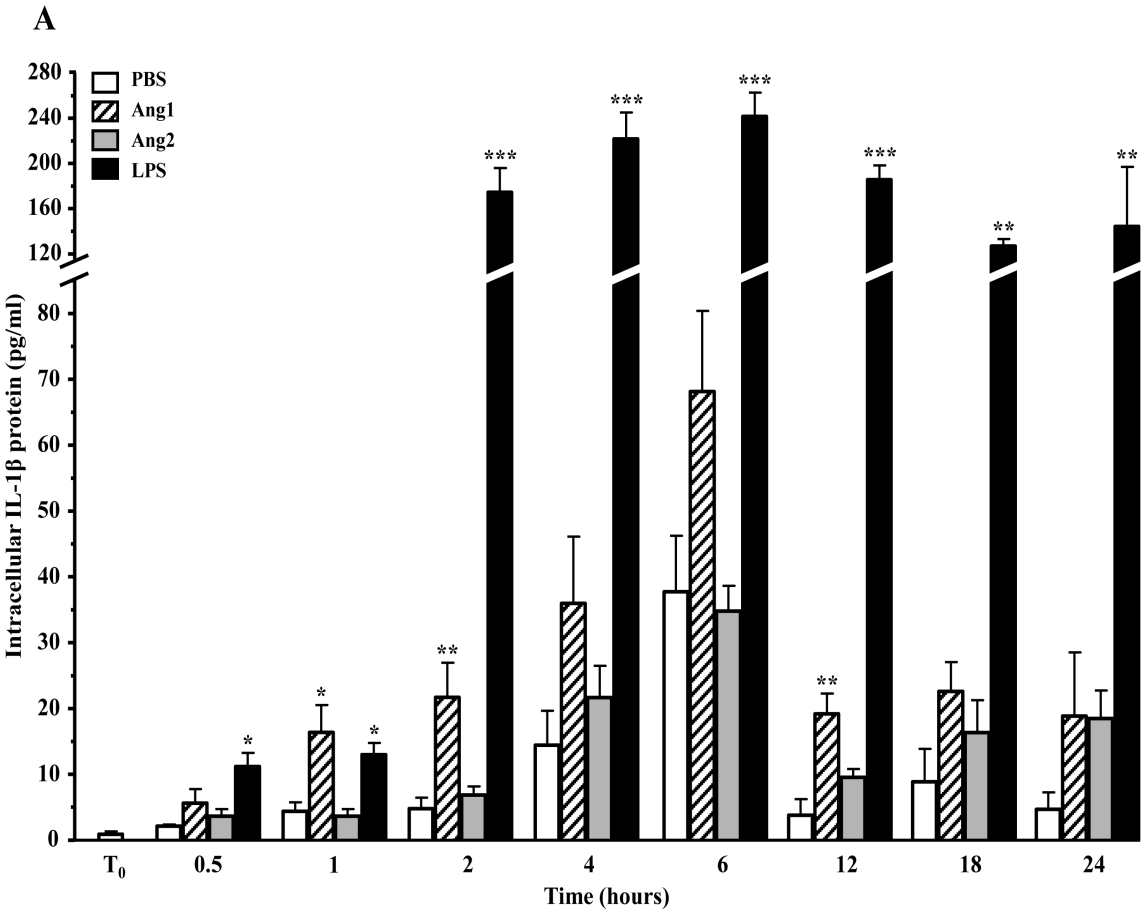
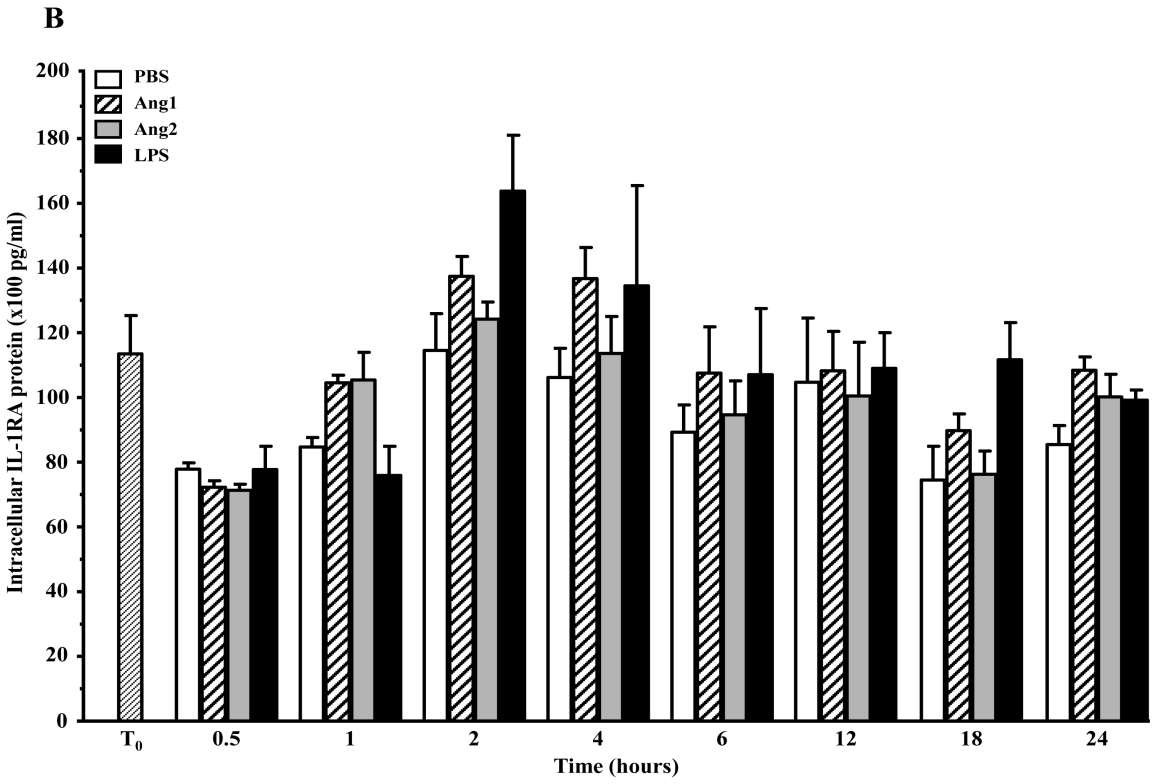


Figure 3 (B-C): IL-1RA protein expression and release



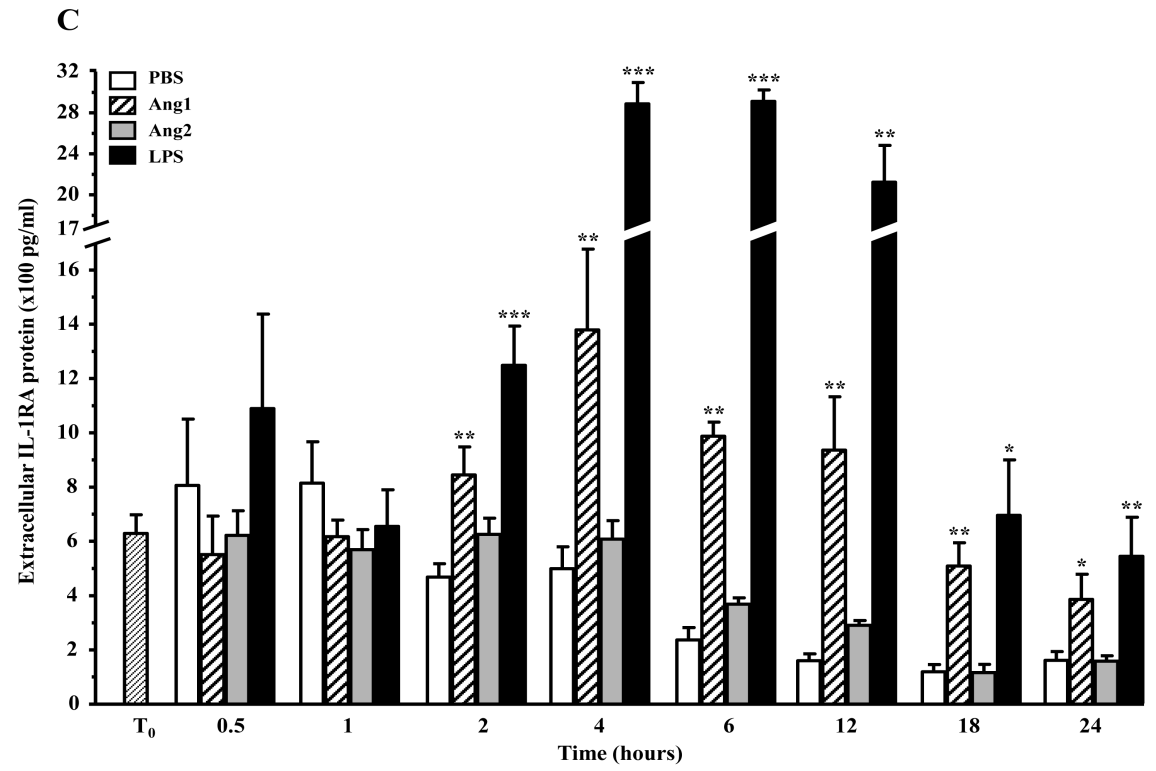


Figure 4: Kinetics of pro-IL-1β protein expression

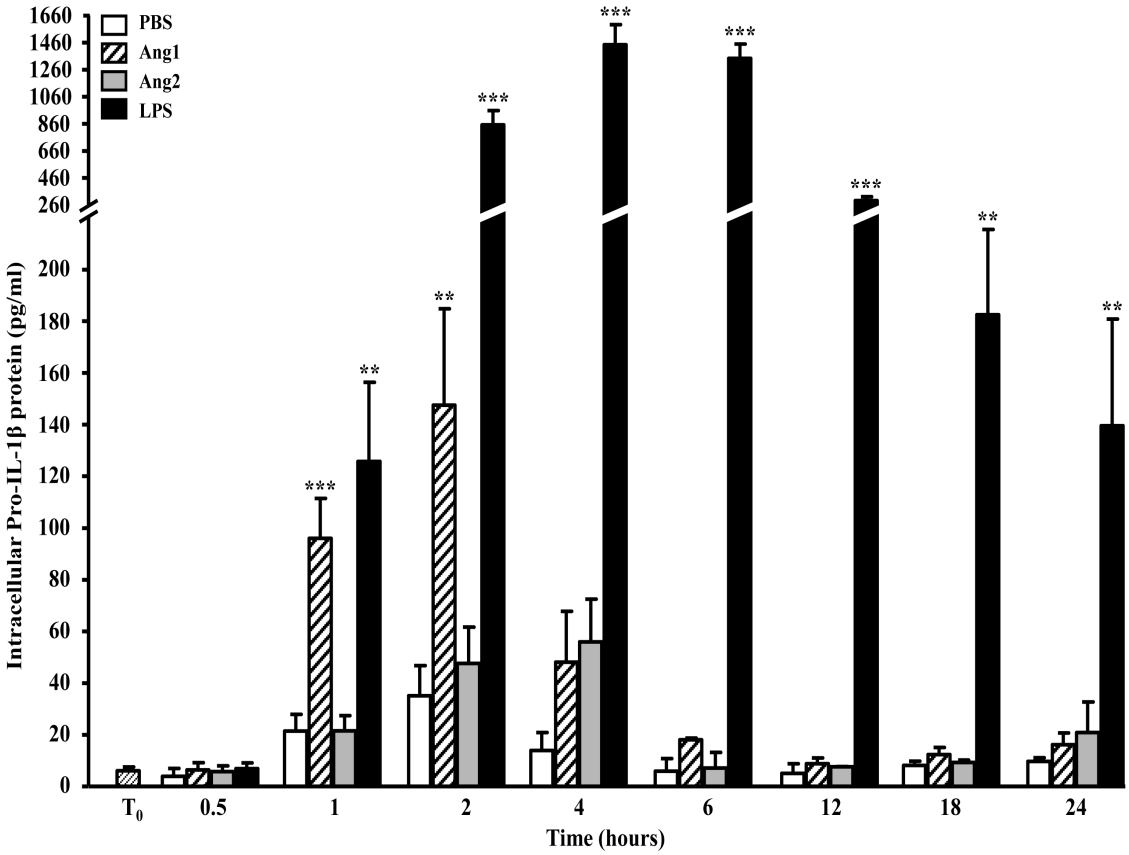


Figure 5: Effect of potassium depletion on IL-1β release

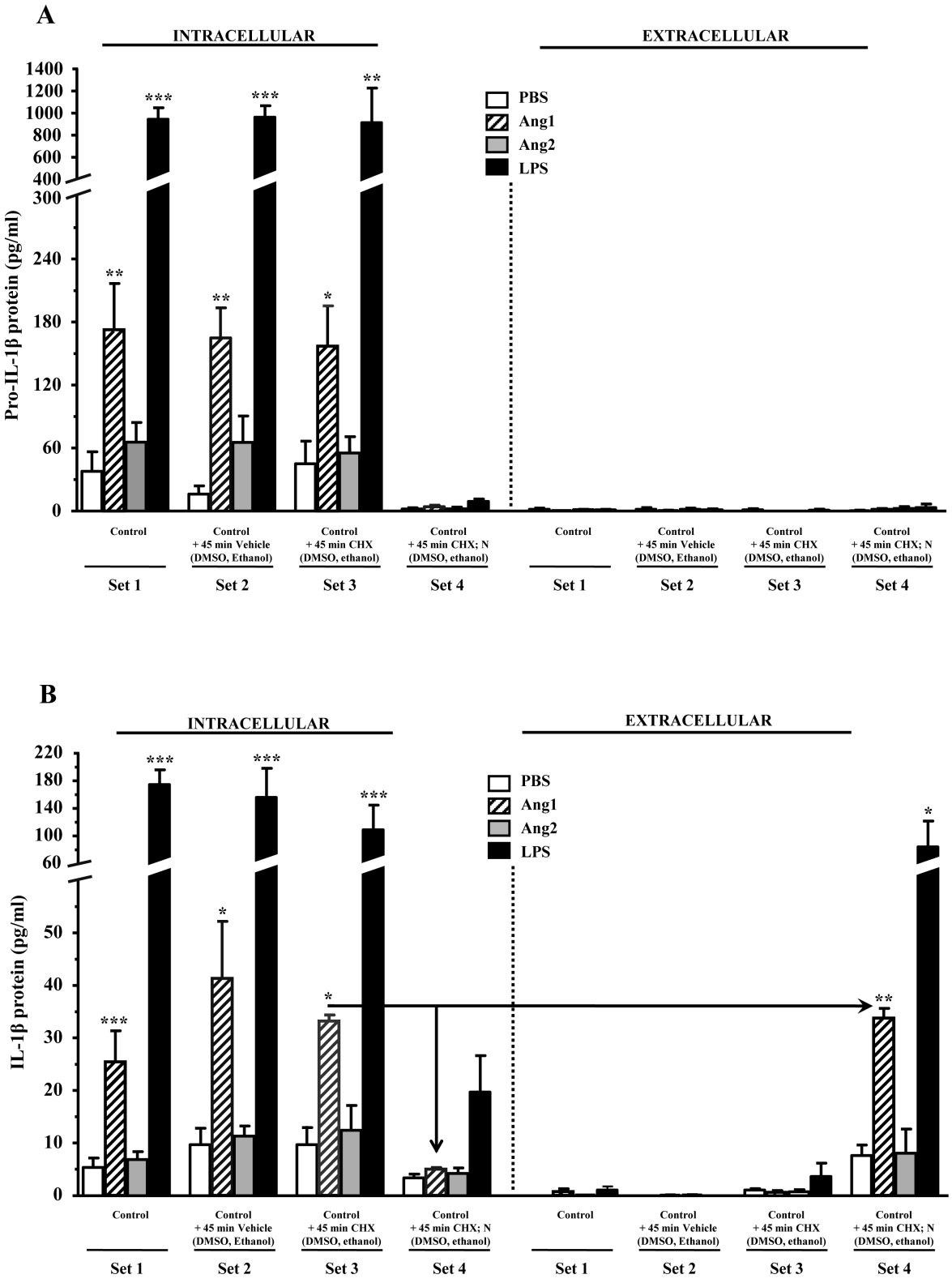


Figure 6: Signalling involed in IL-1 mRNA expression

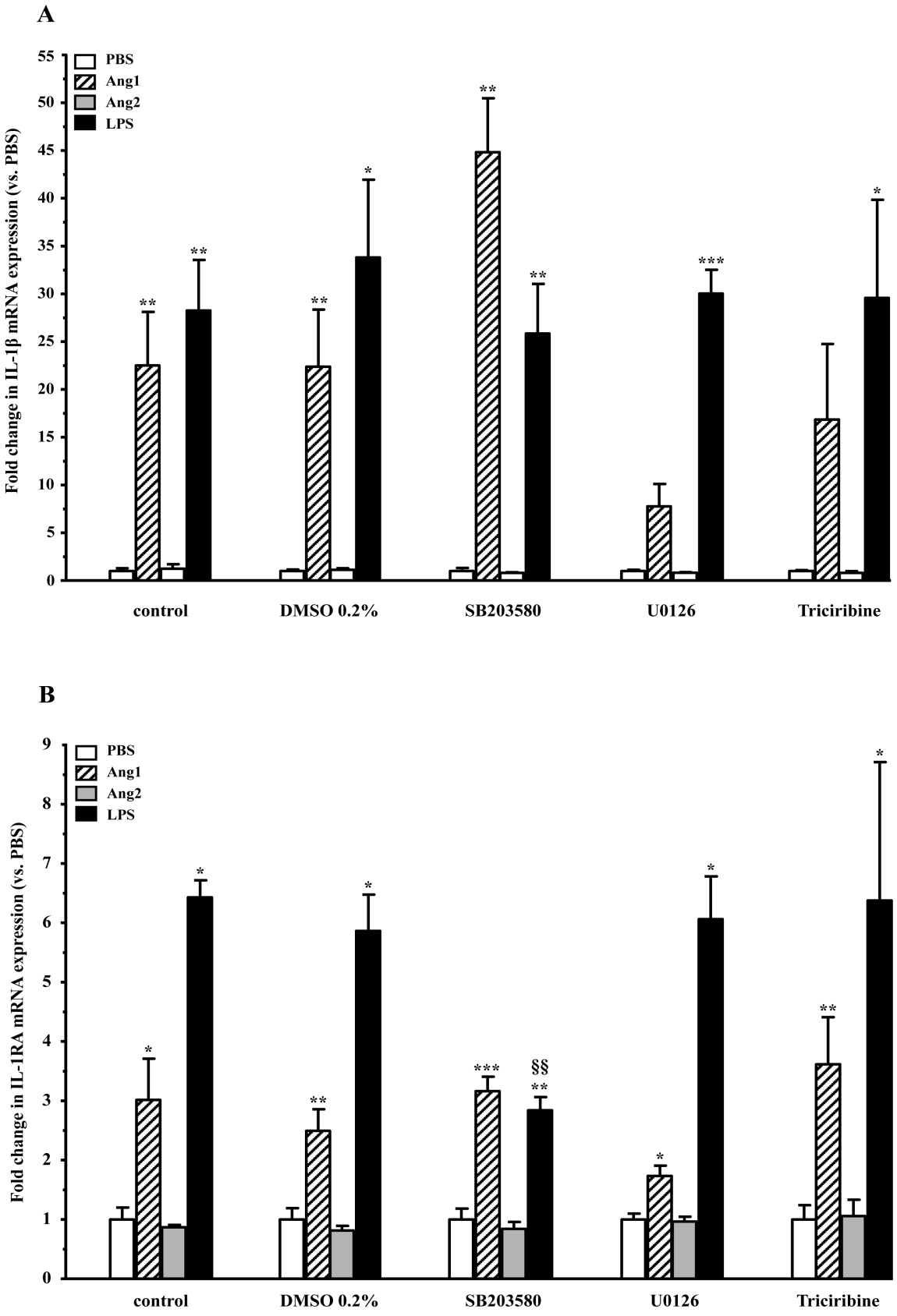


Figure 7 (A-B): Signalling involed in IL-1β protein

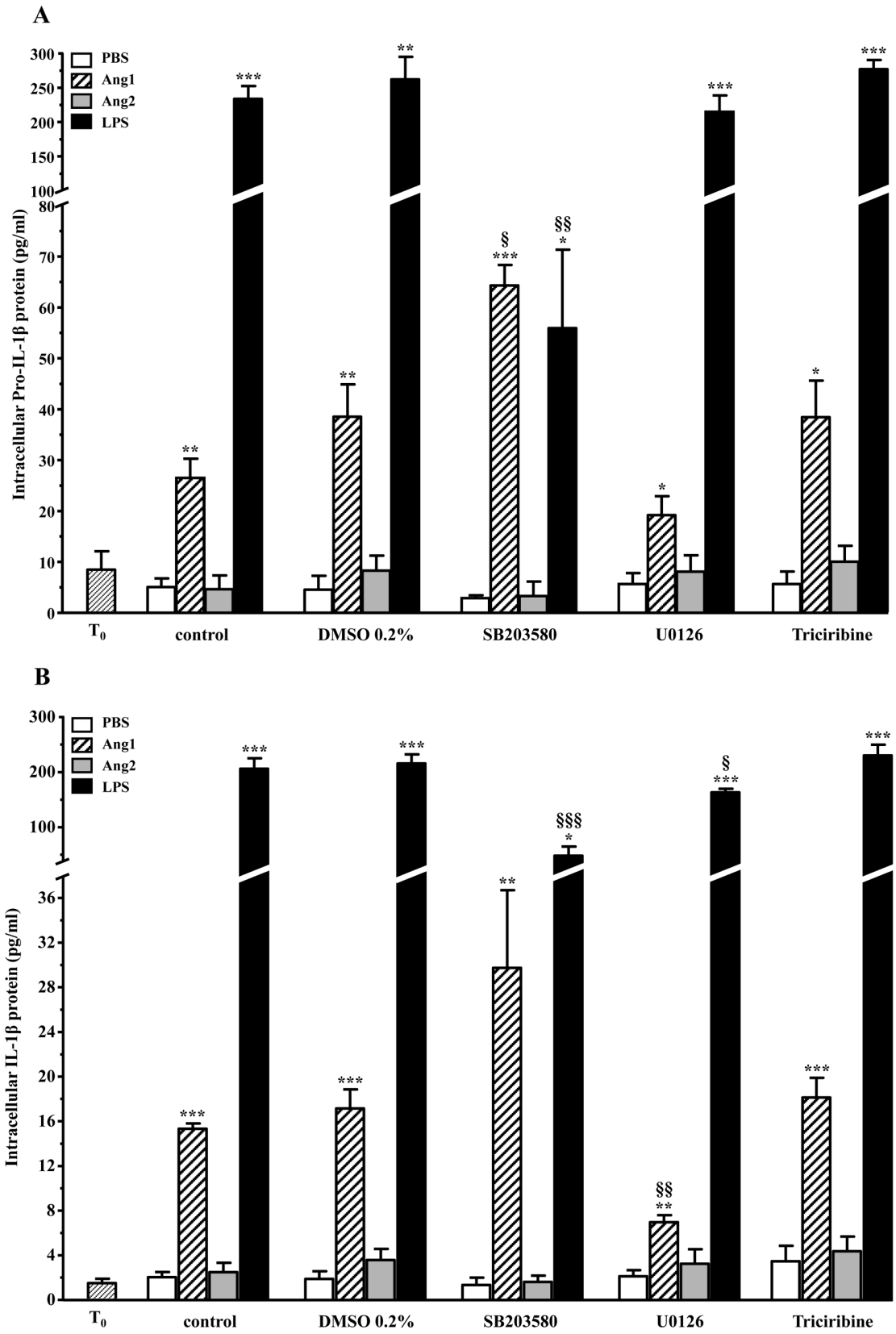
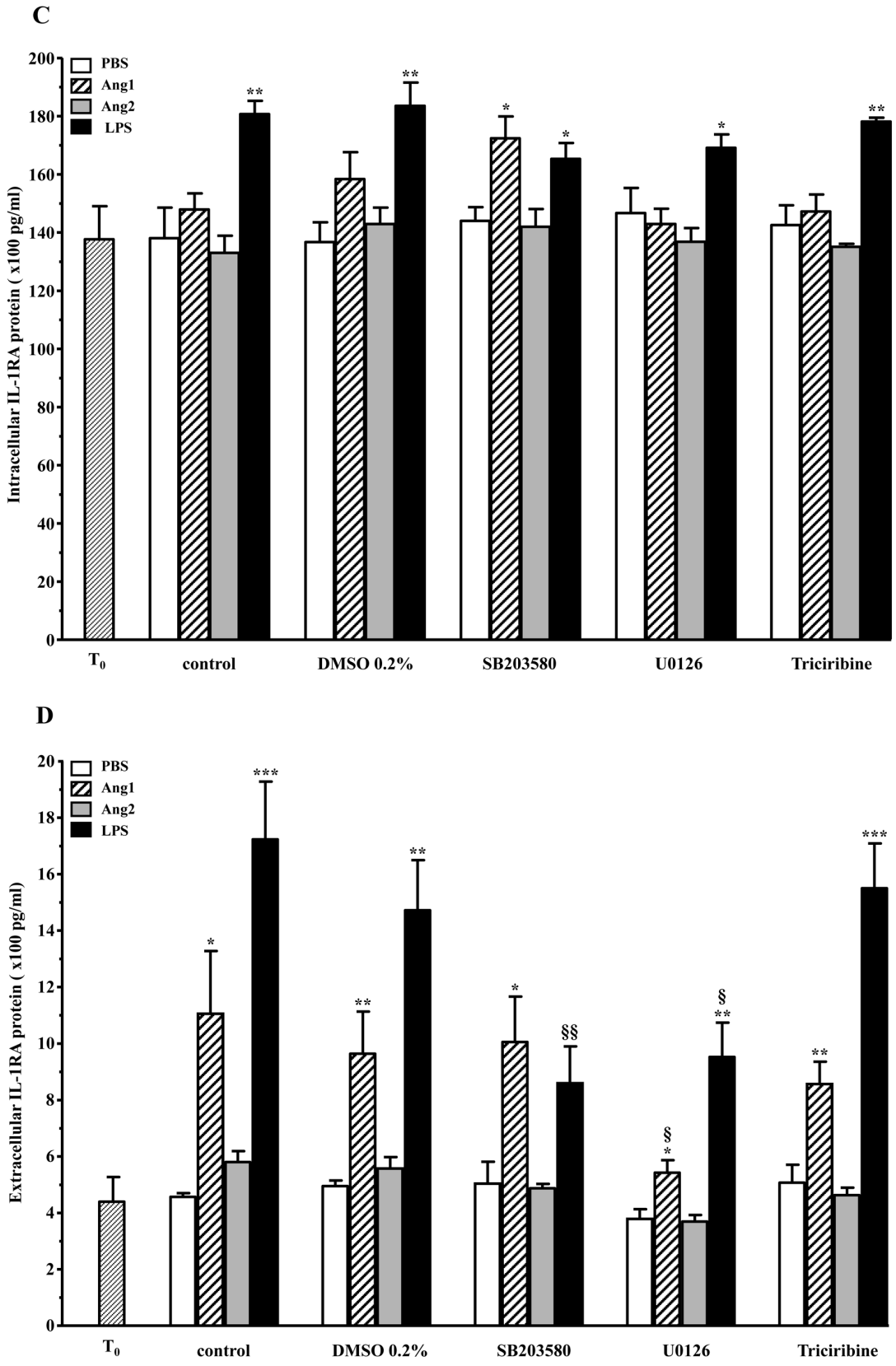


Figure 7 (C-D): Signalling involed in IL-1RA protein



3.0 DISCUSSION

3.1 Angiogenesis and inflammation: an intricate bond

The intimate relationship between blood vessel formation and hematopoietic cell activity is demonstrable early in development and continues throughout life. As this document has attempted to emphasize, the endothelium actively and reactively participates in modulating angiogenic, hemostatic, immune and inflammatory reactions. In each case, a complex network of mediators expressed in a specific spatio-temporal manner coordinates the collaboration between different cellular stakeholders (298). The process of angiogenesis is initiated with EC activation, following which activated ECs undergo junctional and adhesion protein rearrangement (see **1.1 Angiogenesis: Fundamentals**). The subsequent loosening of tight junctions and degradation of basement membrane enable the flow of plasma components, and allow the cells to detach and migrate. During inflammation, a change in the reactivity of the endothelium must occur before blood leukocytes can be effectively recruited to the site of infection (see **1.5 Inflammation**). Indeed, at the onset of the process, both neutrophils and endothelium actively adopt new surface adhesion molecules that initiate a series of cellular and molecular events, ultimately leading to leukocyte capture, rolling and transmigration towards the injurious signal (299). In order to successfully respond to infection, circulating neutrophils must be capable of: 1) sensing stimuli, prioritizing one signal over all others, and migrating towards that reigning signal and 2) retaining a high level of adaptability, such that they can adjust their response in accordance with changes in the biochemical environment (established by mediators released from the inflamed endothelium itself, and by other activated cells). An adequate vasculature and a reciprocal endothelium-leukocyte interaction are therefore key to an effective inflammatory response.

Because inflammation and angiogenesis share a large common cellular denominator – the endothelium – and are initiated by a similar chain of events, it is not surprising to find overlap in the mediators that are involved: Indeed, angiogenesis often accompanies chronic inflammation, which suggests that the biochemical environment present during inflammation is also conducive for neovascularization. The sharing of resources is best exemplified in the host response to endothelial injury, a process that simultaneously engages hemostatic, angiogenic and inflammatory mechanisms. In this particular case, modulators such as VEGF, FGF and

angiopoietins, and cellular actors such as ECs, platelets and leukocytes, are involved in every step of the process, effectively integrating angiogenesis with local inflammatory cues. Finally, many inflammatory mediators, such as IL-6, IL-8 and prostaglandins have direct or indirect angiogenic activities. Likewise, many angiogenic mediators, such as VEGF and NO, influence facets of inflammation. Thus, the polyvalence of the cells and mediators involved makes categorizing them as strictly "angiogenic" or exclusively "inflammatory" a gross oversimplification of two very complex and interrelated processes.

The work presented herein adds to the evidence linking angiogenesis to inflammation, as we have shown that a subset of neutrophils are equipped to detect, and indeed are responsive to, two prototypical families of angiogenic growth factors, the FGFs and angiopoietins. More specifically, the first study showed that in healthy individuals, a certain number of neutrophils express one or more FGF receptors (FGFRs), either at the cytosolic level or at the cell surface. A larger proportion of neutrophils express a single surface-FGFR, FGFR2, which mediates the chemotactic response of neutrophils to the ligands FGF1 and FGF2. Our second study is even more conclusive in terms of bridging the gap between angiogenesis and inflammation. Indeed, we have shown that acute exposure of neutrophils to Ang1 results in intracellular biochemical changes, wherein the neutrophil engages pathways leading to inflammatory gene production. Specifically, Ang1 leads to an intracellular accumulation of one of the most potent pro-inflammatory mediators, IL-1β. At the same time, Ang1 modifies the extracellular environment surrounding the neutrophil by substantially increasing the release of IL-1β's natural antagonist, IL-1RA. Interestingly, while Ang1 tips the balance in favour of blocking IL-1β activity, its activities closely mirror those of the potent pro-inflammatory mediator, LPS. Thus, the ability of angiogenic factors to modulate the activity of the largest cellular component of innate immunity raises a few fundamental questions: Why would these typically inflammatory cells express angiogenic receptors in the first place? Are there subsets of neutrophils fated for specific functions, much like a fraction of monocytes and their polarized progenitors? Because of substantial species differences discussed previously (see **1.5 Inflammation**), the following discussion will focus on a review of human studies where possible.

3.1.1 Neutrophils: heterogeneous and plastic

3.1.1.1 Rationale for neutrophils expressing angiogenic molecules

Why would neutrophils express angiogenic modulators, including growth factor receptors? Just as their myeloid cousins have a proven track record in modulating angiogenesis, there is increasing evidence for direct and indirect neutrophil engagement in this process. In effect, neutrophils have the ability to modulate the angiogenic process during the tissue repair phase that concludes the inflammatory response, by initiating their own programmed cell death and inciting macrophages that engulf them to switch from an inflammatory (M1) to a restorative and anti-inflammatory phenotype (M2) (284). The fact that human neutrophils express functional FGFRs along with other angiogenic growth factor receptors, and store and/or produce substantial quantities of pro-angiogenic effector molecules such as VEGF-A (300), Ang1 (183), IL-8 (190, 293) and MMP-9[27] (301) within their granules, supports a direct and likely active role for these leukocytes in angiogenesis and tissue remodelling. Interestingly, neutrophils are the only cells in the body capable of releasing MMP-9 free of its endogenous inhibitor, the tissue inhibitor of metalloproteinases (TIMP) (301), giving them the ability to directly deliver an immediately effective dose of MMP-9. However, because the above molecules fulfill other functions in the body[28], an argument can be made that their presence in neutrophils does not necessarily mean that they will be used in an angiogenic setting; it is possible that stimulation of vessel growth could simply be a by-product rather than a primary objective for their release. That being said, a direct proangiogenic role for neutrophils has been proposed through *in vivo* studies: in three separate models of angiogenesis (a corneal injury model, a transplantation of pancreatic islets into muscle model, and tumor growth model), neutrophil depletion impairs tissue remodeling, revascularization, and tumor growth, respectively (302-304).

As a counter-measure for hypoxia. On the other hand, it is entirely possible that neutrophils carry an angiogenic payload to facilitate their activity as they fulfill their primary function (i.e. response to inflammation): more blood vessels implies increased blood flow and nutrient supply, meaning a greater number of neutrophils are able to flood into the foci of inflammation and they now have multiple entry points to the infectious site. Additionally, sites of acute inflammation are

[27] The role of MMP-9 in angiogenesis is through its ability to digest ECM, thereby releasing other matrix-bound growth factors (VEGFs, FGFs etc.) and allowing for migration of ECs during tube formation.
[28] For instance, VEGF and MMP-9 could be used to de-stabilize intracellular junctions and to degrade the basement membrane, respectively, thereby allowing for leukocyte extravasation and migration to the site of injury. IL-8, FGF and Ang1 could be deployed as chemotactic agents.

often associated with low oxygen (O_2) availability, placing the surrounding tissues and the masses of recruited cellular infiltrates under the strain of hypoxia. This initiates multiple processes that are aimed at normalizing blood flow (angiogenesis, for instance) and reducing energy expenditure (engaging the anaerobic pathway).

In mammalian organisms, oxygen sensing is a universal cellular event, achieved primarily via the hypoxia inducible factors (HIFs), whose activity is regulated by their α subunits (HIF-1α and HIF-2α) (305). HIFs are major regulators of genes involved in maintaining homeostasis. Ubiquitously expressed HIF-1 enables anaerobic metabolism, as it decreases mitochondrial oxygen consumption while inducing the expression of enzymes and glucose transporters that help cells efficiently produce energy in hypoxic environments (306, 307). Tissue-specific HIF-2 stimulates erythropoiesis and cell survival, likely through the inhibition of p53 (308, 309).

Myeloid response and activity is deeply impacted by O_2 conditions. As these leukocytes migrate from the circulation to sites of inflammation, they have to adapt to functioning in oxygen conditions that are much lower than what is normally available to them in the circulatory system. Both HIF-1α and HIF-2α accumulate in hypoxic human macrophages (310, 311) and neutrophils (312, 313), as well as in the tissue-associated macrophages (TAMs) of various human cancers (311). In response to hypoxia and HIF-2α activation, human macrophages have the ability to increase the transcription of their own proangiogenic genes, such as VEGF, IL-8, PDGF and ANGPTL4 (314). Moreover, HIF-1α has been shown to mediate hypoxia-induced neutrophil survival (315) and is vital in the regulation of myeloid/neutrophil aggregation, motility and bacterial clearing (316), establishing a direct link between hypoxia and neutrophil activity. While the impact of hypoxia on neutrophil angiogenic gene/protein expression is unexplored, it is possible that neutrophils may have retained their angiogenic capabilities as a means to counter low O_2 availability; thus, once hypoxia is detected, neutrophils can aid in the provision of signals necessary for the induction of new blood vessel formation.

As a result of genetic inheritance. Considering that neutrophils share common angiogenic traits with their myeloid cousins, a third explanation is that neutrophils may have simply retained angiogenic surface markers (VEGFR, FGFR, Tie2 etc.) through evolution, by differentiating from a precursor that expressed them. This theory is possible given that neutrophils and endothelial cells are believed to originate from a common precursor, if we go back to the very

beginning of their phylogenic lineages: During early mouse embryogenesis, circa E7.5, hematopoietic cells (HPCs) (precursors to all myeloid cells, erythrocytes and platelets, among others) and EC precursors appear in the blood islands of the yolk sac almost simultaneously, deriving from mesoderm and forming structures in which primitive erythrocytes are surrounded by a layer of angioblasts that give rise to differentiated ECs. The close temporal and spatial relationship between the appearance of the first blood cells and the endothelial scaffold suggests that both cell types may have evolved from the same precursor, or that one could have given rise to the other.

The possible lineage relationship between vascular cells and HPCs is explained in detail in (317). Regardless of the mechanisms leading to the initial phylogenetic split, the two lineages appear to be heavily influenced by the same angiogenic growth factor families (and their receptors), including VEGF (318), FGF (319) and Ang/Tie2 (16). Additionally, although adult HPCs have low VEGFR2 expression (320), they retain certain endothelial markers (321) such as Tie2, CD34[29] and possibly FGFRs, the latter having been detected on the surface of long-term (LT) repopulating murine hematopoietic stem cells (322). Thus, as the progeny of HPCs, neutrophils may have inherited similar surface endothelial markers. However, because there are multiple developmental stages between HPCs and neutrophils, angiogenic markers on the cell-surface of intermediate progenitor cells (myeloblasts, metamyelocytes etc.) in the bone marrow would have to be explored in order to support this theory.

3.1.1.2 Neutrophil plasticity

Are there subsets of neutrophils, such that some would be destined to become inflammatory while others are angiogenic and/or immunosuppressive, much like the divisions in M1/M2 Macrophages? The possible existence of neutrophil subsets provides an attractive explanation for a) why some neutrophils carry a complex angiogenic payload, including growth factors and their receptors, while others do not (Article #1) and b) why these traditionally inflammatory leukocytes would produce anti-inflammatory mediators (Article #2). Indication of neutrophil subdivisions based on phenotypic markers exists in mice (304, 323), but such evidence is very

[29] Human HPCs express CD34 marker whereas mice do not.

scarce in humans (324), owing to the difficulty of identifying surface markers exclusively specific to human neutrophils and tracking the fate of neutrophils *in vivo*.

The possibility that neutrophil subsets exist implies that not all neutrophils are born equal. Adding to this complexity is the observation that neutrophils are capable of altering their phenotypes, shedding or acquiring different surface markers to adapt to the biochemical environment surrounding them. Contrary to monocytes and macrophages, neutrophils had long been considered a terminally differentiated and relatively homogeneous cell population. However, in a human study where systemic inflammation is induced by intravenous endotoxin (LPS) injection, there are two populations of neutrophils distinguished by the level of expression of surface marker CD16 and functionality (325): One population is made of band-like immature neutrophils expressing low CD16 ($CD16^{dim}$), which is rapidly mobilized from the bone marrow and displays decreased receptor expression, respiratory burst and interaction with bacteria; this is in stark contrast to the $CD16^{bright}$ counterparts. Interestingly, after endotoxin treatment, a group of neutrophils splits from within the $CD16^{bright}$ population and reduces its CD62L expression. The new $CD16^{bright}CD62L^{dim}$ subset fulfills an immuno-suppressive function by subduing human T cell proliferation (324). Thus, neutrophils display a great level of plasticity and modify their phenotype in response to a wide range of physiological and pathological cues. Whether this variability is because of maturity levels or because of the existence of subtypes is unclear.

Based on published and unpublished observations from our laboratory, we conclude that there are definite inter- and intra-individual differences in neutrophil characteristics, whether relating to protein expression or to functionality. This is in line with recent assessments highlighting neutrophil heterogeneity, elegantly summarized in (326). For instance, we have noted variability in neutrophil FGFR expression between donors, as well as within the neutrophil population from the same donor (77), whereby only a fraction of the neutrophils will express one or more FGFRs. Similarly, other groups have reported that the expression of surface molecules/receptors such as CD117 (a ligand for PECAM-1), ICAM-1, CXCR4 and CXCR2, is limited to a subset of human neutrophils (summarized in (326)).

Additionally, we and other groups have observed that same-donor neutrophils assayed at different times may exhibit different basal and agonist-induced cytokine production capability[30], which suggests variations in the neutrophils' ability to detect (based on different level of receptor expression?) or to respond (based on different levels of protein pools or components of the protein production machinery?) to stimulus. In effect, the heterogeneity of neutrophil cytokine production has been conclusively demonstrated *in vitro*, with a novel technique that allows visualizing and specifically counting cytokine-producing cells within a larger neutrophil population; in this assay, only a portion of the granulocytes released MIP-1β (9.7%), IL-8 (16.7%) and TNF-α (< 3%) in response to LPS (295).

The heterogeneity in protein expression also appears to extend to functionality. The group that discovered neutrophil NET formation as a bactericidal mechanism also observed that, while the majority of neutrophils in circulation had the potential to make NETs, only a fraction (10-30%) of them actually underwent NETosis (327). This implies that there are mechanisms to regulate which neutrophils start a program that culminates in NET formation. Another example is the fact that only a small subset (<25%) of neutrophils stores Olfactomedin 4 in specific granules, which is a glycoprotein that inhibits the activation of several granular proteases (including cathepsin C & G, neutrophil elastase, and proteinase 3 (PR3)), potentially negatively affecting the bacterial killing ability of this subset.

Aside from these intrinsic differences in neutrophil biology, neutrophils display a great level of plasticity in modifying their phenotype in response to a wide range of biochemical cues (both in physiological and pathological conditions). For instance, expression of adhesion molecules, chemokine receptors, and proteases change according to which stage of the recruitment process the neutrophil is at (see **1.5 Inflammation** and review in (326)). Because only a portion of circulating granulocytes are recruited and eventually transmigrate, there must be selection mechanisms that regulate which neutrophils engage in those functions out of the entire pool of available cells.

Just as other myeloid cells are capable of phenotype changes under different environmental circumstances, a neutrophil also appears capable of switching to another phenotype after certain functions are completed. A number of studies have shown that

[30] Generally, the impact of baseline variability between donors is diluted with the inclusion of additional donors.

neutrophils have the ability to undergo reverse transendothelial migration (rTEM), describing the movement of neutrophils in and out of the transmigration pore or, more rarely, from the subendothelial space back into the vascular lumen (328-330). Interestingly, Buckley *et al* reported that human neutrophils that underwent rTEM display distinct changes in phenotype compared to naïve circulating neutrophils, characterized with a loss of chemokine receptors CXCR1 and CXCR2 and a higher expression of ICAM-1 (ICAM-1^{high}CXCR1^{low}), increased rigidity, a significantly lower tendency to transmigrate again, increased oxidative burst, and decreased susceptibility to undergo apoptosis (330). Thus, rTEM generates a new subset of neutrophils that are returned to the circulation and though unlikely to re-enter tissues at secondary sites of inflammation, might become adherent or physically trapped in microvessels where they could generate powerful oxidative burst.

3.2 Perspectives

Pursuant to our two studies, many unanswered questions remain. The following section will attempt to highlight some of the most interesting venues raised by each article.

3.2.1 Unresolved issues - Article #1

As way of reminder, we showed that purified circulating human neutrophils from healthy individuals express varying levels of FGFRs in their cytosol and at their cytoplasmic membrane. We determined that FGFR2 is the sole cell-surface receptor, while FGFR1 and FGFR4 localize in the cytosol. Human neutrophils do not express any FGFR3. Furthermore, neutrophil FGFRs are active, as FGFR2 (and to a lesser extent FGFR4) mediates FGF1 and FGF2 – induced neutrophil chemotaxis. Based on the results of this study, we propose the following topics for future investigations:

Characterizing FGFRs. This would involve determining what function, if any, cytoplasmic FGFRs fulfill, as well as assessing whether FGFs or other inflammatory mediators could induce receptor translocation from the cytoplasm to the cell-surface. The choice of inflammatory mediators would have to be reflective of the cellular environment during the acute phase of inflammation, and would include IL-8, TNF-α and LPS, all of which have been shown to promote protein translocation to the surface of neutrophils (331, 332). This could also provide

insight into the mechanisms governing the possible interaction between FGF and other inflammatory mediators in the context of neutrophil biology.

Neutrophil adhesion and migration. Besides FGFRs, neutrophils are known to express integrins and HSPGs, both of which are co-receptors for FGFs. This suggests that neutrophils possess the necessary tools to capture FGFs and stabilize interactions at the cell-surface, potentially resulting in changes in neutrophil biological responses. In pilot experiments aimed at exploring the roles of FGFs beyond chemotaxis, we observed that direct FGF1 or FGF2 stimulation has little impact on human neutrophil viability *in vitro* (Haddad *et al*, personal communications, 2011). However, FGFs may still impact other processes, such as neutrophil adhesion and migration across EC monolayers *in vitro*.

Expression of inflammatory genes. Using the same techniques and qPCR arrays outlined in Article #2, we obtained preliminary data showing that FGF1 and FGF2 may be capable of upregulating the mRNA expression of certain members of the CC and CXC families of chemokines (n=2) by human neutrophils (Haddad *et al*, personal communications, 2011). For instance, FGF1 shows a tendency to increase CCL17, CCL 25, CCL26, and CXCL13 four-fold, whereas FGF2 shows an over two-fold increase in CXCL14. While the parameters and conditions for these experiments require additional fine-tuning, the initial results warrant further consideration.

3.2.2 Unresolved issues - Article #2

The observed effect of Ang1 on human neutrophil gene expression showcases the duality of this mediator, inasmuch as it demonstrates a role in both angiogenesis and acute inflammation. As a brief summary, we showed that of the two angiopoietins, only Ang1 leads to a significant induction of mRNA expression of IL-1 family members, particularly of the potent pro-inflammatory agonist IL-1β and its natural endogenous antagonist, IL-1RA. We then confirmed that Ang1 stimulates *de novo* protein synthesis of the precursor form of IL-1β (pro-IL-1β), which, upon maturing into IL-1β, remains trapped inside the cell. In parallel, Ang1 augments the release of IL-1RA, perhaps as a mechanism to counteract the potentially damaging effects if IL-1β is liberated. Interestingly, Ang1 closely mirrors the action of the bacterial endotoxin LPS with

regards to IL-1 production (albeit with less potency), in that LPS also increases *de novo* synthesis of IL-1β and stimulates the release of IL-1RA, but fails to induce IL-1β secretion. These observations clearly illustrate the potential of Ang1 to significantly contribute to the acute inflammatory process.

3.2.2.1 Importance of IL-1

The ligands. The IL-1 family is arguably one of the best examples of the role of cytokines in inflammation and the need for tight control mechanisms. Unlike any other family of cytokines, this one comprises both very potent pro-inflammatory mediators as well as the intrinsic ability to shut them down (**Table IV**, p.154). The IL-1 system includes an interesting array of at least 21 distinct molecules encompassing receptors, co-receptors, ligands, and endogenous antagonists (**Tables IV&V**, p.154-155, adapted from (333)). Notably, the two prototypical IL-1 agonists, IL-1α and β (jointly referred to as IL-1 in early studies), and their endogenously-occurring antagonist IL-1RA have been thoroughly studied *in vitro* and *in vivo* in both health and disease states.

Table IV: List of IL-1 ligands and properties

Ligand	Alternative name	Property
IL-1F1	**IL-1α**	**Agonist**
IL-1F2	**IL-1β**	**Agonist**
IL-1F3	**IL-1RA**	**Receptor Antagonist**
IL-1F4	**IL-18; IFN-γ-inducing factor**	**Agonist**
IL-1F5	**FIL1δ**	**Anti-inflammatory**
IL-1F6	**FIL-1ε**	**Agonist**
IL-1F7	**IL-1H4, IL-18Rβ, IL-1ζ**	**Anti-inflammatory**
IL-1F8	**IL-1H2**	**Agonist**
IL-1F9	**IL-1ε**	**Agonist**
IL-1F10	**IL-1Hy2**	**Receptor antagonist (?)**
IL-1F11	**IL-33**	**Agonist**
IL-18BP		**Anti-inflammatory**

Adapted from Dinarello *et al*, 2009 (333).

The intimate relationship between IL-1 and inflammation is perhaps best illustrated with IL-1β, as there is an entire subgroup of chronic inflammatory disorders, termed "auto-inflammatory", that is specifically responsive to IL-1β blockade, suggesting that increased IL-1β activity is the main culprit in these conditions' pathological progression. In recent years, IL-1β blockade

through the use of Anakinra, the generic name for a non-glycosylated recombinant of human IL-1RA, has become the standard therapy for patients with various systemic and local inflammatory diseases, including systemic-onset juvenile idiopathic arthritis (334) and refractory adult Still's disease (335), and for the management of the symptoms of rheumatoid arthritis (336).

The receptors. The IL-1 receptor family is as fascinatingly diverse as its ligands, with ten known members and a few that remain orphan receptors (**Table V**, p.155). Similarly to the ligands, members of the IL-1 receptors contain activators and suppressors of inflammation. For example, the IL-1 receptor type II (IL-1RII), which exists both in a membrane-bound and a soluble form, has a very high affinity for mature and precursor IL-1β and serves the function of a decoy receptor that prevents IL-1 from binding to and activating IL-1RI. Another example is the single Ig IL-1 receptor-related (SIGIRR), a negative regulator of IL-1α, IL-1β and some Toll-like receptor (TLR) agonists.

Table V: IL-1 Receptors and Accessory proteins

Receptor	Alternative name	Ligands
IL-1RI	CD121a	**IL-1α, IL-1β, IL-1RA**
IL-1RII	CD121b	**IL-1β, pro-IL-1β, weak IL-1α**
IL-1RAcP	IL-1R3 / IL-1RAP	**Co-receptor for IL-1RI, IL-1RII, IL-1Rrp-2 and Fit-1**
Fit-1/ IL-33Rα	IL-1R4	**IL-33**
IL-18Rα	IL-1R5	**IL-18, IL-1F7**
IL-18Rβ	IL-1R7/IL-1RAcPL	**Co-receptor for IL-18Rα**
IL-1Rrp-2	IL-1R6	**IL-1F5, IL-1F6, IL-1F8, IL-1F9**
TIGIRR*-2/IL-1RAPL	IL-1R8	**Unknown**
TIGIRR-1	IL-1R9	**Unknown**
SIGIRR**	TIR8	**Unknown**

* Three immunoglobulin Interleukin-1 receptor-related; ** single Ig IL-1 receptor-related.
Adapted from Dinarello *et al*, 2009 (333).

Signal transduction. Upon binding to IL-1α or to IL-1β, IL-1R1 and the accessory co-receptor IL-1RAcP (IL-1R3) heterodimerize and activate downstream signal transduction. The IL-1R1/IL-1RAcP interaction becomes a scaffold for a signalling complex comprised of myeloid differentiation factor 88 (MyD88), IL-1 receptor-associated kinases (IRAK) and TNF-receptor associated factor 6 (TRAF-6), resulting in the activation of NFκB and mitogen-activated protein kinases (MAPKs) (337). The cytoplasmic portions of IL-1RI and IL-1RAcP contain a similar

domain found in each TLR, termed the "TIR domain", necessary for signal transduction. IL-1RII lacks this TIR domain (IL-1RII activity reviewed in (338)) and is therefore incapable of transducing signals despite being able to engage IL-1RAcP[31] after binding IL-1 (339, 340). Although IL-1RI is constitutively expressed in most cells, expression of IL-1RAcP is not. Cells expressing IL-1RI but deficient in IL-1RAcP can bind IL-1α and IL-1RA normally, but see a 70% reduction in IL-1β ligation. More importantly, in the absence of IL-1RAcP, no biological response is transduced regardless of binding to IL-1α or IL-1β (341).

3.2.2.2 Neutrophil release IL-1RA in response to LPS

Neutrophils have been reported to transcribe and translate both IL-1β and IL-1RA when stimulated with a number of cytokines and growth factors (342-345). Why would the typically pro-inflammatory neutrophils exclusively release substantial quantities of the anti-inflammatory IL-1RA despite the presence of bacterial product (LPS)?

As we have previously seen, there is a vital need for tight control over the inflammation process, in order to prevent unnecessary tissue damage and developing chronic inflammatory diseases. There is increasing evidence that neutrophils participate in dampening the immune response and facilitating tissue repair during the resolution phase of inflammation. This emerging concept is supported by observations that upon stimulation of their TLRs, murine neutrophils fail to produce IL-12 p40, IL-1β, IL-6, or CXCL1. Interestingly, systemic endotoxin treatment in mice results in the recruitment of neutrophils that secrete high amounts of the anti-inflammatory cytokine IL-10, in contrast to macrophages and monocytes. This study also demonstrated that the activity of neutrophils depends on the duration of exposure to the bacterial pathogen: in the acute phase of infection, it appears that the pro- and anti-inflammatory effects of neutrophils even out and therefore do not influence mycobacterial load. In conditions of prolonged chronic infection, neutrophil depletion is actually beneficial to the host, as it promotes inflammation and decreases the mycobacterial burden, suggesting a surprising role for neutrophils in suppressing inflammation and allowing for the persistence of a high mycobacterial burden during infection (346).

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[31] Soluble IL-1RII (sIL-1RII) can bind IL-1 and bind sIL-1RAcP, a product of IL-1RAcP alternative splicing, increasing the affinity of IL-1RII for IL-1 by 100-fold. Current evidence, however, does not support an interaction between IL-1, sIL-1RII and full-length IL-1RAcP (reviewed by Peters *et al*, 2012).

These results from mice appear to be mirrored in human studies, as several groups have demonstrated that the cytokine profile of human neutrophils stimulated with LPS is predominantly anti-inflammatory (293, 295, 347). Additionally, there are reports that human neutrophils in co-cultures with T cells can inhibit the latter populations' production of cytokines (IFN-γ, TNF-α, IL-2 and IL-4) (348) and stunt its proliferation, albeit independently of neutrophil IL-10 or TGF-β production (324). Accordingly, advanced cancer patients display impaired T-cell function (349), correlated with the presence of activated granulocytes that produce hydrogen peroxide (348). If the theorem that the continued presence/activation of neutrophils negatively modulates adaptive immunity responses, this would also explain why granulocyte colony stimulating factor (G-CSF), a stimulant that increases the maturation and mobilization of neutrophils into the circulation from the bone marrow, is reported to have beneficial effects on patients suffering severe infections, without worsening inflammation (350, 351).

3.2.2.3 Neutrophils release IL-1RA in response to Ang1

While the argument that neutrophils restrain the propagation of inflammation in conditions of bacterial infections (*in vitro,* mimicked by prolonged exposure to LPS) can be made, why would they emit substantial quantities of anti-inflammatory IL-1RA in the presence of a much milder and non-infectious agent like Ang1?

An answer to that question comes from taking an intrinsic look at the role of Ang1 in angiogenesis. As we have previously seen (in **1.5 Inflammation**), Ang/Tie2 signalling is intimately involved in the angiogenic process, with Ang1 initiating a return to homeostasis and stabilizing the new vessels. Two thoughts emerge from this process: First, angiogenesis utilizes many mediators that are also common to inflammation, such as VEGF, Ang1, Ang2 and IL-8. Because of their demonstrated chemotactic effects on neutrophils ((151), among others), these mediators have the ability to "accidentally" recruit neutrophils to sites of neovascularization, where they could potentially inflict damage on the newly formed and vulnerable vessels. Second, maintaining vessel integrity and establishing homeostasis means actively dampening or pushing back signals that activate the endothelium or that could damage the new vessel (such as noxious neutrophil content). Thus, at sites of "pure" (read: independent of inflammation) neovascularization, Ang1 would be fulfilling its role of stabilizer by stimulating the release of IL-1RA from the neutrophils that are unintentionally recruited. In doing so, Ang1 ensures that

neutrophils are not accidentally activated where they do not need to be. In inflammatory conditions, because IL-1 is a leukocyte chemoattractant and stimulant as well as strong promoter of endothelial activation, the action of Ang1 on IL-1RA release could potentially serve a dual function critical to the initiation of the resolution phase (and the full engagement of tissue repair): to dampen IL-1-induced endothelial activation and associated cellular responses, and to actively push back neutrophils out of the foci of inflammation. This fits well with the role of Ang1 in maintaining EC quiescence and a non-permissive, un-activated phenotype.

3.2.2.4 The origin of IL-1β

In our studies, we observed a time-dependent accumulation of the IL-1β precursor, pro-IL-1β, almost simultaneously as the mature form. Because the agonists Ang1 and LPS augmented the quantity of IL-1β mRNA transcripts, the appearance of pro-IL-1β is not shocking and can be explained by the induction of IL-1β mRNA translation machinery. However, where did the simultaneous IL-1β come from?

During the execution of the IL-1 experiments described in Article #2, and subsequently during the publication process of said article, we and our reviewers raised the question of possible peripheral blood mononuclear cell (PBMC) contamination, which can skew the results of cytokine production even at concentrations as low as 0.5% of the population (1% contamination is sufficient for detection of IL-1β in supernatants, as reported in (293)). The possibility of contamination would be especially important for IL-1β synthesis and/or secretion, as IL-1β production by human neutrophils is highly contested ((293), among others). However, our isolation conditions result in a neutrophil population with less than 0.1% PBMC contamination, as determined by morphological analysis and flow cytometry. Additionally, monocytes and macrophages release substantial amounts of IL-1β into the medium (295), but we did not detect extracellular IL-1β in any of our assays. This provides strong evidence that PBMC contribution to our results is highly unlikely.

Another possibility is the reported ability of human neutrophils in culture to internalize IL-1β rapidly via IL-1RII (352), with 50–60% of IL-1β internalized within 1 h at 37°C. According to this study, IL-1β internalization reaches a plateau after 1 hour - suggestive of a state of equilibrium in which further uptake of IL-1 is balanced **by release** of intact and/or degraded IL-1 back into the medium. Assuming that the 0.1% PBMC contamination in our population is

capable of producing enough IL-1β, the reported ability of neutrophils to 'scavenge' and endocytose this PBMC-released IL-1β could explain the accumulation of mature IL-1β measured in neutrophil cell lysates. To discount this possibility, we will utilize IL-1β concentrations from our data and what is available in the literature: From Shroder *et al*, PBMCs (10^6 cells/mL) release around 600 pg/mL of intact IL-1β in 4 hours in response to 250 ng of LPS, corresponding to ~ 6 pg/mL for 10^4 cells (293). In our study, our neutrophil culture (10^7 cells/mL) showed an intracellular accumulation of ~ 300 pg/mL of IL-1β in response to 1 μg LPS. A 0.1% contamination of the neutrophil population would correspond to 10^4 PBMCs, which would theoretically produce ~ 6 pg/mL of IL-1β into the medium. Assuming that IL-1β uptake by neutrophils is 50-60%, this means that we should detect ~ 3 pg/mL (50% of 6 pg/mL) of IL-1β in our lysates. While it is true that we used saturating concentrations of LPS, the fact that we detected one hundred times the amount of IL-1β and there was absolutely no release (ref the equilibrium described by (352)) makes endocytosis of PBMC-derived IL-1β an unlikely explanation of the data.

Thus, the mechanisms and players involved in pro-IL-1β conversion to IL-1β in human neutrophils remain unclear. At this stage, it is too early to discount the involvement of caspase-1, or other elements of the inflammasome, and additional studies would have to be carried out before reaching a firm conclusion.

3.2.2.5 Other issues

Ang1, ECs and IL-1. Surprisingly, a survey of the literature revealed that the impact of Ang1 on EC production of IL-1 has never been explored. Given the reported ability of ECs to synthesize IL-1 ligands (353, 354), it is very possible that Ang1 could promote the expression of one or more of the IL-1 family members from ECs. At the very least, Ang1 could induce IL-1RA from ECs, given the ligand's propensity to stimulate endothelial quiescence. This is definitely a venue worth exploring.

IL-1R expression on neutrophils. Assessment of leukocyte expression of IL-1Rs dates back to the mid 1980's-1990, and identified the inactive IL-1RII as natively expressed on neutrophils, B-lymphocytes, monocytes and macrophages (355-357). A more recent assessment using modern detection techniques (western blot, flow cytometry, ELISA) confirmed that IL-1RI is only

weakly expressed on purified human neutrophils and monocytes, with higher detectable levels of soluble IL-1RI and IL-1RII (358). Unfortunately, in this study, surface IL-1RII was not assessed.

Neutrophil IL-1R1 appears to be active, as *in vitro* stimulation with IL-1 induces the transcription of CCL3, CCL4, CCL20, CXCL2 and NFκB1; this effect can be blocked by pre-treatment with IL-1RA. To the best of our knowledge, there appears to be no evaluation of IL-1R3 expression or IL-1RI and R3 heterodimerization in a highly purified population of neutrophils.

Based on our experiments, IL-1RI mRNA appears to be unaffected by short-term treatment with Ang1; however, we did not evaluate IL-1RII mRNA or the protein expression of any of the IL-1 receptors (particularly IL-1RI, IL-1RII, and IL-1R3) in their long or spliced forms. This would be another venue worth looking into.

qPCR array and other interesting targets. As highlighted in Article #2, our qPCR array data has generated other potentially interesting venues for future exploration. For instance, lymphotoxin (LT)-α and -β (LTβ is also known as tumor necrosis factor C), two related cytokines belonging to the TNF superfamily, are essential in the development of lymphoid organs and the differentiation and maturation of several types of immune cells, including T cells, dendritic cells and natural killer cells (359). Thus, LTs play a significant role in both innate and adaptive immune processes (review (360)). In mice, engaging the LTαβ (an LTα and LTβ heterodimer) pathway can potentially activate lymphocyte-organizing chemokines such as CXCL13, CCL19 and CCL21 genes (361). In effect, LT signalling appears to play an important role in inducing the expression of chemokines and adhesion molecules that are crucial for the recruitment and migration of immune cells to sites of infection. The fact that Ang1 shows a tendency to increase LTβ could be significant, and may reshape our assessment of the anti-inflammatory role played by this ligand. The involvement of LT in modulating immune responses may also be a consideration in utilizing Ang1 as an anti-cancer therapy.

Beyond exploring the rest of the potential targets induced by Ang1, what would happen with longer exposure to Ang1/Ang2? Would the expression of some chemotactic receptors/ligands change? Evidence suggests that the answer is yes. Chronic inflammation and continual exposure of neutrophils to cytokine stimulation may alter their protein expression, by inducing the expression of receptors that have normally been attributed to monocyte

function/recruitment (such CCR2 and CCR1) (362). Interestingly, it takes a short-term exposure to Ang1 to upregulate the gene expression of at least one of those receptors, CCR1, by 3.3-fold. If this mRNA increase translates into CCR1 protein expression and presentation to the cell-surface, it would provide evidence that Ang1 can alter leukocyte recruitment profiles, and may influence reactivity of neutrophils to ligands that are not traditionally attributed to neutrophil recruitment, such as RANTES (CCL5), MCP-1 (CCL2) and other CCR1 ligands.

3.3 Final remarks

Because the circulation provides them with extensive access to various tissues and organs throughout the body, myeloid cells are well disposed to act as angiogenic and inflammatory sentinels, adjusting the release of their content as is required for them to carry out their functions, and in accordance with biochemical cues. Thus, myeloid signalling via FGFR, VEGFR and Tie2 integrates angiogenesis and inflammation, and highlights the high level of plasticity required of these leukocytes in carrying their functions.

Our observation that human neutrophils are heterogeneous in their expression of FGFRs prompted us to look at their ability to respond to the prototypical FGF family members, FGF1 and FGF2. While both factors were capable of inducing neutrophil chemotaxis at high doses, their effects on short-term inflammatory gene expression were limited (Haddad *et al*, personal communications, 2011). This is reminiscent of the effects of Ang2 on specific neutrophil responses, whereby the ligand can promote human neutrophil chemotaxis (151) but fails to induce any significant change in inflammatory gene expression (190, 363).

In other myeloid cells such as macrophages, Tie2 ligands are described as neutral (non-inflammatory) permissive agents, whereby they promote "a state" of activation ("priming") rather than exerting a direct activating role themselves (236). However, neutrophils present certain particularities in their Tie2 signalling (such as the absence of Tie1, a lack of antagonism by Ang2, and a wider expression of Tie2 compared to the very small subset of TEMs), which suggests that they could respond differently to angiopoietins compared to their myeloid cousins. In effect, our data provides evidence that Ang1 is not simply a neutral co-stimulator that readies the neutrophil for the actions of a stronger stimulus, but the ligand is capable of significantly impacting neutrophil gene and protein levels, as well as modifying neutrophil biological

behaviour in the absence of other stimuli. That being said, we also recognize that Ang1 does exert a "priming" effect in the context of IL-1β, causing the latter protein to accumulate intracellularly; this could serve to prepare the cells for the eventuality that a yet-to-be-identified second stimulus, or even a set of required conditions, could cause the release of IL-1β. Additionally, the Ang1-induced changes in neutrophil gene and protein expression may manifest differently in the presence of other mediators, as observed for macrophages under a variety of polarizing conditions.

3.3.1 Therapeutic applications

Though the role of a neutrophil in acute inflammation is well documented, many questions remain regarding this leukocyte's biology, particularly during the performance of its duties in an environment where multiple processes and competing signals are taking place simultaneously. Our studies are a good reminder that in the mammalian organism, physiological phenomena rarely occur in isolation; rather, they are the fruit of intricately intertwined and complex networks of tissues, cells and mediators.

By studying the links between angiogenic growth factors and neutrophils, we continue to expand our understanding of the correlation between the phenomena that they drive, specifically between angiogenesis and acute inflammation. Why is this important in the therapeutic realm? Because there is a world of difference between theory and the achieved therapeutic outcome, as most clinical trials tend to demonstrate. For instance, theory dictates that since both processes rely on endothelial activation, compounds that block the angiogenic cascade would theoretically also subdue the inflammatory process, and vice versa. However, this paradigm is not true in the case of VEGF and retinal angiogenesis: If VEGF-induced inflammation is a trigger for pathological angiogenesis (364), then why is it that when Bevacizumab (Avastin) is used to stunt the latter in ocular disease[32](365, 366), severe acute ocular inflammation occurs (367, 368)?

As we have seen in much detail in **1.4 The Angiopoietin family**, there is a body of conflicting data on the role of the angiopoietin-Tie signalling pathway in endothelial survival,

[32] Avastin is a recombinant anti-VEGF antibody used in the treatment of ocular disorders where blood vessel growth is the underlying cause of the pathology, such as macular degeneration (wet variety) (Avery, Pieramici *et al*, 2006) and proliferative diabetic retinopathy (Avery, Pearlman *et al*, 2006).

vessel growth, and vascular maturation. The dichotomies in the reported actions of Ang in angiogenesis make it very difficult to determine therapeutic applications, let alone to predict clinical benefit. Cancer is the perfect example (review (369)): Upregulation of Ang1 has been strongly correlated with tumor malignancy in multiple cancer types, including gliomas, non-small cell lung carcinoma (370), and in ovarian (371), breast (372), and gastric (373) cancers. Furthermore, overexpression of Ang1 in human *in vitro* cell models of cervical cancer (HeLa) and glioblastoma (U87, U373 and U343) has been reported to increase tumor growth (see review (369)). In surprising contrast, overexpression of Ang1 in cell models of human breast (MCF-7) and colon (HT29) tumors, and squamous carcinoma (A431) has been reported to show significant antitumor effect. Overexpression of Ang1 in cell models of human breast (MCF-7), colon (HT29), and skin (A431) cancers has been reported to show significant antitumor effect. It is possible that the tumor-inhibiting effect of Ang1 is related to its anti-inflammatory actions, but this theory is difficult to reconcile with the contradictory findings. In parallel, the role of Ang2 in tumor development is similarly controversial: While overexpression of Ang2 delays tumor growth in lung and mammary carcinomas, it enhances angiogenesis and tumor malignancy in hepatomas, gliomas, and colorectal and gastric carcinomas (see review (369)).

In a scenario where the Dr. Jekyll and Mr. Hyde behaviour of angiopoietins is understood and controlled, there would be clear benefits, such as containing cancer growth by concurrently minimizing inflammation and tumor vascularization. In other cases, we can envisage strategies that take advantage of the pro-angiogenic and anti-inflammatory properties of Ang1. For instance, in brain ischemia, loss or diminishment of blood perfusion to parts of the brain tissue initiates a cascade that rapidly leads to excitotoxicity, tissue damage and inflammation (374). Reperfusion of the affected ischemic tissue is critical for restoring normal function; however, it can paradoxically result in secondary damage, called ischemia/reperfusion (I/R) injury (374), caused by biochemical imbalances (excess reactive oxygen species) and infiltration of innate immunity blood cells that release damaging cytokines, particularly IL-1β (375)**,** which exacerbate tissue injury. In such pathologies, enhancing neovascularization while limiting secondary inflammation is the most desirable therapeutic outcome. To date, over one hundred drugs have been tested for the treatment of acute brain ischemia, yet rt-PA remains the only agent shown to improve stroke outcome in clinical trials (374, 376). Even then, rt-PA benefits are severely limited by the small therapeutic window within which rt-PA must be used (< 3 hours) and the

risks of I/R injuries. Thus, finding mediators that are simultaneously pro-angiogenic yet anti-inflammatory may provide a more optimal treatment of brain stroke.

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5.0 APPENDIX

Original articles:

ARTICLE #1

Haddad, L. E., L. B. Khzam, F. Hajjar, Y. Merhi, and M. G. Sirois. 2011. Characterization of FGF receptor expression in human neutrophils and their contribution to chemotaxis. *American journal of physiology Cell physiology* 301: C1036-1045.

ARTICLE #2

Haddad, L.E. and M.G. Sirois, Angiopoietin-1 upregulates de novo expression of IL-1β and Il1-Ra, and the exclusive release of Il1-Ra from human neutrophils. *PLoS One,* 2014. **9**(2): p. e88980.

Am J Physiol Cell Physiol 301: C1036–C1045, 2011.
First published July 27, 2011; doi:10.1152/ajpcell.00215.2011.

Characterization of FGF receptor expression in human neutrophils and their contribution to chemotaxis

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Submitted 29 June 2011; accepted in final form 26 July 2011

Haddad LE, Bou Khzam L, Hajjar F, Merhi Y, Sirois MG. Characterization of FGF receptor expression in human neutrophils and their contribution to chemotaxis. *Am J Physiol Cell Physiol* 301: C1036–C1045, 2011. First published July 27, 2011; doi:10.1152/ajpcell.00215.2011.—Several members of the fibroblast growth factor (FGF) family are potent endothelial cell (EC) mitogens and angiogenic factors, and their activities can be mediated by four tyrosine kinase receptors (FGFR1–4). In addition, FGFs can induce the release of inflammatory mediators by ECs and the expression of adhesion molecules at their surface, thereby favoring the recruitment and transvascular migration of inflammatory cells such as neutrophils. Neither the expression nor the biological activities that could be mediated by FGFRs have been investigated in human neutrophils. By biochemical and cytological analyses, we observed that purified circulating human neutrophils from healthy individuals expressed varying levels of FGFRs in their cytosol and at their cytoplasmic membrane. FGFR-2 was identified as the sole cell surface receptor, with FGFR-1 and -4 localizing in the cytosol and FGFR-3 being undetectable. We assessed the capacity of FGF-1 and FGF-2 to induce neutrophil chemotaxis in a modified Boyden microchamber and observed that they increase neutrophil transmigration at 10^{-10} and 10^{-9} M and by 1.77- and 2.34-fold, respectively, as compared with PBS-treated cells. Treatment with a selective anti-FGFR-2 antibody reduced FGF-1-mediated chemotaxis by 75% and abrogated the effect of FGF-2, while the blockade of FGFR-1 and -4 partially inhibited (15–40%) FGF-chemotactic activities. In summary, our data are the first to report the expression of FGF receptors in human neutrophils, with FGF-1 and FGF-2 promoting neutrophil chemotaxis mainly through FGFR-2 activation.

inflammation; fibroblast growth factor receptor; cell migration

INFLAMMATION IS AN ESSENTIAL component of host defense against harmful stimuli, involving a complex interplay between vascular tissues and blood leukocytes. Leukocyte recruitment to sites of inflammation occurs via a series of molecular and cellular events, beginning with the tethering and rolling of leukocytes on the endothelium lining blood vessels, followed by firm arrest, diapedesis, and finally leading to extravasation into the vascular wall (31). These cellular responses are accompanied by changes in the expression of effector molecules such as surface adhesion proteins, receptors and mediators secreted by the endothelium and recognized by neutrophils (41). Therefore, the efficacy of neutrophils in reaching infected tissues is dependent on recognizing and responding to alterations of endothelial functions. Interestingly, many of the mediators that neutrophils respond to are also extensively involved in angiogenesis, a phenomenon that leads to the formation of new blood vessels from preexisting vasculature. Factors such as vascular endothelial growth factor (VEGF), angiopoietins (Ang1, Ang2), and interleukins (IL)-1, -6, and -8, all of which are extensively involved at different stages of angiogenesis, have been shown to modulate neutrophil survival, degranulation, respiratory burst, adhesion, and chemotaxis (5, 11, 28, 32, 36).

Another set of potent angiogenic modulators, the family of fibroblast growth factors (FGFs), is also involved in the inflammatory process, but the contribution of specific FGFs to different stages of inflammation remains to be elucidated. Evidence of the involvement of FGFs in inflammation comes from observations that the two most studied members of the family, FGF-1 and FGF-2, are upregulated in inflammatory disorders such as bowel syndrome, Crohn's disease, ulcerative colitis (23), and rheumatoid arthritis (6). Other reports have suggested that FGF-1 and FGF-2 are secreted by and may act as immunoregulators of infiltrating neutrophils, monocytes, macrophages, and T lymphocytes, often in tandem with powerful inflammatory cytokines (3, 7, 33). The notion that FGF-2 alters neutrophil behavior secondary to the activation of endothelial cells (ECs) rather than through direct interaction is supported by evidence that FGF-2 enhances EC surface expression of adhesion molecules ICAM-1/2 (51), E-selectin (34), and monocyte chemoattractant protein-1 (33, 42, 49) before neutrophil rolling and adhesion. Although one study showed that FGF-2 primes neutrophil respiratory burst and increases their surface expression of integrins CD11b/CD18 (42), the effects of direct FGF stimulation, especially those of FGF-1, on neutrophil biological activities remain largely unexplored.

In general, the effects of FGFs, of which there are 23 members, are mediated by binding to four high-affinity tyrosine kinase receptors (FGFR1–4) and their splice variants (22). The diversity in FGF signaling is due, in part, to different FGF/FGFR combinations. Additionally, alternative splicing in the FGFR immunoglobulin (Ig)-like domains generates additional receptor isoforms with novel ligand affinities. Finally, effector cells will usually express different heparan-sulfates at their surface, which are responsible for stabilizing FGF/FGFR complexes and enhancing FGFR downstream signaling (10, 46).

Although it has been shown that cells of hematopoietic origin express functional FGFRs (1), no information has been reported regarding the receptors that mediate the effects of FGF-1 and FGF-2 in neutrophils. Therefore, the aim of the present study was to determine whether neutrophils expressed

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FGFRs and if so, to characterize the contribution of the identified FGFRs to the effects of FGF-1 and FGF-2 on neutrophil chemotaxis.

MATERIALS AND METHODS

Neutrophil purification. Venous blood samples were obtained from healthy donors free from medication for at least 10 days before the start of the experiments. The study has been approved by the human ethical committee of the Montreal Heart Institute, and all subjects provided written informed consent. Neutrophils were isolated using Ficoll-Hypaque gradient, as described previously (32, 36, 44), and resuspended in RPMI medium (Lonza, Allendale, NJ) supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and 1% penicillin-streptomycin. Ninety-eight (98) percent of the isolated cells were polymorphonuclear cells, as determined with a Coulter counter, and viability was found to be >98%, as assessed by Trypan blue dye exclusion assay.

FGF receptor identification by quantitative RT-PCR analyses. Total RNAs were obtained from freshly isolated human neutrophils (10^7 cells) by using the RNeasy extraction kit (Qiagen, Mississauga, ON, Canada). Total RNAs (100 ng) was reverse transcribed using random hexamers and the Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Burlington, ON, Canada) as described by the manufacturer. Reactions were performed on a MX3500P (Stratagene, La Jolla, CA) using 10 ng of cDNAs, Syber Green (Invitrogen), and 300 nM of the primers (Table 1) for each receptor. cDNAs were submitted to 45 cycles of amplification (temperatures for annealing: 60°C; dissociation: 55°C) and gave single peaks for each product.

Reverse transcriptase polymerase chain reaction [quantitative RT-PCR (RT-qPCR)] products were purified on a 2% acrylamide gel, quantified using QIAquick Gel Extraction Kit (Qiagen), and sequenced (Genome Quebec Innovation Centre, McGill University, Montreal, QC, Canada). The concentration of the purified products was measured using a NanoDrop spectrophotometer, and eluted amplicons were used in another set of RT-qPCR reactions as serial dilutions to generate standard curves for each set of oligonucleotides. The number of copies was calculated using the following formula: number of copies = [amount (ng) $\times$ 6.022 $\times$ 10^{23}]/[length (bp) $\times$ 1 $\times$ 10^9 $\times$ 650 (g/mol of bp)]. Standard curves of cDNA copies were generated by RT-qPCR and used to determine the number of mRNA copies for each receptor.

Immunohistochemistry. Freshly isolated human neutrophils (1.2 $\times$ 10^8 cells) were centrifuged, and the pellet was fixed in 10% PBS-buffered formalin overnight, dehydrated in a graded series of ethanol solutions and xylene, and embedded in paraffin. Sections 6 μm thick were rehydrated, blocked in 10% normal goat serum (NGS), and incubated overnight with rabbit polyclonal anti-human FGFR1–4 IgG or normal rabbit IgG (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Following a 30-min incubation with the secondary antibody (goat anti-rabbit IgG, 1% NGS), sections were rinsed three times in Tris buffer and incubated in Vectastain ABC alkaline phosphatase solution (Vector, Burlington, ON, Canada) and revealed with the Vector black alkaline phosphatase substrate solution for 30 min each as recommended by the manufacturer. Slices were rinsed, immerged in methyl green (10 min, 60°C), dehydrated, mounted with resinous medium, and examined with a light microscope under ×400 and ×1,000 magnification. No cross-reactivity was observed between FGFR1–4 antibodies.

Confocal microscopy. Neutrophils were isolated as aforementioned, allowed to adhere on glass coverslips precoated with poly-L-lysine (BD BioCoat; Becton Dickinson, Mississauga, ON, Canada) for 1 h, and fixed with a 2% paraformaldehyde solution. Nonspecific binding of primary antibodies was prevented by preincubating fixed neutrophils with 10% serum from the species used to raise secondary antibodies. Neutrophils were exposed to mouse monoclonal anti-human FGFR1–4 IgG (R&D Systems, Minneapolis, MN) and to a secondary goat anti-mouse antibody coupled to the Cy3-fluorochrome (Invitrogen). No cross-reactivity was observed between FGFR1–4 antibodies. Neutrophil preparations were mounted using DABCO (1,4-diazabicyclo-2–2-2-octane)/glycerol (1:1) solution and glass coverslip. Stained neutrophils were observed by confocal microscopy [Zeiss Axiovert 100 M microscope equipped with a ×63/1.4 Plan-Apochromat oil objective lens (Zeiss, Oberkochen, Germany) and adapted with an LSM 510 confocal system]. Images were recorded with LSM 510 software (Zeiss) and exported in tagged-image file format (TIFF).

Flow cytometry (FACS). Neutrophils (10^7 cells/ml) were rinsed and resuspended in RPMI, and mouse IgG (150 μg/ml) was added for 30 min to prevent nonspecific binding via Fc receptors. Neutrophils were centrifuged, rinsed, resuspended in PBS-BSA (10^6 cells/ml, 1% BSA), and incubated with phycoerythrin (PE)-conjugated mouse monoclonal anti-human FGFR1–4 IgG (25 or 50 μg/ml, R&D Systems), or with control PE-conjugated mouse monoclonal IgG_1 (50 μg/ml, R&D Systems) for 30 min at room temperature. Cells were rinsed and fixed with 2% paraformaldehyde. Flow cytometric analysis (10^5 events) was performed using a FACScan (Becton Dickinson, San Jose, CA).

Neutrophil migration. In vitro chemotactic assays were performed in a 48-well modified Boyden chamber apparatus (Neuro Probe, Gaithersburg, MD) as previously described (5, 26). Briefly, the bottom wells were loaded with RPMI containing PBS, IL-8 (25 nM), FGF-1 (10^{-14} to 10^{-8} M), or FGF-2 (10^{-14} to 10^{-8} M) to a final volume of 27 μl. The top wells were loaded with neutrophils (10^6 cells/ml; 50 μl from a RPMI suspension). The top and bottom wells were separated by a porous polycarbonate membrane filter (3-μm pore size). The modified Boyden chamber apparatus was incubated at 37°C for 1 h in a humidified incubator in the presence of 5% CO_2. At the end of the incubation period, the upper part of the modified Boyden chamber (upper wells) was removed, and the upper side of the polycarbonate membrane was wiped carefully with the rubber scraper provided by the manufacturer. The polycarbonate membrane was

Table 1. *FGF receptor primer sequences*

Receptor	Forward Oligonucleotide	Reverse Oligonucleotide
FGFR-1 (all variants)	CTACAAGGTCCGTTATGCCAC	TGCTACCCAGGGCCACTGTTTTG
FGFR-2 (all variants)	AAGCCCAAGGAGGCGGTCAC	CAGGAACACGGTTAATGTCA
FGFR-2-IIIb	GATAAATAGTTCCAATGCAGAAGTGCT	TGCCCTATATAATTGGAGACCTTACA
FGFR-2-IIIc	AGATTGAGGTTCTCTATATTCGGAATG	CTGTCAACCATGCAGAGTGAAAG
FGFR-α	CTCTCAACCAGAAGTGTACGTGGCTGC	GACATTCACCATGAAGTACCAAG
FGFR-β (same reverse)	CCACATTAGAGCCAGAAGGAGCACC CCACATTAGAGCCAGAAGATGCCA	GCTGGTTTCGTACCTTGTAGCCTCC
FGFR-3 (all variants)	TACTGTGCCACTTCAGTGTGC	ATCCGCTCGGGCCGTGTCCAGTAA
FGFR-4 (all variants)	CCAGCGCATGGAGAAGAAAC	ACCACGCTCTCCATCACGAGAC

FGFR, fibroblast growth factor receptor.

fixed in methanol, colored with the Kwik-Diff staining solution kit (Thermo Shandon, Pittsburgh, PA), mounted on a glass slide, and examined with a light microscope under ×40 magnification. The number of cells in five random fields was counted, and the results are expressed as relative neutrophil migration (number of cells from tested group/number of cells from corresponding control vehicles).

In another set of experiments, neutrophils were pretreated with 0.1 μg/ml of blocking anti-FGFR-1, FGFR-2, or FGFR-4 antibodies (R&D Systems) or with an isotype-matched control IgG for 30 min before a 1-h migration toward PBS, IL-8 (25 nM), FGF-1 (10^{-9} M), or FGF-2 (10^{-9} M). In the latter experiment, maximal effect was set to 100% and corresponds to the number of IgG-pretreated neutrophils migrating toward FGF-1 or FGF-2. The migration of neutrophils pretreated with FGFR antibodies was expressed as a percentage of the maximum FGF-induced response in absence of FGFR antibodies.

Statistical analysis. Data are presented as means ± SE. Statistical comparisons were made by Student's *t*-test or by a one-way analysis of variance, followed by Tukey post hoc where applicable using GraphPad Prism (Mac version 5.0b). Differences were considered significant at $P < 0.05$.

RESULTS

Neutrophil expression of FGFR mRNA. Using neutrophils isolated from venous blood samples of 22 healthy volunteers, we determined the mRNA expression of FGF receptors (1 to 4; FGFR1–4), as described in MATERIALS AND METHODS. Neutrophils expressed an average of 4.72×10^5 copies of total FGFR mRNA, irrespective of the subtypes. FGFR-2 mRNA had the highest level of expression ($3.10 \pm 0.67 \times 10^5$ mRNA copies) followed by FGFR-4 ($1.07 \pm 0.24 \times 10^5$ copies) and FGFR-1 ($0.6 \pm 0.12 \times 10^5$ copies), whereas FGFR-3 mRNA level was undetectable (Fig. 1*A*). As the levels of total FGFR mRNA copies varied between individuals, we represented the distribution of each of FGFR1–4 mRNA per donor. We arbitrarily separated the donors into low ($<4 \times 10^5$ copies; 11/22 donors) and high expressers ($>4 \times 10^5$ copies; 11/22 donors) (Fig. 1*B*); regardless of which group the donors belonged to, FGFR-2 represented the highest proportion of total FGFR mRNA, corresponding to 51.0 ± 9.5% and 67.9 ± 5.1% of the total FGFR mRNA for low and high expressers (Fig. 1*C*).

Neutrophil expression and localization of FGFR proteins. Independently of the above mRNA analyses, neutrophils from 16 different donors were examined for FGFR1–4 protein expression and localization by conventional immunohistochemistry (IHC). Because of the variations in staining intensities and the number of cells that were marked between different donors, we distinguished four staining patterns (Fig. 2, *A–E*) to which we attributed qualitative scores: *score 0* (background staining; Fig. 2*A*), *score 1* (faint but detectable staining in some cells; Fig. 2*E*), *score 2* (high staining in some cells; Fig. 2*B*), or *score 3* (high staining in most cells; Fig. 2*C*). The staining scores for each donor, along with the means of the scores for comparative purposes, are presented in Table 2. FGFR-1 staining showed high staining on a small subset of the neutrophils for most donors (Fig. 2*B*). The majority of donors scored high for FGFR-2, with a much larger fraction of neutrophils intensely stained (Fig. 2*C*). FGFR-3 and FGFR-4 expression fell between *scores 0* and *1* as most donors showed either very faint staining in a minor population of neutrophils or no detection at all (Fig. 2, *D* and *E*). We also observed differences in the localization of the staining, especially between FGFR-2 and FGFR-1 (and to a lesser extent FGFR-4), as shown by the boxed, magnified images in Fig. 2. When viewed at ×1,000 magnification, neutrophils stained for FGFR-2 showed a distinct darker coloration at the cytoplasmic periphery, unlike FGFR-1 or FGFR-4, which showed a rather diffuse intracellular staining pattern. These observations suggest that FGFR-2 is the only neutrophil cell surface receptor, whereas FGFR-1 and FGFR-4 show a cytosolic localization.

Data obtained from flow cytometry analyses reflected the same variability in expression levels of FGFR1–4 from one donor to another. By confocal microscopy, we determined whether the receptors were localized intracellularly or at the cell surface. We observed that FGFR-1 and FGFR-4 staining was perinuclear and diffused across the cytosol of the neutrophils, whereas FGFR-2 staining was mainly confined to a thin strip in close proximity to the cell surface. Once again, FGFR-3 detection was similar to the nonspecific IgG background (Fig. 3).

Characterization of FGFR-2. FGF receptors share the same basic protein structure, which is characterized by up to three extracellular Ig domains (denoted by Ig-I, -II, or -III). These Ig domains define the affinity and responsiveness of FGFRs for different FGF ligands (10, 18, 29). In normal tissues, FGFRs express all three Ig domains and are referred to as "FGFR-α" (Fig. 4*A*). The loss of Ig-I gives rise to "short" forms of FGFRs called "FGFR-β," correlating with transformation to a malignant phenotype and invasiveness (24, 48). FGFR-β forms are thus found in cancerous cell lines, having a 1,000-fold higher affinity to FGF-1 but not for FGF-2. The complexity of the FGFR family is increased by alternative splicing in exons 9 or 8 of Ig-III domain, generating FGFR-IIIb and FGFR-IIIc isoforms, respectively (Fig. 4*B*). These splicing events confer additional ligand-binding properties to FGFRs (45).

Because we identified FGFR-2 as the only cell surface receptor, we further characterized the expression of its different isoforms. We looked at the FGFR-2 mRNA transcript, first to establish whether all three Ig domains were coded, and second to determine which of exons IIIb or IIIc in Ig-III were spliced. As per our previous experiments, we performed RT-qPCR analyses and determined that FGFR-2α is nearly the exclusive isoform (Fig. 4*C*). We then quantified the mRNA of both FGFR-2α-IIIb and FGFR-2α-IIIc subtypes and observed that, in neutrophils, FGFR-2 mRNA was primarily FGFR-2α-IIIc.

In vitro FGF-1 and FGF-2-mediated chemotaxis. Previous studies reported that FGF-2 modulates the recruitment of polymorphonuclear cells, monocytes, and T cells in vivo (51, 52). However, the possibility that FGFs could be direct stimulators of leukocyte recruitment was not addressed. To this end, we used a modified Boyden microchamber model and observed that both FGF-1 and FGF-2 were capable of mediating neutrophil chemotaxis at picomolar concentrations. In addition, treatment with FGF-1 or FGF-2 (10^{-14} to 10^{-8} M) induced a bell-shape response on the number of migrating neutrophils as compared with control PBS-treated cells. The maximal effect was achieved at 10^{-10} M for FGF-1 and 10^{-9} M for FGF-2, corresponding to 1.77- and 2.34-fold increase over PBS-mediated neutrophil migration, respectively. The positive control IL-8 (25 nM) increased migration by 2.72-fold as compared with control PBS-treated neutrophils (Fig. 5*A*).

In another set of experiments, we assessed the contribution of FGFR-1, FGFR-2 and FGFR-4 to FGF-mediated che-

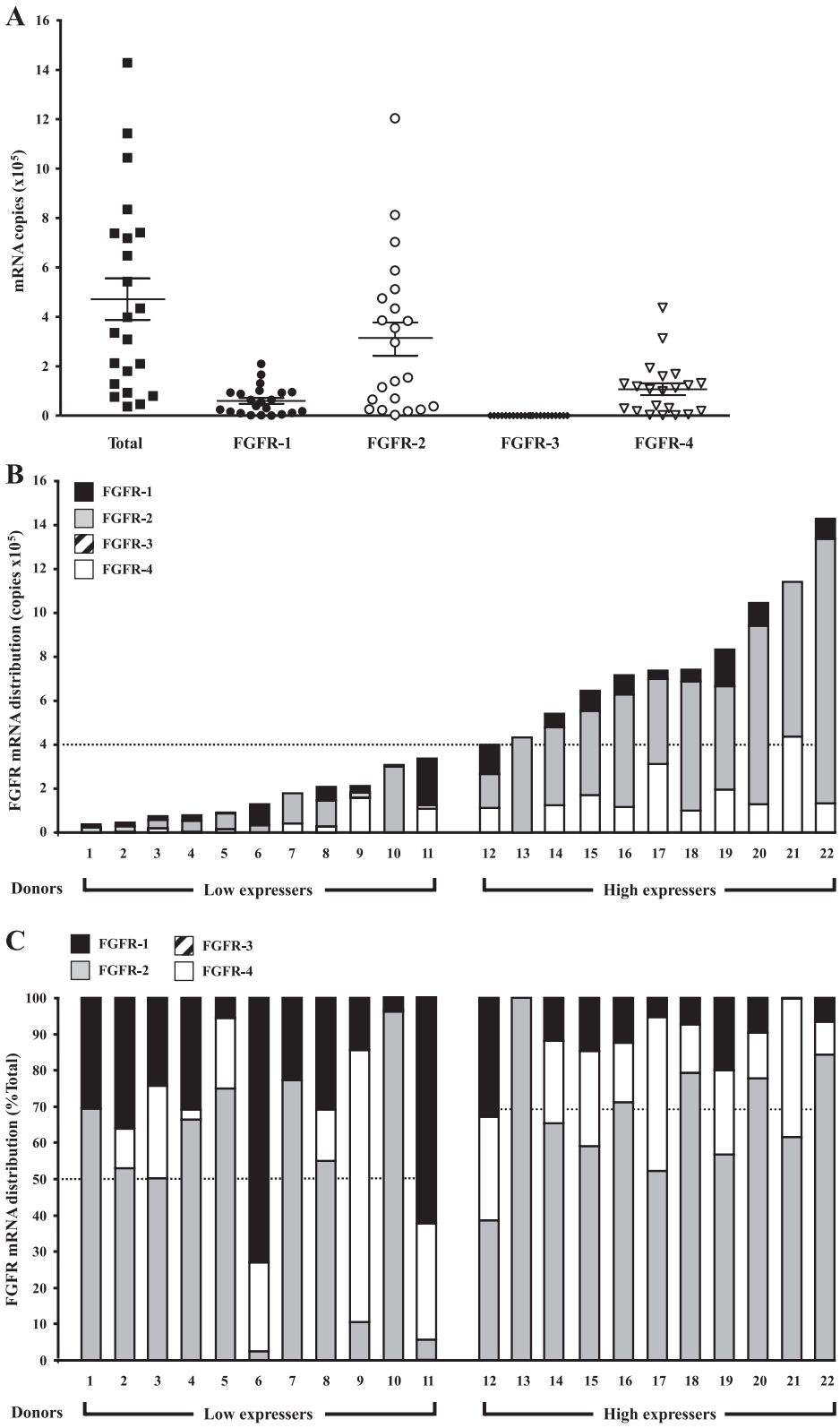

Fig. 1. Fibroblast growth factor (FGF) receptor (FGFR) 1–4 mRNA expression in human neutrophils. Circulating neutrophils isolated from 22 healthy individuals were profiled for FGFR1–4 mRNA expression. *A*: number of FGFR1–4 mRNA copies as well as means ± SE are presented. Donors are the same across columns, with each symbol per column corresponding to a single donor. *B*: distribution of FGFR1–4 mRNA copies per donor is illustrated. Each column represents a single donor, and the peak of the columns corresponds to the total number of FGFR copies. Donors were divided into low ($<4 \times 10^5$ total FGFR mRNA copies) and high ($>4 \times 10^5$ total FGFR mRNA copies) expressers. *C*: distribution of each FGFR1–4 mRNA illustrated in *B* is expressed as a percentage of total FGFR mRNA per individual. Dotted lines indicate the average FGFR-2 percentage observed in low or high expressers.

motaxis. Neutrophils were pretreated with blocking monoclonal antibodies against FGF receptors (FGFR-1, -2, and -4) or with a control isotype-matched IgG (0.1 μg/ml) for 30 min before stimulation with PBS, IL-8 (25 nM), FGF-1, or FGF-2 (10^{-9} M). We observed that the effects of FGF-1 on neutrophil chemotaxis were suppressed by 75.2% with the selective anti-FGFR-2 antibody and partially reduced with the anti-FGFR-1 or anti-FGFR-4 by 39.6% and 31.9%, respectively. FGF-2-induced neutrophil migration was almost completely abrogated by treatment with the anti-FGFR-2 antibody (96% reduction),

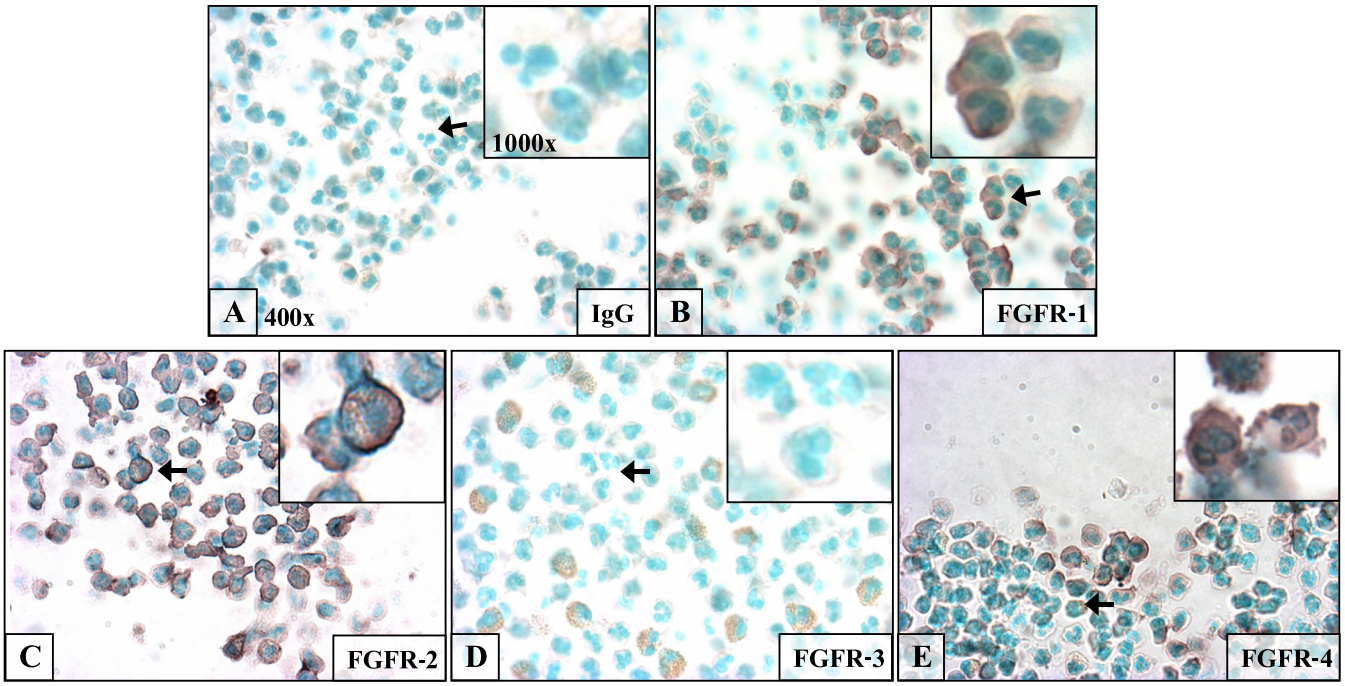

Fig. 2. Expression and localization of FGFR1–4 proteins by immunohistochemistry (IHC). Human neutrophils mounted in paraffin were stained with specific anti-human FGFR1–4 IgG antibodies. Representative IHC staining images for each receptor are illustrated as follows: control IgG (*A*), FGFR-1 (*B*), FGFR-2 (*C*), FGFR-3 (*D*), and FGFR-4 (*E*). The magnified (×100) boxes in the images were chosen from their corresponding fields (black arrows) and show the distinct localization patterns for each receptor.

whereas anti-FGFR-1 and FGFR-4 antibodies reduced migration by 28.9% and 14.6%, respectively (Fig. 5*B*). In contrast, basal and IL-8-induced neutrophil migration was unaffected in the presence of anti-FGFR antibodies (data not shown).

DISCUSSION

In the current study, we demonstrate that circulating neutrophils isolated from the blood of healthy individuals express varying levels of FGFR-1, -2, and -4 mRNA, with FGFR-2 mRNA showing the most consistent and highest level of expression. At the protein level, donors showed a heterogeneous expression of FGF receptors. We identified FGFR-2 as the most expressed and unique neutrophil cell surface receptor, whereas FGFR-1 and FGFR-4 appeared to be cytosol bound. Neither the mRNA nor the protein for FGFR-3 was significantly detectable. In addition, we observed that FGF-1 and FGF-2 were capable of stimulating neutrophil migration under in vitro conditions, mainly through FGFR-2 activation.

Table 2. *Immunohistochemistry scores for FGFR1-4 expression*

Donor	FGFR-1	FGFR-2	FGFR-3	FGFR-4
1	2	3	1	2
2	3	1	0	1
3	1	3	0	0
4	0	0	0	0
5	1	3	0	2
6	3	3	1	2
7	1	2	0	1
8	0	2	0	1
9	2	2	0	0
10	2	2	0	1
11	3	2	0	1
12	1	2	0	1
13	0	2	1	0
14	1	2	1	0
15	1	1	1	0
16	2	2	1	1
Means ± SE	1.43 ± 0.36	2 ± 0.50	0.37 ± 0.09	0.81 ± 0.20

Neutrophils from 16 donors were assessed for the presence and localization of FGFR1-4 proteins by immunohistochemistry. Qualitative scores were attributed per individual for each receptor as follows: IgG background staining (*score 0*), faint but detectable staining in some cells (*score 1*), high staining in some cells (*score 2*), and high staining in most cells (*score 3*). Means ± SE for each receptor are shown in the *bottom row*.

FGF-1 and FGF-2 are potent heparin-binding proangiogenic growth factors that exert their biological functions through the activation of high affinity tyrosine kinase receptors (FGFRs), heparan-sulfate proteoglycans (HSPGs) and integrins expressed on the surface of effector cells (17). Although the presence of FGFRs on human neutrophils has not been reported, these leukocytes from a biological standpoint seem naturally well equipped to respond to FGF stimulation. First, neutrophils express integrins as well as HSPGs, accessory coreceptors that facilitate dimerization of tyrosine kinase receptors and amplify the signaling triggered by HSPG-binding growth factors such as FGFs. Second, neutrophils present at their surface the receptors for at least two other families of angiogenic growth factors with similar properties to FGFs. Indeed, neutrophils were shown to express VEGF receptor-1 and Tie2, which are responsible for VEGF and Ang-mediated neutrophil chemotaxis, respectively (2, 26).

Detection and localization of FGF receptors in human neutrophils. The initial objective of our study was to determine whether neutrophils expressed FGF receptors. Quantitative RT-PCR analyses showed a trend among individuals to express more mRNA for FGFR-2 than any of the other FGF receptor

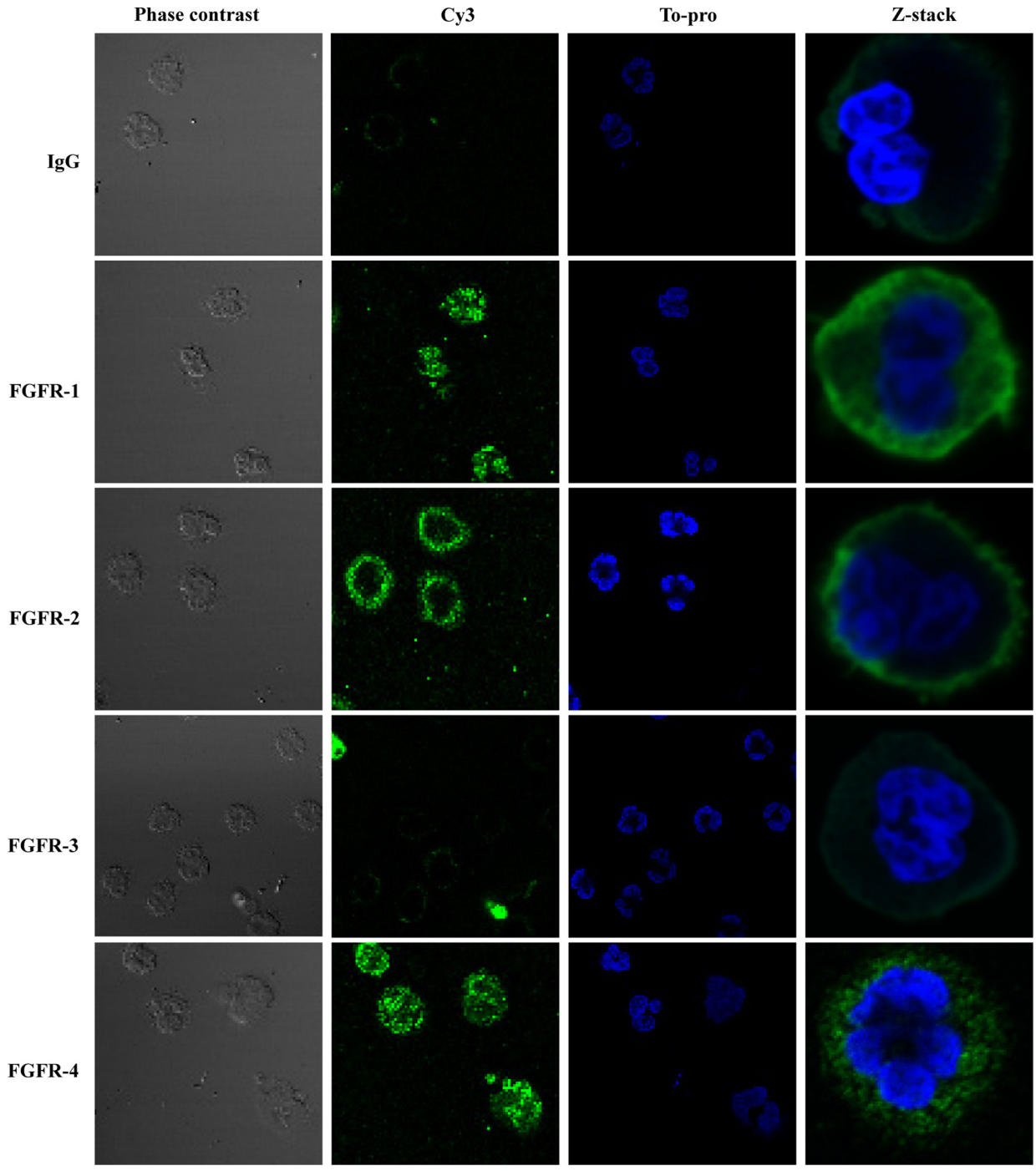

Fig. 3. Localization of FGFR1–4 by confocal microscopy. Human neutrophils were stained with specific anti-human FGFR1–4 antibodies and a secondary Cy3-coupled antibody. Labeled neutrophils were viewed under confocal microscope to assess the protein localization of FGFR1–4. Columns, *left* to *right*, correspond to images of neutrophils taken from random fields: phase contrast showing membrane integrity, specific FGFR staining (secondary antibody coupled to Cy3 dye), and nuclear counterstaining (To-pro). The three-dimensional reconstructions of confocal Z-stack images (*last column*) correspond to neutrophils chosen at random from the larger associated fields.

subtypes. FGFR-1 and FGFR-4 mRNA were also detectable with a generally lower number of copies, whereas FGFR-3 was minimally or not detected. Owing to the fact that the presence of the mRNA does not always coincide with the expression of its corresponding protein, we examined FGFR protein expression by immunostaining and confocal microscopy. FGFR-2 was detected on a large subset of neutrophils for many of the donors, with a staining pattern to the cell surface membrane. FGFR-1 and FGFR-4 showed a less intense but uniformly diffused staining across the cytoplasm of a small fraction of neutrophils, suggesting that if these receptors were indeed cytoplasmic, they were not confined to specific subcellular fractions or to vesicles. FGFR-3 protein detection was absent to very low, in accordance with its corresponding mRNA levels.

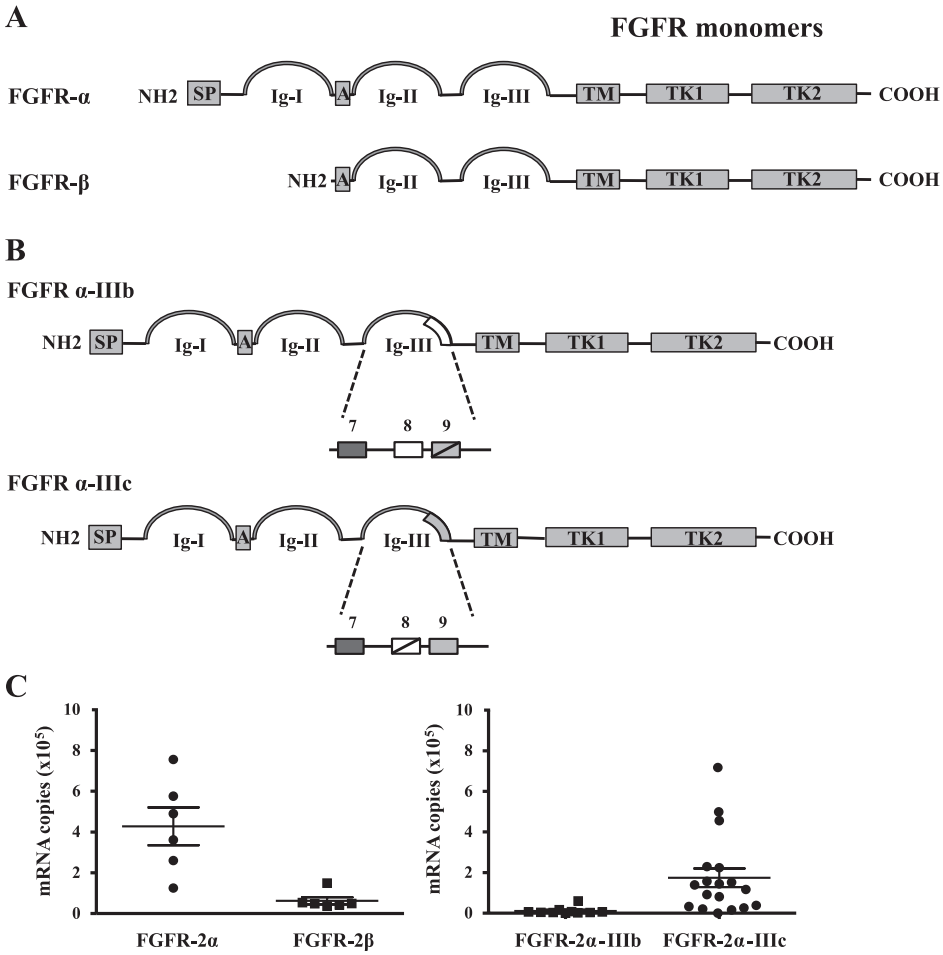

Fig. 4. FGFR-2 subtype and isoform expression in human neutrophils. FGFRs are composed of up to three extracellular Ig-like domains that determine their binding properties to members of the FGF family. *A*: FGFRs can be expressed either as a long form (FGFR-α) containing all three Ig domains or as a shorter form lacking Ig-I (FGFR-β). *B*: alternative splicing in exons 8–9 of the Ig-III domain generates isoforms referred to as "IIIb" (spliced exon 9) or "IIIc" (spliced exon 8) that exert different FGF-binding preferences. *C*: mRNA was isolated from the neutrophils of at least 7 healthy donors and assessed for FGFR-2α and -β subtypes as well as IIIb-IIIc isoforms by quantitative (q)PCR analyses. The number of mRNA copies and means ± SE are shown, with each symbol per column representing a single donor.

Characterization of FGFR-2. The specificity of the FGF-neutrophil interaction is determined not only on the basis of which FGFR is expressed at the cell surface, but also by the subtype and isoform of the surface receptor. Most mammalian FGFRs exist as the FGFR-α isoform, which consists of three extracellular immunoglobulin-like domains (Ig I–III). The lack of Ig-I, which gives rise to short forms of FGFR called "β," leads to a 1,000-fold increase in FGF-1 responsiveness and an important reduction in FGF-2 affinity. FGFs interact with the Ig-II and Ig-III domains, with the Ig-III interactions primarily responsible for ligand-binding specificity. Alternative splicing of exons 9 and 8 in the Ig-III domain gives rise to "IIIb" and "IIIc" variants for FGFR-1, -2, and -3, thus generating seven possible FGFR subtypes at the cell surface that bind a specific subset of FGFs. FGF-1 is capable of activating all seven FGFRs. In contrast, FGF-2 only binds the IIIc forms of FGFRs (9, 10, 14, 15, 20–22, 30). Using qPCR analysis, we confirmed that nearly all the FGFR-2 mRNA from human neutrophils comprises all three Ig domains, coding for the α-isoform. Furthermore, the majority of the FGFR-2 mRNA contains exon 9 in the second part of Ig-III domain, which would thereby generate IIIc isoforms. The presence of FGFR-2α-IIIc isoform on the cell surface of human neutrophils would allow them to interact with FGF-1, FGF-2, FGF-4, and FGF-6 but not with FGF-3, FGF-7, or FGF-10 (4, 14, 29). Given the role of FGFs in angiogenesis, this finding could potentially have important physiological implications; indeed, in cases such as following a cerebral stroke or cardiac ischemia, where an increase in angiogenesis is desired to improve reperfusion while maintaining a minimal influx of inflammatory cells, the choice of FGF ligand could become critical.

Our data regarding FGFR1–4 protein expression and localization are in agreement with previous studies reporting the presence of soluble cytosolic forms for FGF receptors in blood and different cell types (18, 37). The function of the cytoplasmic FGFRs can only be speculative at the moment, but we did consider the possibility that FGF-2 could induce the translocation of its own receptors from the cytosol of the neutrophils to the cell surface. However, when neutrophils were treated with FGF-2 for up to 24 h, FGFR1–4 detection was not significantly increased, as measured by flow cytometry (data not shown). Nevertheless, this observation does not exclude the possibility that FGFR1–4 translocation could be induced by other more potent inflammatory mediators such as IL-8, TNF-α, or bacterial lipopolysaccharide (LPS), all of which can promote protein translocation to the surface of neutrophils (12, 36). It is possible that the soluble forms of FGFR-1 and -4 are proteolytically cleaved by matrix metalloproteases (MMPs) inside the cell before they reach the cytoplasmic membrane, especially since neutrophils synthesize, store, and release large amounts of MMP-2 (13) and MMP-9 (19, 40). This proteolysis can occur in different locations within the cell, especially since MMPs have been shown to cleave substrates in nuclear, mitochondrial, cytoplasmic, and vesicular compartments, including

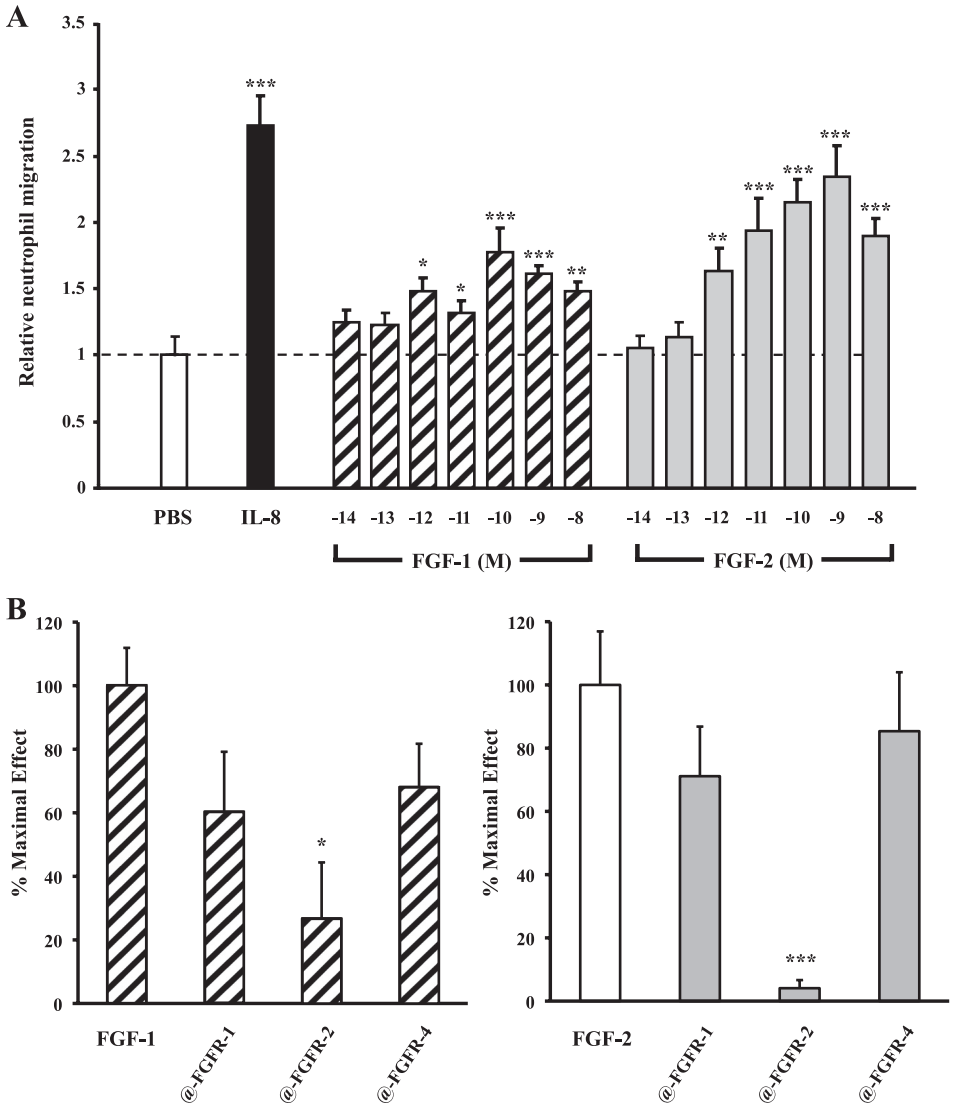

Fig. 5. Effect of FGF-1 and FGF-2 on neutrophil chemotaxis. *A*: untreated human neutrophils were set to migrate in a modified Boyden microchamber toward PBS, IL-8 (25 nM, positive control), or increasing concentrations of FGF-1 or FGF-2 (10^{-14} to 10^{-8} M). Cells from 5 random fields were counted, and migration is expressed relative to control-PBS. *B*: neutrophils were pretreated with 0.1 μg/ml of specific blocking antibodies for FGFR-1, -2, or -4 or an isotype-matched IgG and then set to migrate toward PBS, IL-8, FGF-1, or FGF-2 (10^{-9} M). Migration of IgG-treated neutrophils toward FGF-1 or FGF-2 was set as the maximal effect (100%), and the migration of FGFR-pretreated neutrophils toward FGF-1 or FGF-2 is expressed as a percentage of the maximal effect. Results are presented as means ± SE for at least six independent experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ as compared with control-PBS (*A*) or FGF-1/FGF-2 (*B*).

the intracellular cytoskeletal matrix (8). Furthermore, the presence of FGF-2 in the cytoplasm of neutrophils has been reported (35), which raises the possibility that FGF-2 could have undetermined intracellular functions and could be sequestered by soluble FGFR proteins. Further experimentation will be required before a functional role for these cytoplasmic receptors can be ascertained.

FGF-1 and FGF-2-mediated chemotaxis. Numerous angiogenic factors have been shown to modulate leukocyte behavior under inflammatory conditions. For instance, VEGF has been reported to promote several chronic inflammatory disorders (16, 25, 27, 38, 39, 43, 50). It has been shown that FGF-2 can modulate the interaction of leukocytes with ECs in vitro (6, 47, 51), secondary to the stimulation of adhesion molecule expression on ECs. We addressed the capacity of FGF-1 and FGF-2 to directly modulate neutrophil chemotaxis in an EC-free environment by using a modified Boyden microchamber model. We observed that both FGF-1 and FGF-2 were capable of stimulating neutrophil recruitment, albeit with different potencies. These effects were almost completely abrogated with blocking anti-FGFR-2 antibodies, and only partially blocked by anti-FGFR-1 and FGFR-4 antibodies, suggesting that most of the chemotactic activities of FGFs are mediated by FGFR-2 activation.

In summary, our results demonstrate for the first time the exclusive expression of FGFR-2 at the surface of human neutrophils. The predominance of the FGFR-2α-IIIc isoform suggests that neutrophils respond to only a specific subset of FGF ligands. Accordingly, we observed that FGF-1 and FGF-2, both of which bind strongly to FGFR-2α-IIIc, act as chemotactic agents for the recruitment of neutrophils in vitro, mainly through direct interaction and activation of FGFR-2. Consequently, this study delineates a key inflammatory role for FGF-1 and FGF-2 and supports the possibility of additional functions for FGF/FGFR complex in modulating polymorphonuclear leukocyte proinflammatory activities.

ACKNOWLEDGMENTS

We thank Marie-Elaine Clavet-Lanthier and Dominique Lauzier for technical assistance and expertise in histology, as well as Maya Mamarbashi for assistance in designing PCR oligonucleotide sequences and RT-qPCR exper-

iments. Finally, we thank all of the volunteers who agreed to generously donate blood.

GRANTS

This work was supported by grants from the Canadian Institutes of Health Research (MOP-97943) and from the Heart and Stroke Foundation of Quebec. M. G. Sirois was recipient of a senior scientist scholarship from the Fonds de la recherche en santé du Québec.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Angiopoietin-1 Upregulates De Novo Expression of Il-1β and Il1-Ra, and the Exclusive Release of Il1-Ra from Human Neutrophils

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Abstract

The expression of the angiopoietin (Ang) receptor, Tie2, on both endothelial and inflammatory cells supports the idea that Ang signaling may play a fundamental role in initiating and maintaining the inflammatory response. We have previously shown that Ang1 and/or Ang2 alter the innate immune response by enhancing human neutrophil survival, chemotaxis and production of inflammatory cytokine interleukin-8 (IL-8) *in vitro*. Thus, we hypothesized that Ang1 and Ang2 could modulate other inflammatory signals in neutrophils, a possibility we explored through a gene-based assay looking at changes in the mRNA expression of 84 inflammatory cytokines and their receptors. We observed that Ang1 (10^{-8} M), but not Ang2, increased mRNA expression of prominent pro-inflammatory cytokine IL-1β and its natural antagonist IL-1RA, by up to 32.6- and 10.0-fold respectively, compared to PBS-control. The effects of Ang1 extended to the proteins, as Ang1 increased intracellular levels of precursor and mature IL-1β, and extracellular levels of IL-1RA proteins, by up to 4.2-, 5.0- and 4.4-fold respectively, compared to PBS-control. Interestingly, Ang1 failed at inducing IL-1β protein release or at increasing intracellular IL-1RA, but the ratio of IL-1RA to mature IL-1β remained above 100-fold molar excess inside and outside the cells. The above-noted effects of Ang1 were mediated by MAP kinases, whereby inhibiting MEK1/2 lead to up to 70% effect reduction, whereas the blockade of p38MAPK activity doubled Ang1's effect. These findings suggest that Ang1 selectively alters the balance of neutrophil-derived inflammatory cytokines, favoring the blockade of IL-1 activity, a consideration for future therapies of inflammatory diseases.

Citation: Haddad LE, Sirois MG (2014) Angiopoietin-1 Upregulates De Novo Expression of Il-1β and Il1-Ra, and the Exclusive Release of Il1-Ra from Human Neutrophils. PLoS ONE 9(2): e88980. doi:10.1371/journal.pone.0088980

Editor: Dominik Hartl, University of Tübingen, Germany

Received September 24, 2013; **Accepted** January 14, 2014; **Published** February 12, 2014

Funding: Contract grant sponsor: Canadian Institutes of Health Research (CIHR); Contract grant number: MOP-97943; http://www.cihr-irsc.gc.ca/. Contract grant sponsor: Heart and Stroke Foundation of Québec; http://www.heartandstroke.com/. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Inflammation is characterized by a sequence of events that involve activation of the endothelium, release of endothelial mediators, vascular remodeling to allow for increased permeability and blood flow, and leukocyte – especially neutrophil – recruitment and infiltration into inflamed tissues. Because acute inflammation and angiogenesis can be triggered by the same molecular events, it is not surprising that most molecules that alter permeability, such as vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)-α and nitric oxide (NO), are potent pro-angiogenic factors (review; [1]).

Angiopoietins (Ang) are a family of angiogenic growth factors that play a major role in modulating vascular integrity and maturation. While the expression of the Ang receptor Tie2 on both endothelial and inflammatory cells [2–4] suggests a potential involvement in inflammation, a literature review of the specific contributions of the primary family members, Ang1 and Ang2, reveals a dichotomy of pro- and anti-inflammatory properties that is often influenced by the presence of other inflammatory mediators. From an anti-inflammatory perspective, Ang1 counteracts some components of the activity of pro-inflammatory factors on endothelial cells (ECs), inhibiting increases in EC permeability induced by VEGF, thrombin, bradykinin and histamine *in vitro* [5–7]. Additionally, Ang1 downregulates the release of chemokine IL-8 by ECs [6], and inhibits adherence and transmigration of neutrophils across EC monolayers stimulated with VEGF, TNF-α and thrombin [5,6,8], likely through altering the expression of endothelial E-selectin and intracellular/vascular cell adhesion molecules (ICAM-1/VCAM-1) [5,9]. In mouse skin *in vivo*, co-overexpression of VEGF and Ang1 shows an additive effect on angiogenesis but results in leakage-resistant vessels with little inflammation [10]. In stark contrast, Ang1 exerts certain pro-inflammatory activities: Ang1 through Tie2 activation increases endothelial P-selectin translocation, a protein that mediates the rolling of leukocytes onto the endothelium under inflammatory conditions[11]. Ang1 has also the ability to directly impact leukocyte behavior, stimulating neutrophil IL-8 synthesis and release [12], and acting in a Tie2-dependent manner to recruit neutrophils and eosinophils, to increase neutrophil lifespan, and to promote neutrophil adhesion onto extracellular matrix [3,4,13–15]. The contribution of Ang2 to acute inflammation is even less defined, with some evidence of pro-inflammatory properties such as enhancing TNF-α-dependent adhesion of leukocytes to EC monolayers, as well as TNF-α-induced expression of ICAM-1 and VCAM-1 [16]. Ang2 alone also promotes a transient endothelial

P-selectin translocation and its effects on neutrophil adhesion and chemoattraction are Tie2-dependent and similar to those of Ang1 [4,15]; however, unlike Ang1, Ang2 fails to promote neutrophil IL-8 synthesis and/or release, to increase neutrophil survival, or to counteract the effects of Ang1 on the aforementioned processes [12,13]. Thus, the distinct contributions of Ang1 and Ang2 to acute inflammation remain to be clearly delineated. Neutrophils are generally the first responders at sites of inflammation. They contribute substantially to inflammation through their ability to produce proteases, reactive oxygen species [17,18], and to a lesser extent, cytokines including interleukin (IL)-6, TNF-α and IL-1 receptor antagonist (IL-1RA) [19–23]. Building on our recent findings that Ang1 promotes significant IL-8 production in human neutrophils *in vitro* in a time-dependent manner [12], we broadened our investigation to 84 other pro-inflammatory cytokines and their receptors, and looked at changes in their mRNA expression following angiopoietins stimulation. The first part of this study identified three related targets, all belonging to the IL-1 family of inflammatory cytokines, IL-1α, IL-1β, and IL-1RA, as well as a number of other potential interests unrelated to the IL-1 family. The second part of this study focused on identifying the kinetics and mechanisms that mediate the effects of Ang1 on IL-1 family members in neutrophils.

Materials and Methods

Neutrophil purification

The study was conducted in accordance with the Declaration of Helsinki and approved by the Montreal Heart Institute's ethical committee (Montreal, QC, Canada; ethics No. ICM #01-406). All of the subjects provided written informed consent to the experimental protocol before participating in the study. Venous blood was obtained from healthy donors free from medication for at least 10 days prior to the experiments. Venous blood was obtained by drawing 100 ml (4×25 ml) of blood using a 21G needle into 30 ml syringes prefilled with 5 ml of Anticoagulant Citrate Dextrose Solution USP (ACD) Formula A (Baxter Healthcare; Deerfield, IL). The blood was then transferred into 4×50 ml tubes and spun for 15 min at 200 g at room temperature. Following the centrifugation, the platelet rich plasma (PRP) was removed from the top layer and 20 ml of a 4% Dextran solution (138 mM NaCl, 5 mM KCl, 0.34 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 5.6 mM Glucose, 10 mM HEPES, 12.9 mM Sodium Citrate and 250 mM Dextran; pH 7.4) was added per tube. The tubes were gently mixed and red blood cells were left to sediment for 45 minutes at room temperature. The upper layer containing the white blood cells was collected and gently deposed on a 12.5 ml layer of Ficoll-Paque Plus (GE Healthcare; Baie d'Urfé, QC, Canada) in 50 ml tubes and spun for 28 minutes at 400 g and at room temperature [24–26]. Following this centrifugation, the monocytes and lymphocytes were separated from the neutrophils by Ficoll gradient. The reminiscent red blood cells and neutrophils were found in the pellet. In order to eliminate the red blood cells from the neutrophils, we used a water lysis procedure by which we added 20 ml of distilled water over the neutrophils and red blood cells pellet and mix gently for 20 seconds, followed by the quick addition of 20 ml of HBSS 2X solution while continuing mixing, for a final concentration of HBSS 1X (pH 7.4). Neutrophils were then spun for 10 minutes at 200 g and at room temperature. The pellet was then resuspended in RPMI 1640 medium with Corning Glutagro (Mediatech, Manassas, VA) supplemented with 25 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) and 1% penicillin/streptomycin. Contamination of isolated neutrophil suspension with peripheral blood mononuclear cells was less than 0.1% as determined by morphological analysis and flow cytometry, and viability was found to be greater than 98%, as assessed by Trypan blue dye exclusion assay.

RNA studies

Two RT-qPCR -based techniques were used. The first of these is a gene-based screening method; more specifically, real time quantitative polymerase chain reaction (RT-qPCR) arrays were used to identify targets of angiopoietins stimulation in inflammation. The second method was used to confirm array results and to expand mRNA expression kinetics. Recombinant human Ang1 and Ang2 were obtained from R&D Systems (Minneapolis, MN) and bacterial lipopolysaccharide (LPS) from Sigma-Aldrich (St Louis, MO).

RT-qPCR array analyses. Neutrophils (10^7 cells/ml; 1 ml) from at least three independent donors were treated with PBS, Ang1 (10^{-8} M) or Ang2 (10^{-8} M) for 90 minutes prior to DNAse treatment and total RNA extraction with the RNeasy extraction kit (Qiagen, Mississauga, ON, Canada). RNA samples were evaluated for integrity using a Bioanalyzer 2000 system (Genome Quebec Innovation Centre, McGill University, Montréal, QC, Canada); when all three samples (PBS, Ang1 and Ang2) from the same donor showed an mRNA integrity above 8.5, they were selected for use in arrays. RNA integrity between selected samples differed by less than 0.5. Following isolation, 2 μg of RNA were processed with RT^2 First Strand Kit (SA Biosciences, Frederick, MD) according to manufacturer's instructions. Quantitative PCR analyses of chemokines and receptors were assessed with the Chemokines & Receptors PCR Array (SA Biosciences), RT^2 SYBR® Green qPCR master mix (SA Biosciences) and a Stratagene Mx3500p qPCR System (Stratagene, La Jolla, CA). PCR array data were analyzed by the RT^2 Profiler PCR Array Data Analysis program, available through SA Biosciences' web portal and based on the ΔΔCt method with four different housekeeping genes. Data were normalized to 4 housekeeping genes (B2M, HPRT1, RPL13A and GAPDH) and represented in a volcano plot of fold change in expression of each gene (compared to PBS-control) against its p-value.

RT-qPCR kinetics. Total RNAs (100 ng) from PBS, LPS (1 μg/ml), Ang1 (10^{-9} and 10^{-8} M) or Ang2 (10^{-9} and 10^{-8} M)-treated neutrophils were extracted as mentioned above and reverse transcribed using random hexamers and the Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Burlington, ON, Canada) according to manufacturer's instructions. Reactions were carried out on a MX3500P (Stratagene) using 10 ng of cDNAs, Syber Green (Invitrogen) and 300 nM of specific primers as follows (5′ to 3′): • IL-1α forward (Fwd) TGACCTGGAGGCCATCGCCAA; reverse (Rev) GCAGCAGCCGTGAGGTACTGA, • IL-1β Fwd ACGCTCCGGGACTCACAGCA; Rev TGAGGCCCAAGGCCACAGGT, • IL-1RA Fwd GATGTGGTACCCATTGAGCCTCATGC; Rev ACTGGTGGTGGGGCCACTGT. cDNAs were submitted to 40 cycles of amplification (temperatures for annealing: 60°C; dissociation: 55°C) and gave single peaks for each product. RT-qPCR products were purified on a 2% acrylamide gel, quantified using QIAquick Gel Extraction Kit (Qiagen) and sequenced. Gene expression was normalized using β-microglobulin as the housekeeping gene and results were expressed relative to calibrator T_0 (gene expression at time of isolation) or to the control-PBS at each time point.

In another set of experiments, neutrophils were pretreated with inhibitors of p38 MAP kinase (SB203580; 10 μM), MEK1/2 (U0126; 20 μM), Akt (Triciribine; 5 μM), DMSO (vehicle) or PBS

for 30 minutes prior to a 1-hour stimulation with PBS, LPS (1 μg/ml), Ang1 (10^{-10}–10^{-8} M) or Ang2 (10^{-10}–10^{-8} M). Total RNAs were then extracted and submitted to RT-qPCR analyses as aforementioned.

Quantification of cytokines by ELISA

Purified neutrophils (10^7 cells/ml; 1 ml) were incubated in RPMI and treated with PBS, LPS (1 μg/ml) Ang1 (10^{-10}–10^{-8} M) or Ang2 (10^{-10}–10^{-8} M) for up to 24 hours at 37°C and 5% CO_2. Upon the incubation period, neutrophils were centrifuged at 900 g for 6 minutes and supernatants collected and stored at −80°C. The centrifuged cells were then lysed in ice-cold 1% Triton-RPMI solution containing a cocktail of protease inhibitors. The complete kinetics of synthesis and release of IL-1α, IL-1β and IL-1RA as well as those for pro-IL-1β were evaluated from cell-lysates and supernatants respectively, using Quantikine (pro-IL-1β; R&D Systems) or Duoset ELISA development kits (IL-1α, 1β, 1RA; R&D Systems) and in accordance with manufacturer's instructions.

In another set of experiments, neutrophils were pretreated with DMSO-soluble inhibitors of p38 MAPK (SB203580; 1 and 10 μM), MEK1/2 (U0126; 2 and 20 μM), Akt (Triciribine; 1 and 5 μM), DMSO or PBS for 30 minutes prior to a 2-hour stimulation with PBS, LPS, Ang1 or Ang2. Final DMSO concentration in reaction volumes did not exceed 0.2%. Upon agonist stimulation, supernatants and lysates were collected and the concentrations of cytokines assessed by ELISA.

IL-1β maturation

IL-1β is synthesized in the cytoplasm as a 31-kDa precursor pro-protein (pro-IL-1β) that is cleaved to its mature 17-kDa form by IL-1β-converting enzyme (ICE; also known as caspase-1). Neutrophils (10^7 cells/ml; 1 ml) treated with PBS, LPS (1 μg/ml), Ang1 (10^{-10}–10^{-8} M) or Ang2 (10^{-10}–10^{-8} M) for up to 6 hours were assessed for caspase-1 activity using the Caspase-1 Fluorometric Assay (R&D Systems). Upon each incubation period, neutrophils were centrifuged at 900 g for 6 minutes and the supernatants were gently discarded. Cells were then lysed with 250 μl of lysis buffer (provided by the manufacturer) and protein concentrations were determined with the BCA protein assay. The enzymatic reaction for caspase-1 activity was carried out in a 96-well flat bottom microplate using 50 μl (150 μg) of total protein and the caspase-1 fluorogenic substrate WEHD-AFC. The plates were incubated at 37°C for 2 hours and read on a fluorescent microplate reader (excitation 400 nm, emission 505 nm).

In previous studies, Perregaux *et al.* reported that the potassium ionophore, nigericin is capable of inducing efficient cleavage and release of newly synthesized IL-1β from LPS-treated macrophages [27,28]. We tested this hypothesis in 4 sets of neutrophils (10^7 cells/ml; 1 ml), each set treated with PBS, LPS (1 μg/ml), Ang1 (10^{-8} M) or Ang2 (10^{-8} M), for 2 hours at 37°C and 5% CO_2 to induce maximal accumulation of pro-IL-1β. Upon the first incubation period, neutrophils were centrifuged at 900 g for 6 minutes. Lysates and supernatants were immediately collected from one set; for the remaining three sets, supernatants were carefully removed and replaced with RPMI containing vehicle (DMSO + ethanol), Cycloheximide (CHX; 10 μg/ml; + ethanol) to halt new protein synthesis, or CHX and the potassium-proton ionophore nigericin (N; 20 μM) for a further 45 minutes treatment (37°C and 5% CO_2). Upon this second incubation period, the three sets were centrifuged at 900 g for 6 minutes, and supernatants and lysates were collected and assayed for pro-IL-1β and IL-1β concentrations by ELISA, as previously described. CHX was dissolved in DMSO to a final DMSO concentration that did not exceed 0.1%. Nigericin was dissolved in ethanol to a final ethanol concentration that did not exceed 0.05%.

Statistical analyses

Results are presented as the mean ± SEM of independent experiments performed on neutrophils of at least three independent donors. Statistical comparisons were made by one-way analysis of variance (ANOVA) followed by a Dunnett or Tukey post-hoc test where applicable using GraphPad Prism (Mac version 5.0b). Differences were considered significant at p values less than 0.05.

Results

Expression of inflammatory cytokines and their receptors

We have recently reported that Ang1 (10^{-8} M) promotes the synthesis and release of the inflammatory cytokine IL-8 from neutrophils within 60 minutes and peaking within 2 hours of stimulation, whereas Ang2 has no such effect [12]. Extending these observations, we assessed the potential of angiopoietins to modulate the expression of 84 inflammatory cytokines in neutrophils. Neutrophils were treated with PBS (control vehicle), Ang1 or Ang2 (10^{-8} M; 90 minutes), and total mRNAs were extracted for RT-qPCR array analyses. For follow-up experiments, we selected genes with a nominal p-value <0.05 and a change in expression level $\geq$ 4-fold (Figure 1A and B).

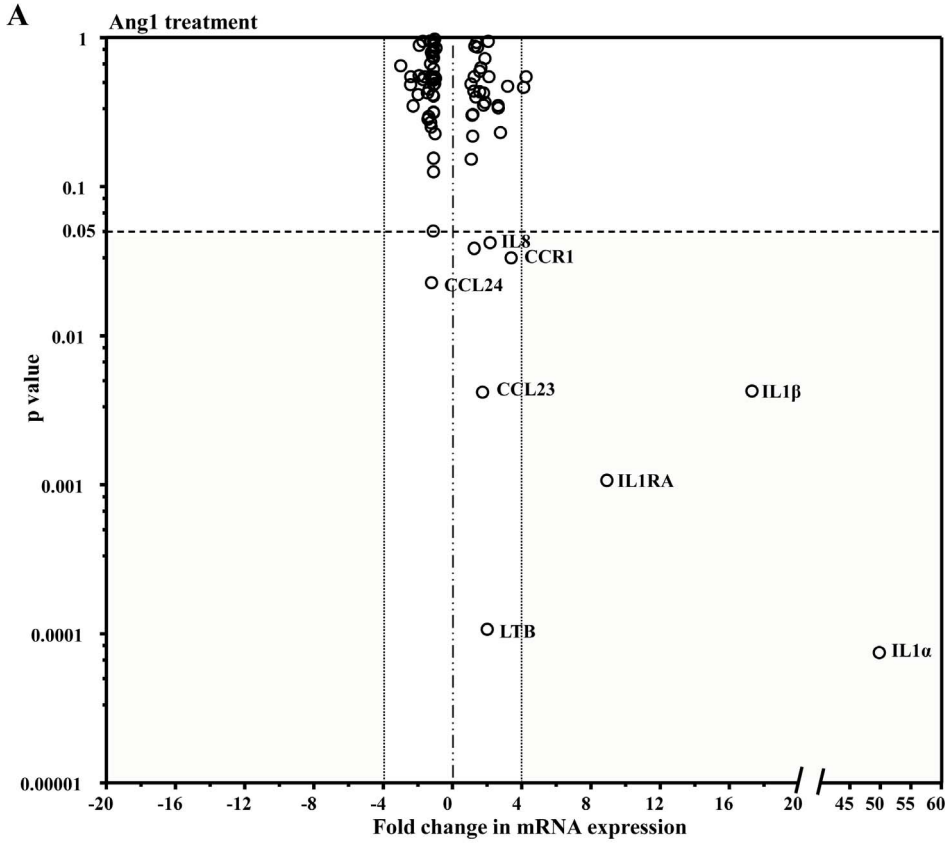
Based on the above criteria, most genes that were assayed did not fluctuate significantly following treatment with either angiopoietins (see Tables 1 and 2 for a list of all genes and their p-value). However, Ang1, but not Ang2, significantly upregulated the expression of 3 genes belonging to the IL-1 family (Figure 1A): IL-1α (49.65-fold increase; $p<0.001$), IL-1β (17.23-fold increase; $p<0.01$) and the endogenous antagonist IL-1RA (8.85-fold increase; $p<0.01$) as compared to PBS-treated cells. Neither other members of the IL-1 family nor the biologically active receptor IL-1R1 varied significantly under our experimental conditions (Table 1).

Under less stringent statistical parameters ($p\approx0.05$ and gene modulation $\geq$ 2-fold change), qPCR arrays identified three potentially interesting targets of Ang1 treatment: IL-8/CXCL8, Lymphotoxin Beta (LTB) and C-C chemokine receptor type 1 (CCR1) (Table 1 and Figure 1A). In parallel, Ang2 showed a tendency to up-regulate IL-8 receptor B (IL-8RB)/CXCR2 (Table 2 and Figure 1B). The significance of these potential targets will be covered briefly in the discussion.

Effect of angiopoietins on the mRNA expression of IL-1α, IL-1β and IL-1RA

Given the strong response of neutrophils in up-regulating IL-1 expression, and the importance of the latter family in initiating and modulating the inflammatory response, we sought to confirm and expand on the above using custom primers for IL-1α, IL-1β and IL-RA. Kinetics were performed by treating neutrophils with PBS, Ang1 (10^{-10}–10^{-8} M), Ang2 (10^{-10}–10^{-8} M) or LPS (1 μg/ml; positive control), for up to 6 hours before mRNA extraction. Given that lower concentrations of angiopoietins (10^{-10}–10^{-9} M) had no significant effect on mRNA expression compared to PBS-control, only the highest concentration of the angiopoietins (10^{-8} M) is represented in the graphs throughout the study. Additionally, because maximal Ang2 (10^{-8} M) had no significant effect compared to PBS-control, only Ang1 (10^{-8} M) is discussed below.

IL-1β mRNA was abundantly expressed in neutrophils, with a cycle threshold (Ct)$<$25 at the time of isolation (T_0), and basal


A
Ang1 treatment
p value
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IL8
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IL1β
IL1RA
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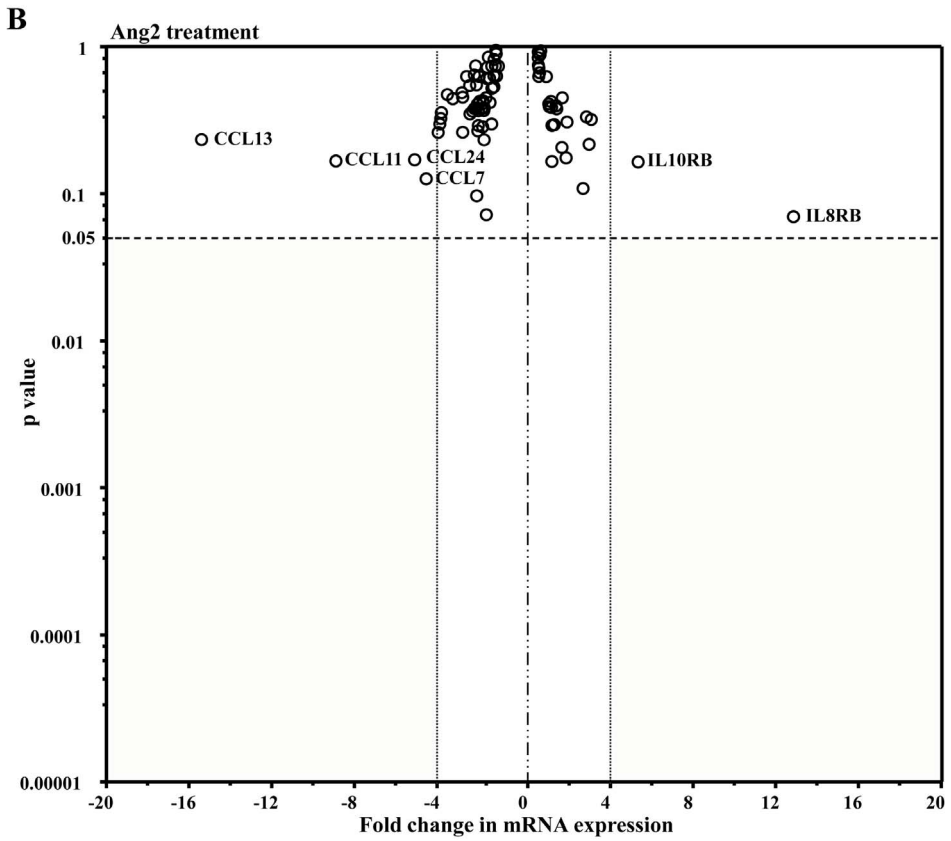
Fold change in mRNA expression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CCL13
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CCL24
CCL7
IL10RB
IL8RB
-20
-16
-12
-8
-4
0
4
8
12
16
20
Fold change in mRNA expression

Figure 1. Expression of inflammatory cytokines and their receptors in neutrophils. Circulating neutrophils isolated from 6 different donors were treated with PBS, Ang1 (10^{-8} M) (**A**) or Ang2 (10^{-8} M) (**B**) for 90 minutes prior to RNA isolation. Data are expressed in a Volcano plot, as fold change in gene expression (x-axis) compared to PBS-treated cells; values outside the dotted vertical lines indicate significant fold increases (positive values) or fold decreases (negative values). Values below the dashed horizontal line ($p<0.05$) underline statistical significance (y-axis). Each circle corresponds to the fold-expression of a single gene.
doi:10.1371/journal.pone.0088980.g001

(PBS) IL-1β mRNA did not significantly change over time. Treatment with Ang1 (10^{-8} M) induced a rapid increase in IL-1β mRNA expression within an hour of stimulation, with 32.6-fold increase compared to PBS-treated cells, after which expression progressively returned to basal values (Figure 2A). Levels of IL-1RA mRNA were also abundant in neutrophils, with a Ct<25 at T_0. Similarly to the potent positive control LPS, Ang1 promoted a significant increase in IL-1RA mRNA expression as early as 1 hour after stimulation, with 3.3-fold expression increase over PBS-control and reaching up to 9.8-fold at 6 hours (Figure 2B). Finally, regardless of treatment, IL-1α mRNA levels were hardly detectable in neutrophils (Table 3), with a Ct>42 at T_0. Subsequent basal and treated Ct values remained above 35 throughout the 6-hour time-period indicating that IL-1α mRNA is barely, if at all, expressed in neutrophils.

Kinetics of protein synthesis and release

Building on the mRNA kinetics studies, we assessed basal expression and *de novo* protein synthesis and release for all three IL-1 family members following angiopoietin treatment. Kinetics studies were extended to a 24-hour period; at each time point, using the same lysates and/or supernatants, the concentrations of IL-1α, IL-1β or IL-1RA protein were simultaneously evaluated by ELISA. For the same reasons as per the mRNA section, only the highest concentration of Ang1 (10^{-8} M) is discussed below.

Intracellular levels of IL-1β in neutrophils (10^7 cells/ml) were almost undetectable at T_0 (Figure 3A). Basal IL-1β protein levels in PBS-treated neutrophils fluctuated over time, starting with 2.1 pg/ml at 30 minutes, reaching a peak of 37.7 pg/ml at 6 hours and declining to 4.7 pg/ml at 24 hours. Ang1 (10^{-8} M) treatment lead to a steady increase in IL-1β synthesis throughout the first 6 hours of stimulation, going from 16.4 pg/ml at 1 hour, up to 68.1 pg/ml at 6 hours, and then stabilizing between 12 and 24 hours at a value below 22 pg/ml.

Several studies performed on macrophages and monocytes *in vitro* reported that LPS and other mediators are capable of promoting IL-1β protein synthesis but fail to induce IL-1β release [27–31]. Our current study shows that circulating human

Table 1. Expression change of inflammatory cytokines and their receptors following Ang1 treatment.

Genes	Fold	P	Genes	Fold	P	Genes	Fold	P	Genes	Fold	P
ABCF1	2.58	0.36	CCL4	1.12	0.31	CXCL14	−1.16	0.16	IL1F9	−1.18	0.05
BCL6	1.83	0.37	CCL5	−1.29	0.26	CXCL2	1.51	0.60	IL-1R1	2.04	0.56
C3	−1.26	0.56	CCL7	−1.78	0.53	CXCL3	1.51	0.44	IL-1RA	8.85	0.001
C4A	−1.04	0.54	CCL8	−1.16	0.13	CXCL5	−1.22	0.77	IL22	−1.32	0.68
C5	−1.16	0.32	CCR1	3.33	0.04	CXCL6	1.73	0.43	IL5	−1.16	0.75
CCL1	−1.16	0.41	CCR2	−1.47	0.29	CXCL9	−1.16	0.90	IL5RA	−1.34	0.97
CCL11	−3.05	0.66	CCR3	4.19	0.56	CARD18	−1.16	0.32	IL8	2.11	0.04
CCL13	−2.47	0.56	CCR4	−1.16	0.54	IFNA2	1.11	0.22	IL8RA	1.23	0.89
CCL15	−1.16	0.56	CCR5	1.02	0.16	IL10	−1.30	0.56	IL8RB	2.72	0.23
CCL16	−1.16	0.84	CCR6	−1.28	0.81	IL10RA	1.19	0.56	IL9	−1.30	0.96
CCL17	−1.09	0.50	CCR7	1.01	0.50	IL10RB	4.05	0.56	IL9R	−1.19	0.42
CCL18	−1.16	0.82	CCR8	−1.43	0.30	IL13	−2.05	0.42	LTA	−1.42	0.46
CCL19	1.73	0.36	CCR9	−1.16	0.49	IL13RA1	1.29	0.40	LTB	1.95	0.0001
CCL2	2.59	0.34	CEBPB	1.07	0.31	IL17C	−2.33	0.35	LTB4R	1.17	0.44
CCL20	3.13	0.48	CRP	−1.32	0.27	IL-1α	49.65	0.000008	MIF	−1.07	0.23
CCL21	−2.48	0.49	CX3CR1	1.33	0.94	IL-1β	17.24	0.004	SCYE1	−1.01	0.87
CCL23	1.67	0.00	CXCL1	2.01	0.96	IL1F10	−1.16	0.96	SPP1	1.39	0.88
CCL24	−1.27	0.02	CXCL10	−1.09	0.99	IL1F5	−1.14	0.96	TNF	1.19	0.04
CCL25	−1.17	0.56	CXCL11	−1.16	0.53	IL1F6	−1.16	0.96	CD40LG	−1.16	0.63
CCL26	−1.99	0.57	CXCL12	−1.98	0.90	IL1F7	1.58	0.64	TOLLIP	1.82	0.74
CCL3	−1.10	0.56	CXCL13	−1.67	0.56	IL1F8	−1.76	0.96	XCR1	−1.50	0.43
B2M*	1.24	0.56	HPRT1*	1.07	0.47	RPL13A*	−1.34	0.40	GAPDH*	1.01	0.04

Human neutrophils from at least 3 different individuals were treated with PBS, Ang1 or Ang2 (Table 2) at 10^{-8} M for 90 minutes. RT-qPCR array analyses were performed to assess expression change of 84 genes involved in the inflammatory response. Each gene from angiopoietin-treated neutrophils was compared to PBS-treated neutrophils and the data expressed as fold change. Negative and positive values denote a decrease and increase in mRNA expression, respectively. Differences were considered significant at Fold ≥ 4 and $p<0.05$. Housekeeping genes are denoted by an asterisk (*). Members of the IL-1 family that satisfied both requirements were considered significantly upregulated by Ang1, and are shaded in grey.
doi:10.1371/journal.pone.0088980.t001

Table 2. Expression change of inflammatory cytokines and their receptors following Ang2 treatment.

Genes	Fold	P	Genes	Fold	P	Genes	Fold	P	Genes	Fold	P
ABCF1	1.48	0.39	CCL4	2.19	0.21	CXCL14	−1.63	0.44	IL1F9	−1.45	0.73
BCL6	1.23	0.64	CCL5	1.04	0.76	CXCL2	3.39	0.34	IL-1R1	1.10	0.68
C3	1.73	0.39	CCL7	−4.43	0.13	CXCL3	1.09	0.91	IL-1RA	1.16	0.95
C4A	−1.09	0.75	CCL8	−1.34	0.62	CXCL5	−3.15	0.34	IL22	−3.17	0.27
C5	−1.16	0.64	CCR1	−1.13	0.64	CXCL6	1.71	0.29	IL5	−1.54	0.40
CCL1	−1.56	0.45	CCR2	1.65	0.43	CXCL9	1.51	0.45	IL5RA	−2.30	0.55
CCL11	−8.00	0.17	CCR3	3.52	0.22	CARD18	−1.47	0.62	IL8	3.21	0.11
CCL13	−12.29	0.24	CCR4	−1.02	0.91	IFNA2	1.06	0.64	IL8RA	2.21	0.46
CCL15	−1.56	0.45	CCR5	−1.31	0.42	IL10	−2.64	0.26	IL8RB	13.48	0.07
CCL16	−1.68	0.45	CCR6	−1.05	0.75	IL10RA	1.12	0.90	IL9	−1.68	0.29
CCL17	−1.24	0.30	CCR7	2.39	0.18	IL10RB	5.03	0.17	IL9R	−1.96	0.12
CCL18	−1.79	0.44	CCR8	−1.80	0.43	IL13	−3.76	0.30	LTA	−1.90	0.27
CCL19	−1.40	0.86	CCR9	−1.22	0.53	IL13RA1	1.70	0.17	LTB	−1.74	0.39
CCL2	−1.99	0.38	CEBPB	−1.51	0.45	IL17C	−2.46	0.64	LTB4R	1.82	0.30
CCL20	1.91	0.40	CRP	−1.91	0.67	IL-1α	3.63	0.32	MIF	−2.01	0.75
CCL21	−2.29	0.35	CX3CR1	−1.23	0.75	IL-1β	2.44	0.31	SCYE1	1.07	0.73
CCL23	1.03	0.94	CXCL1	−1.84	0.64	IL1F10	−1.83	0.44	SPP1	1.55	0.41
CCL24	−4.98	0.17	CXCL10	−1.03	0.96	IL1F5	−1.61	0.23	TNF	−2.69	0.49
CCL25	−1.12	0.83	CXCL11	−1.51	0.44	IL1F6	−1.83	0.44	CD40LG	−1.02	0.60
CCL26	−3.40	0.48	CXCL12	−3.68	0.36	IL1F7	−1.97	0.56	TOLLIP	1.57	0.39
CCL3	1.03	0.86	CXCL13	−3.13	0.45	IL1F8	−1.89	0.29	XCR1	−2.64	0.46
B2M*	1.05	0.74	HPRT1*	−1.22	0.94	RPL13A*	−1.34	0.93	GAPDH*	1.56	0.21

As per Table 1, each gene from Ang2-treated neutrophils was compared to its counterpart from PBS-treated neutrophils and the data expressed as fold-change. Although genes such as CCL7, CCL11, CCL13, CCL24 and IL10RB showed substantial fold-change differences between Ang2 and PBS, statistical significance denoted by the p-value was far from 0.05. Because of its fold-regulation and a p-value close to 0.05, IL-8RB is a promising target. Housekeeping genes are denoted by an asterisk (*).
doi:10.1371/journal.pone.0088980.t002

neutrophils behave in much of the same manner; indeed, under all conditions tested, no IL-1β was detected in the supernatants (Table 3), suggesting that the decreases in intracellular IL-1β levels over time were not due to its release.

Unlike IL-1β, intracellular levels of IL-1RA were substantial at T_0, with detection at 11 350 pg/ml (Figure 3B). We observed a short drop in intracellular IL-1RA in the first 30 minutes of stimulation, with levels fluctuating between 7 130–7 800 pg/ml regardless of treatment. For the remainder of the time-course, variations in intracellular IL-1RA levels were not statistically significant between treatments (Figure 3B), averaging between 7500 pg/ml to up to 16 400 pg/ml. In another stark contrast to IL-1β, we observed that neutrophils constitutively release IL-1RA: At T_0, we detected 629.1 pg/ml of extracellular IL-1RA in the supernatants (Figure 3C). Detection of extracellular IL-1RA under basal conditions continued throughout the entire time-course and corresponded to about 2–10% of total IL-1RA cellular content. Similarly to LPS, Ang1 promoted a statistically significant increase in IL-1RA release as early as 2 hours following stimulation, with 844.6 pg/ml IL-1RA released (vs. 468.2 pg/ml for PBS), after which detection values climbed to a peak of 1 379.3 pg/ml at 4 hours (vs. 498.8 pg/ml for PBS) (Figure 3C). Finally, IL-1α protein was not detected in neutrophil cell lysates or in their corresponding supernatants at T_0 or throughout the time-course under basal conditions or angiopoietin stimulation (Table 3).

Is IL-1β a product of de novo synthesis or maturation from pre-existing pools of precursor?

IL-1β is synthesized in the cytoplasm as an inactive 31-kDa-precursor protein (pro-IL-1β) before being cleaved to its mature 17-kDa form [32]. Thus, we looked at the modulation of precursor pro-IL-1β levels in human neutrophils, and performed an initial assessment of the possible mechanisms governing pro-IL-1β cleavage. For the same reasons as per the mature protein, only the highest concentration of Ang1 (10^{-8} M) is discussed below.

De novo synthesis. As per the mature protein, intracellular levels of pro-IL-1β in human neutrophils were almost undetectable at T_0 (Figure 4). We observed an increase in pro-IL-1β levels under basal (PBS) conditions, reaching as much as 35.1 pg/ml at 2 hours, but subsequently declining to less than 10.0 pg/ml at 24 hours. Treatment with Ang1 (10^{-8} M) promoted a substantial increase in pro-IL-1β synthesis starting at 1 hour, with detection reaching a peak of 147.6 pg/ml at 2 hours, and then declining to 16.0 pg/ml at 24 hours, compared to PBS. Irrespective of the treatment, we did not detect pro-IL-1β proteins in the supernatant of neutrophils (Table 3), consistent with reports that pro-IL-1β is not released from cells [29,30,32-34].

Maturation of pro-IL-1β has been attributed primarily to the activation of caspase-1; however, independent studies reported that the processing of IL-1β might actually occur via a caspase-1-independent mechanism, through enzymes such as serine proteases Cathepsin G, Neutrophil Elastase and Proteinase-3 [35–38]. Using a fluorometric method, we assessed the activity of caspase-1 in human neutrophils treated with PBS, angiopoietins and LPS

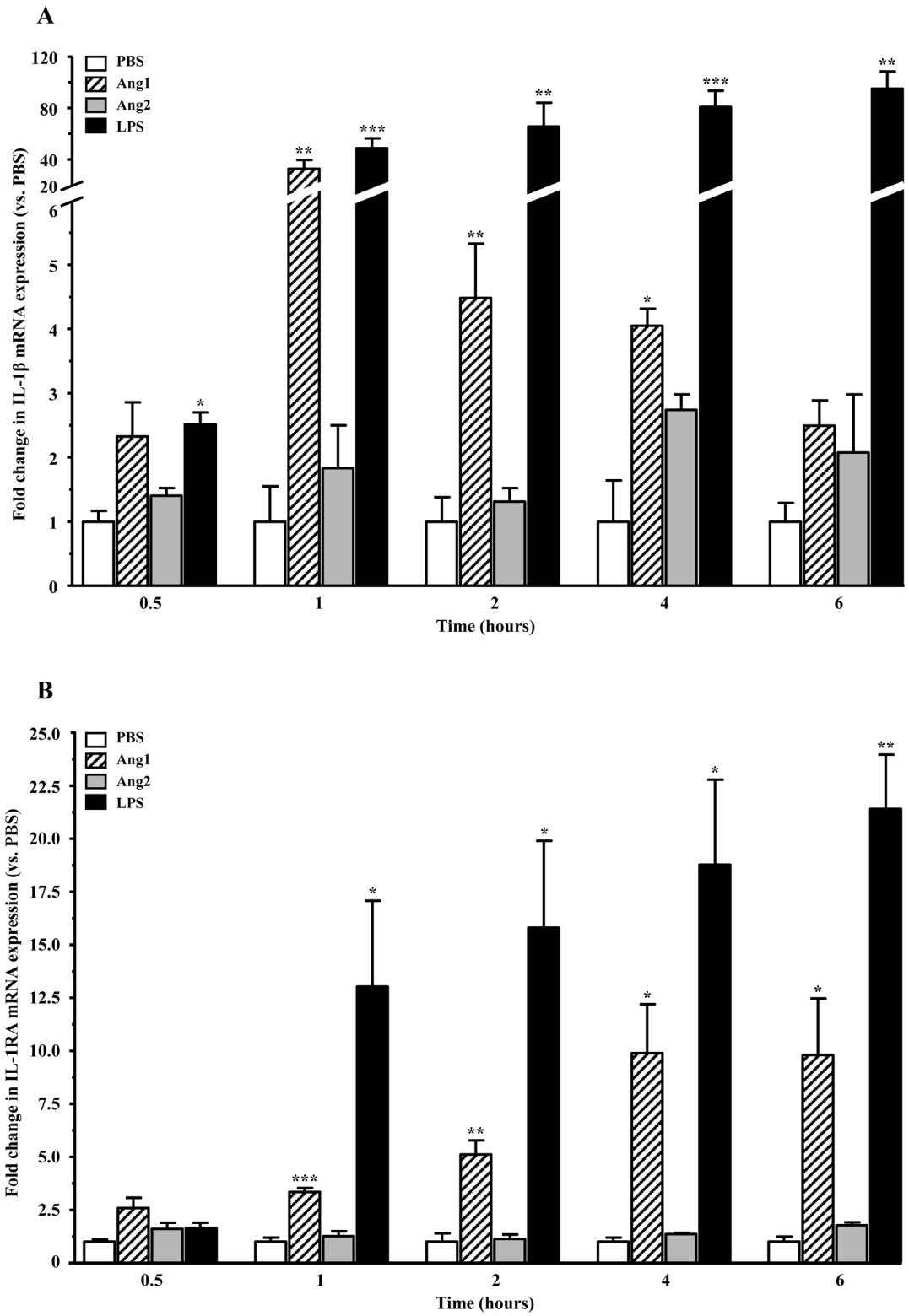


Figure 2. Kinetics of mRNA expression of IL-1β and IL-1RA. Primers were designed to quantify changes in the mRNA levels of IL-1β (**A**) and IL-1RA (**B**), following treatment with PBS, angiopoietins (10^{-8} M) or LPS (1 μg/ml), for up to 6 hours. For each time point, basal (PBS) mRNA expression is set to unitary value, and the data are presented as fold change compared to its corresponding PBS. For angiopoietins, only values resulting from treatment with the highest concentration (10^{-8} M) are shown. Data are represented as the means ± SEM of at least three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. PBS-control (Dunnett's test).
doi:10.1371/journal.pone.0088980.g002

Table 3. IL-1 mRNA and protein expression in freshly isolated and Ang1-stimulated neutrophils.

Genes	mRNA	Protein		
		T_0 Intracellular (S)		Extracellular (S)
IL-1α	N.D.	N.D	N.D	N.D
IL-1β	+	N.D	+	N.D
Pro-IL-1β	+	N.D	+	N.D
IL-1RA	+	+	+	+

T_0 represents content at the time of isolation and reflects the state of circulating human neutrophils in healthy individuals; intracellular and extracellular content are assessed after stimulation with Angiopoietin-1 (Ang1); S: stimulated; N.D.: not detectable. Unstimulated neutrophils do not express or store pools of IL-1α mRNA, and that is not altered by the addition of Ang1. Neutrophils hold large pools of IL-1β mRNA, but Ang1 signal is required for translation. Finally, neutrophils constitutively express and release IL-1RA.
doi:10.1371/journal.pone.0088980.t003

(1 μg/ml) for up to 6 hours. We did not detect any basal caspase-1 activity beyond threshold, and little to no changes following agonist stimulation (data not shown).

Induction of pro-IL-1β maturation and IL-1β release

Studies have reported that the mechanisms leading to the maturation and effective release of IL-1β depend on the subset of leukocytes being investigated. While monocytes readily release IL-1β under LPS treatment [39,40], macrophages require a depletion of intracellular potassium induced by ionophores such as nigericin before efficient IL-1β maturation and subsequent release [27,28]. Because the mechanisms governing IL-1β maturation and release have never been reported in neutrophils, and given that even LPS failed at promoting IL-1β release, we tested the requirement for a secondary stimulus to drive neutrophil processing of pro-IL-1β and release of the mature protein.

Neutrophils were divided into four sets (Figure 5; Sets 1-4) and were treated with agonists for 2 hours, a time when most of the new pro-IL-1β has already accumulated under Ang1 (refer to Figure 4). Upon this first incubation period, supernatants and cell lysates from Set 1 were collected. For Sets 2-4, supernatants were replaced with new media containing vehicles (DMSO + ethanol) or nigericin (N; in ethanol) for an additional 45 minutes as described in **Materials and Methods**. In order to eliminate the contribution of *de novo* synthesis to possible changes in levels of mature IL-1β (i.e. to confirm that any new IL-1β is a result of processing of the accumulated pro-IL-1β), a protein translation inhibitor, cycloheximide (CHX; in DMSO) was added during this step. To preserve sample comparability, supernatants from Sets 2-4 were supplemented with either DMSO or ethanol, as required.

Data from Sets 1 (Figure 5A and B) were used to establish the baseline of protein kinetics, and were comparable to what we had observed in our previous experiments for all the conditions tested. For pro-IL-1β (Figure 5A), neither the addition of vehicles (Set 2, left panel) nor CHX alone (Set 3, left panel) affected the synthesis of pro-IL-1β. Upon addition of nigericin (Set 4, left panel), we observed a near complete loss of detection of intracellular pro-IL-1β, in comparison to Set 3. This loss was not due to the release of pro-IL-1β, since the pro-protein in its native form was not detected in any of the corresponding extracellular fractions (Figure 5A, right panel).

Based on these observations, we hypothesized that nigericin may have indeed induced the processing of pro-IL-1β into IL-1β, as was reported to happen in macrophages [27,28]. However, the concomitant evaluation of IL-1β levels indicated that this was not the case (Figure 5B): While the addition of nigericin (Set 4, left panel) almost completely depleted intracellular IL-1β content compared to neutrophils from Set 3, most of the IL-1β was recovered in the extracellular fraction of nigericin-treated neutrophils (Set 4, right panel). In fact, the amount of IL-1β recovered extracellularly from nigericin-treated neutrophils (Set 4, right panel) nearly matched what had accumulated inside the cells prior to nigericin treatment (Set 3, left panel). Thus, potassium depletion did not promote maturation of pro-IL-1β into IL-1β in human neutrophils, but only the selective exteriorization of IL-1β.

Intracellular mechanisms of IL-1 family synthesis and release

Previous studies reported that the biological activities of angiopoietins can be mediated by PI-3K/Akt, p38 MAPK, and p42/44 MAPK pathways as a function of the cellular activities being solicited [11,14,41–43]. Thus, we wanted to delineate the signaling pathway(s) involved in mediating the effects of the angiopoietins on synthesis and/or secretion of IL-1 family members in human neutrophils. Neutrophils were pretreated with inhibitors of p42/44 MAPK Kinase - MEK1/2 - (U0126; (U0)), p38 MAPK (SB203580; (SB)) or Akt (Triciribine; (T)) for 30 minutes prior to agonist challenge, as previously described [12,44–46]. Inhibitor-pretreated neutrophils were then compared to their vehicle (DMSO; D) counterparts.

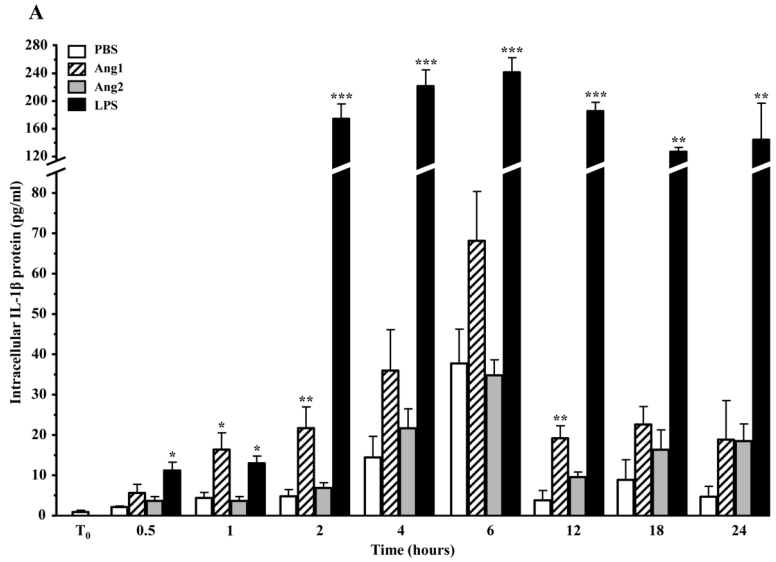
mRNA changes. Because most of the inducible IL-1 mRNA was synthesized within the first hour of Ang1 treatment, we looked at the effects of the inhibitors on mRNA expression after 1 hour of agonist stimulation. Ang2 (10^{-10}–10^{-8} M) and lower concentrations of Ang1 (10^{-10}–10^{-9} M) yielded similar results as PBS-control under all conditions tested; thus, only the highest concentration of Ang1 (10^{-8} M) is discussed below.

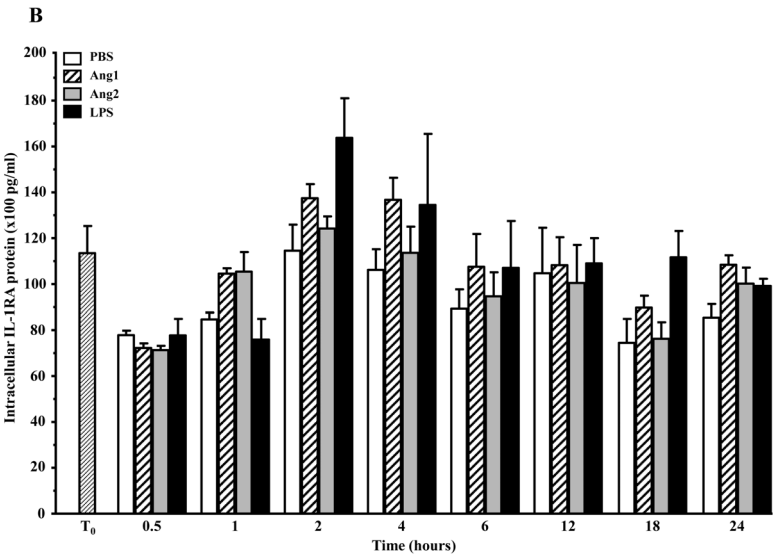
Basal levels of IL-1β mRNA were not affected by either pretreatment with DMSO (vehicle) or with inhibitors (data not shown). Addition of the p38 MAPK inhibitor (SB) significantly increased the effect of Ang1 (10^{-8} M) on IL-1β mRNA expression, from 22.4- (Ang1-D) to 44.8-fold (Ang1-SB) expression (Figure 6A). MEK1/2 inhibition (U0) had the opposing effect, leading to a decrease from 22.4- to 7.8-fold (Ang1-U0) expression, corresponding to a 68% inhibition of Ang1 activity. The Akt inhibitor (T) had no significant effect on the activities of Ang1. Interestingly, none of the inhibitors significantly impacted the effects of LPS on IL-1β mRNA expression (Figure 6A).

As for IL-1RA mRNA expression, only MEK1/2 blockade provided a trend (not significantly) to decrease Ang1 effect (Figure 6B). On the other hand, the blockade of p38 MAPK activity significantly reduced the effect of LPS, from 5.9- (LPS-D) to 2.8-fold (LPS-SB) expression, corresponding to a 63% inhibition. The blockade of MEK1/2 or Akt pathways had no significant effects on the activities of LPS (Figure 6B).

Protein changes. The immediate impact of the aforementioned mRNA changes on the corresponding protein levels was assessed at 2 hours of agonist stimulation, coinciding with the time at which protein synthesis rate was also at its maximum. For the same reasons as per the mRNA experiments, only the highest concentration of Ang1 (10^{-8} M) is discussed below.

Basal (PBS) protein levels were not affected by the addition of DMSO or any of the inhibitors (Figure 7A–D). While p38 MAPK inhibition significantly increased Ang1-induced pro-IL-1β synthesis by 79%, from 38.5 pg/ml (Ang1-D) to 64.3 pg/ml (Ang1-SB), blockade of MEK1/2 lead to a 60% inhibition, with pro-IL-1β protein levels decreasing from 38.5 pg/ml (Ang1-D) to 19.2 pg/ml (Ang1-U0) (Figure 7A). As for LPS, blockade of p38 MAPK lead


A
PBS
Ang1
Ang2
LPS
Intracellular IL-1β protein (pg/ml)
Time (hours)


B
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Ang1
Ang2
LPS
Intracellular IL-1RA protein (x100 pg/ml)
Time (hours)

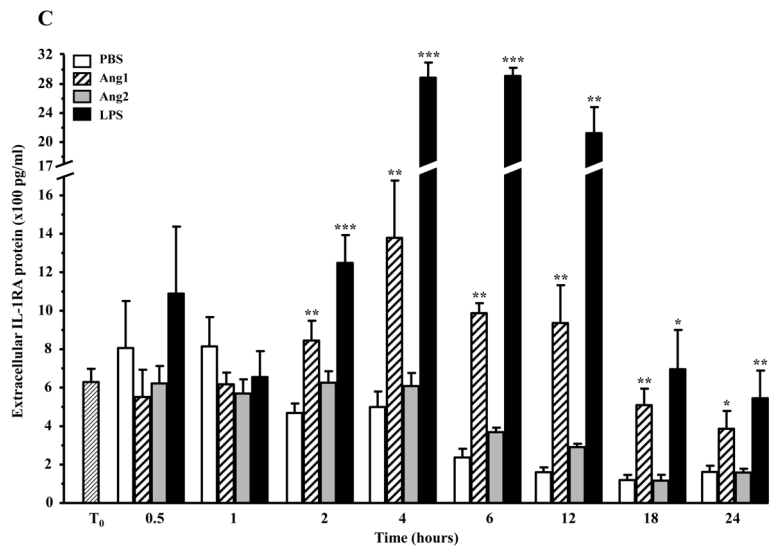
C
PBS
Ang1
Ang2
LPS
Extracellular IL-1RA protein (x100 pg/ml)
Time (hours)

Figure 3. Kinetics of IL-1β and IL-1RA protein expression. Neutrophils were treated with PBS, angiopoietins (10^{-8} M) or LPS (1 μg/ml), for up to 24 hours. Intracellular IL-1β (**A**), IL-1RA (**B**) and extracellular IL-1RA (**C**) were quantified by ELISA. For angiopoietins, only values resulting from treatment with the highest concentration (10^{-8} M) are shown. No IL-1β was detected extracellularly. Data are represented as the means ± SEM of at least three independent experiments. *p<0.05, **p<0.01, *** p<0.001 vs. PBS-control (Dunnett's test).
doi:10.1371/journal.pone.0088980.g003

to a marked inhibition by 80% of pro-IL-1β protein expression, as levels dropped from 262.1 pg/ml (LPS-D) to 55.9 pg/ml (LPS-SB), despite similar treatment having no effect at the mRNA level. Blockade of MEK1/2 or Akt had no effect on LPS-driven pro-IL-1β levels (Figure 7A).

The inhibition pattern for mature IL-1β mimicked that of pro-IL-1β for both Ang1 and LPS (Figure 7B). Blockade of p38 MAPK increased Ang1-driven IL-1β protein levels by 86%, as levels jumped from 17.3 pg/ml (Ang1-D) to 29.9 pg/ml (Ang1-SB). Blockade of MEK1/2 lead to a 68% inhibition, as IL-1β levels decreased from 17.3 pg/ml (Ang1-D) to 7.1 pg/ml (Ang1-SB). In the case of LPS, as per the precursor protein, mature IL-1β levels were deeply affected by the blockade of p38 MAPK, witnessing a 77% effect inhibition as levels dropped from 219.2 pg/ml (LPS-D) to 52.0 pg/ml (LPS-SB). Surprisingly, the blockade of MEK1/2 activity had a partial but significant effect on IL-1β protein, corresponding to 24% inhibition, as levels went from 219.2 pg/ml (LPS-D) to 166.9 pg/ml (LPS-U0). For both Ang1 and LPS, the Akt pathway did not modulate IL-1β levels significantly. It should be noted that no pro- or mature IL-1β proteins were detected in the extracellular fraction, regardless of treatment (data not shown).

Intracellular IL-1RA protein levels were maintained between 13–18 ng/ml across treatments, with only a very slight increase and decrease in Ang1 and LPS-driven levels respectively, following p38 MAPK inhibition (Figure 7C). The lack of effect on the intracellular stores of IL-1RA protein following p38 MAPK blockade is noteworthy in the case of LPS, especially given the 63% reduction in the corresponding mRNA; these data demonstrate that the cell holds IL-1RA mRNA in large excess, and actually utilizes less than 40% of the total mRNA quantity it produces to convert into protein. However, the impact of inhibitors was immediately noticeable at the level of the release of IL-1RA (Figure 7D), suggesting that the neutrophil prioritizes of a having a constant pool of intracellular IL-1RA and will modify the amount released in response to different conditions. First, under Ang1, the dynamics of p38 MAPK-MEK1/2 mediation differed from those of IL-1β, in that only the blockade of MEK1/2 had a significant impact, equivalent to 65% inhibition, on extracellular IL-1RA levels, as levels dropped from 963.4 pg/ml (Ang1-D) to 542.5 pg/ml (Ang1-U0). Meanwhile, the blockade of p38 MAPK had no important impact on Ang1-mediated IL-1RA release (Ang1-SB), in line with the apparent lack in p38 MAPK contribution at the mRNA and the intracellular protein levels. For LPS, both the blockade of p38 MAPK and MEK1/2 exerted a negative effect on IL-1RA release: inhibition of p38 MAPK lead to a marked 75% inhibition, with levels dropping from 1 471.9 pg/ml (LPS-D) to 749.3 pg/ml (LPS-SB). Furthermore, blockade of MEK1/2 resulted in a marked 41% inhibition, with levels decreasing to 952.4 pg/ml (LPS-U0). Finally, for both Ang1 and LPS, the Akt pathway did not modulate IL-1RA release significantly.

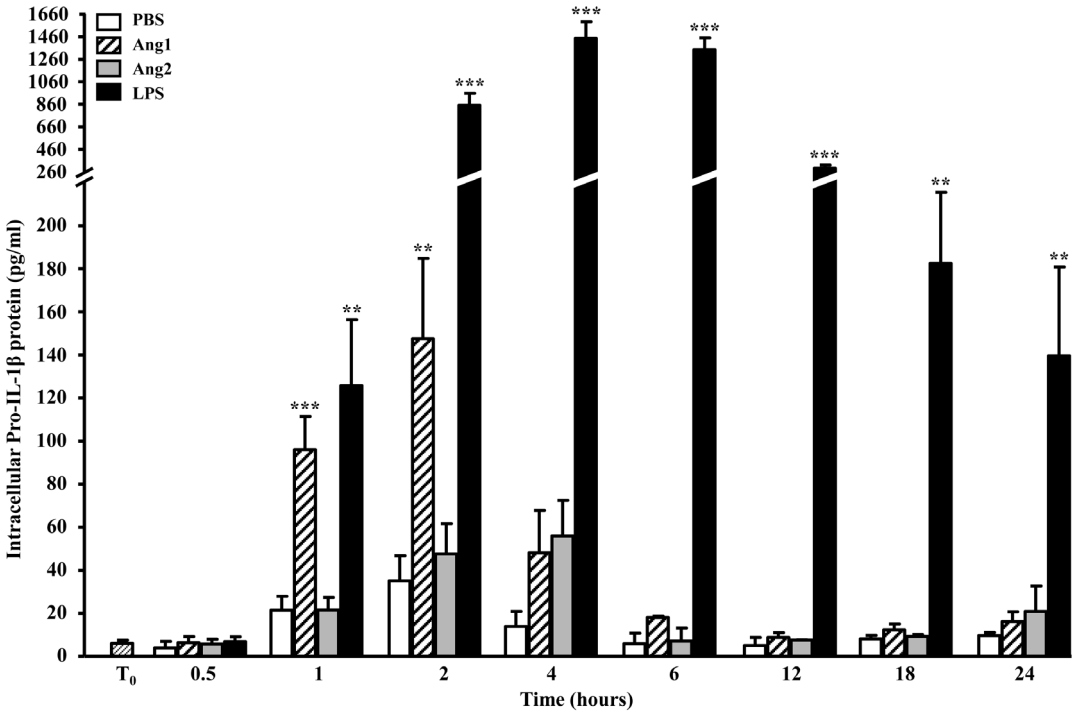

Figure 4. Kinetics of pro-IL-1β protein expression. Neutrophils were treated with PBS, angiopoietins (10^{-8} M) or LPS (1 μg/ml), for up to 24 hours. Only intracellular levels of pro-IL-1β were detectable, as no pro-IL-1β was detected in the supernatants at any time points and under any of the conditions tested. For angiopoietins, only values resulting from treatment with the highest concentration (10^{-8} M) are shown. Data are represented as the means ± SEM of at least three independent experiments. *p<0.05, **p<0.01, *** p<0.001 vs. PBS-control (Dunnett's test).
doi:10.1371/journal.pone.0088980.g004

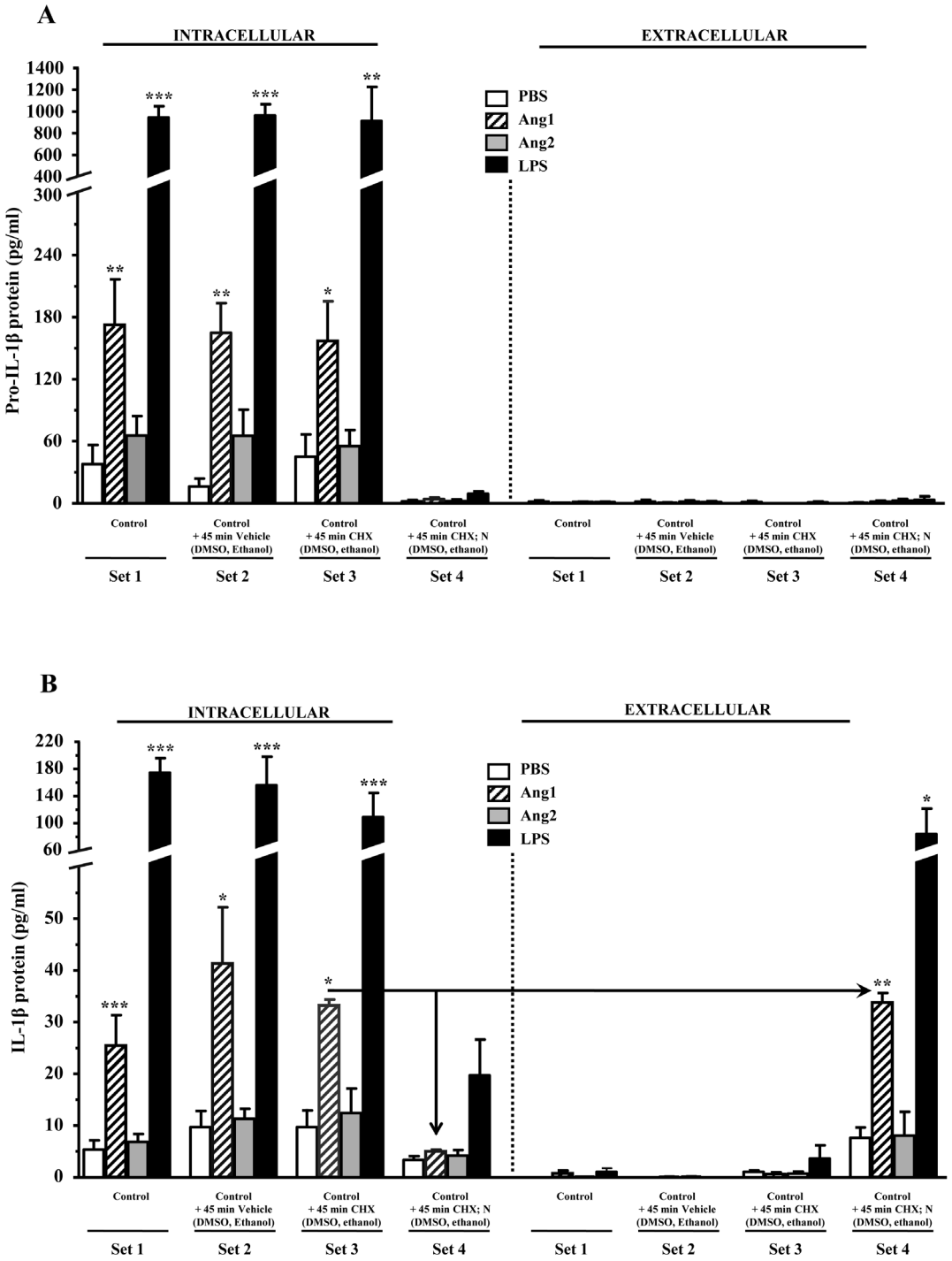

Figure 5. Effect of potassium depletion on IL-1β release. Neutrophils were treated with PBS, angiopoietins (10^{-8} M) or LPS (1 μg/ml), for two hours, followed by an additional 45-minute treatment with potassium ionophore nigericin, CHX, or appropriate vehicles. Changes in intracellular (left panels) and extracellular (right panels) levels of pro-IL-1β (**A**) and IL-1β (**B**) before and after ionophore addition were quantified by ELISA. CHX: Cycloheximide. N: Nigericin. Vehicles: DMSO, ethanol. Data are represented as the means ± SEM of at least three independent experiments. *$p<0.05$, **$p<0.01$, *** $p<0.001$ vs. PBS-control (Dunnett's test).
doi:10.1371/journal.pone.0088980.g005

Discussion

Vessel destabilization, increase in permeability and leukocyte infiltration are hallmarks of both inflammation and angiogenesis. Under normal physiological conditions, these processes undergo a natural resolution or removal of inciting signals, a critical step in preventing disorganized vascular network formation and a sustained inflammatory reaction. During the resolution step, changes in the microenvironment through local mediators produced lead to an active "push-back" of infiltrating neutrophils, and serve to limit the activity of destabilizers such as VEGF, nitric

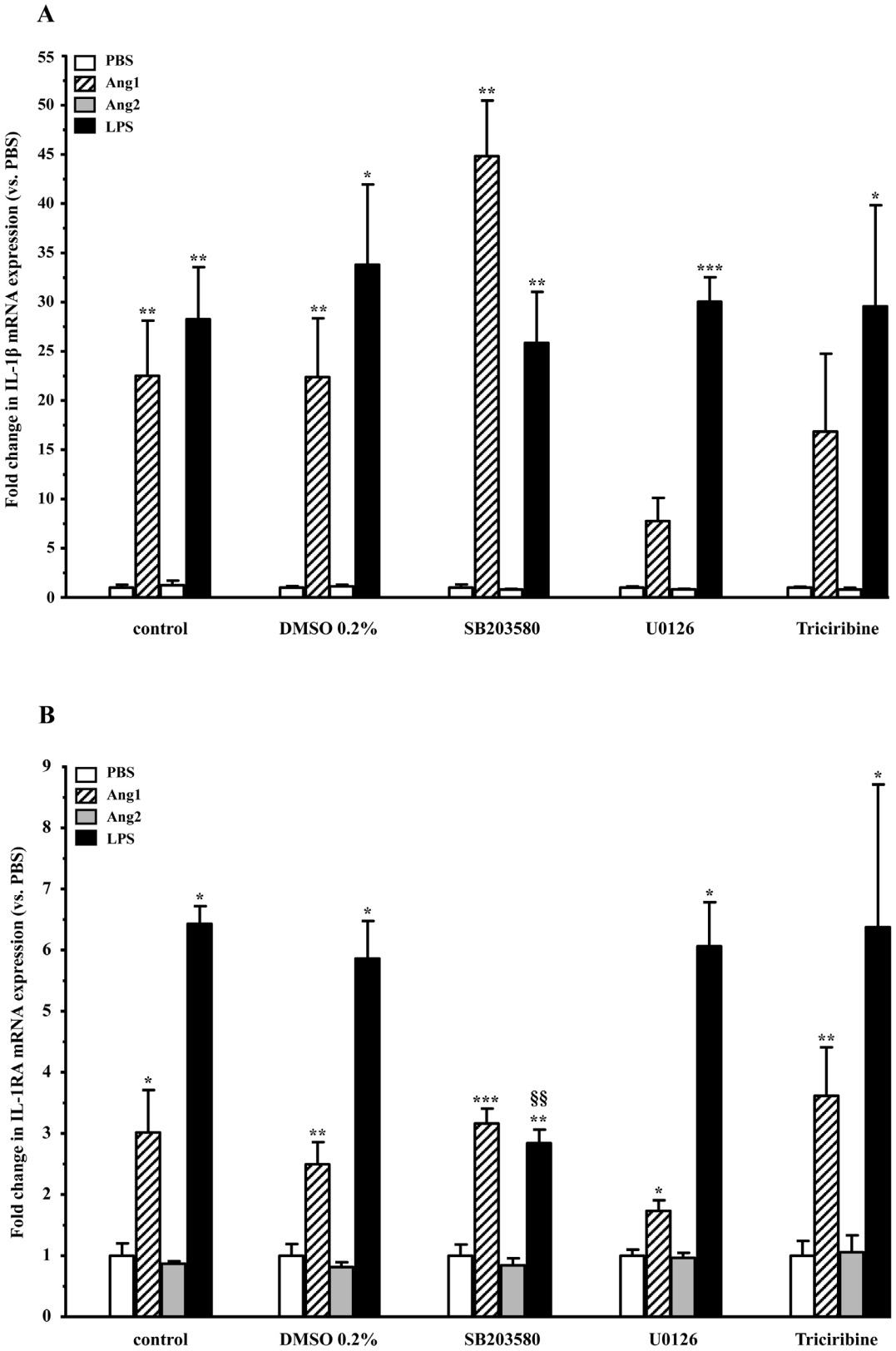

Figure 6. Effect of downstream signaling inhibitors on IL-1β and IL-1RA mRNA expression. Neutrophils were pretreated with inhibitors of Akt (Triciribine; 5 μM), p38 MAPK (SB203580; 10 μM), and p42/44 MAPKK (U0126; 20 μM), vehicle-DMSO (0.2%) or PBS for 30 minutes prior to a 1-hour agonist challenge. Total mRNA was used in RT-qPCR for assessment of mRNA expression of IL-1β (**A**) and IL-1RA (**B**). Data are presented as mean ± SEM of at least three independent experiments. *$p<0.05$, **$p<0.01$, *** $p<0.001$ vs. PBS-control within each set (Dunnett's test); §$p<0.05$, §§$p<0.01$ vs corresponding agonist-DMSO (Tukey's test).
doi:10.1371/journal.pone.0088980.g006

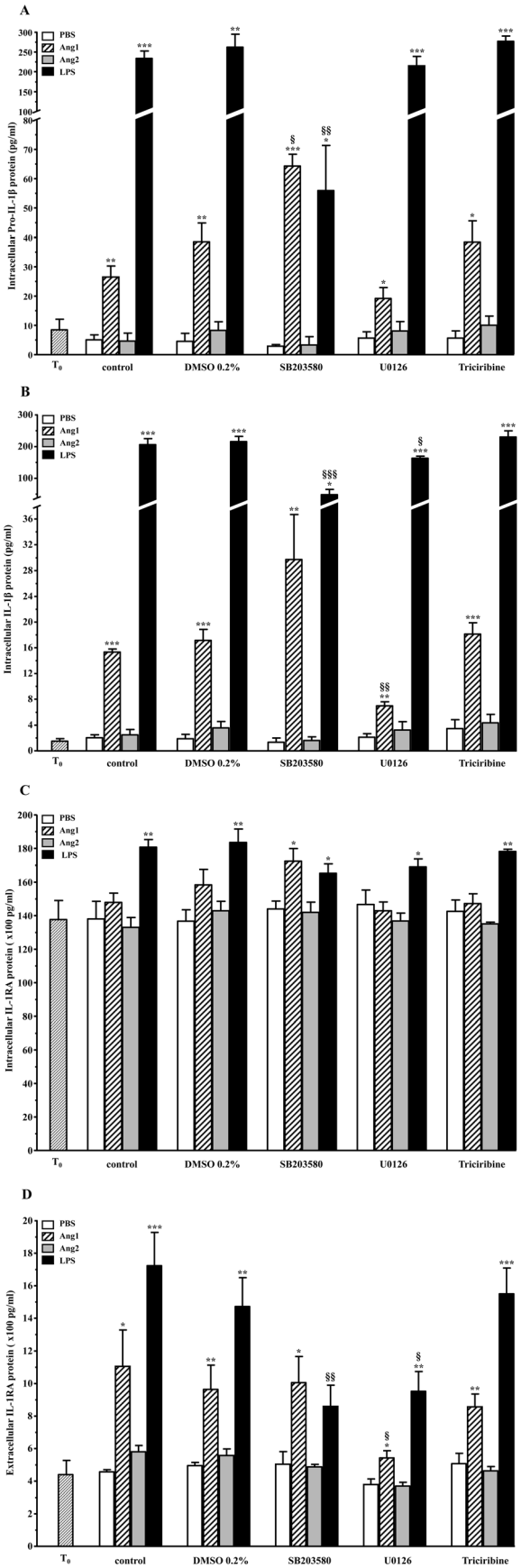

Figure 7. Effect of downstream signaling inhibitors on IL-1β and IL-1RA protein synthesis and release. Neutrophils were pretreated with inhibitors of Akt (Triciribine; 5 μM), p38 MAPK (SB203580; 10 μM), and p42/44 MAPKK (U0126; 20 μM), vehicle-DMSO (0.2%) or PBS for 30 minutes prior to a 2-hour agonist challenge. Concentrations of intracellular pro-IL-1β (**A**), IL-1β (**B**), IL-1RA (**C**) and released IL-1RA (**D**) were quantified by ELISA. Data are represented as mean ± SEM of at least three independent experiments. *$p<0.05$, **$p<0.01$, *** $p<0.001$ vs. PBS-control within each set (Dunnett's test); §$p<0.05$, §§$p<0.01$ and §§§$p<0.001$ vs corresponding agonist-DMSO (Tukey's test).
doi:10.1371/journal.pone.0088980.g007

oxide (NO) and Ang2 while increasing stabilizing elements such as Ang1 (resolution reviewed in [47];[48]). A previous study suggested that neutrophils might actually contribute to the resolution of inflammation based on their ability to produce endogenous anti-inflammatory mediators but little pro-inflammatory cytokines [23]. The present study supports those findings, as we show that during a 24-hour lifespan, neutrophils constitutively release endogenous anti-inflammatory mediator IL-1RA from a pool of stored protein that is continuously replenished, but no IL-1 agonists are produced or secreted. While we observed that Ang1 and LPS "prime" neutrophils to synthesize IL-1β *de novo*, in the absence of other signals, both precursor and mature IL-1β stores are retained within the intracellular compartment and are degraded over time. Additionally, the intracellular spikes in IL-1β levels were accompanied by parallel increases in the release of IL-1RA. Thus, we propose that neutrophils from healthy individuals naturally and intrinsically curtail the activity of IL-1 agonists, and "put the brakes" on the propagation of IL-1 mediated inflammation.

Of the 11 members of the IL-1 family of ligands, IL-1α and IL-1β are two major agonists with a demonstrated role in inflammation, angiogenesis, and hematopoiesis [49]. Both agonists bind to and activate IL-1 Receptor Type 1 (IL-1R1), and their activity is competitively antagonized by the endogenous IL-1RA. IL-1α and IL-1β are synthesized as precursor proteins; however, while IL-1α is active in both the precursor and the mature form upon release, IL-1β requires cleavage for activation and subsequent secretion. The importance of tight control over IL-1 production/processing is underlined by a number of serious inflammatory diseases, termed "autoinflammatory" (reviewed in [50]), that are closely correlated with deregulation in bioactive IL-1β secretion, and where the use of recombinant IL-1RA (anakinra) has clear therapeutic benefits. Details on the processing of IL-1β and its release are still unclear, but several groups have pointed to mechanistic cell-dependent differences. In monocytes, the IL-1β processing enzyme, caspase-1, is constitutively active, and mature IL-1β is released in large quantities (1 500 pg/ml) upon stimulation with LPS [39,40] at a rate that is less than 20% of the total precursor pool [50]. For macrophages, an LPS challenge is insufficient; a second intracellular potassium (K^+)-depleting stimulus is required to trigger the assembly of a complex called the inflammasome, the subsequent activation of caspase-1, and the processing and release of IL-1β [40,49,51,52]. Based on our current data, we show that processing of pro-IL-1β in neutrophils is neither contingent on caspase-1 activation, nor on K^+ depletion. Strictly speaking, K^+ emptying did lead to mature IL-1β being detected outside the cells; however, these levels were the result of a simple externalization of already-accumulated mature IL-1β, with no active role *per se* for K^+ depletion in the maturation step. These results provide evidence that IL-1β maturation in human neutrophils is distinct from the release process, and could be mediated by a mechanism other than caspase-1, as suggested by Greten *et al.* [36], or K^+ efflux. The contribution of other

components of the inflammasome to IL-1β processing in neutrophils, however improbable, should be considered and further explored.

Intracellular mechanisms

Several studies have suggested that p38 MAPK regulates the synthesis and release of cytokines by many types of blood cells. For example, inhibition of the p38 MAPK pathway in monocytes, macrophages and neutrophils blocked LPS-induced protein transcription (including that of IL-1β), translation and subsequent cytokine release [53–57].

In general, the p38 MAPK pathway responds weakly to growth signals and is preferentially recruited by pro-inflammatory cytokines, whereas p42/44 MAPKs have been shown to be strongly activated by growth factors and growth-promoting hormones. Such is the case for Ang1 in mediating IL-8 *de novo* synthesis in neutrophils, a process that occurs through a p42/44 MAPK-dependent mechanism, and independently of p38 MAPK or Akt activity (56). Along the same lines, the present study suggests that p42/44 MAPK mediates most of the effects of Ang1 on IL-1 production in neutrophils. However, IL-1RA regulation appears to be less stringent than that of IL-1β: while antagonist *de novo* synthesis is not affected by any inhibition, and the release of IL-1RA is mostly regulated by a single signal transduction pathway (p42/44 MAPK), control of agonist production is two-fold involving not only p42/44 MAPK, but also p38 MAPK that exerts a negative regulatory role on the entirety of the IL-1β *de novo* synthesis process. The negative regulation exerted by p38 MAPK over the IL-1β synthesis process is likely a second insurance that IL-1β production remains tightly controlled when one of the two kinase pathways is unavailable, such as in the presence of a stronger pro-inflammatory signal.

A look into the downstream signaling governing the effects of LPS on IL-1 highlights differences that could be attributed to the potency of the inflammatory signal. According to our data, none of the studied pathways played a role in LPS-mediated IL-1β transcription, which is especially surprising for p38 MAPK given its similar role in macrophage cell lines [56]. However, p38 MAPK impacted both IL-1β translation and processing, as the precursor and the mature proteins were significantly down-regulated with p38 MAPK blockade. LPS also recruited p42/44 MAPK for IL-1β maturation, and both kinase pathways had a significant contribution to IL-1RA release. Thus, in the context of neutrophil IL-1 production, the recruitment of downstream signaling effectors is stimulus-dependent. Finally, because neutrophils maintained their constitutive synthesis of IL-1RA at the same level despite the inhibitors, it is likely that other signal transduction effectors mediate this process.

Other potential targets of angiopoietin stimulation

qPCR arrays identified four additional potential targets of angiopoietin stimulation whose genetic changes could be rendered significant with more exhaustive kinetics studies: IL-8, CCR1 and Lymphotoxin B (LTB) for Ang1, and IL-8RB for Ang2. For instance, providing validation that the above targets might be significant is a recent finding that, when stimulated with Ang1 for 2 or more hours, neutrophils increased their IL-8 *de novo* synthesis[12], an effect that did not extend to Ang2. Lu P *et al.* demonstrated that IL-1α and IL-1β induced the production of a CCR1 ligand, CCL3, from human hepatomas [58]; while neither CCL3 nor any of the other CCR1 ligands (CCL4/MIP-1β and CCL5/RANTES) were affected by Ang1 treatment, it is possible that increases in IL-1β could drive CCR1 expression, increasing neutrophil responsiveness to surrounding tissue-derived corresponding ligands. As for Ang2, we have previously shown that it has similar agonistic capacity to Ang1 in mediating PAF synthesis, CD11b/CD18 activation and chemotaxis in neutrophils [4,14], but this is the first time we report that Ang2 may modulate protein transcription. While the cross-talk between angiopoietins and the aforementioned proteins remains to be elucidated, the involvement of CCL3/CCR1 and IL-8RB in neutrophil migration could offer additional insight into the mechanisms governing differences in Ang1 and Ang2-driven neutrophil chemotaxis.

In conclusion, the identification of several inflammatory targets of angiopoietin stimulation provides further evidence of the implication of angiopoietins in acute inflammation. We showed that Ang1, a blocker of vessel permeability, induces transcription, translation and maturation of one pro-inflammatory IL-1 agonist, IL-1β. Perhaps to counter the damaging activities of IL-1β in the presence of a potential release signal, or perhaps to initiate resolution or to push back any additional neutrophil infiltration, neutrophils upregulate their release of IL-1RA in response to both Ang1 and the more potent pro-inflammatory signal LPS, as well as observed under TNF-α treatment [59]. These initial observations shed light on the complex interplay of inflammatory cells and mediators at the final stages of angiogenesis and acute inflammation, and provide a possible role for Ang1 in attenuating IL-1–related pathologies.

Acknowledgments

We would like to thank Mr. Fadi Hajjar and Mrs. Maya Mamarbashi for their invaluable insight in all matters pertaining to RT-qPCR experiments, as well as the volunteers that have agreed to generously donate blood.

Author Contributions

Conceived and designed the experiments: MGS. Performed the experiments: LH. Analyzed the data: LH MGS. Contributed reagents/materials/analysis tools: MGS. Wrote the paper: LH MGS.

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