A close-up on neutrophils

Visualizing the mechanisms of their in vivo recruitment and function

SARA MASSENA
Dissertation presented at Uppsala University to be publicly examined in A1:107a, Biomedicinskt Centrum, Husargatan 3, Uppsala, Friday, 18 December 2015 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Randall S. Jonhson (Department of Physiology, Development and Neuroscience, University of Cambridge; and Department of Cell and Molecular Biology, Karolinska Institute).

Abstract

A successful immune response depends on prompt and sufficient recruitment of leukocytes from the circulation to infected or injured sites. Mobilization of leukocytes to hypoxic tissues is vital for angiogenesis, i.e. the formation of new blood vessels from preexisting vasculature, and thus crucial for tissue growth and regeneration. Deviations from normal leukocyte recruitment drive a variety of pathologies, including chronic inflammation, autoimmune diseases and cancer, for which therapeutic options are limited or unspecific. Understanding the mechanisms by which the body controls leukocyte recruitment is therefore critical for the development of novel therapeutic strategies.

The present investigations focused on delineating the mechanisms behind leukocyte mobilization from the bloodstream to afflicted sites, by means of in vivo imaging techniques and in vitro assays. We demonstrate that, in response to inflammation, increased vascular permeability enhances transendothelial transport of tissue-released chemokines. Within the vasculature, chemokines form a chemotactic gradient sequestered on heparan sulfate, which directs crawling neutrophils and expedites their extravasation to the inflamed tissue. Consequently, gradient formation grants efficient bacterial clearance. Citrullination of chemokines by leukocyte-derived PAD enzymes in the inflamed tissue prevents chemokine transport into blood vessels, which dampens further neutrophil recruitment and thereby controls the amplitude of the inflammatory response. Moreover, the mechanisms of neutrophil recruitment in response to proangiogenic factors released during hypoxia are revealed to differ from those observed during classical inflammation. Particularly, VLA-4 integrin and VEGFR1 expressed on a defined subset of neutrophils, along with endothelial VEGFR2, are required for efficient neutrophil recruitment to hypoxia. Rather than stimulus-induced phenotypic changes on neutrophils, specific neutrophil subtypes with innate proinflammatory or proangiogenic functions (respectively, CD49d-VEGFR1lowCXCR4low and CD49d+VEGFR1highCXCR4high) coexist in the circulation of humans and mice.

In summary, this dissertation provides relevant information on specific steps of neutrophil recruitment to inflamed or hypoxic tissues, which may represent future means to down-regulate aberrant immune responses during chronic inflammation and autoimmune diseases; to increase angiogenesis during ischemia; or to limit pathological angiogenesis, a characteristic of tumor growth and of several chronic inflammatory disorders.

Keywords: angiogenesis, chemokine, chemotactic gradient, citrullination, hypoxia, inflammation, intraluminal crawling, intravital imaging, PAD enzymes, permeability, proangiogenic, proinflammatory

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ISSN 1651-6206
ISBN 978-91-554-9401-8
urn:nbn:se:uu:diva-265203 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-265203)
List of Papers

This doctoral dissertation is based on the following studies, which are referred to in the text by their Roman numerals:


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<th>Description</th>
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<tbody>
<tr>
<td>CD31</td>
<td>platelet-endothelial cell adhesion molecule-1 (PECAM-1)</td>
</tr>
<tr>
<td>Cit</td>
<td>citrulline</td>
</tr>
<tr>
<td>CX₃CR₁&lt;sup&gt;GFP/GFP&lt;/sup&gt;</td>
<td>mice expressing green fluorescent protein (GFP) reporter gene on the fractalkine receptor gene (CX₃CR1), thereby expressing GFP on all monocytes</td>
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<tr>
<td>CXCL12</td>
<td>CXC chemokine 12 (stromal derived factor-1, SDF-1)</td>
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<tr>
<td>CXCL8(1-77)</td>
<td>CXC chemokine 8 (IL-8, interleukin-8), intact isoform</td>
</tr>
<tr>
<td>CXCL8(1-77)Cit₅</td>
<td>CXC chemokine 8, citrullinated isoform (citrullination of arginin in position 5)</td>
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<td>CXCR4</td>
<td>CXC chemokine receptor 4 (CD184)</td>
</tr>
<tr>
<td>ECs</td>
<td>endothelial cells</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>Flt-1&lt;sup&gt;tk⁻&lt;/sup&gt;</td>
<td>Flt-1 (fms-like tyrosine kinase-1)-deficient mice, i.e. mice which lack the tyrosine kinase domain of the VEGFR1</td>
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<tr>
<td>FI</td>
<td>fluorescent intensity</td>
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<tr>
<td>GPCRs</td>
<td>G-protein coupled receptors</td>
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<tr>
<td>HIF-1</td>
<td>hypoxia inducible factor-1</td>
</tr>
<tr>
<td>hpa-tg</td>
<td>Hpa (heparanase)-overexpressing transgenic mice, characterized by structurally modified and significantly shorter heparan sulfate chains</td>
</tr>
<tr>
<td>HS</td>
<td>heparan sulfate</td>
</tr>
<tr>
<td>HSPGs</td>
<td>heparan sulfate proteoglycans</td>
</tr>
<tr>
<td>HUVECs</td>
<td>human umbilical vein endothelial cells</td>
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<tr>
<td>i.a.</td>
<td>intraarterial administration</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1 (CD54)</td>
</tr>
<tr>
<td>i.s.</td>
<td>intrascrotal administration</td>
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<td>LFA-1</td>
<td>lymphocyte function-associated antigen-1 (CD11a/CD18, α&lt;sub&gt;1&lt;/sub&gt;β&lt;sub&gt;2&lt;/sub&gt; integrin)</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>Mac-1</td>
<td>macrophage antigen-1 (CD11b/CD18, α&lt;sub&gt;4&lt;/sub&gt;β&lt;sub&gt;2&lt;/sub&gt; integrin)</td>
</tr>
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MFI  mean fluorescence intensity
MIP-2  macrophage inflammatory protein-2 (CXCL2 chemokine)
NETs  Neutrophil extracellular traps
PAD  peptidylarginine deiminase
SF  superfusion buffer
tsad⁻/⁻  TSAd (T cell-specific adapter protein)-deficient mice, characterized by disrupted VEGFR2-Src kinase signaling
VCAM-1  vascular cell adhesion molecule-1 (CD106)
VE-cadherin  vascular endothelial-cadherin (CD144)
VEGF  vascular endothelial growth factor
VEGFR1  VEGF receptor 1 (Flt-1)
VEGFR2  VEGF receptor 2 (KDR/Flk-1)
VLA-4  very late antigen-4 (CD49d/CD29, α₄β₁ integrin)
WT  wild-type mice
Introduction

A successful immune response to injury or infection is dependent on prompt recruitment of leukocytes from the blood circulation to the afflicted site [1-3]. However, over-exuberant leukocyte recruitment and activation drive diverse disorders, including chronic inflammation and autoimmune diseases. For many of these conditions, therapeutic options are limited and unspecific. Leukocyte accumulation in hypoxic tissues has been described, and there is now growing evidence that recruited leukocytes promote blood vessel growth at these sites, contributing to tissue growth, maintenance of tissue integrity, and healing of damaged tissue [4-9]. Nevertheless, abnormal or impaired blood vessel growth contributes to a variety of pathologies such as myocardial infarction, cancer and inflammatory disorders [9-13]. To comprehend the underlying signals and involved mechanisms by which the immune response is controlled is therefore critical for the development of more efficient treatments.

The work presented in this dissertation focuses on delineating the mechanisms by which circulating leukocytes are recruited to afflicted foci during inflammation or hypoxia. To investigate these dynamic events in their natural environment, advanced in vivo imaging techniques were used enabling direct visualization of the interactions between leukocytes, endothelial cells and chemokines.

The multistep recruitment of leukocytes from blood to inflamed tissue

During infection or injury, locally released stimuli (e.g. bacterial peptides, complement fragments C5a, macrophage-released chemokines, and DAMPs [damage-associated molecular patterns]) activate ECs (endothelial cells) in the nearby venules to upregulate adhesion molecules on the plasma membrane (e.g. selectins, ICAM-1 [intercellular adhesion molecule-1, CD54] and VCAM-1 [vascular cell adhesion molecule-1, CD106]). Activation of ECs aids interactions between circulating leukocytes and the vascular endothelium, and orchestrates a multistep cascade of events which ultimately leads to leukocyte transmigration to the inflamed tissue.
Leukocyte tethering and rolling

Leukocyte tethering and rolling along the vessel wall are mediated by selectins, a family of long adhesive molecules, which extend from the plasma membrane and facilitate attachment of circulating leukocytes to the endothelium [15, 16]. L-selectin (CD62L) is constitutively expressed by leukocytes, whereas P- and E-selectin (CD62P and CD62E, respectively) are expressed by inflamed ECs [3]. Increased expression of P- and E-selectin on activated venular endothelium tethers leukocytes [1, 17, 18] by binding to their ligands on the leukocyte surface [19-21]. Once tethered, leukocytes initiate a slow rolling motion on the vascular surface, by rapidly releasing and again engaging selectin-ligand bonds, a phenomenon which requires shear stress [3, 19, 22]. L-selectin participates redundantly with P- and E-selectin, supporting both capture and rolling of leukocytes in blood vessels [1, 17].

Leukocyte activation and adhesion to the endothelium

During rolling along the venular endothelium, leukocytes encounter chemokines sequestered on the endothelium. Interactions between sequestered chemokines and rolling leukocytes (via binding to GPCRs [G-protein coupled receptors] expressed on the leukocyte cell membrane) lead to adhesion to the endothelium, an event mediated by specialized leukocyte integrins [23].
Integrins are a family of noncovalently associated heterodimeric cell surface adhesion molecules expressed by most circulating leukocytes in a low affinity state [24]. Upon GPCRs activation by chemokines, a complex intracellular signaling network is triggered within milliseconds [3, 25] allowing integrins to undergo an almost instantaneous change in avidity and ligand affinity [21, 25-28], which is necessary for rapid formation of shear-resistant bonds with its ligands (e.g. ICAM-1 and VCAM-1) expressed on ECs [3]. Binding of the \( \beta_2 \)-integrin LFA-1 (lymphocyte function-associated antigen-1, CD11a/CD18, \( \alpha_L \beta_2 \) integrin) to endothelial ICAM-1, mediates neutrophil firm adhesion to the vascular endothelium in vivo [1, 3, 25, 29].

**Intraluminal leukocyte crawling**

Upon adhesion to the vessel wall, leukocytes have been described to crawl significant distances in the vessel lumen in search for optimal sites for transmigration [29-32]. Preventing leukocytes from crawling greatly decreases leukocyte transmigration to the tissue [29].

Neutrophil crawling on the stimulated endothelium is dependent on the leukocyte \( \beta_2 \)-integrin Mac-1 (macrophage antigen-1, CD11b/CD18, \( \alpha_M \beta_2 \) integrin) and its ligand ICAM-1 on ECs [29, 30] and occurs in two distinct stages. In the initial stage, a mechanotactic signal provided by shear stress induces neutrophil crawling perpendicular to blood flow until an endothelial cell junction is found [32]. In a second stage, when a junctional site is encountered, the shear stress signal is ignored and neutrophils begin to follow the path of the junction until they finally transmigrate.

How crawling neutrophils find the optimal sites for transmigration is not yet known neither is the physiological relevance of this event.

**Leukocyte extravasation out of the blood vessels**

Leukocyte extravasation out of the blood vessels involves transmigration through the endothelium and across the subendothelial basement membrane surrounding the venules.

Leukocyte transmigration through the endothelium occurs predominantly paracelluarly (i.e. between adjacent ECs) and is mediated by numerous endothelial cell junctional molecules (e.g. CD31 [platelet-endothelial cell adhesion molecule 1, PECAM-1], CD99 [transmembrane glycoprotein MIC2], and ESAM [endothelial cell-selective adhesion molecule]) [1, 3, 33-37], but also by integrins expressed on leukocytes [1, 3, 36]. Leukocyte transcellular migration across the endothelium (i.e. through ECs) has also been reported to happen [29, 33, 37-40]. Interestingly, the transmigration route was found to be dependent on the ability for leukocytes to crawl to optimal transmigration sites at the endothelial cell junctions [29]. When neutrophils are unable
to crawl, as seen in Mac-1-deficient mice, transcellular transmigration predominates [29].

To overcome the barrier of the blood vessel and finally reach the inflamed tissue, leukocytes have to transmigrate across the subendothelial basement membrane surrounding the venular endothelium. This has been shown to occur in areas with low expression of collagen IV, laminin-10 and nidogen-2, associated to gaps between pericytes [41]. Interestingly, leukocytes have been observed to initiate transmigration through endothelium at sites superimposing these specific regions [41].

How intravascular crawling leukocytes can detect these areas on the other side of the endothelium remains unknown.

Chemokine transport and presentation to circulating leukocytes

Chemokine interactions with GCPRs on the surface of rolling leukocytes trigger activation of leukocyte integrins which enables the following steps of the leukocyte recruitment cascade. In order to be presented to rolling leukocytes, chemokines produced in the tissue in response to infection and other inflammatory stimuli need to be transported across the venular wall.

Following transport into venules, immobilization of chemokines on the luminal endothelium is essential to avoid them to be washed away by the blood flow and away from the site of inflammation [42-44].

Heparan sulfate

Heparan sulfate proteoglycans (HSPGs) are complex negatively charged polysaccharide chains (HS, heparan sulfate) conjugated to a protein core expressed on most cell types. HSPGs are diverse and can be transmembrane (syndecans), bound by a glycosyl phosphatidylinositol (GPI) linkage to plasma membrane lipid (glypicans), or secreted and deposited in the extracellular matrix (perlecan, agrin) [45]. HSPGs can bind a variety of positively charged proteins through electrostatic interactions [46-48], inclusively chemokines [49].

Sequestration of chemokines on the long endothelial HSPGs chains increases the local concentration of chemokines and facilitates chemokine binding to its receptors on leukocytes under shear flow [42-44, 47, 48, 50]. Binding of chemokines to HS stabilizes the chemokine structure and prolongues its biological activity, and has been suggested to protect chemokines from enzymatic degradation [51]. Moreover, endothelial HS acts as a ligand for L-selectin aiding neutrophil slow rolling [44], and thereby increases the propensity for leukocyte-chemokine encounters within blood vessels.
Transcytosis *versus* paracellular transport

For tissue-derived chemokines (*e.g.* released by perivascular leukocytes such as tissue-resident macrophages and mast cells) to be displayed on the luminal surface of ECs, they need to be transported across the endothelial cell barrier. Theoretically, there are several ways in which abluminal-to-luminal transport of chemokines may happen: 1) transcytosis via an endocytic compartment; 2) paracellular diffusion between the ECs; or 3) lateral movement in the cell membrane, while associated with cell surface molecules. If in fact these three distinct pathways occur (either simultaneously or depending on type of endothelium, tissue, and/or chemokine) is subject to ongoing debate and proof of evidence is limited by the methods used.

Strong data supporting abluminal-to-luminal chemokine transcytosis have been published [52]. In these immuno-electron microscopy studies the chemokine IL-8 (interleukin-8, CXCL8), was found to bind HS basolaterally of the ECs, where after these complexes could be detected within endothelial cell caveolae. Tissues were collected and fixed at different time points post intradermal administration of the chemokine. Caveolae containing IL-8-HS complexes were found over time closer and closer to the lumen, suggesting that transport of tissue-derived chemokines across the endothelium occurs through caveolae-dependent transcytosis of chemokine-HS complexes [52]. Nevertheless, since the experimental demonstration of chemokine transcytosis relies on electron microscopy studies, the fate of chemokines within ECs cannot unequivocally be revealed. Furthermore, soluble chemokines transported by diffusion through intercellular gaps would be lost during tissue processing prior to examination.

Results disputing this theory have also been published [53]. Comparable concentrations of tissue-administered chemokines (IL-8) were detected by ELISA (enzyme-linked immunosorbent assay) in the bloodstream of wild-type mice (WT) and of mice lacking caveolae (*cav-1*−/− [caveolin-1 deficient mice]) 1.5 hours *post* IL-8 injection into the flank skin [53]. This observation demonstrates that caveolae-dependent mechanisms are dispensable for chemokine transport over the endothelium and suggest alternative routes of transport. Moreover, the detection of tissue-derived chemokines free in the plasma supports the theory that chemokines can be transported across the endothelium by diffusion, probably through intercellular gaps.

Taken together, the current data for chemokine transport over the vessel wall are incomplete and contradictory and more robust methods that provide *in vivo* evidence of the occurrence of distinct pathways are required for clarification of this paradigm.
Vascular permeability

Increased vascular permeability and consequent leakage of plasma and macromolecules (e.g. proteins and antibodies) through endothelial cell junctions results in local edema, a hallmark of inflammation. This event primarily occurs via changes in the integrity of endothelial cell junctions, depending on destabilization of the VE (vascular endothelial)-cadherin-catenins complex and its interaction with the actin cytoskeleton. Disturbances in this complex lead to endothelial cell contraction, which in turn creates more space between endothelial cell junctions allowing molecules and fluid to pass [54-57]. The physiological relevance of increases in vascular permeability for recruitment of circulating leukocytes has been topic of debate during recent years.

Chemokines and other inflammatory mediators (e.g. TNF-α [tumor necrosis factor-alpha]) can compromise VE-cadherin cell-cell junctions and increase vascular permeability by direct actions on ECs [58, 59]; or indirectly upon mast cell activation via chemokine receptors on the cell surface. Stimulated mast cells rapidly secrete histamine from their intracellular granules [60, 61] which in turn acts on ECs increasing vascular permeability within minutes [62].

Augmented vascular permeability in the presence of inflammatory mediators is accompanied by leukocyte adhesion and diapedesis [63]. In fact, early increases in vascular permeability during in vivo inflammation occur already in 5 min, i.e. preceding recruitment of leukocytes from the bloodstream which takes place firstly 30 minutes after start of stimulation (intravital microscopy of the stimulated rat mesentery) [64]. These results demonstrate that chemokines and other inflammatory mediators quickly induce vascular permeability, a prerequisite for their consequent promigratory effect on leukocytes. Interestingly, during sterile inflammation vascular permeability and leukocyte extravasation have been shown to be uncoupled [65, 66].

The mechanisms by which increased vascular permeability expedite recruitment of leukocytes to inflamed tissues are still unknown. Intuitively, opening the gaps between adjacent ECs would allow paracellular transport of solutes in both directions. Augmented vascular permeability during inflammation may therefore facilitate transendothelial transport of chemokines into venules and consequently leukocyte recruitment.

Hypoxia and angiogenesis

Proper oxygen delivery to tissues is vital; ECs are therefore equipped with transcription factors that respond locally and rapidly to changes in available oxygen, e.g. the transcription factor HIF-1 (hypoxia inducible factor-1). Dur-
ing circumstances where the tissue is deprived of sufficient oxygen supply (e.g. wounding, ischemic diseases, chronic inflammatory processes, tumor growth, stroke), an elaborated hypoxia-sensitive signaling pathway renders ECs responsive to proangiogenic signals triggering angiogenesis [67]. Angiogenesis, meaning the growth of new blood vessels from preexisting vasculature, is a fundamental physiological process for tissue growth and regeneration. However, deviations from normal vessel growth contribute to a variety of pathologies. Insufficient angiogenesis underlies conditions such as stroke, myocardial infarction and delayed wound healing, and abnormal angiogenesis can fuel cancer and inflammatory disorders [9].

Recruitment of leukocytes with proangiogenic functions

During hypoxia, recruitment of leukocytes with proangiogenic functions and attenuated inflammatory properties has been shown to be crucial for angiogenesis both during physiologic as pathologic conditions. During the menstrual cycle, physiologic angiogenesis depends on neutrophils recruited to the endometrium [4]. Rather than exerting proinflammatory functions, recruited neutrophils were reported to contribute to endometrial vascular growth by releasing VEGF (vascular endothelial growth factor)-A at the site [4]. Leukocyte involvement in angiogenesis and tissue healing has also been revealed in studies of muscle regeneration following induction of ischemia in mice [7] and following myocardial infarction [5]. Interestingly, sequential mobilization of two distinct subsets of monocytes is required for tissue healing following myocardial infarction [5]. Recruited Ly-6Chigh monocytes exhibit proteolytic activity and inflammatory functions, whereas Ly-6Cslow monocytes contribute to angiogenesis and have attenuated inflammatory properties. Moreover, a new leukocyte subtype (Gr-1dimCD11b+) was demonstrated to enhance angiogenesis in ischemic hind-limb muscle [6]. Furthermore, it has recently been found that neutrophils recruited to avascular islets of Langerhans transplanted to striated muscle are crucial for angiogenesis, as transplantation of islets to neutropenic mice resulted in complete inhibition of islet revascularization [8]. Leukocytes have also been shown to be recruited to hypoxic tumor microenvironment where they are required for induction and maintenance of pathological angiogenesis and tumor growth [10-13].

If specific proangiogenic leukocytes are recruited from the circulation, or if recruited leukocytes become proangiogenic in the current microenvironment remains unknown, as well are the driving factors and distinctive mechanisms of their recruitment from the circulation to the hypoxic sites.
Vascular Endothelial Growth Factor-A

VEGF-A is the major regulator of angiogenesis. In response to a hypoxic environment, VEGF-A expression is highly upregulated in a HIF-1 dependent manner [67-69], which triggers angiogenesis, and thereby contributes to tissue recovery and survival. Abnormal VEGF-A expression has been related to the development of pathologic conditions by promoting uncontrolled angiogenesis, as seen in solid tumors [67, 68, 70].

VEGF-A triggers angiogenesis by direct activation of ECs through two VEGF receptor tyrosine kinases: VEGFR1 (fms-like tyrosine kinase 1, Flt-1) and VEGFR2 (in humans, kinase insert domain receptor, KDR; in mice, fetal liver kinase 1, Flk-1) [70-72]. Besides its direct proangiogenic actions on ECs, a role for VEGF-A in leukocyte recruitment to hypoxic sites has been suggested. Rapid upregulation of adhesion molecules ICAM-1 and VCAM-1 accompanied by increased adhesion and transmigration of neutrophils, has been reported on HUVECs (human umbilical vein endothelial cells) stimulated by VEGF-A [73]. Increased expression of ICAM-1 after stimulation with VEGF-A was also reported for colonic microvascular ECs and correlation between VEGF-A levels and increased neutrophil and T-cell adhesion was demonstrated in vitro [74, 75]. Indeed, a recent study has confirmed in vivo the ability of VEGF-A to induce recruitment of leukocytes with proangiogenic properties [76]. Interestingly, in addition to being expressed on ECs, VEGF receptors are found on a variety of other cells including leukocytes [77] and neutralization of VEGFR1 was seen to suppress not only angiogenesis but also vascular inflammation and accumulation of immune cells at the afflicted sites [78, 79].

If VEGF-A is able to activate VEGF receptors on circulating leukocytes and in this way induce leukocyte recruitment to hypoxic tissues remains to be unraveled.
Aims

The overall aim of this dissertation was to delineate the mechanisms by which chemoattractants released in the tissue during inflammation or hypoxia are able to regulate leukocyte recruitment from blood circulation to afflicted sites.

More specifically, the work in the three different studies was focused on:

Study I

1. Determining whether localized release of inflammatory chemokines in tissue guides intraluminal crawling neutrophils to optimal transmigration sites;

2. Analyzing whether an intravascular haptotatic chemokine gradient is formed intravascularly and if it is sequestered on endothelial HS;

3. Defining the relevance of intravascular gradient formation for efficient bacterial clearance.

Study II

1. Investigating if increased vascular permeability during inflammation facilitates the influx of tissue-released chemokines to postcapillary venules;

2. Examining, in vivo and in real time, the route of chemokine transport into venules;

3. Establishing if influx of chemokines across the venular wall is altered by posttranslational modifications of chemokines (citrullination).
Study III

1. Investigating the mechanisms that underlie VEGF-A-induced leukocyte recruitment;

2. Determining the identity and specific surface markers of the VEGF-A-recruited leukocyte population;

3. Assessing, in an in vivo model of hypoxia, the importance of the identified VEGF-A-responsive leukocyte population for angiogenesis.
Methods

Animals

WT (C57Bl/6: Study I: B & K Universal, Sweden; Studies II-III: Taconic M & B, Denmark); B6 CD45.1, Pep Boy mice (WT CD45.1⁺; The Jackson Laboratory); Hpa (heparanase)-overexpressing transgenic mice (hpa-tg) [80]; CX3CR1GFP/GFP (The Jackson Laboratory); TSAd (T cell-specific adapter protein)-deficient mice (tsad⁻/-; kindly provided by Professor Jeffrey A. Bluestone, UCSF, San Francisco, CA) [81]; and Flt-1 (fms-like tyrosine kinase-1)-deficient mice (Flt-1 tk⁻/-; kindly provided by Professor Yihai Cao, Karolinska Institute, Stockholm, Sweden) [82], weighing between 20-35g, were used. All genetically modified animals were on a C57Bl/6 background. All procedures were approved by the Uppsala Regional Ethical Committee on Animal Research.

Exposure of the cremaster muscle for in vivo microscopic evaluation

The anesthetized mouse (isoflurane in a mixture of air and oxygen) was placed in supine position onto a viewing stage (37°C). An incision was made in the scrotal skin and the left cremaster muscle was carefully separated from associated connective tissue fascia. A lengthwise incision was made on the ventral surface of the muscle using a thermal cautery. Testis and epididymis were separated from the cremaster muscle and pushed back into the abdominal cavity. The edges of the muscle were secured with suture silk to hold the cremaster opened and flattened onto the stage [29, 83]. The muscle was constantly kept warm and moistened with a bicarbonate-buffered saline solution (pH 7.4, 37°C). A catheter was inserted in the left femoral artery for allowing retrograde infusion close to the muscle (i.a. [intraarterial administration]).

Homogenous versus localized stimulation

Stimuli were either added to the bicarbonate buffer superfusing the cremaster muscle or loaded into a gel placed on the surface of the cremaster (400
µm away from the venule of interest) to mimic a homogenous or a localized inflammatory source, respectively.

A full description of administered doses and analyzed parameters can be found in Massena et al. Blood. 2010 [84].

Investigation of the receptors and adhesion molecules involved in VEGF-A-induced neutrophil recruitment

For assessing the receptors and adhesion molecules involved in neutrophil recruitment, animals were pretreated (i.a., 30 min before start of the experiment) with neutralizing antibodies towards the molecules of interest. Control experiments were performed following administration of appropriate isotype control antibodies.

A full description of the antibodies and administered doses used can be found in Massena et al. Blood. 2015 [85].

Inhibition of caveolae-mediated transcytosis in the living animal

For inhibiting transendothelial caveolae-mediated transport of chemokines to the vessel lumen, mice were treated with filipin (Filipin complex from Streptomyces filipinensis, Sigma-Aldrich) added to the bicarbonate-buffer superfusing the exposed cremaster muscle (1 µg/ml, at a 1 ml/min rate). Filipin binds to cholesterol and disrupts caveolar structure and transport function [86-91].

Bone marrow transplantation

Bone marrow cells were isolated from iliac bones, femurs and tibias, and 1.5x10⁶ cells were transplanted into congenic recipients who had been beforehand irradiated with a split dose of 10 Gy in a ¹³⁷Cs irradiator (MDS Nordion) to grant myeloablation. The two isoforms of CD45, CD45.1 and CD45.2 were used to distinguish between donor (CD45.2⁺) and recipient (CD45.1⁺) derived blood cells. Peripheral blood chimerism was determined by flow cytometry 5 weeks posttransplantation using antibodies towards CD45.1 and CD45.2 (anti-mouse CD45.1 FITC and anti-mouse CD45.2 PE, Affymetrix, eBioscience). Neutrophil recruitment experiments were performed on the created chimeric mice (donor cell chimerism >85%) 16 weeks after bone marrow transplantation.
In vivo model of hypoxia-driven angiogenesis

Mouse pancreatic islets were isolated, cultured overnight and transplanted to the cremaster muscle of syngeneic mice, as previously described [8, 76, 92]. Leukocyte recruitment and islet graft neovascularization were examined in vivo 4 days after transplantation.

Making things glow: Fluorescent tagging

Chemokines
Chemokines (recombinant mouse MIP-2 [macrophage inflammatory protein-2; CXCL2], R&D Systems; recombinant human IL-8, intact form, i.e. CXCL8[1-77], Peprotech; or the in house citrullinated form of this chemokine, i.e. CXCL8[1-77]Cit5 [93], were fluorescently labeled in house (Study I: Fluorotag FITC conjugation kit, Sigma-Aldrich; Study II: Microscale protein labeling kit, Alexa Fluor 647, Molecular Probes, Thermo Fisher Scientific).

Blood vessels
To allow visualization of the vasculature, endothelial cell junctions were labeled with monoclonal antibodies (mAbs) to CD31 (anti-mouse CD31 [PECAM-1] functional grade purified, clone 390, Affymetrix, eBioscience; i.a.) conjugated in house to Alexa Fluor dye 555 (Alexa Fluor Antibody Labeling Kit, Molecular Probes, Thermo Fisher Scientific).

Neutrophils
Neutrophils were distinguished from other leukocyte subtypes upon administration of Ly6G mAbs (anti-mouse Ly6G [Gr1] functional grade purified, clone RB6-8C5, or clone 1A8, both from Affymetrix, eBioscience; i.a.) conjugated in house to Alexa Fluor Dye 488, 555 or 647 (Alexa Fluor Antibody Labeling Kits, Molecular Probes, Thermo Fisher Scientific).

Monocytes
Monocytes were detected by CD115 mAb (anti-mouse CD115 [c-fms] functional grade purified, clone AFS98, Affymetrix, eBioscience; i.a.) conjugated in house with Alexa Fluor Dye 488 (Alexa Fluor Antibody Labeling Kit, Molecular Probes, Thermo Fisher Scientific) or by using CX3CR1<sup>F/F</sup> or CX3CR1<sup>GFP/GFP</sup>
mice. This mouse strain is genetically encoded for expressing green fluorescent protein on all monocytes (targeted insertion of green fluorescent protein [GFP] reporter gene on the fractalkine receptor gene [CX3CR1]) [94].

Vascular Permeability

For assessing vascular permeability, mice were administered with Alexa Fluor Dye 488-labeled bovine serum albumin (Sigma-Aldrich; i.a.) and the leakage of fluorescent albumin from cremasteric postcapillary venules to tissue (permeability index) detected over time [64, 65, 95].

Transplanted pancreatic islets

For visualizing transplanted pancreatic islets on the cremaster muscle, isolated mouse islets were fluorescently labeled with the intracellular probe Celltracker Blue CMAC (7-amino-4-chloromethylcoumarin) dye (Molecular Probes, Thermo Fisher Scientific) immediately before transplantation.

Intravital video-microscopy

An intravital microscope (Study I: Leitz Ortholux II with a 25×/0.6W [Leitz] or a 40×/0.8W [Carl Zeiss] objective; Study I-III: Leica DM5000B with a 20×/0.5 W or a 40×/0.5W HCS Apo objective, Wetzlar) connected to a video camera (Study I: Hamamatsu C3077 or Hamamatsu C10600; Studies II-III: Hamamatsu Orca R2) and to a computer with Apple iMovie HD acquisition software (Study I) or Improvision Volocity acquisition software (Perkin Elmer; Studies I-III) was used. MATLAB software (MathWorks) was used to generate the crawling plots (Study I).

Intravital microscopes allow detailed in vivo examination of cellular and biological dynamic processes by visualizing these unique events over time in their natural environment. A requirement of this technique is that the tissue examined is translucent.

High-speed confocal laser scanning microscopy

A line-scanning confocal microscope (Zeiss LSM 5 Live) with a Plan Apochromat 20×/0.8 ∞/0, a W Plan Apochromat 40×/1.0 DIC VIS-IR ∞/0 or a W Plan Apochromat 63×/1.0 VIS-IR ∞/0 objective, and Zeiss Zen 2009 software was used. Analysis of data was performed using image analysis software Imaris (Bitplane).

Confocal line-scanning microscopes are fast imaging systems which enable to analyze, with high resolution, the dynamics of physiological events.
deep within biological tissues. By acquiring confocal images at video rates, this technique allows in vivo high-resolution examination of high-speed processes in depth.

Noninvasive whole body imaging
Mice were injected subcutaneously with bioluminescent bacteria (Staphylococcus aureus strain Xen29, Perkin Elmer). At different time-points after inoculation, mice were anesthetized with isoflurane in a whole body imaging device (IVIS Spectrum imaging system, Perkin Elmer) and bacteria-derived bioluminescence quantified using Living Image imaging software (Perkin Elmer).

The IVIS Spectrum in vivo imaging system uses an optical imaging technology which enables noninvasive high-sensitivity detection of bioluminescent and fluorescent reporters in living animals. Whole body scanning can be performed at several time points in the same animal, allowing monitoring of physiological and pathological processes over time e.g. disease progression.

In vitro studies
A full description of the in vitro methods used can be found in Massena et al. Blood. 2015 [85].

Isolation of neutrophils
Neutrophils were isolated from human or mouse blood, as described earlier [96, 97].

Detection of mRNA receptor expression
For detection of relative mRNA receptor expression, mRNA was isolated from purified neutrophils using a RNeasy mini kit (Qiagen) and real-time RT-PCR (reverse transcription polymerase chain reaction) performed on a LightCycler System (Roche Diagnostics) with LightCycler software.

Neutrophil-receptor activation assay and flow cytometric studies
Neutrophil-receptor activation was assayed using standard western blotting techniques, following direct in vitro stimulation, and neutrophil phenotypes were determined using a BD LSRII or a FACSCalibur flow cytometer system (both from BD Biosciences).
Chemotaxis assay

For studying chemotactic ability of the different neutrophil populations to gradients of VEGF-A, neutrophil subtypes were sorted on a FACSARia II system (BD Biosciences) and chemotaxis assessed using the Cell Director 2D chemotaxis assay (Gradientech).

Statistics

All data are presented as mean plus or minus SEM. Unpaired Student's t-test or analysis of variance (ANOVA one- or two-way) with Bonferroni correction was used for comparison between 2 or more groups, respectively. For verifying single variable changes with time and within one group, paired student's t-test or analysis of variance (ANOVA repeated measures) with Bonferroni correction was used for comparison between two or more time points, respectively. Statistical significance was considered for probability values ($P$) less than 0.05. Statistical analysis was performed using Prism software (GraphPad Software).
Results and discussion

Neutrophils chemotax already within vessels towards an extravascular inflammatory source (*Study I*)

During infection or tissue injury, chemoattractants released at the inflammatory focus (*e.g.* chemokines and bacterial products) form extravascular chemotactic gradients. This results in activation of ECs in nearby venules, which initiates the multistep leukocyte recruitment cascade [3]. Following transmigration to the perivascular tissue, leukocytes are guided by these extravascular chemotactic gradients and chemotax towards the inflammatory focus [83, 98].

We investigated if intraluminal crawling neutrophils could, already within blood vessels, detect the chemotactic source and thereby efficiently chemotax to optimal transmigration sites closer to the origin of the inflammation. A chemokine-releasing gel was placed on the cremaster muscle of mice to create an extravascular chemokine gradient. Neutrophil recruitment was evaluated by intravital video-microscopy and compared to that induced by a homogenous extravascular chemokine concentration.

When muscles were stimulated by homogenous extravascular MIP-2 concentrations (*i.e.* MIP-2 added to the superfusion buffer [SF]), neutrophils crawled in all directions within postcapillary venules (*Fig. 2 A*). However, when MIP-2 was administered in a gel placed on the muscle 400 µm away from the vessel of interest, it was evident that neutrophils could detect the extravascular chemokine origin and crawled intravascularly towards the gel (*Fig. 2 B*). Chemotaxis within the vasculature resulted in neutrophils transmigrating closer to the chemokine source. Furthermore, the time between initiation of adhesion and neutrophils exiting the vessel was much shorter when crawling neutrophils were guided by a chemotactic gradient (MIP-2 gel: 9±1 minutes; MIP-2 SF: 15±1 minutes).
Figure 2. **A localized extravascular chemokine source guides crawling neutrophils to optimal transmigration sites.** Displacement of crawling neutrophils from the adhesion point (the center of the circle) to transmigration site (marked dots), during activation by (A) homogenous extravascular chemokine concentration (MIP-2 SF; n = 4 mice, 32 neutrophils tracked) or by (B) localized extravascular chemokine release (MIP-2 gel, placed extravascularly at 90°; n = 5 mice, 57 neutrophils tracked). The red arrows indicate the direction of the blood flow. These plots were originally published in *Blood* [84]. © the American Society of Hematology.

Chemokine sequestration by endothelial HS is imperative for guidance of crawling neutrophils (*Study I*)

Luminal immobilization of chemokines on the endothelium has been shown in several *in vitro* models [42-44, 52], and chemokines have been suggested to be sequestered by HS expressed on ECs [47, 48].

To explore the role of intravascular HS in chemokine sequestration *in vivo*, neutrophil recruitment was investigated in mice that overexpress heparanase (hpa-tg), an endo-β-D-glucuronidase responsible for endoglycosidic cleavage of HS. Transgenic overexpression of this enzyme in mice results in structurally modified and significantly shorter HS chains with reduced binding properties [99].

The different steps of the neutrophil recruitment cascade were investigated in the cremasteric microcirculation of hpa-tg after stimulation by MIP-2. Alongside MIP-2 stimulation, neutrophils adhered in similar numbers in postcapillary venules of both hpa-tg and WT, which indicates that ECs and neutrophils could still be activated in hpa-tg and the recruitment cascade initiated. However, in hpa-tg, the number of neutrophils which transmigrated to the inflamed tissue was decreased almost 60% after 90 minutes of MIP-2 superfusion compared to WT.
Random intraluminal neutrophil crawling in hpa-tg. Crawling neutrophils in hpa-tg cremasteric venules showed no preferred directionality upon localized extravascular chemokine release (MIP-2 gel, placed extravascularly at 90°), resulting in a similar number of cells crawling towards and away from the chemokine gel. N = 5 mice; 48 neutrophils tracked. The center of the circle corresponds to the site where neutrophils adhered and the blue dots represent the transmigration sites. The red arrow indicates the direction of the blood flow. This plot was originally published in Blood [84]. © the American Society of Hematology.

We found that the impaired neutrophil transmigration observed in hpa-tg was due to a poor guidance of crawling neutrophils towards the chemotactic source (MIP-2 gel). Intravascular crawling neutrophils were not able to detect a chemotactic gradient within the hpa-tg venules and crawled in apparently random directions (Fig. 3), which ultimately resulted in an impaired ability to initiate diapedesis.

Interstitially released chemokines form intravascular haptotatic gradients sequestered on endothelial HS (Study I)

To undeniably certify that interstitially released chemokines were being intravascularly sequestered by endothelial HS and thereby presented to circulating neutrophils, MIP-2 was conjugated with a fluorophore prior to administration in the gel.

Confocal microscopy of the cremaster muscles revealed that the chemokine was being presented not only in the extravascular compartment, but also within venules close to the chemokine loaded gel. In hpa-tg, much less chemokine could be detected on the venular endothelium compared with WT (Fig. 4 A-B). Indeed, quantification of the MIP-2-derived FI (fluorescence intensity) within the venules of the two mouse strains revealed 5-fold more...
MIP-2 sequestered in venules of WT compared to hpa-tg. In agreement with the results obtained by intravital video-microscopy, the reduced intravascular chemokine sequestration observed in hpa-tg was accompanied by lower numbers of transmigrated neutrophils in the perivascular tissue (Fig. 4 A-B).

Figure 4. Chemokine gradients are sequestered on the venular lumen in WT but not in hpa-tg. Representative confocal microscopy acquired images of (A) WT’s and (B) hpa-tg’s cremaster muscles activated in vivo by fluorescent MIP-2 (green) loaded gels. Endothelial cell junctions were stained (i.a.) with anti-CD31 mAb (red) and neutrophils with anti-Ly6G-RB6-8C5 (Gr-1) mAbs (blue). These images were originally published in Blood [84]. © the American Society of Hematology.

Taken together, these results demonstrate that a gradient of chemokines originated from a localized extravascular source is sequestered on the vessel lumen by endothelial HS and provides directional cues to crawling neutrophils. By following this gradient, neutrophils efficiently and rapidly find optimal transmigration sites close to the origin of the inflammation. Whether all chemokines can establish these haptotactic gradients remains unclear since different chemokines bind with different affinity to heparan sulfate [47, 49].

Disruption of the intravascular chemotactic gradient leads to a decreased ability to clear infections (Study I)

The physiological relevance of the establishment of an intravascular chemotactic gradient, which efficiently guides crawling neutrophils to optimal
transmigration sites, was determined by following bacterial clearance in WT versus in hpa-tg. Mice were infected subcutaneously with a bioluminescent strain of *Staphylococcus aureus* (Xen 29) and the emitted luminescence was followed during 2 weeks by noninvasive whole body scan in an IVIS imaging system.

**Figure 5.** Random crawling in hpa-tg venules results in a decreased ability to clear bacterial infections. Representative images of the detection of the bioluminescence emitted by *S. aureus* strain Xen 29 in WT and hpa-tg at different time points after subcutaneous inoculation (noninvasive whole body scan, IVIS Spectrum). These images were originally published in *Blood* [84]. © the American Society of Hematology.

Infected hpa-tg had a significant defect in immune response, seen by significantly higher bacterial levels compared to infected WT. This was clear already on day 1 after infection (Fig. 5). Ultimately, 80% of the hpa-tg failed to clear the infection during the studied 2 week-period, whereas all WT cleared the infection during the same time period. Thus, these results demon-
strate that disruption of the chemotactic gradient presented by HS leads to a decreased ability to clear infections, as seen in hpa-tg.

Altogether, this study reveals that chemokines originated from an extravascular inflammatory focus form an intravascular haptotactic gradient sequestered by HS on ECs. Intraluminal crawling neutrophils follow this gradient, which expedites their recruitment through efficient diapedesis closer to the origin of the inflammation, granting efficient bacterial clearance.

Increased vascular permeability facilitates the influx of chemokines into postcapillary venules and expedites neutrophil recruitment (Study II)

Transport of chemokines across the endothelium has been described to occur through transcytosis in complexes with HS [44, 52] and haptotactic chemokine gradients sequestered on luminal endothelial HS are required for efficient leukocyte recruitment [84]. Still, tissue-derived chemokines can be detected freely in the plasma [53, 100]. Natural dissociation of complexes between HS and proteins, such as chemokines, takes place within lysosomes in order for following recycling or degradation of the diverse components to happen [101]. Soluble heparin inhibits the biological effects of chemokines, at least in part, by interference with the HS-protein interactions [51]. Indeed, dissociation of chemokine-endothelial HS bounds following heparin treatment (i.a.) has been shown in vivo [84]. Nevertheless, endogenous heparin is produced almost exclusively by activated mast cells and is rarely found in blood vessels. Thereby the presence of tissue-derived chemokines free in plasma is most likely not a result of intravascular dissociation of HS-chemokine complexes following transcytosis, suggesting other mechanisms of chemokine transendothelial transport.

Increased vascular permeability and consequent plasma leakage through endothelial paracellular gaps is a physiological response to inflammation [54], an event that precedes leukocyte recruitment by inflammatory mediators [64, 95]. Furthermore, vascular permeability was demonstrated in vivo to be linearly correlated to subsequent increases in number of adherent and emigrated leukocytes [64].

We investigated if increases in vascular permeability expedited chemokine influx into the vasculature and ergo chemokine presentation to circulating leukocytes. The mouse cremaster muscle was exposed to fluorescently labeled MIP-2 alone or together with the permeability-increasing mediators histamine or VEGF-A (extravascular administration i.e. added to the SF). MIP-2 influx into venules and permeability index (i.e. leakage of fluorescent albumin from circulation to tissue [64, 65, 95]) were assessed by confocal microscopy.
Figure 6. Increased vascular permeability facilitates chemokine transport over the vessel wall. (A) The increase in vascular permeability induced by MIP-2, histamine and VEGF (measured in vivo as extravasation of fluorescent albumin to the perivascular interstitium, i.e. permeability index) correlates with (B) increased influx of fluorescently labeled MIP-2 into postcapillary venules. (C) Representative images of in vivo detection of chemokine influx into a postcapillary venule over time acquired by confocal microscopy. Fluorescent MIP-2 (green) added to the buffer superfusing the cremaster muscle (t = 0 min), and CD31 mAbs (red) administered 15 min prior to experiment (i.a.). Scale bar = 35 µm. (D-G) MIP-2-induced neutrophil recruitment (Ly6G-1A8+ leukocytes) was potentiated by the hastened chemokine transport over the vessel wall. N = 6 mice per treatment group and experimental set. *, P < 0.05 compared to MIP-2 alone.
Simultaneous monitoring of these events in postcapillary venules demonstrated that MIP-2-induced increase in venular permeability (Fig. 6 A) precedes chemokine influx and sequestration on the luminal endothelium of the same microvessels (Fig. 6 B-C). Moreover, increased venular permeability induced by histamine or VEGF-A accelerated and correlated in magnitude to influx of MIP-2 to postcapillary venules. In turn, subsequent neutrophil recruitment was hastened and amplified (Fig. 6 D-G). The potentiated MIP-2-induced neutrophil recruitment observed when vascular permeability was augmented by histamine or VEGF-A was not seen when mice were treated with the permeability increasing mediators in the absence of chemokine stimulation, indicating that the correlation between vascular permeability and neutrophil recruitment is indirect and dependent on permeability-aided chemokine influx into blood vessels.

#### Figure 7. Increased vascular permeability results in chemokine accumulation at endothelial cell junctions.

Chemokine sequestration at venular endothelial cell (EC) junctions was assessed in vivo by confocal microscopy and compared to sequestration at non-junctional areas in mice treated with fluorescent MIP-2 alone or together with histamine (i.e., 30 min stimulation). MIP-2: n = 5 mice; 53 venular segments analyzed; MIP-2 + histamine: n = 5 mice; 70 venular segments analyzed. *, P < 0.05 versus MIP-2-derived FI at endothelial cell junctions.

Within blood vessels, crawling neutrophils are guided by mechanotactic signals [32] and by intravascular haptotactic gradients [84] to optimal transmigration sites located predominantly at endothelial cell junctions [29].

Interestingly, when chemokine influx into postcapillary venules had been incremented by histamine-induced vascular permeability, we found that MIP-2 primarily accumulated at endothelial cell junctions (Fig. 7), suggesting paracellular transport of tissue-derived chemokines either by diffusion or associated with surface molecules.

If the chemokine accumulation observed on endothelial cell junctions correlates with formation of the chemotactic gradient which guides neutrophils towards optimal transmigration sites, remains to be uncovered.
Altogether our results demonstrate that, during inflammation, increased vascular permeability aids the influx of chemokines into postcapillary venules and contributes to rapid sequestration of chemokines at endothelial cell junctions, which expedites neutrophil recruitment to the inflamed tissue.

Chemokine transport into postcapillary venules is independent of caveolae-mediated transcytosis (Study II)

We demonstrated that increased venular permeability during inflammation facilitates the influx of chemokines into cremasteric postcapillary venules (most likely by paracellular transport in response to opening of endothelial cell junctions). Our results challenge earlier reports showing that chemokine transport from the apical to the luminal side of the endothelium occurs through transcytosis by way of caveolae [44, 52, 102, 103].

The contribution of caveolae-dependent transcytosis for chemokine transport to the vessel lumen was therefore investigated following inhibition of caveolae formation by filipin [86-91] added to the buffer superfusing the exposed cremaster muscle (30 min prior to MIP-2).

In vivo inhibition of caveolae-mediated transcytosis had no effect on the magnitude neither on the dynamics of the influx of fluorescent MIP-2 into postcapillary venules (Fig. 8 A). Our results are congruent with studies of chemokine transport in mice lacking caveolae (cav-1⁻/⁻), where similar concentrations of IL-8 could be measured by ELISA in the bloodstream as in WT, 1.5 h post chemokine injection into the flank skin [53]. Taken together these results imply that chemokine transport across the endothelium can occur in a caveolae-independent way.

The unaltered chemokine transport across the venular wall observed in the filipin-treated animals, resulted in MIP-2-induced neutrophil adhesion within postcapillary venules to the same extend as in control animals (i.e. treated with MIP-2 alone; Fig. 8 B). Nevertheless, the numbers of emigrated neutrophils were significantly reduced when caveolae-mediated transcytosis had been blocked (Fig. 8 C). Closer examination of the interactions between endothelium and neutrophils in filipin-treated mice revealed that, following firm adhesion to the endothelium, neutrophils crawled to and along junctions where they initiated diapedesis. However, transmigrating neutrophils were trapped on endothelial cell domes, failing to finalize transmigration to the inflamed tissue (Fig. 8 D-E).

These results indicate a novel role for caveolae-mediated transcytosis in neutrophil transendothelial migration.
Figure 8. Chemokine influx into postcapillary venules is independent of caveolae-mediated transcytosis. (A) Treatment with filipin (added to the buffer superfusing the cremaster muscle starting at 30 min prior to MIP-2 treatment; 1 µg/ml) to block caveolae-mediated transcytosis, had no significant effect on chemokine influx into postcapillary venules. MIP-2: n = 6 mice; MIP-2 + filipin: n = 5 mice. *, $P < 0.05$ compared to MIP-2 alone. (B) MIP-2-induced neutrophil adhesion (Ly6G-1A8+ leukocytes) was unaffected when caveolae-mediated transcytosis was blocked by filipin. (C) Significantly lower numbers of neutrophils emigrated from postcapillary venules in the filipin-treated group than in the control group (i.e. treated with MIP-2 alone). N = 6 mice per treatment group. *, $P < 0.05$ compared to MIP-2 alone. (D-E) Transmigrating neutrophils (Ly6G-1A8+ leukocytes; yellow) get trapped on their way out from venules (labeled with CD31 mAbs; red). Images acquired by confocal microscopy. Scale bar = 10 µm (D) or 3 µm (E).
Citrullination of chemokines in the inflamed tissue dampens their influx across the venular wall and abrogates neutrophil recruitment (Study II)

Citrullination of proteins is a natural occurring posttranslational modification of an arginine (Arg) to a citrulline (Cit) residue catalyzed by PAD (peptidyl arginine deiminase) enzymes, a highly conserved family of enzymes, which are expressed in a wide range of cells [104]. The role of citrullination in inflammation has become increasingly apparent since elevated amounts of PAD enzymes (mainly PAD2 and 4) and of their citrullinated products associate with autoimmune disorders, chronic inflammation and cancer [104-106]. Interestingly, leukocytes are a source of PAD2 and the major source of PAD4, and have previously been shown to be able to citrullinate several chemokines (e.g. CXCL8, CXCL5, CCL17, CCL26) [93] which results in altered chemokine activity [93, 107].

Citrullination of CXCL8 by leukocyte-derived PAD enzymes was shown to reduce the ability of this chemokine in inducing neutrophil extravasation to the peritoneal cavity of mice [93]. Defects in direct neutrophil activation and guidance could not explain the observed decrease in the in vivo chemotactic activity of CXCL8, since citrullination of this chemokine did not affect the its binding affinity to its receptors CXCR1 and CXCR2 on neutrophils neither consecutive increases of [Ca²⁺]. Furthermore the citrullinated CXCL8 isoform was still able to induce neutrophil chemotaxis in vitro (Boyden chamber assay) [93]. We speculate that the decreased in vivo chemotactic activity of the citrullinated CXCL8 may reflect reduced bioavailability and impaired transport over the venular wall.

We investigated whether citrullination could alter the ability of chemokines to be transported into venules and thereby reduce the levels of sequestered chemokines presented to circulating leukocytes. Mice were treated extravascularly (i.s., single dose) with fluorescently labeled CXCL8, either in its intact form (CXCL8[1-77]) or citrullinated (CXCL8[1-77]Cit₅), whereafter the cremasteric vasculature was imaged.

Citrullination of CXCL8 resulted in a drastically reduced chemokine influx and luminal sequestration in postcapillary venules (observed as a marked decrease in CXCL8-derived FI), compared to what was detected in mice treated with the intact CXCL8 isoform (Fig. 9 A-B). The low chemokine levels observed intraluminally in mice treated extravascularly with CXCL8(1-77)Cit₅ translated into impaired neutrophil adhesion and recruitment to the inflamed cremaster muscle (Figs. 9 C-D). This is in agreement with the previously mentioned study showing decreased ability of this citrullinated chemokine in inducing neutrophil extravasation to the peritoneal cavity of mice [93].
Our results suggest that citrullination of chemokines by leukocyte-derived PAD enzymes in the inflamed tissue inhibits chemokine traffic and intraluminal sequestration and thereby locally down-regulates neutrophil recruitment.

Figure 9. Citrullination of chemokines inhibits their ability to be transported into venules. (A) Representative images of in vivo detection of fluorescent CXCL8 (green) and of neutrophils (Ly6G-1A8+ leukocytes; yellow) in postcapillary venules (CD31+; red) and perivascular tissue, acquired after 30 min of stimulation with fluorescent CXCL8(1-77) or fluorescent CXCL8(1-77)Cit5 (i.s.). Scale bars = 25 µm. (B) Chemokine influx into postcapillary venules was quantified from the acquired images. CXCL8(1-77): n = 6 mice, 82 vessel segments analyzed; citrullinated CXCL8 (CXCL8[1-77]Cit5): n = 5 mice, 46 vessel segments analyzed. *, P < 0.05 compared to CXCL8(1-77). (C) CXCL8-induced neutrophil adhesion to postcapillary venules and (D) emigration to the perivascular tissue was abolished when CXCL8 had been citrullinated. CXCL8 added to the buffer superfusing the cremaster muscle (t = 0 min). Control: n = 4; CXCL8(1-77): n = 8 mice; CXCL8(1-77)Cit5: n = 6 mice. *, P < 0.05 compared to control or to CXCL8(1-77), as indicated.

PAD activity does not occur under normal physiological conditions but is regulated by the external microenvironment [104]. For example, PAD2 and
PAD4 activity can be triggered during inflammation by alterations in calcium homeostasis in activated cells and in the inflamed tissue [108]. PAD4 is highly expressed in the nucleus and within cytoplasmic granules of neutrophils, and PAD2 has mainly a cytoplasmic location [109]. During inflammation, these deiminases can though be detected extracellularly in a free diffusible form [109, 110] with preserved activity for citrullinating extracellular proteins [110, 111]. Due to its natural intracellular location, the presence of PAD4 in the extracellular milieu during inflammation can be explained, for example, by the release of enzymes from granules by recruited neutrophils, and by the formation of NETs (neutrophil extracellular traps) which involve the release of the neutrophil cellular contents [110]. Likewise PAD4, PAD2 has been reported to be released by neutrophils in association with NETs [110].

We speculate that the release of PAD enzymes by recruited leukocytes and subsequent citrullination of chemokines in the inflamed tissue may be a physiological feedback mechanism to regulate the amplitude and duration of inflammation.

Interestingly PAD4 has been shown to be significantly expressed in a vast range of tumor tissues and to be associated with tumorigenesis and protection against tumor cell apoptosis [112]. The mechanisms are though unclear. Modulation of chemokines in the tumor microenvironment may be a way for tumors to suppress the recruitment of proinflammatory leukocytes at the tumor site and in that manner escape immunosurveillance.

Our findings demonstrate novel means to control inflammation. Posttranslational modification of chemokines in inflamed tissues may be a natural way for the innate immune system to down-regulate chemokine traffic and activity, to constrain leukocyte recruitment and thereby the magnitude of the immune response.

Neutrophils express VEGFR1, which is crucial for their recruitment to VEGF-A (Study III)

VEGF-A is induced by local hypoxia [67, 77] and is well known for its potent proangiogenic effects through activation of ECs. VEGF-A expression at hypoxic sites has been related to recruitment of leukocytes with proangiogenic properties.

We investigated the distinctive mechanisms behind VEGF-A-induced leukocyte recruitment. Using intravital video-microscopy of the mouse cremaster muscle, we found that VEGF-A specifically recruited neutrophils (Ly6G-1A8+ leukocytes) from the bloodstream to the muscle, and that VEGF-A-induced neutrophil recruitment occurred as quickly as within 30 minutes of stimulation (Fig. 10 A-B; WT). The rapid response to VEGF-A
addition to the superfusate suggests a direct effect of VEGF-A on circulating neutrophils. Indeed, we found VEGFR1 mRNA expression as well as functional cell surface receptors on circulating neutrophils, reinforcing our hypothesis of a direct chemotactic action of VEGF-A on neutrophils.

Induction of VEGF-A overexpression in different organs has been shown to induce monocyte recruitment in parallel with VEGF-A-induced perivascular expression of the chemokine CXCL12 (i.e. SDF-1, stromal cell-derived factor-1) [113]. However, under the experimental conditions of our study and during the studied period of 90 minutes, recruitment of monocytes (CX3CR1+/CD115+ leukocytes) by VEGFA was not observed.

Figure 10. Activation of VEGFR1 on neutrophils and of VEGFR2 on endothelium is necessary for in vivo VEGF-A-induced neutrophil recruitment. (A-B) Specific knock-down of either VEGFR1 tyrosine kinase (Flt-1 tk⁻⁻) or of VEGFR2-TSad-Src signaling (tsad⁻⁻) inhibited VEGF-A-dependent neutrophil recruitment to the cremaster muscle. N = 4-7 mice per group. (C-D) VEGF-A dependent neutrophil recruitment was inhibited in WT transplanted with Flt-1 tk⁻⁻ bone marrow whereas neutrophil recruitment to VEGF-A was unaffected in WT transplanted with tsad⁻⁻ bone marrow. N = 5-6 mice per group. #, P < 0.05 compared to basal values within the same group; *, P < 0.05 compared to other groups, as indicated. These diagrams were originally published in Blood [85]. © the American Society of Hematology.

To establish the involvement of the different VEGF receptors, the recruitment experiments were repeated in mice without functional VEGFR1 (Flt-1 tk⁻⁻) or VEGFR2 (tsad⁻⁻). By using these transgenic mice, we demonstrated that VEGF-A-recruitment of neutrophils is dependent on both VEGFR1 and VEGFR2 (Fig. 10 A-B). These results were confirmed in WT treated with
neutralizing antibodies towards these receptors (Flt-1, clone MF1 towards VEGFR1 and Flk-1, clone DC101 towards VEGFR2).

The origin of the involved receptors (*i.e.* endothelial-*versus* neutrophil-expressed) was assessed by creating chimeric mice with impaired VEGFR1- or VEGFR2-signaling specifically on hematopoietic cells (*Flt1 tk*−/− to WT and *tsad*−/− to WT, respectively).

In irradiated WT transplanted with *Flt-1 tk*−/− bone marrow (*Flt-1 tk*−/− to WT), no neutrophils were recruited in response to VEGF-A (*Fig. 10 C-D*), demonstrating that VEGFR1-signaling on neutrophils is indispensable for VEGF-A-induced neutrophil recruitment. Contrasting results were observed in *tsad*−/− to WT chimeric mice as neutrophils adhered to, and transmigrated through the venular wall to a similar extent as in WT transplanted with WT bone marrow (WT to WT; *Fig. 10 C-D*). Thus, the reduced neutrophil recruitment observed in *tsad*−/− in response to VEGF-A was due to loss of VEGFR2 signaling in non-hematopoietic cells, most probably ECs. Upregulation of adhesion molecules upon activation of endothelial VEGFR2 may explain the observed role of VEGFR2 in neutrophil recruitment.

Our results reveal that VEGF-A-induced neutrophil recruitment involves direct activation of VEGFR1 on circulating neutrophils in parallel with activation of VEGFR2 on ECs.

**VLA-4 integrin plays an indispensable role in VEGF-A-induced neutrophil recruitment (Study III)**

During classical inflammation, neutrophil recruitment to the inflamed tissue depends on β2-family integrins: LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) [29]. It is generally accepted that, in the circulation, surface expression of the integrin VLA-4 (very late antigen-4, CD49d/CD29, α4β1 integrin) is restricted to other leukocytes than neutrophils [114, 115]. However, there is growing body of evidence to suggest the existence of β2-independent neutrophil recruitment mechanisms which rely on VLA-4 [116-118].

To investigate if different surface integrins were employed by neutrophils when recruited by a proinflammatory versus a proangiogenic stimulus, the different steps of the neutrophil recruitment cascade were followed by intravital video-microscopy of the mouse cremaster muscle stimulated with MIP-2 or VEGF-A (added to the SF), following pretreatment with neutralizing antibodies towards the alpha subunits of the integrins LFA-1, Mac-1 and VLA-4 (*i.e.* anti-CD11a mAb, anti-CD11b mAb or anti-CD49d mAb, respectively; i.a.).
Figure 11. Neutrophils employ VLA-4 integrin when recruited by VEGF-A. The role of the three major leukocyte integrins (LFA-1, Mac-1 and VLA-4) in neutrophil recruitment (Ly6G-1A8+ leukocytes) induced by VEGF-A was assessed by intravital video-microscopy of the cremaster muscle in mice pretreated with neutralizing mAbs directed to CD11a, CD11b or CD49d. (A) Number of adherent and (B) emigrated neutrophils, as well as the fractions of (C) adherent neutrophils that crawl and (D) of crawling neutrophils that detach from the venular wall. Data collected following 90 min of stimulation with VEGF-A. N = 4-5 mice per treatment group. *, P < 0.05 compared to other groups, as indicated. This figure was originally published in Blood [85]. © the American Society of Hematology.

During activation by the proinflammatory chemokine MIP-2, neutrophil firm adhesion depended exclusively on LFA-1 and the involvement of Mac-1 was restricted to neutrophil crawling, in agreement with previous studies [29]. As expected, inhibition of VLA-4 by treatment with anti-CD49d mAbs had no effect on neutrophil recruitment during MIP-2 activation. These results are in contrast to what was observed following stimulation of the cremaster muscle with the proangiogenic factor VEGF-A. Specifically, a striking reduction in the number of adherent neutrophils was observed in mice pretreated with anti-CD11a or with anti-CD11b (Fig. 11 A), leading to decreased numbers of emigrated neutrophils in both treatment groups (Fig. 11 B). Furthermore, anti-CD49d treatment greatly impaired neutrophil transmigration in response to VEGF-A, despite no significant effect on neutrophil adhesion (Fig. 11 A-
Detailed analysis of the adherent cells revealed that VLA-4 was involved in intraluminal crawling of neutrophils. We observed a 65% reduction in the number of adherent neutrophils that initiated crawling within venules of anti-CD49d-treated mice compared to isotype-treated animals (Fig. 11 C). Furthermore, neutralization of this integrin caused the majority of crawling cells to detach (Fig. 11 D). Consequently, neutrophil emigration to the tissue was completely inhibited by anti-CD49d mAbs in VEGF-A activated muscles.

These data indicate that, in response to VEGF-A, VLA-4 is important to maintain neutrophil firm adhesion and to prevent detachment during intraluminal crawling, but the downstream mechanisms are still unknown. The finding that particular integrins are involved in neutrophil recruitment to VEGF-A, may provide specific means to target angiogenesis without compromising the recruitment of proinflammatory neutrophils, thus limiting potential side effects.

A VEGF-A-responsive neutrophil subpopulation (CD49d⁺VEGFR1<sub>high</sub>CXCR4<sub>high</sub>) exists naturally in the circulation of humans and mice (Study III)

The dramatic effect of anti-CD49d treatment on VEGF-A-induced neutrophil recruitment implies that either a CD49d⁺ circulating neutrophil subtype exists or that VEGF-A stimulation can induce changes in neutrophil integrin expression.

We studied the phenotype of blood neutrophils by flow cytometry and found that 3.2±0.5% of the circulating human neutrophil population (n=12 healthy donors) is positive for this integrin (Fig. 12 A). The identified CD49d⁺ neutrophils expressed higher levels of VEGFR1 than CD49d⁻ neutrophils (Fig. 12 B) demonstrating that an innate pool of circulating VEGF-A-sensitive CD49d⁺ neutrophils exist. This population was also found in the circulation of unchallenged mice. Furthermore, the isolated human CD49d⁺ neutrophils responded in vitro to VEGF-A stimulation with increased chemokinesis whereas CD49d⁻ neutrophils did not respond to the stimulus (Fig. 12 C-D), supporting the concept of a specific circulating neutrophil subtype, which is specifically recruited by VEGF-A to hypoxic sites.

As previously mentioned, leukocyte accumulation in VEGF-A overexpressing tissues has been reported to rely on VEGF-A-induced CXCL12 perivascular expression [113]. These leukocytes were found to express the receptor CXCR4 (CXC chemokine receptor 4, CD184) and to chemotax in vitro to CXCL12 [113]. Furthermore, CXCL12 signaling through neutrophil CXCR4 was reported to increase in vitro VLA-4-dependent adhesion of bone marrow isolated neutrophils to VCAM-1 [119].
Figure 12. A VEGF-A-responsive CD49d⁺VEGFR1⁺CXCR4⁺ neutrophil subset exists naturally in the circulation. (A) Representative flow cytometry plot of human neutrophils isolated from blood (and gated in FSC/SSC and on CD16 expression), labeled with mAb towards CD49d. (B) VEGFR1-derived mean fluorescence intensity (MFI) on CD49d⁺ and CD49d⁻ neutrophils was quantified following staining with VEGFR1 mAbs. N = 12 healthy individuals. (C-D) Polar plots displaying the trajectories of CD49d⁺ or CD49d⁻ neutrophils migrating in stable gradients of VEGF-A. Gradients were formed with high concentration to the right side of the plots. N = 4 independent trials. (E) CXCR4-derived MFI on CD49d⁺ and CD49d⁻ neutrophils was quantified following staining with CXCR4 mAbs. N = 8 healthy individuals. *, P < 0.05. These diagrams were originally published in Blood [85]. © the American Society of Hematology.
Using flow cytometry, we found that the CD49d$^+$ human neutrophils expressed higher levels of CXCR4 compared to CD49d$^-$ neutrophils (Fig. 12 E), suggesting that CXCL12 may be an additional signal for the recruitment of neutrophils to hypoxic sites. Similarly, higher CXCR4 levels were found on CD49d$^+$ mouse neutrophils.

Taken together these results demonstrate that, in both humans and mice, CD49d$^+$VEGFR1$^\text{high}$CXCR4$^\text{high}$ neutrophils constitute a specific circulating neutrophil subtype responsive to direct activation by VEGF-A.

**Specific blockade of the recruitment of circulating CD49d$^+$VEGFR1$^\text{high}$CXCR4$^\text{high}$ neutrophils to hypoxic tissue leads to impaired vessel neoformation (Study III)**

To examine the ultimate contribution of the identified circulating neutrophil subtype CD49d$^+$VEGFR1$^\text{high}$CXCR4$^\text{high}$ to angiogenesis and the possibility to intervene with this process, we used our previously established in vivo model of hypoxia-driven islet graft revascularization (avascular pancreatic islets transplanted to striated muscle) [8, 76]. VLA-4 integrin, demonstrated to be crucial for the recruitment of the characterized neutrophil subset, was targeted during the islet engraftment period (intravenous administration of anti-CD49d mAbs; once-daily, starting 24 h prior to transplantation).

Grafts were examined in vivo by confocal microscopy 4 days after transplantation (Fig. 13 A-D). In mice where CD49d had been neutralized, less than half of the amounts of neutrophils (Ly6G-RB6-8C5 [Gr-1]$^+$ leukocytes, blue) were found at the site of islet engraftment compared with isotype-treated animals. Preventing the recruitment of CD49d$^+$ neutrophils by anti-CD49d treatment decreased the numbers of newly formed islet-juxtaposed vessels to almost 50% (i.e. blood vessels surrounding each islet graft within 30 µm from the islet perimeter; CD31$^+$, red). In addition, the newly formed vasculature often formed larger vascular plexa rather than distinct capillaries, indicating delayed pruning of vessels.

Altogether, we demonstrated that distinct proinflammatory and proangiogenic neutrophil subsets (CD49d$^-$VEGFR1$^\text{low}$CXCR4$^\text{low}$ and CD49d$^-$VEGFR1$^\text{high}$CXCR4$^\text{high}$, respectively) coexist in the circulation of humans and mice and that specific inhibition of the recruitment of the proangiogenic subset to hypoxic tissue impairs vessel neoformation. Thus, angiogenesis can be modulated by targeting cell-surface molecules specifically involved in the recruitment of proangiogenic neutrophils, without compromising the recruitment of the neutrophil population involved in the immune response to pathogens.
Figure 13. Recruitment of CD49d^{+}VEGFR1^{high}CXCR4^{high} neutrophils is important for angiogenesis. (A-B) Representative confocal Z-projections of transplanted pancreatic islets (blue, dashed lines), recruited neutrophils (Ly6G-RB6-8C5 [Gr-1]+ leukocytes, green) and newly formed vasculature (CD31+, red) in the cremaster muscle of mice treated with isotype control or with anti-CD49d mAbs during islet engraftment, and (C-D) respective surface renderings. Images acquired in vivo by confocal microscopy 4 days after transplantation. Scale bars = 50 µm. These images were originally published in Blood [85]. © the American Society of Hematology.
In the first study of this dissertation we have shown, for the first time *in vivo*, that localized interstitial chemokine release leads to formation of an intravascular haptotactic gradient dependent on chemokine sequestration by endothelial HS. Establishment of this chemotactic gradient within the vasculature was found to be crucial for effective guidance of crawling neutrophils to optimal transmigration sites localized closer to the inflammatory source. Finally, we have shown that disruption of this intravascularly formed gradient leads to random intraluminal neutrophil crawling and to decreased neutrophil recruitment to the inflamed tissue, which ultimately results in a severely reduced ability to clear bacterial infections.

In the second study, we have demonstrated a pivotal role for increased vascular permeability during inflammation, particularly in facilitating paracellular transport of tissue-released chemokines into postcapillary venules and thereby neutrophil recruitment to the inflamed tissue. Furthermore, we have shown that the influx of tissue-released chemokines across the venular wall is dampened *in vivo* upon chemokine citrullination by PAD enzymes. As a result, neutrophil recruitment to the inflamed tissue is abrogated.

In the third and final study, we have identified, in humans and mice, a previously undefined proangiogenic circulating subset of neutrophils (CD49d$^+$VEGFR$^1_{\text{high}}$CXCR4$^4_{\text{high}}$) recruited to hypoxic tissue by the proangiogenic factor VEGF-A via activation of VEGFR1 on neutrophils and VEGFR2 on ECs. We found that proangiogenic and proinflammatory neutrophils engage different adhesion molecules during recruitment to tissue, and VLA-4 integrin was shown to be particularly required for crawling and emigration of the proangiogenic subset. When targeted *in vivo*, neutralization of this integrin led to specific inhibition of the recruitment of circulating proangiogenic neutrophils to hypoxic tissue, and to impaired vessel neoformation.

Altogether, the results presented in this dissertation shed new light on the mechanisms by which proinflammatory and proangiogenic stimuli released in the tissue during inflammation or hypoxia are able to regulate neutrophil recruitment from the blood circulation to afflicted sites. These findings open up new possibilities to understand and to control the immune response.
Future perspectives

To study endothelial characteristics (e.g. proteoglycan composition) which may account for the differences in chemokine sequestration across the vascular tree.

To study chemokine transport and sequestration in venular regions with low expression of basement membrane components, reported to be “hot spots” for neutrophil transmigration.

To further understand the role of chemokines within ECs; of their mobilization to the luminal surface; and the involvement of HS and of DARC (Duffy antigen receptor for chemokines) in this process.

To delineate the role of caveolae-dependent endocytosis/transcytosis in leukocyte transmigration to inflamed tissues.

To measure how the ratio between intact and citrullinated CXCL8 changes over time following CXCL8 induction.

To test the ability of proangiogenic neutrophils and proinflammatory neutrophils to induce citrullination and truncation of chemokines.

To assess the ability of truncated IL-8, reported to be proangiogenic, in recruiting the distinct neutrophil subtypes.

To further study the heterogeneity of the two identified neutrophil subsets regarding gene expression profile, ROS-production, phagocytosis ability, chemokine-receptor expression etcetera.

To study neutrophil recruitment and function in a multistimuli environment involving both proinflammatory and proangiogenic stimuli mimicking complex situations like chronic inflammation and tumor growth.
Acknowledgements

The majority of the work included in this dissertation was carried out at the Department of Medical Cell Biology, Division of Integrative Physiology, Uppsala University, Sweden.

I would like to express my gratitude to each and every one who made this work possible, namely:

My supervisor Professor Mia Phillipson;

My co-supervisors Professor Lena Holm and Associate Professor Charlotte Rolny;

The co-authors to the studies: Nora Ausmees, François Binet, Gustaf Christoffersson, Lena Claesson-Welsh, Pär Gerwins, Antoine Giraud, Karin Gustafsson, Carmen Herrera Hidalgo, Elina Hjertström, Johan Kreuger, Jin-Ping Li, Jalal Lomei, Anneleen Mortier, Mia Phillipson, Paul Proost, Charlotte Rolny, Cédric Seignez, Masabumi Shibuya, Israel Vlodavsky, Evelina Vågesjö, Michael Welsh, Simone Weström, and Eyal Zcharia;

The heads of the department, present and formers: Professor Nils Welsh, Professor Arne Andersson and Professor Erik Gylfe;

My colleagues at the Division of Integrative Physiology;

The animal facility staff;

The students involved in the projects: Monica Eriksson, Petra Gradin, Tomas Jinnerot, Oscar Johansson, Cecilia Jäder, Amin Mottahedin, Malin Thyselius, and Hanna Tolf;

Sonchita Bagchi and Laura Gualandi for skilled technical assistance;

The people who over the years have helped me with all sorts of problems and technicalities: Annika Andersson, Jesper Andersson, Mieke De Buck, Ing-Mari Dohlk, Angelica Fasching, Pernille Husberg, Annika Jägare, Mari-
The colleagues who have shared an office with me: David Ahl, Sara Bohman, Gustaf Christoffersson, Stephanie Franzén, Nina Funa, Annika Jägare, Haoyu Li, Åsa Nilsson, Ulrika Pettersson, Olof Schreiber, Cédric Seignez, Anna Siemiatkowska, Sara Stridh and Evelina Vägesjö;

My friends;

Above all my family.

The work on this dissertation was supported by grants from the A. Naumann and B. Carlsson Foundation for Cardiovascular Diseases, the A. and D. Sundqvist Foundation for Cardiovascular and Rheumatic diseases, the Clas Groschinsky Memorial Foundation, the E. and M. Schedin Foundation for Cancer Research, the Harald and Greta Jeansson Foundation, the Family Olinder-Nielsen Foundation, the J. and L. Carlsson Foundation for Research on Cardiovascular Diseases, the Knut and Alice Wallenberg Foundation, the Lars Hierta Memorial Foundation, the Magnus Bergvall Foundation, the Ministry of Education, Culture, Sports, Science and Technology of Japan (Special Project Research on Cancer-Bioscience Grant-in-Aid), the National Cancer Institute, the National Institutes of Health, the P. A. and A. A. Pettersson Foundation for Medical Research, the Royal Swedish Academy of Sciences, the Ragnar Söderberg Foundation, the Regnell Trust for the Medical Faculty, the Sture Hansson Memorial Foundation for Cancer Research, the Swedish Cancer Society, the Swedish Diabetes Foundation, the Swedish Medical Research Council, the Swedish Society for Medical Research, and the Åke Wiberg Foundation.
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Acta Universitatis Upsaliensis

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Editor: The Dean of the Faculty of Medicine

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