From the DEPARTMENT *of* PHYSIOLOGY *and* PHARMACOLOGY
KAROLINSKA INSTITUTET, Stockholm, Sweden

NEUTROPHIL-INDUCED ENDOTHELIAL BARRIER DYSFUNCTION *in* ACUTE INFLAMMATION – MECHANISMS *and* THERAPEUTIC STRATEGIES

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STOCKHOLM 2020

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Published by Karolinska Institutet.
Printed by E-PRINT AB 2020.
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ISBN 978-91-7831-598-7

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Karolinska Institutet

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THESIS FOR DOCTORAL DEGREE (Ph.D.) BY

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Public defense February 14th 2020, 09.00
Samuelssonsalen, Tomtebodavägen 6, Solna

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ABSTRACT

The acute inflammatory response is characterized by recruitment of leukocytes and increased vascular permeability, resulting in the cardinal signs of inflammation; redness, heat, swelling, pain and loss of function. Permeability changes of the vascular wall are important in functional immune responses and host defense. On the other hand, derangement of the vascular barrier is a principal cause for plasma leakage and edema formation in severe disease states such as sepsis, substantially accounting for high morbidity and mortality by contributing to organ dysfunction and circulatory failure. Neutrophil granulocytes, a subtype of leukocytes, are first on sight in the acute inflammatory response where they adhere to, and migrate through, a monolayer of endothelial cells that constitute the innermost layer of the vascular wall. Neutrophil-derived proteins, released from activated neutrophils, cause endothelial barrier disruption via partly unknown mechanisms. Since neutrophil activation and degranulation are considered central in the pathogenesis of acute inflammatory disease states, and novel treatment strategies are sought after, this thesis work aimed to further expand our understanding of mechanisms regulating neutrophil-evoked alterations of the endothelial barrier.

In **paper I**, the role of the kallikrein-kinin system (KKS) in neutrophil-induced vascular leakage was investigated. The KKS is a pro-inflammatory protein complex found in plasma that is responsible for formation of bradykinin (BK), a known inducer of vascular hyperpermeability via binding of bradykinin receptors on endothelial cells. In three different *in vivo* models of acute inflammation in two different species, we found that inhibition of KKS attenuated neutrophil-mediated plasma leakage. Further, *in vitro* studies with isolated human neutrophils and endothelial cells showed that factors secreted from activated neutrophils caused BK-mediated endothelial barrier disruption, and that neutrophil-derived heparin-binding protein facilitated KKS activation caused by neutrophil granule proteases.

In **paper II**, we investigated the therapeutic potential and mode of action of the heparin derivative sevuparin in neutrophil-mediated vascular leak caused by group A *Streptococcus*. *In vivo* and *in vitro* studies showed that sevuparin attenuated endothelial barrier disruption and lung plasma leakage by neutralizing neutrophil-derived proteins. Affinity chromatography and mass spectrometry were utilized to identify proteins targeted by sevuparin, confirming the previously established disruptive role of several neutrophil-derived proteins on endothelial barrier function.

In **paper III**, we tested the hypothesis that platelet-derived polyphosphates (polyP) activate neutrophils, and investigated polyP as a potential therapeutic target in acute inflammation. During inflammation, interaction of activated platelets with neutrophils results in neutrophil activation. Also, platelets are known to release polyP that have been attributed roles in both inflammation and coagulation. We found *in vitro* that polyP induced neutrophil degranulation and *in vivo* that systemic administration of polyP caused lung plasma leakage in a neutrophil-dependent manner. Furthermore, inhibition of polyP decreased lung plasma leakage in a mouse model of acute systemic inflammation induced by group A *Streptococcus*.

In **paper IV**, we set out to investigate the effect of phenylbutyrate (PBA), a short-chain fatty acid suggested to have immunomodulatory properties, on the inflammatory response in a mouse model of pneumonia with *Pseudomonas aeruginosa*. PBA treatment altered the kinetics of neutrophil recruitment in lungs in response to *P. aeruginosa* resulting in enhanced initial mobilization of neutrophils followed by a more rapid decline in cell recruitment compared to no treatment. Coincident with the decline in cell recruitment, lung edema and protein leakage was reduced. *In vitro*, PBA was found to promote release of neutrophil chemotactic factors from lung epithelium.

In conclusion, this thesis work provides new insights into mechanisms regulating endothelial barrier function in neutrophilic inflammation and suggests potential therapeutic strategies.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Akut inflammation uppstår som en konsekvens av infektion med exempelvis bakterier eller virus, eller på grund av vävnadsskada, och kännetecknas av rodnad, värmeökning, **svullnad**, smärta och nedsatt vävnadsfunktion. Den akuta inflammationsreaktionen har en viktig funktion i att bekämpa infektioner och läka skador, men vid allvarliga sjukdomstillstånd såsom sepsis (tidigare kallat blodförgiftning), lunginflammation eller stort trauma kan inflammationssvaret bli så starkt att det i sig bidrar till ökad sjuklighet och dödlighet. Den akuta inflammationsreaktionen börjar med att skadad eller infekterad vävnad skickar ut signalmolekyler som aktiverar blodkärl, som i sin tur aktiverar en viss typ av vita blodkroppar, så kallade **neutrofiler**, som cirkulerar i blodet. Detta leder till att neutrofiler fäster till **endotelceller i kärlväggen** för att sedan vandra ut från blodkärlet till den infekterade eller skadade vävnaden. När detta sker så **orsakar neutrofiler, genom att påverka funktionen hos endotelceller, ett läckage av vätska och proteiner** ut från blodkärlet vilket resulterar i svullnad. Denna svullnad kan i vissa fall, exempelvis vid chocklunga (ARDS) orsakad av sepsis eller lunginflammation, vara så omfattande att den försvårar andning och ger irreversibla skador på lungorna.

I mitt avhandlingsarbete undersökte vi hur plasmaläckage och vävnadssvullnad orsakad av aktiverade neutrofiler uppstår vid akut inflammation, med målet att hitta nya sätt att motverka inflammationssvaret. För att studera detta användes djurmodeller vilka representerade olika inflammatoriska tillstånd, framförallt inflammation i lungvävnad, samt isolerade endotelceller och neutrofiler från människa.

I **delarbete I** fann vi att neutrofil-orsakat plasmaläckage till stor del sker genom att aktiverade neutrofiler frisätter proteiner som i sin tur leder till bildning av bradykinin. Bradykinin är ett ämne som man sedan tidigare vet kan orsaka plasmaläckage genom att påverka endotelceller. Vidare fann vi att HBP, ett protein som frisätts från aktiverade neutrofiler, har en särskild roll genom att förstärka bildningen av bradykinin. I **delarbete II** undersökte vi ifall läkemedlet sevuparin har effekt på neutrofil-inducerat plasmaläckage och vävnadssvullnad. Vi fann att sevuparin motverkar svullnad genom att binda specifika proteiner som frisätts från aktiverade neutrofiler och hämma aktiviteten hos dessa.

Blodplättar finns i blodet och är viktiga för levring av blodet vid blödning. Utöver denna funktion så är de också inblandade i den inflammatoriska processen. I **delarbete III** undersökte vi om ämnet polyfosfat (polyP), som frisätts från aktiverade blodplättar, kan aktivera neutrofiler. PolyP har tidigare visats kunna orsaka vävnadssvullnad med det är inte klarlagt hur. Vi fann att polyP aktiverar neutrofiler vilket leder till frisättning av neutrofil-proteiner och nedsättning av kärlendotelets barriärfunktion.

I delarbete I-III undersöktes olika mekanismer och potentiella behandlingsstrategier med det generella syftet att dämpa ett alltför kraftigt inflammationssvar. En annan lovande strategi, som är relevant framförallt vid inflammation orsakad av infektion, är behandling med läkemedel som kan förbättra immunförsvarets förmåga att bekämpa infektion och främja utläkning. I **delarbete IV** undersökte vi effekten av läkemedlet fenylbutyrat (PBA) på inflammationssvaret. Vi fann att PBA modifierar inflammationssvaret genom att initialt påskynda ansamlingen av neutrofiler i vävnaden, för att sedan orsaka en snabbare minskning av dem. Denna minskning sammanföll med minskad vävnadssvullnad.

Sammantaget så ger denna avhandling ökad kunskap om mekanismer bakom neutrofil-orsakat plasmaläckage och vävnadssvullnad vid akut inflammation, och pekar på ett flertal potentiella angreppspunkter för att motverka läckage från blodkärl vid inflammatoriska sjukdomstillstånd.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following articles, which are referred to in text by their Roman numerals:

I. Kenne E, **RASMUSON J**, Renné T, Vieira ML, Müller-Esterl W, Herwald H, Lindbom L.
(2019)
Neutrophils engage the kallikrein-kinin system to open up the endothelial barrier in acute inflammation
FASEB J. 33(2): 2599-2609

II. **RASMUSON J**, Kenne E, Wahlgren M, Soehnlein O, Lindbom L.
(2019)
Heparinoid sevuparin inhibits Streptococcus-induced vascular leak through neutralizing neutrophil-derived proteins
FASEB J. 33(9): 10443-10452

III. **RASMUSON J**, Kenne E, Lindbom L.
Platelet polyphosphates activate neutrophils and cause lung plasma leakage in acute systemic inflammation
Manuscript.

IV. **RASMUSON J**, van der Does AM, Koppelaar E, Agerberth B, Hiemstra PS, Lindbom L, Kenne E.
Phenylbutyrate treatment ameliorates Pseudomonas aeruginosa-induced lung inflammation in mice
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

AA	Arachidonic acid
AC	Affinity chromatography
ADP	Adenosine diphosphate
AJ	Adherens junction
ALI	Acute lung injury
AMP	Antimicrobial peptide
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
BK	Bradykinin
CAMP	Cathelicidin antimicrobial peptide
CG	Cathepsin G
COX	Cyclooxygenase
CRAMP	Cathelicidin-related antimicrobial peptide
DIC	Disseminated intravascular coagulation
DNAse	Deoxyribonuclease
EC	Endothelial cell
ECP	Eosinophil cationic protein
EPO	Eosinophil peroxidase
FXII	Factor XII
GAG	Glycosaminoglycan
HBP	Heparin-binding protein/azurocidin
HK	High-molecular weight kininogen
hkGAS	heat-killed group A *Streptococcus*
hkPAO1	heat-killed *Pseudomonas aeruginosa* O1
HMGB1	High mobility group box 1
HUVEC	Human umbilical vein endothelial cells
ICAM	Intercellular adhesion molecule
IEJ	Interendothelial junction
IL	Interleukin
KKS	Kallikrein-kinin system
LOX	Lipoxygenase
LT	Leukotriene
MLC	Myosin light chain
MLCK	Myosin light chain kinase

MPO	Myeloperoxidase
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa B
NSAID	Non-steroidal anti-inflammatory drug
PBA	Phenylbutyrate
PG	Prostaglandin
PK	Plasma prekallikrein
PMN	Polymorphonuclear leukocyte
PolyP	Polyphosphate
PSGL1	P-selectin glycoprotein ligand 1
P3	Proteinase 3
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
SCFA	Short-chain fatty acid
TGF-β	Transforming growth factor beta
TJ	Tight junction
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VE-cadherin	Vascular endothelial cadherin

1. INTRODUCTION

1.1 INFLAMMATION

In the case of injury or infection, the host response termed inflammation is mobilized. This complex system of events has the common purpose to protect and restore tissue function by eliminating infectious pathogens and damaged cells, and a functional inflammatory response is crucial for survival. Some common causes for initiation of inflammation are infections with microorganisms, trauma, allergy, autoimmunity and ischemia (Kumar et al., 2007). Although the inflammatory response aims to protect and repair, in some cases the response is misdirected and/or uncontrolled, which leads to host tissue damage so significant that inflammation becomes a major part of pathogenesis. Inflammation can be divided into two types, acute and chronic. Acute inflammation, which is the focus of this thesis work, is initiated rapidly and its severity ranges from minor wounds to critical systemic inflammatory conditions with high mortality. Chronic inflammation on the other hand, is a prolonged process due to an inability to eliminate the inflammatory factor, involving coincident tissue injury and healing.

1.1.1 THE ACUTE INFLAMMATORY RESPONSE

The acute inflammatory reaction takes place in the microcirculation and encompasses elements of the vascular wall such as endothelial cells (EC) and the extracellular matrix, as well as blood-borne elements such as leukocytes, platelets and plasma components (Kumar et al., 2007). The classic clinical manifestations of inflammation were first established by Aulus Cornelius Celsus in the 1st century AD, namely heat (*calor*), redness (*rubor*), swelling (*tumor*) and pain (*dolor*), and in the 19th century Rudolf Virchow contributed with the fifth sign: loss of function (*functio laesa*) (Silva, 1978). All these cardinal signs originate from cellular and molecular events in the microcirculation. Arteriolar dilation generates increased blood flow causing redness and heat, and increased permeability of the vessel wall leads to swelling and edema formation by leakage of plasma into extravascular tissue (Kumar et al., 2007). Coincidently, release of chemical factors mediates pain by the stimulation of nociceptors.

When tissue becomes injured or infected, the cells of the innate immune system in the near environment are exposed to DAMPs or PAMPs (damage/pathogen-associated molecular pattern molecules). DAMPs, also known as danger signals or alarmins, are endogenous factors such as high mobility group box 1 protein, (HMGB1), deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and S100 proteins that are released from cells upon stress, trauma or ischemia. PAMPs on the other hand are pathogen-derived molecules such as lipopolysaccharide (LPS), peptidoglycan and single-stranded RNA (ssRNA) that are expressed on or released from pathogens.

PAMPs and DAMPs are recognized by pathogen recognition receptors (PRRs) such as Toll-like receptors (TLR) on immune cells. Tissue-resident immune cells such as macrophages, mast cells and dendritic cells then initiate the inflammatory response by releasing nitric oxide (NO), histamine, prostaglandins, leukotrienes, cytokines and chemokines that affect the cells within the vascular wall. NO, histamine and prostaglandins contribute to vasodilation by affecting smooth muscle cells, and cytokines stimulate ECs to express leukocyte adhesion molecules (Newton and Dixit, 2012). These events then contribute to the recruitment of leukocytes that leads to extravasation of leukocytes from blood to injured or infected tissue. Moreover, prior to and concurrent with leukocyte adhesion to the endothelium, vasoactive mediators that increase the permeability of the EC barrier are released from affected tissue and leukocytes, leading to leakage of plasma out to the extravascular space. Extravasated plasma leads to edema formation and contains important components of the immune system such as complement factors and immunoglobulins. The fact that the acute inflammatory response at its core is a functional response is important to emphasize. Inflammation is crucial for a successful immune response since it kills pathogens, engulfs damaged cells and paves the way for the adaptive immune system. Also, it initiates wound healing and tissue repair.

1.1.2 ACUTE SYSTEMIC INFLAMMATION AND LUNG INJURY

However functional the inflammatory response might be, there are occasions where it becomes misdirected or uncontrolled. Acute systemic inflammation can develop as a consequence of bacterial infection as well as from sterile inflammation caused by for example trauma or acute pancreatitis, to name a few. Acute systemic inflammation is characterized by increased levels of proinflammatory cytokines such as interleukin-1 (IL-1), interleukin 6 (IL-6) and tumor necrosis factor (TNF). They are in large responsible for the clinical signs fever, leukocytosis and an increase in acute-phase proteins such as C reactive protein. In severe cases of both infectious and noninfectious disease, cytokine release is so massive that it is referred to as a cytokine storm (Tisoncik et al., 2012). This leads to uncontrolled inflammation and distributive shock due to systemic effects on blood vessel tone, endothelial permeability and leukocytes, resulting in hypotension, leaky vessels and the risk of multiple organ dysfunction syndrome (MODS). Organ systems that are commonly affected in MODS are lungs, kidneys, liver, the cardiovascular system and central nervous system.

Respiratory failure as a consequence of an acute systemic inflammatory response can be defined as acute lung injury (ALI), or the more severe form acute respiratory distress syndrome (ARDS). In clinical practice, ARDS is diagnosed and graded according to the Berlin definition (Ranieri et al., 2012),

a classification system that takes into account the time span of onset of respiratory symptoms, radiographic findings on chest x-ray, noncardiogenic pulmonary edema and hypoxemia. Further, the severity is graded based on the partial pressure of arterial oxygen as a ratio of fraction of inspired oxygen. ALI was the term for the mildest form of ARDS in earlier definitions, but is still used frequently in animal studies. ALI/ARDS are severe disease states that represent a major clinical challenge in intensive care with a mortality rate of up to 45% (Maca et al., 2017). A central concept in the pathogenesis of ALI/ARDS is dysregulated inflammation. The progression of ALI/ARDS comprises deterioration of endothelial and epithelial barriers that lead to an increased alveolar-capillary barrier permeability. This results in extravasation of plasma into interstitial and alveolar compartments, consequently impairing oxygenation and respiratory function. The formation of lung edema coincides with accumulation of leukocytes, in particular neutrophil granulocytes, which have a decisive function in innate immune host defense against invading pathogens. However, besides their protective capacity, neutrophils are also known to cause disruption of endothelial barriers and to thereby contribute to the pathophysiology of ALI/ARDS (Matthay et al., 2012).

Sepsis is a common cause of acute systemic inflammation and ALI/ARDS (Monahan, 2013). Sepsis is according to the latest definition, a "life-threatening organ dysfunction resulting from dysregulated host responses to infection", and furthermore septic shock is a more severe form of sepsis "in which underlying circulatory, cellular and metabolic abnormalities are profound enough to substantially increase the risk of mortality" (Cecconi et al., 2018). The mortality rate for sepsis is up to 30% and for septic shock it is up to 60% (Cecconi et al., 2018). Sepsis can be caused by basically any pathogenic microorganism and some of the most common pathogens are Gram-positive bacteria such as *Staphylococcus aureus*, and Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* (Cecconi et al., 2018). Another pathogen that causes sepsis and septic shock is the Gram-positive *Streptococcus pyogenes*, also known as group A *Streptococcus* (GAS). GAS causes common infections such as 'strep throat', impetigo, scarlet fever and erysipelas, all fairly easily cured with antibiotics. However, GAS is also responsible for more severe infections such as pneumonia and necrotizing fasciitis that at times lead to the life-threatening septic condition streptococcal toxic shock syndrome (STSS) (Stevens and Bryant, 2016).

1.2 THE MICROCIRCULATION IN ACUTE INFLAMMATION

1.2.1 THE VASCULAR WALL AND ENDOTHELIAL CELLS

The mechanisms of the acute inflammatory response, ultimately leading to edema formation, take place in the microcirculation. The microcirculation comprises arterioles, capillaries and venules. The vascular wall within the microcirculation is built up by a monolayer of endothelial cells that are attached to a basement membrane, and to a various extent pericytes and smooth muscle cells (Alberts et al., 2002). The endothelium constitutes a semipermeable barrier that under normal physiological conditions regulates the in- and outflow of fluid, plasma solutes and immune cells between intra- and extravascular spaces, making it a decisive factor in maintaining tissue-fluid homeostasis (Sukriti et al., 2014). The EC barrier has a sifting role, allowing water, electrolytes and other small solutes to pass freely through the interendothelial junctions, named the paracellular route. Larger molecules like albumin can in a controlled manner also pass through the healthy EC barrier by vesicular transport with caveolae, named the transcellular route (Sukriti et al., 2014).

Endothelial cells form a single monolayer with cobblestone appearance at the innermost layer of the vascular wall. They are attached to one another with different types of interendothelial junctions (IEJs). Tight junctions (TJs) are IEJs that are highly expressed in arteries and throughout the blood-brain-barrier and have a stabilizing effect on the EC barrier. They are formed by the proteins occludin, claudin and junctional adhesion molecules (JAMs) (Sukriti et al., 2014). Adherens junctions (AJs), another type of IEJs, have an important role in the regulation of EC permeability to plasma proteins. They are formed by homotypic adhesion of the transmembrane protein vascular endothelial cadherin (VE-cadherin). TJs and AJs are in close contact with the intracellular actin cytoskeleton that is highly involved in inflammatory increases in permeability. Besides TJs and AJs, gap junctions also connect endothelial cells. They are formed by the protein connexin and mainly seem to have a role in direct cell-to-cell transport of solutes and signal molecules (Sukriti et al., 2014). On the basolateral side of EC is the basement membrane that is composed of collagens, laminins and fibronectin. The endothelial cells bind to the basement membrane via cell-matrix focal adhesions with integrins, and these junctions are also known to be involved in the regulation of vascular permeability (Yuan et al., 2012).

On the luminal surface of the endothelium is a gel-like layer of glycoproteins, proteoglycans and glycosaminoglycans (GAGs) called the glycocalyx. The majority of the GAGs (e.g. heparan sulfate and chondroitin sulfate) are negatively charged and therefore bind several plasma proteins by

electrostatic forces. In normal physiology, the glycocalyx composes a barrier that limits vascular permeability, and that maintains an anti-adhesive and anti-coagulant layer. In inflammatory disease states, such as sepsis, degradation of the glycocalyx may contribute to increased vascular permeability (Uchimido et al., 2019).

1.2.2 BLOOD FLOW IN INFLAMMATION

The two cardinal signs redness and heat are both a consequence of arteriolar vasodilation in the microcirculation. Vasodilation is achieved by vasoactive mediators released from residing immune cell, endothelial cells and blood-borne leukocytes. NO and prostaglandins, such as prostaglandin I_2, are released from ECs and immune cells, and histamine and bradykinin are released from leukocytes. The arteriolar vasodilation leads to an increased blood flow that in turn enhances the hydrostatic intravascular pressure, partly contributing to an extrusion of fluid and proteins (Pober and Sessa, 2014) according to Starling's equilibrium.

1.2.3 VASCULAR PERMEABILITY IN INFLAMMATION

Edema formation in inflammation is brought on by leakage of plasma into the extravascular space due to an increase in vascular permeability. This allows the passage of blood components to the extravascular environment and aids in clearing tissue from harmful stimuli. Vascular permeability is increased by alterations of the integrity of the EC barrier. Upon stimulation with edemagenic agents, reviewed further in the next section, the IEJs of ECs open up and cytoskeletal reorganization cause ECs to contract, resulting in formation of interendothelial gaps. The cytoskeletal change induced in inflamed endothelium is known as stress fiber formation, a typical appearance caused by polymerization of actin and myosin filaments (Sukriti et al., 2014). Binding of a permeability-increasing mediator to an EC induces actomyosin contractile activity via myosin light-chain (MLC). MLC is phosphorylated by myosin light-chain kinase (MLCK) that can be activated by multiple signals, including increased cytosolic Ca^{2+}, protein kinase C and tyrosine kinases. Furthermore, Rho-associated kinase (ROCK) is activated by RhoA, a small GTPase, and contributes to increased activity of MLC (Rigor et al., 2013). Besides contraction of ECs, there is also a disassembly of IEJs following stimulation with inflammatory mediators. Of the IEJs, especially AJs are suggested to be involved in increases in permeability due to phosphorylation of VE-cadherin (Dejana and Vestweber, 2013). In summary, both contraction of cells due to actomyosin activation and retraction of cells caused by disassembly of IEJs, are important mechanisms of the formation of interendothelial gaps.

1.2.4 MEDIATORS OF VASCULAR HYPERPERMEABILITY

There are several known inflammatory mediators that increase vascular permeability via direct effects on EC. Thrombin, histamine, bradykinin, cysteinyl leukotrienes, vascular endothelial growth factor (VEGF) and TNF are all known to destabilize the endothelial barrier (Mehta et al., 2014, Duah et al., 2013). Our work centered around the role of bradykinin in acute inflammation.

1.2.4.1 Bradykinin

Bradykinin (BK) is a short-lived nonapeptide (9 amino acids) that is known to increase endothelial permeability. BK is formed upon activation of the kallikrein-kinin system (KKS), an inflammatory response mechanism constituted by the plasma proteins prekallikrein (PK), factor XII (FXII) and high molecular weight kininogen (HK) (Schmaier, 2016). Activation of FXII in turn activates PK that subsequently cleaves HK that result in BK formation. Besides FXII-dependent activation of KKS, PK can also be activated by prolylcarboxypeptidase (PRCP) located on ECs (Shariat-Madar et al., 2002), and neutrophil-derived proteases have been found to directly liberate BK from kininogens (Imamura et al., 2002, Stuardo et al., 2004, Kahn et al., 2009). Besides HK, there is also low molecular weight kininogen (LK) and both have the common domain 4 that is the part that forms BK upon proteolytic cleavage (Schmaier, 2016). The plasma concentration of HK is about 100 μg/ml and it binds to ECs, neutrophils and platelets via heparan sulfate, urokinase receptor (uPAR) and others. Furthermore, HK binds M protein, a membrane-bound virulence factor on group A *Streptococcus* (Ben Nasr et al., 1995). The main function of HK is thought to be liberation of BK, which in turn induces EC barrier disruption by binding the G-protein-coupled bradykinin B_1 and B_2 receptors on ECs. Bradykinin B_2 receptor is constitutively expressed and bradykinin B_1 receptor is upregulated during inflammation (Schmaier, 2016). BK-induced increase in endothelial permeability has been suggested to involve both ROCK and MLC signaling, leading to cytoskeletal stress fiber formation (Ma et al., 2012), as well as disassembly of VE-cadherin (Orsenigo et al., 2012).

1.2.4.2 Others

Leukotrienes (LTs) and prostaglandins (PGs) are lipid mediators with numerous roles in inflammatory processes, and they are formed by enzymatic processing of arachidonic acid. Whereas PGs mainly have functions regulating vasodilation, coagulation and induction of fever, and are formed by cyclooxygenases (COX) by several cell types (Ricciotti and FitzGerald, 2011), leukotrienes are more involved in immune cell recruitment and alterations in EC permeability, and are formed by lipoxygenases (LOX)

mainly in immune cells. Leukotriene B_4 (LTB_4) is a potent chemoattractant for neutrophils and induces neutrophil activation and degranulation. LTC_4, LTD_4 and LTE_4, called the cysteinyl leukotrienes, have, amongst other effects, a known capacity to induce vascular hyperpermeability (Busse, 1998).

Thrombin is a serine protease formed from prothrombin that has a key role in both primary hemostasis by activating platelets as well as secondary hemostasis by mediating fibrin formation (Posma et al., 2016). Furthermore, thrombin causes EC barrier disruption by binding protease-activated receptor 1 (PAR-1) on ECs, leading to RhoA and MLCK activation (Sukriti et al., 2014).

Histamine is a hormone and an inflammatory mediator predominantly originating from mast cells and basophil granulocytes. Upon pathogenic or allergenic antigen binding to membrane-bound IgE antibodies, histamine is released and binds histamine H_1 receptor on ECs, which induces EC barrier disruption by increasing intracellular Ca^{2+}, MLCK activation and also by phosphorylation of AJs and TJs (Sukriti et al., 2014).

As compared to the previously mentioned mediators, TNF is known to destabilize the EC barrier in a more delayed fashion by the induction of nuclear factor kappa B (NF-κB) transcription, increased cytokine production and upregulation of leukocyte adhesion molecules such intercellular adhesion molecule 1 (ICAM1) (Sukriti et al., 2014).

1.3 LEUKOCYTES

Leukocytes are the main defenders of the human body and they appear in many different forms. They are divided into polymorphonuclear (PMNs) and mononuclear leukocytes. The PMNs have irregularly shaped nuclei and they are all granulocytes since they contain cytoplasmic granules. The PMNs are further divided into neutrophils, eosinophils and basophils. Neutrophil granulocytes are the most abundant leukocytes, constituting 50-70% of leukocytes, and will be further reviewed below. Neutrophils are often referred to as PMNs, since they normally constitute about 95% of the PMNs. Eosinophil granulocytes are involved in the host response against parasitic infections and also, like basophil granulocytes, in allergic inflammation. The mononuclear leukocytes are the monocytes and the lymphocytes (such as B- and T-cells). Furthermore, there are tissue-resident leukocytes such as macrophages, dendritic cells and mast cells (Boron and Boulpaep, 2012).

1.3.1 LEUKOCYTE RECRUITMENT

Extravasation of leukocytes, which is mainly restricted to the postcapillary venules, has been studied for almost 200 years (first described by Dutrochet in 1824). The mechanisms regulating recruitment of leukocytes during acute inflammation is known as the leukocyte adhesion cascade and is divided into a series of steps; margination, capture, rolling, slow rolling, arrest, adhesion strengthening and spreading, intravascular crawling, and paracellular and transcellular migration (Ley et al., 2007).

Initially, smooth muscle cell relaxation and vasodilation enables increased contact between leukocytes and endothelium, termed margination. When in contact with EC, leukocyte rolling is mediated predominantly by the selectins (L-, P- and E-selectin) interacting with P-selectin glycoprotein ligand 1 (PSGL1) and other as yet unknown ligands. L-selectin is expressed on leukocytes and P- and E-selectins are expressed on activated EC. P-selectin is also upregulated on activated platelets. Slow rolling occurs as a next step due to interaction of PSGL1 and β_2 integrins on leukocytes with E-selectin and ICAM1 on activated ECs. Two important β_2 integrins on PMNs are CD11a/CD18 (lymphocyte function-associated antigen 1, or LFA1) and CD11b/CD18 (macrophage receptor 1, or MAC1) (Ley et al., 2007). During slow rolling, leukocytes are activated by chemotactic mediators such as interleukin-8 (IL-8) and LTB_4 presented on EC. Activation of leukocytes results in a rapid activation of integrins (conformational change by inside-out signaling) that then bind ICAM1 and vascular cell adhesion molecule 1 (VCAM1) on EC. This leads to arrest and firm adhesion of leukocytes (Ley et al., 2007). β_2 integrins are essential for adhesion of neutrophils, as adhesion as well as subsequent transmigration is abolished upon blocking the function of CD18 (Arfors et al., 1987). Before they migrate through the vessel wall, leukocytes crawl on the endothelium to find a suitable site for extravasation, and intraluminal crawling has been shown to be dependent on MAC1 and ICAM1 interaction (Phillipson et al., 2006). Two distinct pathways for leukocytes through the endothelium have been found: a paracellular route where leukocytes migrate through interendothelial junctions, and a transcellular route where they pass through the body of the ECs (Ley et al., 2007).

1.3.2 NEUTROPHIL GRANULOCYTES

Neutrophils are the main effectors of acute inflammation and they are first on site following tissue injury or infection. In homeostasis, they are continuously released from bone marrow and circulate the blood for a short period of time before they end up in for example liver or spleen and go into apoptosis (Kubes, 2018). During inflammation, the release of neutrophils

from bone marrow is increased up to ten times. In humans, neutrophils constitute 50-70% of leukocytes in blood, in difference to mice where they make up 10-25%. Neutrophils have a segmented nucleus and contain secretory vesicles and three types of cytoplasmic granules. The granules are formed in a specific order during neutrophil maturation and are traditionally divided into azurophilic (primary), specific (secondary) and gelatinase (tertiary) granules (Cowland and Borregaard, 2016). The granules and secretory vesicles together contain several hundreds of proteins.

For the eradication of pathogens and noxious stimuli, neutrophils can apply both intra- and extracellular methods. Intracellular microbial killing following phagocytosis is performed with the use of reactive oxygen species (ROS) and granule-derived bactericidal proteins in the phagolysosome (Kolaczkowska and Kubes, 2013). Reactive oxygen species such as superoxide anion ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl anions (OH^-), hydroxyl radicals ($^{\bullet}OH$) and hypochlorous acid (HOCl) are produced by nicotinamide adenine dinucleotidephosphate (NADPH) oxidase. ROS production in inflammation is to a considerable degree caused by neutrophils, and besides actions in the phagolysosome, ROS can also be released extracellularly, called a respiratory burst (Meegan et al., 2017).

Degranulation and release of granule content is another mechanism of neutrophils to extracellularly combat pathogens. Degranulation is mediated following activation of neutrophils by for example IL-8, LTB_4 or bacterial components that via membrane-bound receptors mediate intracellular signaling involving increase in cytosolic Ca^{2+} and actin cytoskeletal remodeling. This results in granule exocytosis with release of soluble granule-derived proteins and presentation of membrane bound granule-specific receptors on the neutrophil surface (Lacy, 2006). It is generally considered that the secretory vesicles are the first to be released, followed in an orderly fashion by gelatinase, specific and finally azurophilic granules. In table 1, a selection of the most abundant granule proteins in their respective granule subset is shown.

The secretory vesicles are considered to be formed by endocytosis since they contain plasma proteins such as albumin and α-1-antitrypsin. Further, they house membrane bound receptors towards pathogens and factors of the complement system, as well as β_2 integrins (Rorvig et al., 2013). The contents of the gelatinase and the specific granules somewhat overlap. Matrix metalloproteinase-9 (MMP9) that cleaves gelatin and collagen is found in gelatinase granules. Cathelicidin antimicrobial peptide (CAMP), which is cleaved into LL-37 upon release, is located in both types of granules and have antimicrobial effects. Neutrophil gelatinase-associated lipocalin (NGAL) is found in specific granules and has, amongst other, bacteriostatic effects. The

azurophil granules contain the serine proteases neutrophil elastase (NE), proteinase 3 (P3), cathepsin G (CG) and the enzymatically inactive heparin-binding protein (HBP/azurocidin), together called the serprocidins. They have various antimicrobial effects and are also involved in recruitment of immune cells (Cassatella et al., 2019). Furthermore, they are known to increase endothelial permeability. HBP has also been localized in secretory vesicles of neutrophils (Tapper et al., 2002). Also, in the azurophil granules myeoloperoxidase (MPO), the defensins (human neutrophil peptides 1-3) and bactericidal permeability-increasing protein (BPI), all with antimicrobial effects, are found (Cassatella et al., 2019).

Beyond respiratory burst of ROS and degranulation of granule proteins, neutrophils can undergo neutrophil extracellular trap (NET) formation, where the neutrophil extrudes a web of DNA, histones, granule proteins and cytoplasmic proteins, which has the ability to trap and kill pathogens (Kolaczkowska and Kubes, 2013). NETs were first discovered in 2004 as a novel pathogen-killing mechanism (Brinkmann et al., 2004), and since then both pathogens and host factors have been found to induce NET formation.

Table 1. Neutrophil granule subsets and most abundant proteins in each type of granules and secretory vesicles.[A]

Azurophil granules	Specific granules	Gelatinase granules	Secretory vesicles
Myeloperoxidase (MPO) Cathepsin G (CG) Proteinase 3/Myeloblastin (P3) Neutrophil elastase (NE) Heparin-binding protein (HBP)/Azurocidin Neutrophil serine protease 4 (NSP4) Bactericidal permeability-increasing protein (BPI) Defensins (HNPs) Lysosomal proteases	Lactoferrin Collagenase Lysozyme Neutrophil gelatinase-associated lipocalin (NGAL) Pentraxin 3 Haptoglobin Cathelicidin antimicrobial peptide (CAMP)/LL-37 Olfactomedin-4 Complement 3a receptor	Gelatinase B/Matrix metalloproteinase-9 (MMP9) Ficolin-1 Cathelicidin antimicrobial peptide (CAMP)/LL-37	β_2 integrins Heparin-binding protein (HBP)/Azurocidin Complement receptor 1 Formyl peptide receptor (FPR) Plasma proteins Toll-like receptors (TLRs)

[A] Based on previous work (Rorvig et al., 2013, Cowland and Borregaard, 2016, Cassatella et al., 2019).

1.4 NEUTROPHIL-MEDIATED VASCULAR LEAKAGE

In acute inflammation, when neutrophils adhere to activated endothelium of a vessel wall in the microcirculation, endothelial permeability increases and plasma starts to leak out to the interstitial space. That neutrophils induce plasma leakage has been known for a long time (Wedmore and Williams, 1981). Following these observations it was found that adhesion of neutrophils to EC is mediated via β_2 integrins, and that mere adhesion, and not necessarily extravasation of neutrophils, is enough to induce plasma leakage (Arfors et al., 1987, Gautam et al., 2000).

There are several mechanisms suggested to be involved in neutrophil-induced microvascular leakage. Upon binding of β_2 integrins to ICAM1, intracellular signaling in EC ultimately result in interendothelial gap formation. MLC phosphorylation by MLCK, as well as RhoA and ROCK activation, is involved in neutrophil-induced EC stress fiber and interendothelial gap formation (Yuan et al., 2002, Breslin and Yuan, 2004). Furthermore, tyrosine phosphorylation of focal adhesion kinase (FAK) was also shown in EC upon neutrophil stimulation (Guo et al., 2005). Neutrophils also have known disruptive effects on the interendothelial junctions by affecting VE-cadherin (Tinsley et al., 2002, Wessel et al., 2014).

Following stimulation with for example LTB_4 or complement factor 5a (C5a), or upon engagement of β_2 integrins, neutrophils are activated resulting in release of mediators, such as granule proteins, with effects on the EC barrier. This can occur either as a consequence of neutrophil adhesion, whereby the endothelium is affected both via ICAM1-signaling as well as via paracrine mechanisms, or it can occur in non-adherent neutrophils. Supernatants from neutrophils stimulated with LTB_4 or C5a have been found to contain granule proteins and to induce EC hyperpermeability (Breslin and Yuan, 2004, Di Gennaro et al., 2009). In a study by Gautam and colleagues, antibody cross-linking of β_2 integrins, as a way of mimicking neutrophil adhesion to EC, resulted in outside-in signaling and release of cationic neutrophil-derived proteins that induced EC stress fiber formation and plasma leakage (Gautam et al., 2000). In line with this, M protein, a virulence factor of GAS, was shown to induce neutrophil degranulation of all subsets of granules via binding of β_2 integrins (Soehnlein et al., 2008a).

Upon activation via soluble mediators or receptor engagement, neutrophils release granule proteins, ROS as well as NETs, and they have all been found to contribute to EC barrier disruption. Granule proteins such as the serine proteases NE, CG and P3 are released from activated neutrophils and increase endothelial permeability. They cleave AJs and components of the extracellular matrix (Sharony et al., 2010), and also cleave receptors, thereby

mediating intracellular signaling promoting cytoskeletal rearrangement (Korkmaz et al., 2010). HBP/azurocidin, which is enzymatically inactive, has also been shown to increase permeability (Gautam et al., 2001, Herwald et al., 2004, Bentzer et al., 2016). The granule protein myeloperoxidase (MPO) is abundantly expressed in neutrophils and facilitates formation of microbicidal reactive oxidants. MPO catalyzes the formation of hypohalous acids, such as hypochlorous acid, that has damaging effects on EC and increase EC permeability (Patterson et al., 2014). Eosinophil peroxidase (EPO) and eosinophil cationic protein (ECP), granule proteins mainly considered to originate from eosinophil granulocytes but that are also found in neutrophils, are other mediators that increase vascular permeability (Minnicozzi et al., 1994). Inhibition of ROS production or treatment with antioxidants decreased neutrophil-induced inflammation (Zhu and He, 2006, Boueiz and Hassoun, 2009), and both effects on IEJs and effects on intracellular signaling and cytoskeletal reorganization has been proposed (Mittal et al., 2014).

Data over the last decade has indicated that NETs can affect endothelial permeability (Ma et al., 2019). NETs have destructive effects on ECs *in vitro* (Saffarzadeh et al., 2012), and were suggested to cause plasma leakage *in vivo* (Caudrillier et al., 2012). NETs are constituted by nuclear, granular and cytosplasmic proteins (Urban et al., 2009). Nuclear-derived histones are found in NETs and have been shown to cause endothelial and epithelial disruption (Abrams et al., 2013, Saffarzadeh et al., 2012, Wildhagen et al., 2014). Furthermore, the cytoplasmic calcium-binding proteins S100A8, A9 and A12 are also found in NETs and they all increase endothelial permeability (Wang et al., 2014, Wittkowski et al., 2007).

Transendothelial migration of neutrophils and increased vascular permeability has previously been considered as coupled events whereby neutrophils create holes in the EC barrier as they extravasate that lead to leakage of plasma. As of today, this concept is revised following several studies showing spatial and temporal uncoupling of these events (for a review, see (He, 2010)). In addition, it was recently shown that regulation of leukocyte extravasation and vascular permeability differed in terms of tyrosine phosphorylation of VE-cadherin (Wessel et al., 2014). As a mechanistic basis for how the EC barrier remains its integrity during neutrophil diapedesis, Heemskerk and colleagues found that EC form tight pores involving F-actin that allows transmigration without leakage of plasma (Heemskerk et al., 2016).

1.4.1 NEUTROPHILS IN ACUTE SYSTEMIC INFLAMMATION AND LUNG INJURY

Neutrophil recruitment and activation are considered key events in the development of ALI and ARDS, feared complications of either direct pulmonary insult or of indirect acute systemic inflammatory conditions (Grommes and Soehnlein, 2011, Williams and Chambers, 2014, Rebetz et al., 2018). Neutrophils have been called double-edged swords, due to that they possess both important protective antimicrobial effects as well as tissue-destructive capacity. Sepsis and septic shock can lead to the development of ARDS, and in the pathogenesis of sepsis and septic shock, neutrophils play a major role (Sonego et al., 2016, Stiel et al., 2018). In patients with ARDS, neutrophil count in broncho-alveolar lavage (BAL) fluid was found to correlate with ARDS severity (Aggarwal et al., 2000). Furthermore, neutrophil depletion in different animal models of ALI has been found to improve pulmonary microcirculation and to prevent plasma leakage (Park et al., 2019, Looney et al., 2006). In support of a role for neutrophil-derived proteins in ALI/ARDS, intravenous administration of streptococcal M protein was found to cause ALI by inducing neutrophil degranulation (Soehnlein et al., 2008a). Also, α-defensins were found to disrupt the capillary-epithelial barrier and cause lung injury in mice (Bdeir et al., 2010), and neutrophil elastase has been found to take part in ALI pathogenesis (Kawabata et al., 2002). The dysregulated activation of neutrophils during septic shock, including release of NETs, also contributes to activation of coagulation that can develop into septic shock-induced coagulopathy. Immunothrombosis is a mechanism of intravascular immunity that allows for capture of microorganisms in microthrombi and that involves NETs. However, uncontrolled activation of immunothrombosis, suggested to be mediated by neutrophils, can lead to disseminated intravascular coagulation (DIC), a feared complication in septic shock (Stiel et al., 2018).

1.5 PLATELETS IN NEUTROPHILIC INFLAMMATION

Platelets are anuclear cell fragments derived from megakaryocytes that have important functions in restricting bleeding from vessels following injury – a process termed hemostasis and that also include coagulation factors. Platelets and coagulation factors also take part in thrombosis, the pathological formation of blood clots in the vessel lumen. Besides their role in hemostasis and thrombosis, platelets are also highly involved in inflammation and host response to infections (Deppermann and Kubes, 2018). Platelets adhere to endothelium during inflammation and are suggested to be important in the progression of ALI (Zarbock and Ley, 2009). When activated, platelets and neutrophils form platelet-neutrophil

complexes that contribute to neutrophil-mediated lung edema. Inhibition of platelet-neutrophil complex formation, and also depletion of either neutrophils or platelets, has been found to reduce lung edema in ALI (Zarbock et al., 2006, Looney et al., 2009). Furthermore, in models of sepsis and ALI, neutrophil activation resulting in NET formation was found to be dependent on platelet-neutrophil interactions (McDonald et al., 2012, Caudrillier et al., 2012).

Both paracrine and receptor-mediated mechanisms for platelet-induced neutrophil activation have been found. Neutrophils are activated upon binding of P-selectin to PSGL-1 on neutrophils, as well as by interaction of the membrane glycoprotein GPIbα or platelet integrins with β_2 integrins on neutrophils (Lisman, 2018). Also, platelet-mediated NET formation was shown to be induced via HMGB1 presented on the surface of platelets (Maugeri et al., 2014). Furthermore, the chemokines CXCL7 and CCL5-CXCL4 heteromers displayed paracrine effects on neutrophils following release from activated platelets, thus contributing to ALI in mice (Bdeir et al., 2017, Grommes et al., 2012). Another paracrine mediator of platelet-induced neutrophil activation is serotonin that recently was found to induce neutrophil degranulation and to take part in myocardial ischemia-reperfusion injury (Mauler et al., 2019).

Platelets contain two types of granules, α-granules and dense granules, which house more than 300 membrane-bound and soluble mediators that can be mobilized upon platelet activation (Golebiewska and Poole, 2015). Inorganic polyphosphates (polyP) are linear polymers of inorganic phosphate residues that are located in dense granules of platelets (Ruiz et al., 2004). PolyP is furthermore found in granules of mast cells and basophil granulocytes (Moreno-Sanchez et al., 2012). PolyP was shown to be released from activated platelets and to stimulate both coagulation and inflammation (Morrissey and Smith, 2015), and also to mediate bradykinin formation and plasma leakage by activating FXII (Muller et al., 2009). Furthermore, polyP was found to increase EC permeability, enhance adhesion molecule expression on EC and to induce neutrophil recruitment (Bae et al., 2012, Hassanian et al., 2015). Besides residing in mammalian cells, inorganic polyphosphates are also found in prokaryotes and can differ in length from only a few phosphate residues to up to thousands. Polymer length has been found to affect the capacity of polyP to induce coagulation as well as its proinflammatory potential (Morrissey and Smith, 2015, Brown and Kornberg, 2004).

1.6 RESOLUTION OF INFLAMMATION

Following an inflammatory response, the immune system initiates a resolving phase aimed at restoring tissue function. After neutrophils have arrived to a site of injury or infection, they actuate a second swell of immune cells by recruiting inflammatory monocytes that aid in neutralizing the cause for inflammation (Soehnlein et al., 2008b). When this task is fulfilled, neutrophils become apoptotic and release mediators that signal to abort further infiltration of neutrophils (Ortega-Gomez et al., 2013, Soehnlein and Lindbom, 2010). Chemokine depletion by enzymatic cleavage or sequestration terminates the recruitment of neutrophils and monocytes, and apoptotic neutrophils release annexin A1, that promotes further apoptosis, and lactoferrin, that has anti-inflammatory properties (Li et al., 2012, Ortega-Gomez et al., 2013). Pro-inflammatory macrophages start engulfing apoptotic neutrophils, a term called efferocytosis, and at the same time switch their phenotype and become pro-resolving. They stop producing pro-inflammatory cytokines and eicosanoids such as TNF and LTB_4, and instead begin to release interleukin 10 (IL-10) and transforming growth factor β (TGF-β), two cytokines with anti-inflammatory effects. Following this switch, the resolution-phase macrophages also increase their ability to present antigen and stimulate recruitment of B- and T-cells (Ortega-Gomez et al., 2013).

Eicosanoids, lipid mediators derived from arachidonic acid (AA) or other polyunsaturated fatty acids (PUFAs), play a central role in both initiation and resolution of inflammation. Prostaglandins and leukotrienes, derived from AA with cyclooxygenases (COX) and lipoxygenases (LOX), respectively, induce vasodilation and increase in vascular permeability, and promote neutrophil activation and recruitment during the initiation of inflammation (Serhan et al., 2008). Whereas 5-LOX mainly converts AA into pro-inflammatory leukotrienes, 12-LOX and 15-LOX instead can convert AA into lipoxins that are mediators with both anti-inflammatory and pro-resolving activities. Furthermore, LOX can convert ω3-PUFAs into resolvins and protectins that have similar activities as lipoxins. The difference between anti-inflammatory and pro-resolving activities is that pro-resolution mediators are not immunosuppressive, but stimulate resolution by enhancing recruitment of monocytes, promoting macrophages to phagocytose apoptotic cells and microbes, as well as by inducing expression of antimicrobial mediators (Serhan et al., 2008).

1.7 MODULATION OF NEUTROPHILIC INFLAMMATION

Since inflammation is a significant factor in the pathogenesis of several disease states, ways to control inflammation have been studied extensively. As of today, there are numerous anti-inflammatory agents targeting different pathways that are used to inhibit the inflammatory response. However, new approaches are still sought after due to limited and/or adverse effects. An intricate challenge is that on the one hand there is a need to find better ways to control the inflammatory host response, and on the other not to hamper host defense. In terms of infectious disease, strategies that can enhance host defense are suggested to improve treatment. For example, patients afflicted with sepsis that survive the hyperinflammatory cytokine storm in some cases develop immunoparalysis with impaired neutrophil function that is associated with increased mortality (Tisoncik et al., 2012). Below, examples of various treatment strategies are reviewed.

1.7.1 INHIBITION OF MEDIATOR PRODUCTION

Reducing the production of inflammatory mediators can inhibit the inflammatory response. Glucocorticoids mainly exercise their anti-inflammatory effects by binding intracellular glucocorticoid receptors that in turn hinder gene expression of pro-inflammatory mediators such as cytokines and cyclooxygenases via inhibition of the transcription factors NF-κB and activator protein-1 (AP-1). Furthermore, glucocorticoids mediate non-genomic actions by decreasing the release of AA (Ramamoorthy and Cidlowski, 2016). In all, glucocorticoids broadly target inflammation at the base of the signaling pathways, generating inhibition of the increase of pro-inflammatory cytokines, prostaglandins as well as the recruitment of immune cells. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit inflammation by targeting COX-1 and/or COX-2, thus hindering the formation of prostaglandins that are responsible for inducing inflammatory vasodilation and sensitization of nociceptors. Furthermore, NSAIDs also have an antipyretic effect (Diaz-Gonzalez and Sanchez-Madrid, 2015). Another group of drugs with anti-inflammatory effects are disease-modifying antirheumatic drugs (DMARDs), which are mainly used for their immunosuppressive effects. An example of these is the synthetic DMARD methotrexate that inhibits the immune system on DNA level (Brown et al., 2016). A downside with treatments that inhibit production of inflammatory mediators is that it increases the risk of disseminating infection and that it impairs resolution of inflammation (Voiriot et al., 2019).

1.7.2 INHIBITION OF NEUTROPHIL FUNCTION

Recruitment of leukocytes is a hallmark of the inflammatory response, taking place in the postcapillary venules by the interaction of several cell adhesion molecules (CAMs) expressed on leukocytes and EC. Due to its crucial role in inflammation, targeting the leukocyte adhesion cascade has therefore been suggested as a promising approach to antagonize inflammation. Inhibitors of selectins, β_2 integrins (CD11/CD18) and ICAM have shown promise in preclinicial studies, but results from clinical trials evaluating treatment of inflammatory disease states such as ischemia-reperfusion and myocardial and cerebral ischemic injury have been inconsistent (Ulbrich et al., 2003). The fact that inhibiting CD18-mediated adhesion with blocking antibodies mimics the genetic disorder leukocyte adhesion deficiency (LAD), characterized by recurrent bacterial infections due to impaired neutrophil recruitment, might indicate potential adverse effects of such treatment strategies.

Neutrophils are highly involved in ARDS pathogenesis and a multitude of different neutrophil-associated targets have been tested in preclinical studies of acute lung injury. To name a few, antagonists against TLRs, cytokines and chemokines, inhibition of the granule proteins neutrophil elastase and different matrix metalloproteinases, deoxyribonuclease I (DNAse I) treatment targeting NETs and treatment with antioxidants against ROS have been tested (Potey et al., 2019).

Bradykinin formation via activation of the KKS is known to increase vascular permeability, and neutrophil-derived proteases have previously been shown to induce BK formation (Imamura et al., 2002, Stuardo et al., 2004, Kahn et al., 2009). Hereditary angioedema is a disease in which mutations in the gene encoding C1 esterase inhibitor result in exaggerated bradykinin formation. This leads to attacks of cutanenous and mucosal edema that can be treated with a bradykinin B_2 receptor antagonist (HOE 140/icatibant) or a plasma kallikrein inhibitor (DX88/ecallantide) (Longhurst and Bork, 2019).

Heparin is a strongly negatively charged polysaccharide with anticoagulant properties, which alongside low molecular weight derivatives is extensively used in clinical practice to treat and prevent thrombosis. Heparin also possesses documented anti-inflammatory properties such as inhibition of different aspects of neutrophil activation (Mulloy et al., 2016). The anti-inflammatory activities have been found to function independent of the anticoagulant, which are mainly confined to a specific pentasaccharide sequence that potentiates the effect of antithrombin III. Therefore, chemical modifications of heparin have been made that reduce the anticoagulant quality while retaining the anti-inflammatory. Such low anticoagulant

heparin derivatives have been found to attenuate acute inflammation by inhibiting neutrophil-derived proteins (Rao et al., 2010, Wildhagen et al., 2014).

1.7.3 ENHANCEMENT OF HOST DEFENSE

Due to a suggested increase in the prevalence of immunosuppression, explained by expanded use of immunosuppressive drugs and increased life expectancy (Harpaz et al., 2016), as well as the increase in antibiotic resistance, there is a need for finding new ways to treat infectious disease. An optimal therapeutic against inflammation caused by infection with a pathogen would enhance the host defense whilst controlling the neutrophil-associated tissue-damaging inflammatory response.

A promising target is inositol hexakisphosphate kinase 1 (IP6K1), an enzyme involved in modulating neutrophil functions such as phagocytosis and ROS production, and that also regulates the production of polyP in platelets. It was recently shown that mice deficient in IP6K1 subjected to bacterial pneumonia had enhanced bacterial killing whilst having reduced neutrophil infiltration and lung damage (Hou et al., 2018). This study also found that platelet polyP had a major role in inducing neutrophil activation with subsequent pulmonary inflammation.

Another potential therapeutic strategy with both anti-inflammatory and immune modulating effects is treatment with short-chain fatty acids (SCFAs). The SCFAs butyrate, propionate and acetate are shown to be involved in regulating inflammation (Li et al., 2018). In several studies, sodium butyrate and its analogue phenylbutyrate (PBA) were found to attenuate inflammatory responses (Ni et al., 2010, Vieira et al., 2012, Venkatraman et al., 2003, Liang et al., 2013, Ono et al., 2017, Kim et al., 2013). Furthermore, sodium butyrate and PBA has been shown to induce expression of antimicrobial peptides (AMPs) (Schauber et al., 2004, Steinmann et al., 2009, Mily et al., 2013, Sarker et al., 2011). In humans, the AMPs are the α- and the β-defensins and the cathelicidin LL-37. They are expressed by immune cells and epithelial cells, and have antimicrobial as well as immune-modulating and pro-resolving effects (Steinstraesser et al., 2011).

2. AIMS

The overall aim of this thesis was to investigate the mechanisms of neutrophil-induced vascular hyperpermeability in acute inflammation, with an attempt to find novel treatment strategies.

Specifically, the aim of each study was as follows:

Study I: Investigate the role of the kallikrein-kinin system in neutrophil-evoked endothelial barrier disruption.

Study II: Investigate the effect and mode of action of heparinoid sevuparin on neutrophil-induced plasma leakage in acute systemic inflammation

Study III: Investigate the role of platelet-derived polyphosphates in neutrophilic inflammation.

Study IV: Investigate the effect of phenylbutyrate treatment on the inflammatory response and the role of cathelicidin in murine pulmonary inflammation.

3. EXPERIMENTAL PROCEDURES

For detailed descriptions, see methods sections in paper I-IV.

3.1 ETHICAL STATEMENT

All animal experiments were performed in accordance with ethical applications approved by Stockholm Ethics Committee for Animal Experiments. Collection of blood from healthy donors was performed in accordance with ethical application approved by the Regional Ethics Review Board in Stockholm.

3.2 *IN VIVO* METHODOLOGY

In the research field of immunology and inflammation, animal models are widely used to increase our understanding of the human immune system. Although animal models have many limitations and do not fully reflect human diseases, their use has overall been invaluable for increasing our knowledge in human functions in health and disease, and also for the development of novel treatments. The immune system, especially the innate immune system that is the focus of this thesis, is highly conserved throughout evolution and has enabled the development of a multitude of animal models of inflammation. Inflammation research has employed animal models for almost a century, resulting in considerable advances in understanding and treatment of human inflammatory disease (Webb, 2014). When utilizing animal models there are numerous factors that can influence the results. With regards to models of inflammation and neutrophils, some examples are; the gender of the animals (Kay et al., 2015), the time of day for experiment (Adrover et al., 2019) and the method of anesthesia (Cruz et al., 2017).

Two different mouse strains were used throughout this thesis work. In paper I and IV, female C57bl/6 were utilized, with the knockout mice strains *BdkB2*$^{-/-}$ and *F12*$^{-/-}$ in paper I being on a C57bl/6 background. In paper II and III, male Balb/c mice were used. In all cases, wild type mice were from Harlan. C57bl/6 and Balb/c are both inbred strains, meaning that the genotype is near identical in between individual animals. These strains were chosen since they are both commonly used for research on inflammation. The use of inbred strains is favorable when high reproducibility is wanted, the downside being that results might not in the same manner translate into the genetically diverse human setting.

Below, the *in vivo* models utilized for this thesis work will be reviewed.

3.2.1 INTRAVITAL MICROSCOPY OF HAMSTER CHEEK POUCH

Intravital microscopy (IVM) is a suitable method when real-time high-resolution visualization of physiological and pathophysiological processes is wanted, and is the only method that allows detailed spatiotemporal assessment of the inflammatory process at the microvascular level. In a historical context, IVM has played a crucial role in our understanding of immunology and physiology (Secklehner et al., 2017). IVM can be performed on a multitude of different tissues and in different species. For example, cremaster muscle, skin, liver, lung and mesentery can all be studied in mice with IVM. The cheek pouch of hamsters is another example, well suited for detailing plasma extravasation, and is the one that was used in paper I in this thesis.

IVM of the hamster cheek pouch is a well-established method to study the microcirculation *in vivo*. It allows for detailed assessment of leukocyte recruitment and alterations in vascular permeability in real-time (Raud and Lindbom, 1994). Briefly, in an anaesthetized hamster, the cheek pouch is turned inside out and pinned down on to a transparent plate. The microcirculation within the pouch can then be observed with light microscopy. To study leukocyte recruitment, chemoattractants can be administered in various ways such as topical application onto tissue or by intravenous injection. To study the alteration in vascular permeability that occurs subsequent to leukocyte adhesion, a fluorescent plasma marker is administered intravenously and plasma leakage is observed using fluorescence microscopy. A challenge in utilizing IVM is to exteriorate and prepare the tissue without inducing an inflammatory response caused by the surgical procedure. Also, it is important to mimic physiological conditions regarding temperature and pH. Limitations of IVM of the hamster cheek pouch are that it is a time-consuming method that requires surgical skills for preparation of tissue, and that there is a risk of bias when selecting areas of tissue to be studied. To ensure representative data, five different microvascular sections were studied in each animal. In paper I, topical administration of LTB_4 was used to induce neutrophil adhesion, and vascular permeability was assessed through monitoring leakage of FITC-dextran following different treatments.

3.2.2 MODELS OF ACUTE INFLAMMATION IN MICE

3.2.2.1 Pleurisy in mice

This is a cavity model, meaning that an inflammatory stimulus is injected into either a created or an already existing body cavity. Other common cavity models are the peritonitis model and the subcutaneous air pouch model (Moore, 2003). The pleurisy model allows for parallel quantitative

assessment of immune cell recruitment as well as exudation of plasma. Pleurisy is induced by injection of an inflammatory stimulus into the pleural cavity of anaesthetized mice. In paper I, LTB_4 and thioglycollate were used. Intravenous access was allowed by placing a catheter in the left jugular vein. This was performed to administer the plasma tracer FITC-dextran at the start of the experiment as well as to control depth of anesthesia during the four-hour incubation. Following incubation, mice were euthanized and the pleural cavity was accessed by opening the thorax. The pleural exudate was collected and analyzed for volume (V), fluorescence intensity (FI) and neutrophil count. Neutrophil count was assessed by flow cytometry and plasma leakage was quantified by calculating the plasma clearance volume (permeability index, PI) based on the formula $PI = FI_{exudate} \times V_{exudate} / FI_{serum}$. The use of PI for quantification takes into account both the volume of fluid that has leaked out into the pleural space as well as the degree of macromolecular leakage. Some limitations of this model are that mice are anesthetized throughout the experiment, that blood pressure is not monitored, and that there is a risk of causing a contaminating hemorrhage both during injection of inflammatory stimulus and during the surgical opening of the thorax. To minimize the risk of bleeding when accessing the pleural cavity the mice were bled by cutting the abdominal vena cava and aorta prior to collection of the exudate.

3.2.2.2 Acute systemic inflammation in mice

This method was used to investigate the acute inflammatory reaction in lung in response to a systemically administered stimulus. The model aims to mimic acute lung injury induced by systemic inflammation such as sepsis, and allows for assessment of immune cell accumulation and plasma leakage in lung. The protocol was based on previous work in our group (Soehnlein et al., 2008a), with some modifications. Female C57bl/6 mice were used in paper I and male Balb/c mice were used in paper II and III. Mice were anaesthetized with intraperitoneal injection of a mixture of ketamine and xylazine and were kept anaesthetized on a temperature-controlled heating plate throughout the experiment that lasted for 30 minutes. For intravenous access, a catheter was placed in the left jugular vein. Mice were stimulated with intravenous administration of heat-killed group A *Streptococci* (hkGAS) (paper I, II and III) or with synthetic polyphosphates (paper III). Evans blue dye, which binds albumin and becomes a marker for macromolecular leakage, was injected intravenously at the start of the experiment. After 30 minutes, the jugular vein catheter was connected to a syringe pump, the abdomen was cut open and the abdominal aorta and vena cava were exposed. Both aorta and vena cava were clamped and an incision was quickly made in the aorta proximal to the clamp. Following this, the pump was started (600 µl/min) and mice were perfused with 4 ml PBS supplemented with heparin.

Lungs where then collected and all lobes but the right medial lobe were dried over night for quantification of Evans blue in lung tissue. The right medial lobe was passed through a cell strainer and lung neutrophil accumulation was quantified by flow cytometry. Limitations with this model are similar to those in the pleurisy model as the mice as anesthetized and blood pressure is not monitored. Furthermore, it cannot discriminate between adhered and extravasated neutrophils since cell analysis is performed on homogenized lung tissue.

3.2.2.3 Pulmonary inflammation in mice

Several mouse models have been developed to study the inflammatory response during infectious and non-infectious pulmonary inflammation. Inflammatory stimuli can be administered by intratracheal instillation, whereby the stimulus is delivered through the trachea following surgical preparation in anaesthetized mice. Another, less invasive, method is administration of aerosolized stimuli in an aerosol inhalation chamber, which allows mice to be unrestrained and awake. Furthermore, inflammatory stimuli can be administered by intranasal inoculation whereby droplets placed on the nostrils are aspirated by the animal (Bielen et al., 2017). This method only requires brief anesthesia and a short duration of restraint. Following induction of pulmonary inflammation or infection, mice are then observed for a period of time and depending on the purpose and the read-out of the experiment mice are then sacrificed and samples are collected. When subjecting awake animals to pathogens or noxious stimuli, it is of high importance to have an established humane endpoint that may not be exceeded, and therefore animals should be observed for early detection of symptoms throughout the experiment. Sample collection can be done in many ways depending on the research question. A frequently used method is bronchoalveolar lavage (BAL) where a tube is inserted into the trachea and lungs are flushed with saline solution that is aspirated. This allows for collection of sample from the alveoli and is often used to analyze immune cell recruitment as well as protein content. Another option is to excise the lung tissue for immunohistochemistry or to homogenize the lung tissue for cell analysis or gene expression. A common way of quantifying edema formation is to excise and weigh the lung tissue in a “wet” state and then later on after drying, weighing it in a dry state, rendering a wet-dry weight. In paper IV, heat-killed *Pseudomonas aeruginosa* strain PAO1 (hkPAO1) was given by intranasal inoculation to mice briefly anaesthetized with Isoflurane. Mice were then observed and evaluated according to the score sheet for humane endpoint, and sacrificed at different time points. Following sacrifice, BAL was performed and BAL fluid was analyzed for immune cell content by flow cytometry as well as for protein content with ELISA. Lung tissue was then

harvested and homogenized, and subjected to quantitative polymerase chain reaction (qPCR). In separate experiments, mouse lungs were perfused in a similar manner as in the acute systemic inflammation model and then lung lobes were excised and weighed to quantify edema formation.

3.2.2.4 Neutrophil and platelet depletion

In paper I-III, neutrophil depletion was achieved by intraperitoneal injection of anti-Gr1 monoclonal antibody (Soehnlein et al., 2008b), and neutropenia was confirmed by peripheral blood cell count prior to experiment. In paper III, platelet depletion was accomplished by intraperitoneal injection of anti-mouse thrombocyte serum (McDonald et al., 2012).

3.3 *IN VITRO* METHODOLOGY

3.3.1 FLOW CYTOMETRY

Flow cytometry was utilized to quantify leukocytes in pleural exudate (paper I), in homogenized lung tissue (paper I-IV) and in BAL fluid (paper IV) *in vivo*, and to count transmigrated neutrophils *in vitro* (paper IV). For the *in vivo* experiments, cell suspensions were stained with anti-Gr-1 (Ly-6G/Ly-6C) and anti-F4/80 antibodies and leukocytes were identified based on morphology with forward (cell size) and side (cell granularity) scatter, and then gated to exclude non-leukocytes. Neutrophils (Gr-1+, F4/80-), monocytes (Gr-1+, F4/80+) and macrophages (Gr-1-, F4/80+) were characterized based on different expression of Gr-1 and F4/80.

3.3.2 ENDOTHELIAL AND EPITHELIAL CELLS

Cultured endothelial cells (EC) are commonly used for research on inflammatory processes such as reorganization of the endothelial cytoskeleton and alterations in endothelial barrier function. Both immortalized endothelial cell lines and primary endothelial cells are frequently used, and may be more or less suitable depending on the research question. Studying the endothelial barrier *in vitro* has benefits in that it allows high-throughput testing of effects of various stimuli and treatments. Also, cell-type specific molecular mechanisms can by studied more easily than *in vivo*. However, there are some obvious limitations with studying an isolated cell type that normally is in contact with a basement membrane, extracellular matrix and other cell types such as pericytes. *In vivo*, EC are also continuously exposed to fluid shear stress that affects EC functions. Another factor is the heterogeneity of EC throughout the vasculature.

EC activation can be studied in real-time by loading EC with calcium indicators that emit fluorescence during increases in cytosolic calcium. Further, staining of the actin cytoskeleton in fixed cells can show actin stress fiber formation as a measure of EC activation. Barrier integrity of an EC monolayer and alterations in permeability can be assessed with measurement of transendothelial electrical resistance (TEER) that measures the resistance of an EC monolayer cultured on a semipermeable membrane. A transwell system, also known as a Boyden chamber, can be used to measure EC permeability through measuring the flux of dyes such as FITC-dextran or Evans blue-conjugated albumin from an upper to a lower compartment separated by an EC monolayer. Such a transwell system can be used also to monitor transmigration of cells, such as neutrophils, across a naked membrane or a cell monolayer.

In paper I and II, human umbilical vein endothelial cells (HUVEC) were used to study EC activation with cytoskeletal reorganization and intercellular gap formation following stimulation with isolated neutrophils together with hkGAS, or with supernatants from hkGAS-stimulated neutrophils. This was performed in the presence of purified HK. HUVEC are primary endothelial cells that were the first type of EC to be cultured *in vitro* (Nachman and Jaffe, 2004). This method was chosen since it was stable and reproducible, and allowed a rather high throughput with several different treatments. A limitation of this method is that it entails an element of judgment when selecting areas for gap area quantification. Also, the excessive gap formation might not directly reflect the situation *in vivo* since there is no basement membrane that can resist the contraction of EC. However, quantifying in vitro endothelial gap formation is still an eligible method for assessing EC activation and has been used in previous studies (Duah et al., 2013).

In paper I, HUVEC were utilized when studying the proteolysis of EC-bound HK, and also the human endothelial hybrid cell line (EA.hy926) for studying the displacement of HK from ECs. EA.hy926 is a hybrid cell line of HUVEC and carcinoma cells with preserved EC properties such as contact inhibition and expression of common endothelial markers (Bouis et al., 2001). In this experiment, the endothelial cells were considered as a scaffold with GAGs for HK to interact with.

In paper IV, the human lung epithelial cell line BEAS-2B was stimulated with phenylbutyrate (PBA) and heat-killed *Pseudomonas aeruginosa* (hkPAO1), and supernatant was used in a chemotaxis assay with neutrophils.

3.3.3 NEUTROPHILS AND PLATELETS

3.3.3.1 Neutrophil experiments

Neutrophils, or PMNs, were obtained from venous blood collected in EDTA-coated tubes. The protocol for isolation was based on work previously described (Nauseef, 2007). Isolation is a three-step process where whole blood is first mixed with dextran that initiates red blood cell (RBC) sedimentation. An upper layer of leukocyte-rich plasma can then be aspirated and layered on top of Ficoll-Paque. Ficoll-Paque is a density gradient medium and following centrifugation neutrophils and some remaining RBC are located in the pellet. The supernatant that contains lymphocytes, monocytes, basophils and platelets, is discarded. The final step is to get rid of the remaining RBC by hypotonic cell lysis with H_2O.

For preparation of neutrophil secretion (paper I and II), isolated human neutrophils were stimulated with hkGAS under gentle rotation for 30 minutes, centrifuged, and the supernatant was stored at -20°C until use.

A neutrophil adhesion assay was employed in paper II. Neutrophils were allowed to adhere to bovine serum albumin (BSA)-coated plates, with or without hkGAS and sevuparin. After washing of plate wells, remaining adherent neutrophils were lysed and MPO activity was measured to quantify neutrophil adhesion.

Neutrophil degranulation following stimulation with hkGAS, synthetic polyP or platelet supernatant was studied in paper II and III. Subsequent to stimulation, with or without different treatments, neutrophil suspensions were centrifuged and MPO activity in supernatant was analyzed as a measure of degranulation.

In paper IV, the chemotactic activity of PBA was investigated in a transwell system with an upper and a lower chamber separated by a membrane (pore size 3 μm). In brief, PBA together with hkPAO1, or supernatant from lung epithelial cells stimulated with PBA and hkPAO1, was added to the lower chamber. Isolated neutrophils were then added to the upper chamber and after two hours incubation, migrated neutrophils were quantified by flow cytometry.

3.3.3.2 Neutrophil stimulation with platelet secretion

In paper III, blood from healthy volunteers was collected in sodium citrate tubes and prostaglandin E_1 was directly added to avoid platelet activation. Platelet-rich plasma was collected following centrifugation and platelets were pelleted and resuspended. Platelet concentration was determined with manual cell count following stain with Stromatol. Platelet secretion was

prepared by stimulating platelets with adenosine diphosphate (ADP) and supernatant was collected after centrifugation. Neutrophils and platelet secretion at a ratio of 1:100 from the same donor were then incubated with or without treatment with polyP inhibitor and MPO activity was analyzed as a measure of neutrophil degranulation.

3.3.4 BACTERIA

In paper I-III, heat-killed group A *Streptococcus* (GAS, *Streptococcus pyogenes*) (clinical isolate, *emm* 87), and in paper IV, heat-killed *Pseudomonas aeruginosa* (PAO1) were used for *in vivo* and *in vitro* experiments. Bacteria were cultured in Brain Heart Infusion broth at 37°C, washed and resuspended in PBS. Heat-killing was performed by incubation at 100°C for 1 hour (PAO1) or a minimum of 3 hours (GAS).

3.3.5 PROTEIN ASSAYS

3.3.5.1 Western blot of HK

Western blot is a common method for detecting specific proteins in homogenized tissue samples. In paper I, western blot was used to assess proteolysis of EC-bound HK. HUVEC were incubated with human citrated plasma together with neutrophils or granule proteins. EC were washed and lysed followed by separation by gel electrophoresis. Proteins were transferred to a nitrocellulose membrane and HK was detected by enhanced chemiluminescence with sheep anti-human HK antiserum (AS88) that detects heavy and light chains of HK. Signal intensity of HK was measured by densitometry with Quantity One software. To control for even loading, protein concentration in cell lysate or a second stain of the membrane for the housekeeping protein β-actin was performed.

3.3.5.2 HK competition assay

In paper I, we used an assay for quantifying competitive binding of proteins to immobilized heparan sulfate and cultured EC. The protein of interest, HK, was radiolabeled with Iodine-125 (^{125}I-HK) and incubated together with different concentrations of FXIIa, neutrophil elastase or HBP onto either heparan sulfate or EC. Following incubation, plates were washed and the amount of bound HK to either scaffold was quantified using a gamma counter.

3.3.5.3 Enzyme activity assays

In paper I, the enzymatic activity of PK was analyzed using the chromogenic substrate S-2302 following incubation with supernatant from hkGAS-stimulated neutrophils. Cleavage of chromogenic substrates results in

formation of color that can be analyzed with a spectrophotometer. The rate by which color appears is proportional to the enzymatic activity, making this a simple and useful tool for assessment of activity of various enzymes in different settings and following treatment with different inhibitors.

In paper II, enzymatic activity of neutrophil elastase was analyzed in supernatant from hkGAS-stimulated neutrophils following incubation with different doses of sevuparin. The fluorogenic substrate MeOSuc-AAPV-AMC was used for quantification of enzymatic activity, which corresponds to the rate of increase in fluorescence intensity measured in a fluorometer.

In paper II and III, the activity of MPO was measured. In the neutrophil adhesion assay in paper II, MPO was measured in cell lysate as a means to quantify the number of adhered neutrophils. In paper II and III, neutrophil degranulation was quantified by measuring MPO activity in neutrophil supernatant. This assay was previously described (Suzuki et al., 1983) and in brief, it utilizes the capacity of MPO to form hypochlorous acid (HOCl) out of hydrogen peroxide (H_2O_2), that in turn oxidizes the substrate TMB (3,3',5,5'-tetramethylbenzidine). TMB then develops into a blue reaction product that can be measured with a spectrophotometer. By using an MPO standard dilution series in parallel, the MPO activity can be determined.

3.3.5.4 Enzyme-linked immunosorbent assay (ELISA)

In paper IV, ELISA was employed to measure concentrations of murine TNF, IL-6 and IL-10 in BAL fluid. ELISA is a plate-based assay frequently used for determining quantitative measures of antigens and antibodies, and can be performed in many different ways depending on the analyte in question. For these experiments we used ready-made ELISA kits from Thermo Fisher Scientific.

3.3.6 AFFINITY CHROMATOGRAPHY (AC)

In paper II, two different AC techniques were utilized to assess which neutrophil-derived proteins are bound by sevuparin and heparin. AC can be designed in many different ways to suit the task at hand, but essentially it is a method for separation of molecules from a complex solution. Supernatant from hkGAS-stimulated neutrophils was passed through a commercial column of heparin-coated sepharose and flow-through was collected. Also, neutrophil secretion was mixed with a sevuparin-coated sepharose matrix, and after incubation and centrifugation the supernatant was collected. This AC technique is called a batch procedure.

3.3.7 MASS SPECTROMETRY

In paper II, liquid chromatography-mass spectrometry (LC-MS) was used to perform proteomic analysis of supernatant from hkGAS-stimulated neutrophils (carried out by Proteomics Karolinska). Protein content in untreated supernatant was compared with supernatant subjected to either sevuparin or heparin AC in terms of fold change of individual protein abundance. LC-MS is a common method used to identify components of a complex biological sample such as serum, urine or supernatants from cell suspensions. In brief, the mixture is subjected to protein digestion and the resulting peptides are separated with LC. The peptides are then eluted and transferred to a mass spectrometer that ionize and analyze the peptide's mass-to-charge ratio. The proteins can then be identified by checking the MS spectra against a database for MS spectra and human proteins such as the UniProt human database. Relative quantity of the identified proteins can also be retrieved.

3.3.8 POLYMERASE CHAIN REACTION (PCR)

In paper IV, reverse transcription (RT)-PCR and quantitative (q) PCR were performed to analyze the gene expression levels of *Cramp*, *Tnf*, *Cxcl1*, *Alox5*, *Alox15* and *Ptgs2* (COX-2) in homogenized lung tissue at several time-points following administration of hkPAO1 and PBA. In brief, RNA was isolated from tissue by passing tissue lysate through a commercial column (RNeasy kit, Qiagen), followed by cDNA synthesis using RT-PCR. Next, cDNA was subjected to qPCR and alterations in gene expression were assessed and normalized for the housekeeping genes *Hprt*, *Hmbs* and *Gapdh*.

3.4 STATISTICS

Results are presented as means ± standard error of the mean (SEM). Statistical differences were analyzed with GraphPad Prism 5 using 1-way ANOVA with Tukey's multiple comparisons test, repeated measures ANOVA followed by Bonferroni's or Tukey's post-hoc tests, one-tailed Mann-Whitney U test or two-tailed unpaired t-test. Differences were considered significant if $P < 0.05$.

4. RESULTS & DISCUSSION

This thesis work focused on the role of neutrophils in regulation of endothelial barrier function in acute inflammation. Mechanisms contributing to neutrophil-mediated alterations of vascular permeability have been studied extensively by our research group and others, and although several mechanisms are already established the map is far from complete. In the following section, a synopsis of the results that constitute this thesis will be discussed. More detailed information is available in the individual papers.

4.1 NEUTROPHIL-INDUCED EC BARRIER DISRUPTION AND PLASMA LEAKAGE

Neutrophils induce plasma leakage (Wedmore and Williams, 1981) following adhesion via β_2 integrins (Arfors et al., 1987). In paper I, neutrophil-dependent increase in vascular permeability was assessed in the microcirculation of the hamster cheek pouch and in the mouse pleurisy model with the neutrophil chemoattractant LTB_4. Topical application of LTB_4 onto the hamster cheek pouch induced leukocyte adhesion to the vessel walls of postcapillary venules and concurrent plasma leakage as imaged by light and fluorescence microscopy respectively (**Figure 1A**). In the mouse pleurisy model, which allows for quantification of neutrophil extravasation and plasma leakage, LTB_4 injected into the pleural cavity induced neutrophil recruitment and plasma leakage. Plasma leakage, as measured by the volume of exudate as well as the amount of the fluorescent plasma tracer FITC-dextran, was averted in mice subjected to neutrophil depletion and following blockage of the β_2 integrin-subunits CD11a and CD11b (**Figure 1B-C**).

In paper I, II and III, neutrophil-dependent plasma leakage was also demonstrated in a mouse model of acute systemic inflammation with hkGAS. M protein, a virulence factor of GAS, has previously been shown to activate neutrophils via β_2 integrins (Herwald et al., 2004). Intravenous administration of hkGAS induced accumulation of neutrophils and an increase in plasma exudation in lung tissue, and in neutropenic mice, plasma leakage was abolished (**Figure 2C-D**).

To study the effects of neutrophils on the endothelial barrier function in a human setting, cultured primary endothelial cells from human umbilical vein (HUVEC) were stimulated with human neutrophils together with hkGAS (paper I and II). Incubation of endothelial cells with either quiescent neutrophils or hkGAS alone caused no alterations of the integrity of the endothelial barrier. However, simultaneous stimulation with neutrophils and hkGAS induced EC remodeling and formation of interendothelial gaps. Binding of β_2 integrins, either by adhesion to EC or with streptococcal

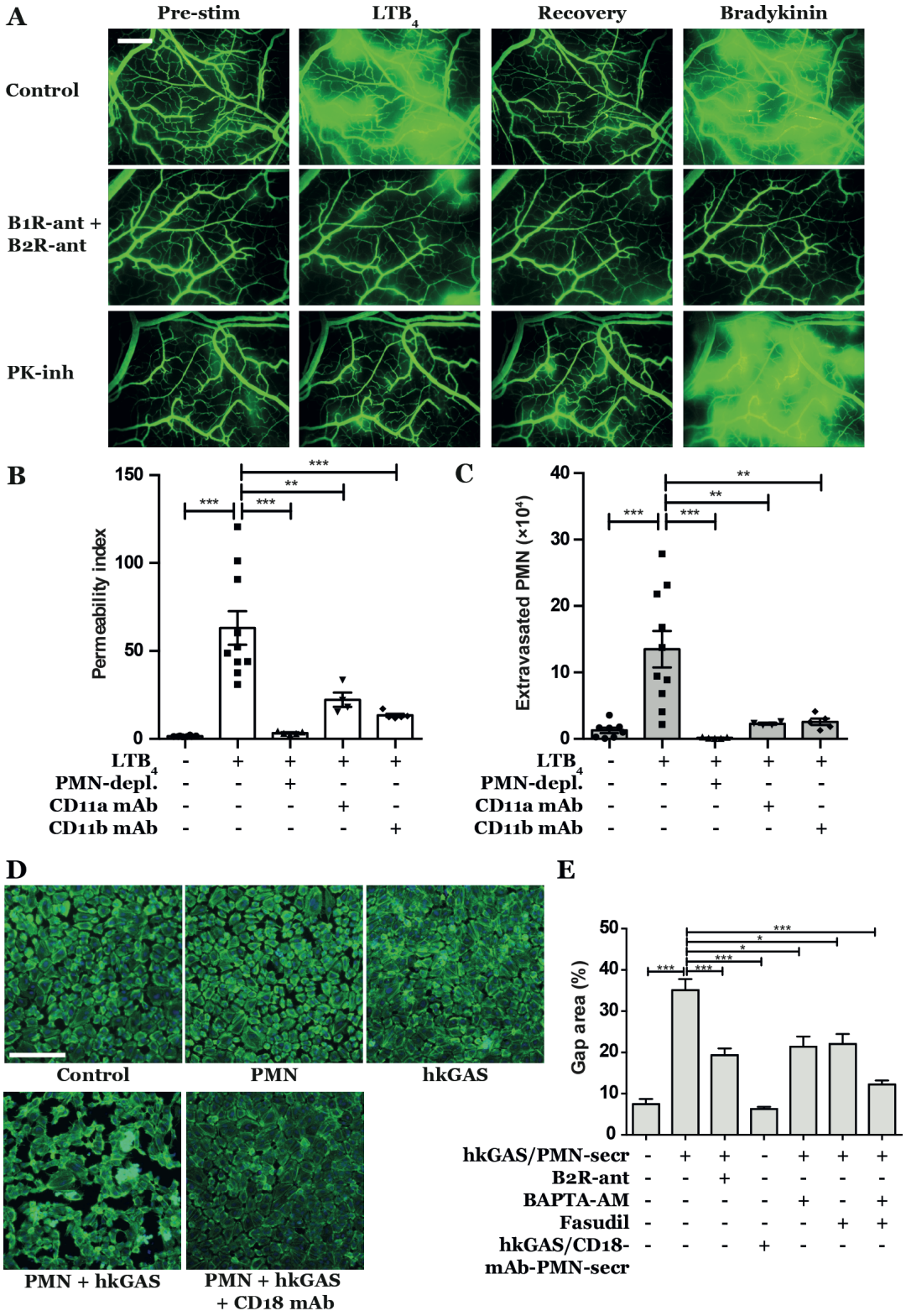

Figure 1. Neutrophil-induced endothelial gap formation and vascular leakage. **A)** Vascular leakage in postcapillary venules of hamster cheek pouch following stimulation with LTB_4 and bradykinin. B_1R-ant=B_1 receptor antagonist, B_2R-ant=B_2 receptor antagonist, PK-inh=plasma kallikrein inhibitor **B)** Plasma leakage (permeability index) and **C)** neutrophil recruitment in LTB_4-induced pleurisy in mice. PMN-depl = neutrophil depletion. **D)** HUVEC monolayers stained for F-actin after stimulation with human neutrophils (PMN) and hkGAS. **E)** Gap formation quantification in HUVEC monolayers following stimulation with secretion from hkGAS-activated human neutrophils.

M protein, results in neutrophil degranulation, which is known to induce vascular leakage and lung damage (Gautam et al., 2000, Herwald et al., 2004, Soehnlein et al., 2008a). Indeed, blocking β_2 integrins on neutrophils with an anti-CD18 antibody prior to stimulation with hkGAS completely prevented EC barrier disruption (**Figure 1D**).

Neutrophil-derived proteins such as HBP are released from activated neutrophils and cause derangement of the EC barrier (Gautam et al., 2001, Herwald et al., 2004, Di Gennaro et al., 2009). In line with this, we stimulated HUVEC with supernatant from neutrophils incubated with hkGAS and found that supernatant from either unstimulated neutrophils or from hkGAS alone did not affect the EC layer, but supernatant from hkGAS-stimulated neutrophils induced EC barrier disruption (paper I and II). Pretreatment of neutrophils with an anti-CD18 antibody prior to stimulation with hkGAS rendered the supernatant inactive on EC, confirming the role of β_2 integrins for neutrophil degranulation with streptococcal M protein (**Figure 1E**).

To investigate whether the effect of neutrophil secretion on EC was simply cytotoxic or involved previously established cell-signaling pathways, EC were pre-treated with the calcium chelator BAPTA-AM and the Rho kinase inhibitor fasudil prior to stimulation with neutrophil secretion. Both BAPTA-AM and fasudil partly inhibited EC gap formation and the combination of both had an additive effect, resulting in near complete inhibition (**Figure 1E**). These results suggest that neutrophil secretory products cause EC remodeling and gap formation via Rho kinase and increased actomyosin interaction, and are in line with previous findings (Breslin and Yuan, 2004).

4.2 NEUTROPHIL-EVOKED PLASMA LEAKAGE IS MEDIATED BY THE KALLIKREIN-KININ SYSTEM

Neutrophil-derived proteins released upon activation disrupt the endothelial barrier and cause vascular leakage. Furthermore, bradykinin (BK) and similar kinins, the end-products of the KKS, are known to increase vascular permeability, and neutrophil-derived proteases such as neutrophil elastase and proteinase 3 have previously been shown to liberate vasoactive kinins from kininogen (Imamura et al., 2002, Stuardo et al., 2004, Kahn et al., 2009). In paper I, we therefore investigated a potential role of the KKS in neutrophil-mediated vascular hyperpermeability.

In the previously stated *in vivo* models of acute inflammation with neutrophil-dependent plasma leakage, we found that inhibition of the KKS attenuated plasma leakage. In the hamster cheek pouch, antagonizing BK

receptors and inhibition of PK resulted in decreased plasma leakage (**Figure 1A**), and the inhibitors had similar effects in the pleurisy model and in the model of acute systemic inflammation with hkGAS (**Figure 2C-D**). Furthermore, mice lacking either the BK B_2 receptor or FXII presented with attenuated plasma leakage as compared to wild-type mice in the pleurisy model (**Figure 2A-B**).

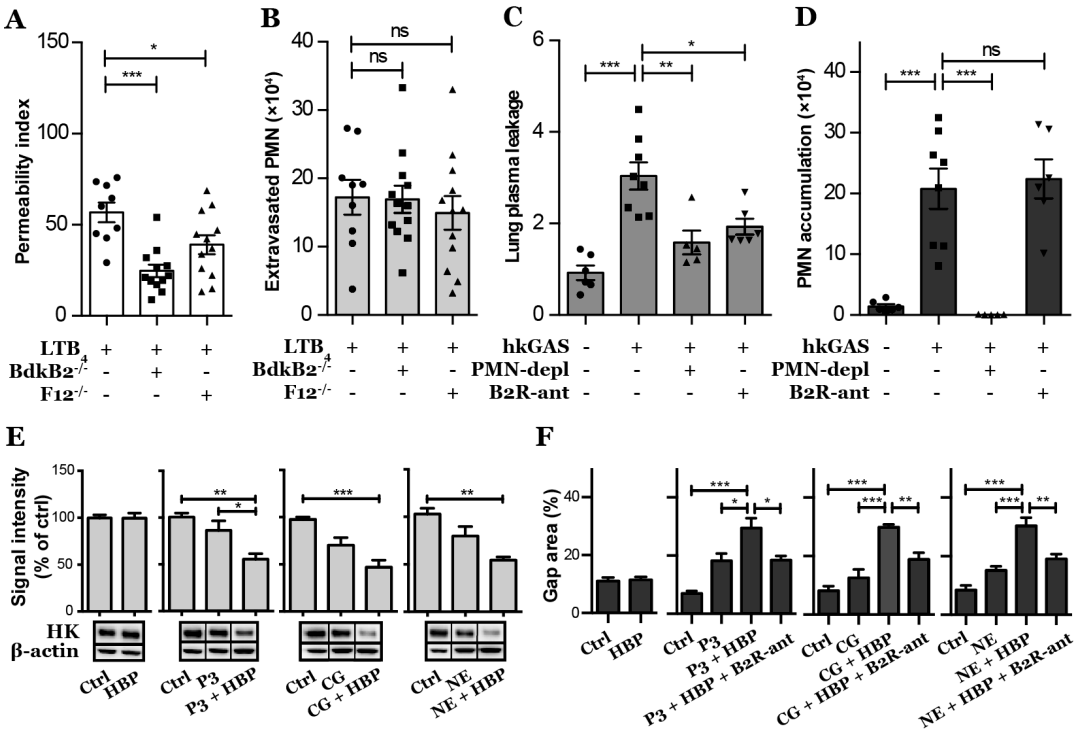

Figure 2. Neutrophil-induced vascular leakage involves the KKS. A) Plasma leakage (permeability index) and **B)** neutrophil recruitment in LTB_4-induced pleurisy in mice. BdkB2$^{-/-}$ = BK B2 receptor knockout mice, F12$^{-/-}$ = factor XII knockout mice. **C)** Lung plasma leakage and **D)** lung neutrophil accumulation in hkGAS-induced acute systemic inflammation in mice. B_2R-ant = B_2 receptor antagonist, PMN-depl = neutrophil depletion. **E)** Western blot signal intensity of EC-bound HK normalized to mean control for each experiment. HBP = heparin-binding protein, P3, = proteinase 3, CG = cathepsin G, NE = neutrophil elastase. **D)** HUVEC monolayer gap formation quantification.

To further investigate the mechanisms by how the KKS mediates neutrophil-evoked plasma leakage, HUVEC (pre-incubated with purified HK) were treated with the B_2 receptor antagonist and stimulated with human neutrophils and hkGAS, and also with supernatant from hkGAS-activated neutrophils. In both cases, blockage of the B_2 receptor inhibited EC remodeling and gap formation (**Figure 1E**). Bradykinin B_2-receptor-mediated endothelial cell signaling has previously been suggested to involve both actomyosin contraction (Ma et al., 2012), which is confirmed by our data, and VE-cadherin disassembly (Orsenigo et al., 2012). Our results do not rule out VE-cadherin disassembly and possibly both mechanisms contribute.

Next, proteolysis of HK on HUVEC, with implicit kinin formation, was assessed following neutrophil activation. HUVEC were pre-incubated with human plasma (as a source of HK) and stimulated with hkGAS or LTB4 with or without neutrophils. Alternatively, HUVEC were incubated with TNF prior to addition of neutrophils. TNF activates EC and thereby stimulates neutrophil adhesion and degranulation. A significant degradation of HK, as measured by Western blot, was found with all three stimuli of neutrophil activation implicating neutrophil-induced kinin formation. Furthermore, incubation of PK and FXII, proteases known to mediate HK cleavage, with supernatant from hkGAS-activated neutrophils induced plasma kallikrein activation. Previous studies have shown that neutrophil granule proteins can induce kinin formation by direct proteolysis of HK (Imamura et al., 2002, Stuardo et al., 2004, Kahn et al., 2009), and our results suggest an additional indirect pathway for HK cleavage whereby neutrophil-derived proteins trigger activation of the PK/FXII loop.

With the aim to further characterize the mechanisms by which neutrophil-derived proteins act on HK, we utilized an assay for competitive binding of HK to GAGs. HK is known to bind GAGs on EC (Renne et al., 2000), and as a regulatory mechanism for BK formation a previous study found that HK is protected from proteolysis whilst bound, but is made available upon displacement from GAGs (Renne et al., 2005). HBP and the other serprocidins (NE, P3 and CG) have all previously been shown to increase EC permeability (Gautam et al., 2001, Bentzer et al., 2016, Peterson et al., 1987, Peterson, 1989). In contrast to the other serprocidins, HBP lacks proteolytic activity (Campanelli et al., 1990), but its structure favors binding to negatively charged GAGs (Olofsson et al., 1999). We hypothesized that HBP binds to GAGs following neutrophil degranulation and displaces HK that is then made available for proteolytic cleavage. Indeed, HBP was found to dose-dependently outrival HK from binding to heparan sulfate and EC, in contrast to the proteases NE and activated FXII. Furthermore, supporting our hypothesis, HBP potentiated the effects of NE, P3 and CG in causing proteolysis of EC-bound HK (**Figure 2E**) and in mediating BK B_2 receptor-mediated EC gap formation in HUVEC monolayers (**Figure 2F**). Together these results suggest that during neutrophil adhesion and subsequent degranulation, HBP paves the way for BK formation by displacing HK and making it accessible to proteases such as PK, NE, P3 and CG. This event possibly only occurs in the sheltered compartment created between the EC and the adherent leukocyte, where an optimal milieu with lesser amounts of plasma protease inhibitors is created (Loike et al., 1992) (**Figure 3**). In support of this, EC gap formation induced by hkGAS-stimulated neutrophils was prevented in the presence of human plasma, as compared to incubation with buffered saline and purified HK (unpublished results).

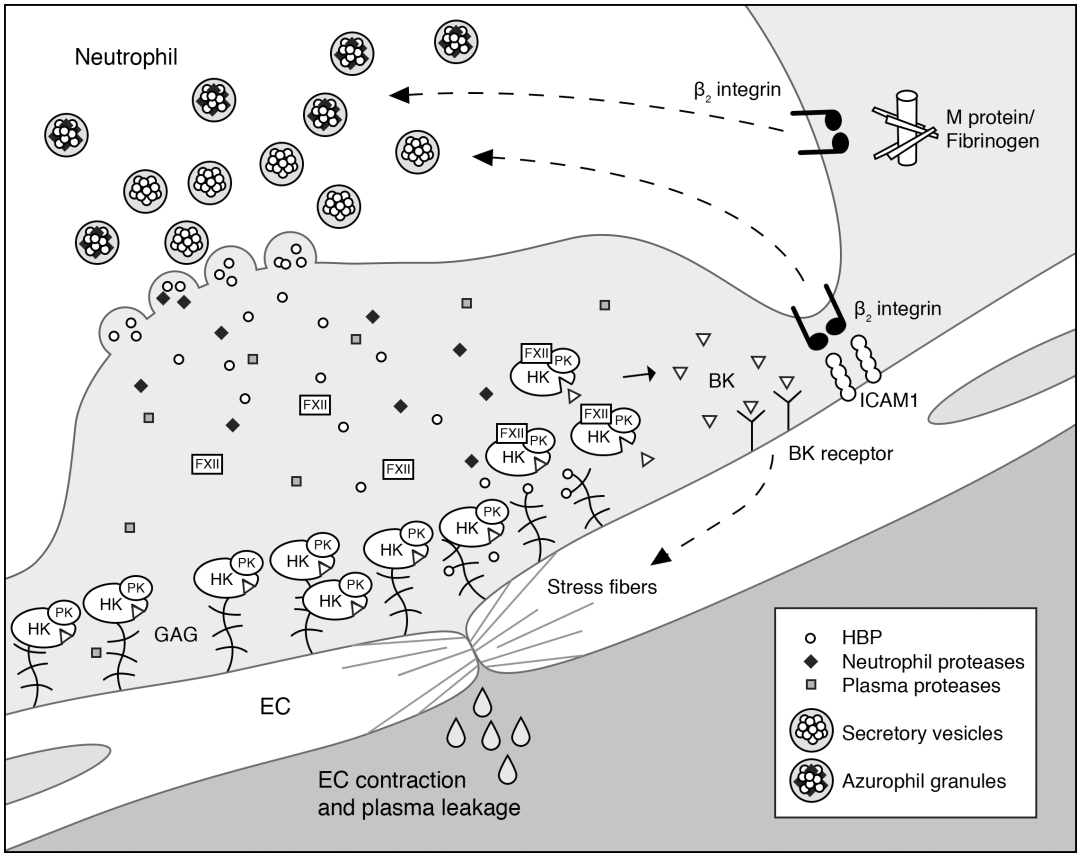

Figure 3. Proposed mechanism for neutrophil-induced vascular leakage. Neutrophil activation via β_2 integrins results in release of granule proteins into a shielded compartment between neutrophil and EC. Here, HBP displaces the HK/PK complex from GAGs which enables proteolytic processing of HK/PK by neutrophil and plasma proteases. Proteolysis of HK liberates BK that induces EC actin stress fibers and gap formation via BK receptors.

A previous study suggested that TNF is released from activated neutrophils and is responsible for causing neutrophil-induced plasma leakage (Finsterbusch et al., 2014). We investigated these findings in our model systems and found no such effects either *in vitro* with stimulation of TNF on EC, as well as treatment with a TNF-inhibitor on PMN-secretion-stimulated EC, or *in vivo* using a TNF-inhibitor in mice subjected to intravenous hkGAS.

Vascular leakage is a central feature in severe microbial infections such as sepsis, and neutrophil degranulation and HBP release following stimulation with GAS-derived M protein has previously been shown to cause lung injury (Herwald et al., 2004, Soehnlein et al., 2008a). Furthermore, the KKS together with HBP has previously been found to take part in the pathogenesis of erysipelas caused by GAS (Linder et al., 2010). However, neutrophil activation and release of HBP is not confined to infections with GAS, as several studies have suggested HBP as a promising biomarker of hypotension and organ dysfunction in sepsis with different pathogens (Fisher and Linder, 2017). Neutrophils and the KKS are also suggested as mediators of vascular leakage in the pathogenesis of infection with hantavirus (Koma et al., 2014,

Taylor et al., 2013). Supporting this, two case reports of patients with hantavirus infection with capillary leakage syndrome and respiratory failure describe significant clinical recovery after treatment with the BK B_2 receptor antagonist HOE 140/icatibant (Antonen et al., 2013, Laine et al., 2015). Collectively, there is evidence by us and others that indicates the KKS as a potential target in treatment of neutrophilic inflammatory disease conditions with vascular leakage. However, in contrast to this, a study of porcine Gram-negative sepsis with *N. meningitis* found no improvement in capillary leakage after treatment with icatibant (Barratt-Due et al., 2011). Possibly, the KKS is predominantly involved in mediating vascular leakage during infections with pathogens that strongly stimulate neutrophil activation and degranulation. Neutrophils do respond differently depending on the pathogen, and GAS has been found to induce neutrophil degranulation and HBP release more efficiently than *S. aureus* or *E. coli* (Snall et al., 2016).

In all, paper I shows a role for the KKS in mediating neutrophil-evoked plasma leakage in acute inflammation and propose a mechanistic basis for how neutrophil-derived proteins act in concert to induce BK formation and EC barrier disruption. Further, it presents a possible target for preventing excessive vascular leak in neutrophilic inflammation.

4.3 SEVUPARIN INHIBITS *STREPTOCOCCUS*-INDUCED VASCULAR LEAKAGE BY NEUTRALIZING NEUTROPHIL-DERIVED PROTEINS

In paper II, the role of neutrophil-derived proteins in the acute inflammatory response was further investigated. We hypothesized that the heparin derivative sevuparin could inhibit the activity of neutrophil-derived proteins and thus prevent neutrophil-induced vascular leakage caused by hkGAS. Sevuparin is a low-anticoagulant heparinoid developed for preventing vaso-occlusion in malaria and sickle cell disease (Vogt et al., 2006, Telen et al., 2016). It is a derivative of heparin where the high-affinity antithrombin III-binding pentasaccharide has been removed, rendering it inactive on factor Xa and thrombin.

The effect of sevuparin was investigated in the mouse model of acute systemic inflammation with hkGAS. Sevuparin attenuated lung plasma leakage to the same extent as neutrophil depletion, whilst not affecting neutrophil accumulation (**Figure 4A-B**). In line with this, sevuparin significantly inhibited *in vitro* EC gap formation caused by hkGAS-stimulated neutrophils.

To further elucidate by which mechanism sevuparin altered neutrophil-evoked effects on the endothelial barrier, *in vitro* neutrophil adhesion and degranulation assays were employed. Sevuparin did not inhibit either adhesion or degranulation of neutrophils, in contrast to previous studies showing inhibition of these activities with other heparin derivatives (Peter et al., 1999, Brown et al., 2003). However, sevuparin treatment of secretion from hkGAS-stimulated neutrophils significantly decreased the enzymatic activity of NE and attenuated EC gap formation, suggesting that sevuparin targets components released from activated neutrophils (see paper II).

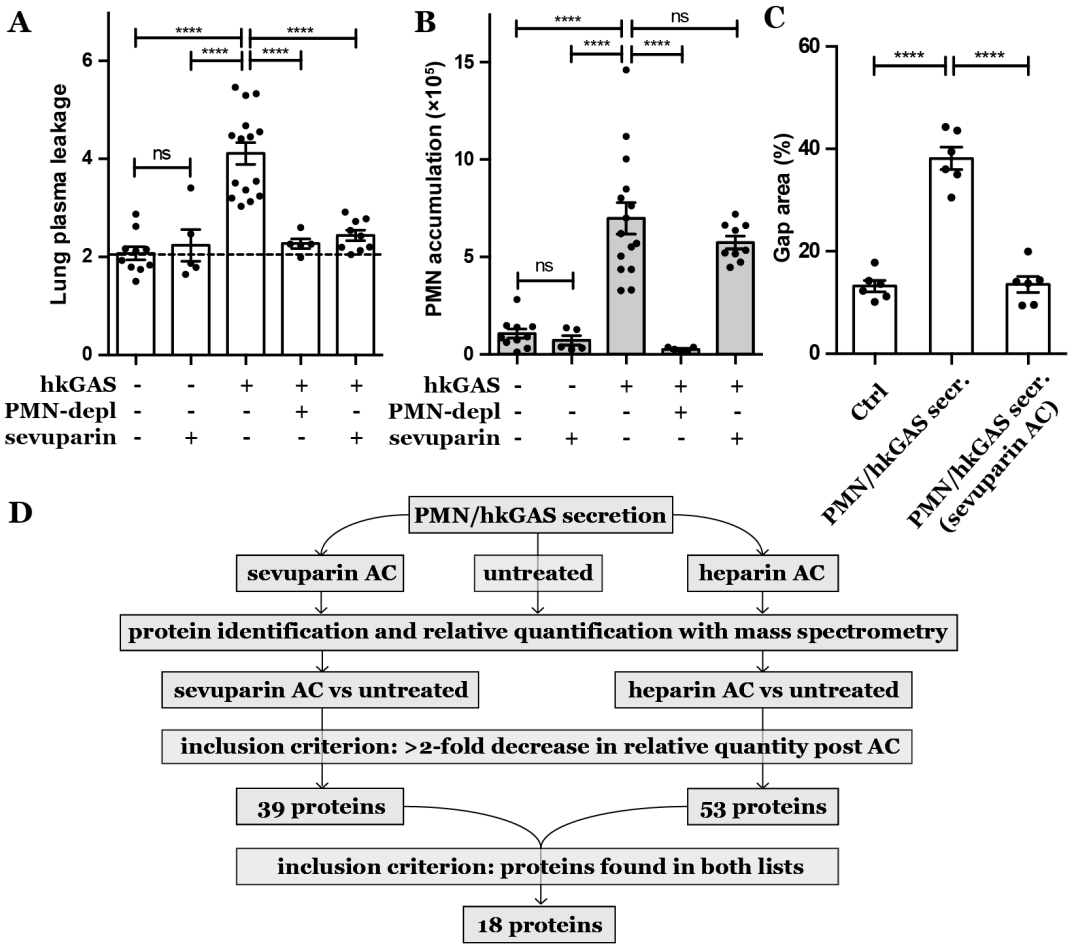

Figure 4. Sevuparin attenuates lung plasma leak, and affinity chromatography (AC) with sevuparin-coated beads renders neutrophil secretion inactive. A) Lung plasma leakage and **B)** lung neutrophil accumulation in hkGAS-induced acute systemic inflammation in mice. PMN-depl = neutrophil depletion. **C)** HUVEC monolayer gap formation following stimulation with secretion from hkGAS-activated human neutrophils, with or without AC with sevuparin-coated beads. **D)** Flowchart for identifying neutrophil-derived proteins with EC barrier-disrupting effects using AC and mass spectrometry.

Next, secretion from hkGAS-activated neutrophils was subjected to affinity chromatography (AC) with sevuparin-conjugated sepharose beads or with a heparin column. Post-AC supernatants from both sevuparin- and heparin-AC completely lacked disruptive effects on EC, indicating that the neutrophil secretion components responsible for effects on EC were eliminated by binding the polysaccharides (**Figure 4C**). Mass spectrometry analysis of neutrophil secretion and post-AC supernatants was then performed to find out which neutrophil-derived proteins that interacted with sevuparin and heparin, implicitly the proteins responsible for EC barrier disruption. Over 400 proteins were identified in the secretion from hkGAS-activated neutrophils and the relative quantity of the individual proteins was compared before and after AC with sevuparin and heparin. Comparison rendered a list in order of how much the proteins had decreased in quantity following AC. An assumption was made that the proteins responsible for the disruptive effects on EC were proteins that were most efficiently removed by AC (inclusion criterion of >2-fold change in relative quantity). Also, since post-AC supernatants from either sevuparin or heparin AC had no effects on EC, it was concluded that proteins found in only one of the clusters could not be responsible for the disruptive effects on EC. After including proteins that had decreased in quantity more than 2-fold after either sevuparin or heparin AC, and after excluding proteins only found in one of the lists, 18 proteins remained (**Figure 4D and Table 2**).

Several of the proteins in table 2 have previously established disruptive effects on EC. The granule-derived serprocidins (HBP, NE, P3, CG) were all found to effectively bind sevuparin. Furthermore, the granule protein MPO, as well as EPO and ECP, also bound sevuparin to a high degree. Besides proteins of granular origin with documented permeability-increasing effects on EC, the nucleus-derived histone H4 and the cytoplasmic proteins S100A8, S100A9 and S100A12 all strongly interacted with sevuparin. Protein S100A8 and S100A9 together form the heterodimer complex calprotectin with known antimicrobial effects and that is used as a biomarker for inflammatory bowel disease (Burri and Beglinger, 2014). In a study by Urban and colleagues, proteomic analysis of the constituents of neutrophil extracellular traps (NETs) identified proteins of granular, nuclear as well as cytosolic origin (Urban et al., 2009). Of the NET-associated proteins they found, several were the same as the proteins that we identified. Although we did not investigate the presence of NETs in paper II, these results imply that hkGAS stimulated NETosis. In support of this, GAS has previously been found to induce release of NETs (Lauth et al., 2009). Deoxyribonuclease I (DNAse I) is known to break down NETs by cleaving DNA strands and is frequently used in experimental studies investigating the role of NETs. In the mouse model of acute systemic inflammation with hkGAS, DNAse I treatment only had a

minor inhibitory effect on lung plasma leakage and no effect on lung neutrophil accumulation, whereas cell-free DNA in serum was substantially decreased as compared to untreated mice (unpublished results).

Table 2. Neutrophil-derived proteins bound by both sevuparin and heparin.

Protein name	UniProt	Fold change post AC[A] (% remaining)		Known disruptive or protective effect on EC barrier
		Sevuparin	Heparin	
EPO	P11678	Infinite (0.0%)	3.0 (33.2%)	**Disruptive** (Minnicozzi et al., 1994)
Bactericidal permeability-increasing protein[B]	P17213	Infinite (0.0%)	2.4 (41.5%)	**Protective** (Arditi et al., 1994)
Cathepsin G	P08311	269.3 (0.4%)	4.5 (22.4%)	**Disruptive** (Kenne et al., 2019)
Azurocidin, HBP	P20160	38.5 (2.6%)	18.9 (5.3%)	**Disruptive** (Gautam et al., 2001)
Protein S100-A12	P80511	22.4 (4.5%)	11.5 (8.7%)	**Disruptive** (Wittkowski et al., 2007)
Lactotransferrin	P02788	14.5 (6.9%)	51.6 (1.9%)	**Protective** (Erga et al., 2001)
Annexin A3	P12429	9.2 (10.9%)	2.2 (45.7%)	**No known**
Histone H4	P62805	9.0 (11.1%)	Infinite (0%)	**Disruptive** (Xu et al., 2009)
MPO	P05164	5.85 (17.1%)	23.3 (4.3%)	**Disruptive** (Patterson et al., 2014)
Neutrophil elastase	P08246	5.7 (17.7%)	21.7 (4.6%)	**Disruptive** (Peterson et al., 1987)
ECP, RNAse 3	P12724	5.3 (18.8%)	9.4 (10.7%)	**Disruptive** (Minnicozzi et al., 1994)
Cathelicidin antimicrobial peptide, LL-37	P49913	5.0 (20.2%)	5.9 (17.1%)	**Protective** (Byfield et al., 2011)
Metalloproteinase inhibitor 2, TIMP-2	P16035	4.3 (23.1%)	Infinite (0%)	**Protective** (Kim et al., 2012)
Myeloblastin, Leukocyte proteinase 3	P24158	2.9 (34.2%)	4.8 (20.7%)	**Disruptive** (Kenne et al., 2019)
Protein S100-A8	P05109	2.6 (38.2%)	33.1 (3.0%)	**Disruptive** (Wang et al., 2014)
Protein S100-A9	P06702	2.4 (42.2%)	36.8 (2.7%)	**Disruptive** (Wang et al., 2014)
Olfactomedin-4	Q6UX06	2.2 (45.6%)	2.8 (35.8%)	**No known**
Platelet basic protein	P02775	2.0 (49.8%)	2.1 (47.3%)	**No known**

[A]Values from 2 separate analyses comparing neutrophil secretion after sevuparin and heparin AC with untreated neutrophil secretion.

The effects of heparin on the inflammatory and coagulative processes involved in sepsis are under investigation, and as of today, heparin's role as a therapeutic in sepsis remains conflicting (Li and Ma, 2017). Modified low-anticoagulant derivatives of heparin might prove beneficial due to a reduced risk of adverse bleeding. Similar to the effects we find with sevuparin, other variants of low-anticoagulant heparin have also been found to bind neutrophil-derived proteins such as HBP, S100 proteins and histones, as well as to prevent lung damage and improve survival in sepsis (Rao et al., 2010,

Wildhagen et al., 2014). Sevuparin is negatively charged and electrostatic charge interaction is therefore likely the mechanism for neutralizing the cationic neutrophil-derived proteins.

Taken together, paper II confirms the significant role of neutrophil secretory products in causing vascular leakage in acute inflammation and presents sevuparin as a potential therapeutic.

4.4 POLYPHOSPHATES ACTIVATE NEUTROPHILS AND CAUSE LUNG PLASMA LEAKAGE

Besides neutrophils, platelets are also highly involved in the acute inflammatory response by forming platelet-neutrophil complexes and contributing to neutrophil activation through several proposed mechanisms. Inorganic polyphosphates (polyP) are released from granules upon platelet activation, and has been shown to increase EC barrier permeability by direct effects on EC, but also via activation of the KKS. Furthermore, a recent study suggested platelet polyP as a novel regulator of neutrophil activation by inducing NET formation in thromboinflammation (Chrysanthopoulou et al., 2017).

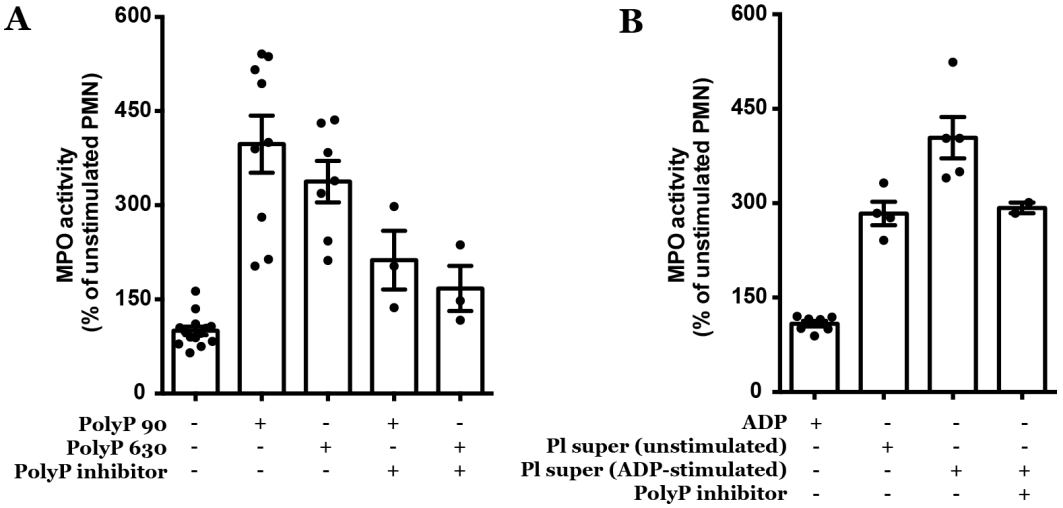

Figure 5. Platelet polyphosphates (polyP) induce neutrophil degranulation. A) Myeloperoxidase (MPO) activity in in supernatant of human neutrophils stimulated with polyP of different polymer lengths. PolyP 90 = 90 phosphate units, PolyP 630 = 630 phosphate units, PolyP inhibitor = PAMAM dendrimer generation 1.0. **B)** MPO activity in supernatant of neutrophils stimulated with either adenosine diphosphate (ADP) or supernatant (Pl super) from unstimulated or ADP-stimulated platelets, with or without polyP inhibitor.

In paper III the aim was to investigate platelet polyP as an activator of neutrophils and its potential role in neutrophilic systemic inflammation. Isolated neutrophils were incubated with synthetic polyP of various polymer lengths and MPO release was analyzed as a measure of neutrophil degranulation. PolyP released from platelets was previously shown to be 60-100 phosphate residues long (Ruiz et al., 2004, Muller et al., 2009) and longer polymers of platelet polyP have been suggested to be presented on the surface of activated platelets as insoluble nanoparticles (Verhoef et al., 2017). PolyP with polymer lengths of 90 and 630 phosphate units induced neutrophil degranulation that was attenuated in the presence of a polyP inhibitor (PAMAM dendrimer generation 1.0) (**Figure 5A**) previously found to target polyP by charge interaction (Smith et al., 2012). Furthermore, neutrophils were incubated with supernatant from activated platelets with or without polyP inhibitor, with similar results. (**Figure 5B**).

Next, the potency of polyP in mediating neutrophil-induced lung edema was assessed in mice. Intravenous administration of synthetic polyP 630 induced lung plasma leakage that was prevented in both neutropenic mice and in mice treated with the polyP inhibitor (**Figure 6A-B**). Furthermore, polyP 630 increased lung neutrophil accumulation that was not diminished with the polyP inhibitor. In contrast, polyP 90 did not induce any increases in neutrophil accumulation or lung plasma leakage. Previous studies, investigating the impact of polyP polymer length on its proinflammatory and procoagulant capacity, have shown that longer polymers (i.e. >500 units) increase EC permeability at lower concentrations than shorter polymers (i.e 60-100 units), and that longer polymers induce FXII activation to a higher degree than shorter polymers (Dinarvand et al., 2014, Smith et al., 2010, Wang et al., 2019). With regards to the discrepancy of our results with polyP 90 and 630 *in vivo*, since *in vitro* neutrophil degranulation did not differ, a possible explanation might be that polyP 90 is more easily degraded by endogenous phosphatases.

Targeting polyP has previously been suggested as a therapeutic strategy in inflammatory disease (Smith et al., 2012). After having shown that polyP induced neutrophil degranulation and neutrophil-mediated vascular leakage, we therefore investigated the role of platelet polyP in the more clinically relevant mouse model of acute systemic inflammation with hkGAS. Platelet-depletion decreased lung neutrophil accumulation as compared to platelet-competent mice, whereas effect on plasma leakage was difficult to interpret due to focal hemorrhages in lung tissue (**Figure 6C-D**). Treatment of mice with the polyP inhibitor decreased lung plasma leakage, but also increased lung neutrophil accumulation (**Figure 6C-D**). That lung plasma leakage was decreased supports a role for polyP in the systemic inflammatory

response caused by hkGAS. Furthermore, that lung neutrophil accumulation was increased with the polyP inhibitor might indicate cytotoxicity, which is a known adverse effect of PAMAM dendrimers (Sadekar and Ghandehari, 2012, Durocher and Girard, 2016).

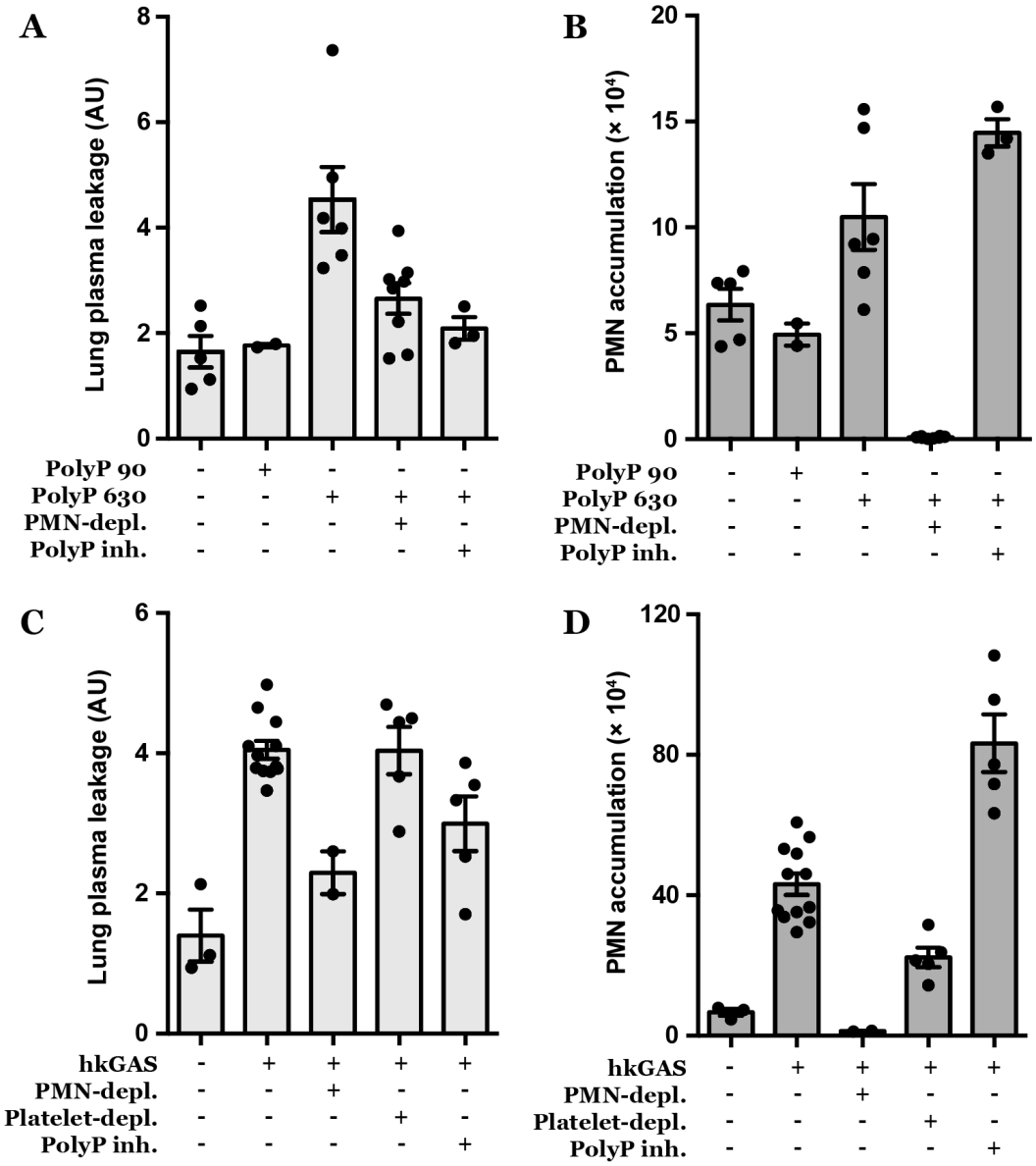

Figure 6. PolyP inhibition decreases lung plasma leakage in acute systemic inflammation in mice. A and C) Lung plasma leakage and **B and D)** neutrophil accumulation in mice subjected to intravenous injection of polyP 90, polyP 630 or hkGAS. PMN-depl = neutrophil depletion, PolyP inh = PAMAM dendrimer, Platelet-depl = platelet depletion.

Paper I showed that neutrophil-mediated vascular leakage involves activation of KKS and BK formation. FXII, the initiator of KKS activation together with PK, also initiates the intrinsic pathway of coagulation. In a previous study, platelet polyP was suggested as the missing link between primary and secondary hemostasis by activating FXII, and also as a novel mediator of inflammation by inducing BK formation (Muller et al., 2009). These findings were however questioned by others that concluded platelet-polyP not to be a physiologically relevant FXII activator (Faxalv et al., 2013). Since then, another study has found that platelet-size polyP only has minor direct effects on FXII, but that it can accelerate FXII activation by PK (Wang et al., 2019). In paper III, we found that neutrophil-induced vascular leakage, previously found to involve the KKS, is decreased with a polyP inhibitor and that polyP induces neutrophil degranulation. Given ours and others findings, it is intriguing to speculate that the role of platelet polyP in causing vascular leakage, besides the direct effects on EC, is by both KKS activation and by direct effects on neutrophils that in turn activates the KKS (**Figure 7**). An additional hypothesis is that polyP-mediated KKS activation leads to BK-mediated neutrophil activation, which is supported by findings that neutrophils express BK receptors (Bockmann and Paegelow, 2000) and that LPS-induced neutrophil-platelet aggregation could be prevented with a BK receptor antagonist (Hou et al., 2018). In line with our findings, Hou and colleagues also found that platelet polyP activates neutrophils.

In conclusion, paper III shows that polyP activates neutrophils and is involved in causing lung vascular leakage in acute systemic inflammation.

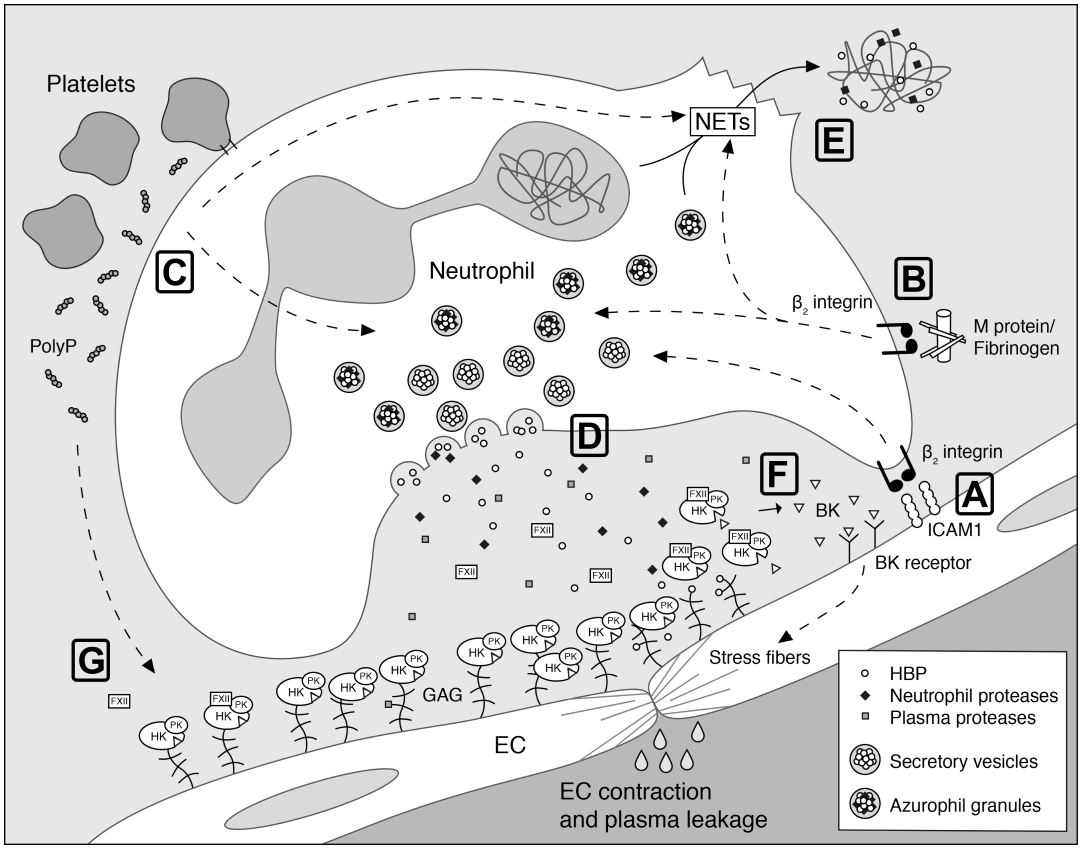

Figure 7. Suggested pathways for neutrophil activation and neutrophil-induced increases in endothelial permeability. A) Neutrophil adhesion to endothelial cells, **B**) streptococcal M protein-fibrinogen complex bind β_2 integrins, **C**) platelet-induced neutrophil activation (receptor-mediated or paracrine effect with e.g. polyphosphates (polyP)), **D**) neutrophil degranulation, **E**) neutrophil extracellular trap (NETs) formation, **F**) granule proteins activate the kallikrein-kinin system with consequent bradykinin (BK) formation, **G**) platelet polyP has direct effects on endothelial cells and/or activates the kallikrein-kinin system.

4.5 PHENYLBUTYRATE TREATMENT MODULATES THE HOST RESPONSE IN *PSEUDOMONAS AERUGINOSA*-INDUCED PULMONARY INFLAMMATION

In paper IV the aim was to study the effects of phenylbutyrate (PBA) on the inflammatory response in a mouse model of pulmonary inflammation with heat-killed *Pseudomonas aeruginosa* (hkPAO1). The short-chain fatty acid (SCFA) PBA is in clinical use for treatment of urea cycle disorders, and by its capacity to reduce endoplasmic reticulum stress and as a histone deacetylase inhibitor it is used for treatment in type-2 diabetes and various forms of cancer. Furthermore, PBA has both anti-inflammatory and host defense-enhancing properties, and is suggested as a therapeutic in infectious disease (Coussens et al., 2015).

Inflammation was induced by intranasal instillation of hkPAO1 with or without PBA, and after incubation for different time periods mice were sacrificed and bronchoalvelolar lavage (BAL) fluid was analyzed for leukocyte content. HkPAO1 induced lung neutrophil recruitment, and treatment with PBA altered the kinetics of neutrophil influx to the lungs. At 4 h, neutrophil recruitment was significantly increased in PBA-treated mice compared to vehicle, and at 24 h it was significantly decreased (**Figure 8A**). Since the kinetics of neutrophil influx and consecutive clearance appeared faster with PBA treatment, the inflammatory edema in lungs was evaluated. Protein levels in BAL fluid and edema formation in lung tissue, assessed by wet-dry weight of whole lung, were decreased in PBA-treated mice 48 h post bacterial instillation (**Figure 8B-C**). These results support previous studies showing that PBA has anti-inflammatory effects (Ono et al., 2017, Kim et al., 2013).

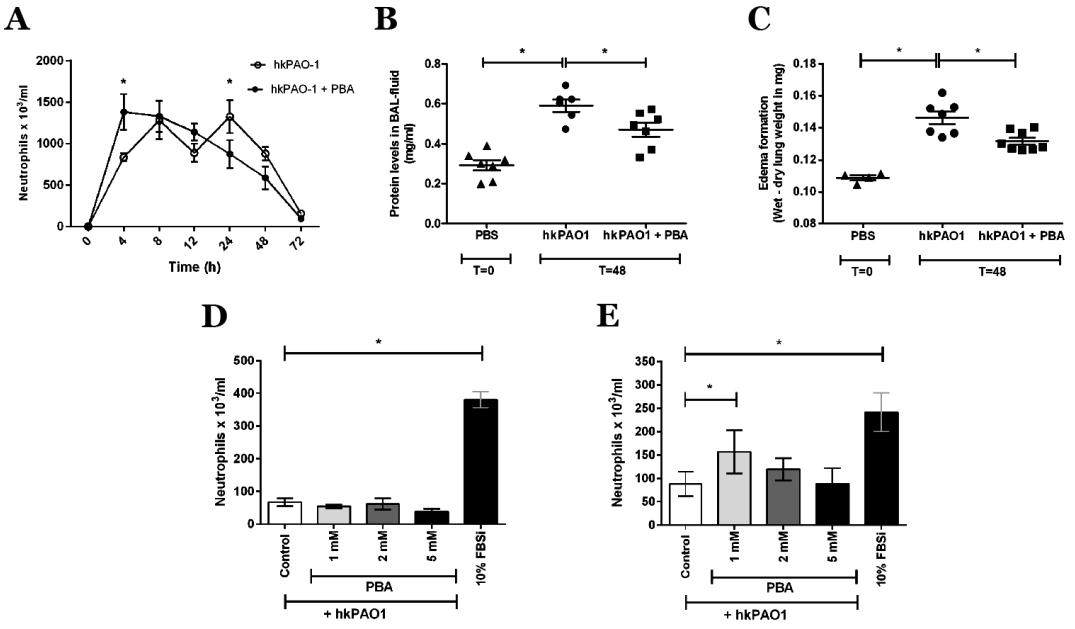

Figure 8. Phenylbutyrate (PBA) modulates the kinetics of the host response in hkPAO1-induced pulmonary inflammation in mice. A) Neutrophil accumulation in BAL fluid of mice subjected to intranasal administration of hkPAO1 at several time points with or without treatment with PBA. **B)** Protein levels in BAL fluid and **C)** wet-dry weight of lung tissue at T=0 and 48 hours after stimulation with hkPAO1. **D and E)** Chemotaxis assay with human isolated neutrophils showing that PBA does not have **D)** direct chemotactic activity, but **E)** stimulate lung epithelium to release factors that induce neutrophil chemotaxis. FBSi = heat-inactivated fetal bovine serum.

SCFAs were previously shown to have direct chemotactic properties towards neutrophils (Vinolo et al., 2011), and since neutrophil recruitment was increased at 4 h in PBA-treated mice, PBA's chemotactic potential was investigated. Intranasal treatment with PBA in unstimulated mice did not increase neutrophil recruitment as measured in BAL fluid, and an *in vitro*

chemotaxis assay showed no increase in migration towards PBA in the presence of hkPAO1 (**Figure 8D**). However, supernatant from cultured lung epithelium treated with PBA and hkPAO1 increased neutrophil migratory capacity, suggesting that PBA induces release of chemotactic factors from lung epithelium that could explain our findings *in vivo* (**Figure 8E**). Two typical neutrophil chemoattractants potentially involved are CXCL1 (mouse homologue of IL-8) and LTB_4, but gene expression analysis of *Cxcl1* and *Alox5* in homogenized whole lung tissue showed no difference between PBA-treated and vehicle mice.

PBA has previously been found to enhance host defense by increasing expression of antimicrobial peptides (AMPs) such as the cathelicidin LL-37 (Steinmann et al., 2009, Mily et al., 2013, Sarker et al., 2011). For that matter, gene expression of cathelicidin-related AMP (*Cramp*), the mouse homologue to cathelicidin, was measured in homogenized whole lung tissue at several time-points following hkPAO1-instillation with and without PBA. However, no significant increase was found in hkPAO1-stimulated mice and PBA did not alter the expression. Since PBA treatment dampened the inflammatory response at later time points, we hypothesized that PBA might expedite resolution of inflammation and therefore we measured the anti-inflammatory cytokine IL-10 in BAL fluid and the gene expression of *Alox15* in lung tissue. *Alox15* encodes 15-LOX, which mediates the formation of the pro-resolving lipoxins. IL-10 was not detected in BAL fluid and *Alox15* expression was unchanged at all time points. Gene expression of *Ptgs2* (COX-2) was also measured in lung tissue, and PBA-treated mice had significantly decreased expression of COX-2 at 4 h, supporting an anti-inflammatory role of PBA.

In summary, in a pulmonary inflammatory response caused by hkPAO1, PBA modulates the kinetics of the cellular host response by enhancing initial neutrophil recruitment, supposedly by stimulating lung epithelium to release chemotactic factors, and then causing a faster decline in neutrophil influx coincident with an attenuation of lung damage.

5. CONCLUDING REMARKS

Major conclusions drawn based on the experimental work that makes up this thesis:

- The kallikrein-kinin system is involved in mediating neutrophil-induced vascular leakage in acute inflammation (paper I)

- The neutrophil-derived protein HBP act in concert with neutrophil serine proteases to activate the kallikrein-kinin system causing endothelial barrier disruption (paper I)

- Heparinoid sevuparin inhibits lung plasma leakage in hkGAS-induced acute systemic inflammation by neutralizing neutrophil-derived proteins (paper II)

- Platelet-derived polyphosphates activate neutrophils and contribute to neutrophil-induced lung plasma leakage in acute systemic inflammation (paper III)

- Phenylbutyrate treatment modulates the host response in pulmonary inflammation caused by hkPAO1 by altering the kinetics of neutrophil recruitment and by attenuating pulmonary edema (paper IV)

Neutrophils are key players in infectious and inflammatory conditions such as ALI/ARDS. This thesis work present data confirming the central role of neutrophils in mediating inflammatory vascular leakage, and provides additional insights into mechanisms regulating neutrophil activation and endothelial barrier permeability. Targeting different functions of neutrophils might prove successful for improving outcome in inflammatory disease conditions. As of today, several different strategies are employed to treat inflammatory disease. One is to attack the stimuli that trigger the inflammatory reaction, e.g. with antibiotics or antivirals if the cause is infectious. Another option, complementary to the use of antibiotics and highly relevant due to the increase in antibiotic resistance, is to enhance the host response with immune modulators. In paper IV, the previously suggested immune modulator PBA was found to both accelerate the inflammatory response and to have anti-inflammatory effects in a murine model of pulmonary inflammation. Immune modulation for treatment of infection and for improving resolution of inflammation is a promising strategy with great potential. In non-infectious inflammatory disease, a common strategy is to counteract the inflammatory reaction by inhibiting the

production or activity of inflammatory mediators. In this thesis, various mediators of neutrophil-induced plasma leakage (**Figure 7**) were effectively antagonized with different pharmacological inhibitors. A possible advantage with this treatment strategy, as compared to more generalized anti-inflammatory treatments, is that selectively targeting an inflammatory mediator may leave other functions of host defense intact. In support for this view we find that the neutrophils ability to adhere to EC and to extravasate to surrounding tissue is not seemingly impaired following inhibition of KKS constituents or treatment with sevuparin in paper I and II. These results might indicate a preserved cellular immune response whilst tissue is protected from excessive edema, suggesting that these treatment strategies could be beneficial as a complement to anti-infectious treatment in pathogen-induced inflammatory disease where the inflammatory response strongly contributes to morbidity and mortality.

This thesis has focused on the role of neutrophils in acute inflammation, and has investigated the involvement of both platelets and the kallikrein-kinin system in this context. That several mechanisms of inflammation and coagulation are intertwined is becoming more and more clear, and new insights into the roles of the different players, namely neutrophils, platelets, coagulation factors, the kallikrein-kinin system and the endothelium, will lead to better understanding of these processes and hopefully to improved treatment strategies. Besides acting as a selective barrier between blood and tissue, the endothelium is also a thromboregulator that in health maintains an anti-thrombotic state. In inflammatory conditions such as sepsis, the endothelium increases expression of adhesion molecules and release factors that promote activation of neutrophils and platelets, as well as coagulation factors. Intravascular activation of the host response system leads to thromboinflammation that involves, amongst others, the aforementioned players (Ekdahl et al., 2016). In a recent study investigating the role of neutrophils in thromboinflammation, platelet polyP was found to induce NET formation (Chrysanthopoulou et al., 2017), and in paper III we found that polyP induces neutrophil degranulation and neutrophil-induced lung plasma leakage. The host defense mechanism immunothrombosis, involving activation of neutrophils, platelets and coagulation factors, is important for successfully combating intravascular pathogens by capture in microthrombi. However, exaggerated neutrophil activation in sepsis can cause uncontrolled immunothrombosis progressing into DIC (Stiel et al., 2018). Furthermore, a recent study proposed bacterial-type long-chain polyP as a factor for development of DIC by inducing FXII-dependent platelet aggregation and consumption (Zilberman-Rudenko et al., 2018). Taken together this suggests that inhibitors targeting polyP of both platelet and bacterial origin might be a

treatment strategy for conditions involving both inflammation and coagulation.

Heparin, due to pleiotropic effects on both coagulation and inflammation, is a promising candidate as a therapeutic in inflammatory disease conditions such as sepsis (Li and Ma, 2017). In paper II, sevuparin attenuated lung plasma leakage in hkGAS-induced acute systemic inflammation. Unlike heparin, sevuparin lacks anticoagulant effects and might therefore be advantageous with regards to the risk of adverse bleeding. However, in sepsis with coagulopathy, heparin may be beneficial. Further studies investigating the effects of heparin, sevuparin and other low-anticoagulant derivatives in acute systemic inflammatory conditions are anticipated.

The studies within this thesis were carried out in animal and cell culture-based models of both noninfectious and infectious inflammatory conditions. In patients with e.g. ARDS there are numerous factors that differ from experimental models and that might influence treatment efficacy. For example, it may vary if the cause is infectious or noninfectious, and if infectious it matters which pathogen that is responsible. That neutrophils respond differently in terms of degranulation and NET formation towards different bacterial strains has previously been shown (Snall et al., 2016, Bystrzycka et al., 2018). In this thesis work, effects on survival with different treatments were not investigated, and only heat-killed bacterial strains were used so assessment of bacterial dissemination was not feasible. It ought to be emphasized also that treatment during an already established inflammatory response was not tested. In a clinical context, pre-treatment does not occur and therefore the time-dependent efficacy is also a factor that warrants further examination.

To sum up this thesis I would like to end with a definition of inflammation by Mauricio Rocha e Silva that I think encapsulates its complexity: “a multi-mediated phenomenon, of a pattern type in which all mediators would come and go at the appropriate moment to play their roles in increasing vascular permeability, attracting leucocytes, producing pain, local edema and necrosis, in which the predominance of any would be fortuitous or depending on its specific capabilities of producing symptoms, some directly, some indirectly, some by potentiating or by releasing other agents” (Silva, 1978).

6. ACKNOWLEDGEMENTS

I would like to acknowledge and express gratitude to a number of individuals that in different ways have been more or less involved in my meandering scientific journey.

First and foremost I wish to thank my main supervisor **Lennart Lindbom**. For teaching me to do research, for always having an open door and seemingly endless time to discuss both work and private life, for being more deliberate and thorough than me and thereby improving our work, and for helping me wrap this thesis up although I sort of overstayed my welcome at the department. Sorry for postponing your retirement!

Equally important is my "main" co-supervisor **Ellinor Kenne**. You taught me everything in the lab during the early years, and you have been an important positive force for both me and my PhD project throughout the years. Thank you so much E!

I also wish to thank my other co-supervisors Professor **Eddie Weitzberg** and Professor **Oliver Söhnlein** for input and support.

Erik Lundeberg, my previous roommate, now I consider you my clinical mentor. **Anne van der Does**, thanks for all the tough years spent in the lab. Your directness seasoned me in a good way.

Friends and former corridor colleagues **Joshua and Ebba Gregory**.

Sofia Petterson for all the nice chats.

Niklas Ivarsson, so sudden, so unexpected, I wish you great things on the other side.

Other Fyfa colleagues that I will remember, **Eva-Karin Gidlund**, **Mia Ydfors**, **Susanna Wallman Appel**, **Malene Lindholm**, **Gustaf Wigerblad**, **Anja Finn**, **Milana Kokosar**, **Romina Fornes**, **Bo Rydqvist**, **Håkan Westerblad**, **Eva Lindgren**, **Peter Wolf**, **Mats Rundgren**, **Michael Elm**, **Joseph Bruton**, **Magdalena Kegel**, **Cecilia Jädert**.

Alexander Viktorin for helping with the thesis layout.

My dear friends from outside work and **my classmates** from medical school, thanks for putting up with me whining about the thesis throughout the years.

My family, especially my brother **Joakim Rasmuson** for designing the figures in paper I and in the thesis frame.

Finally, my own family, **Malin**, Majken and Konrad. For making this possible. You are my world and I cannot thank you enough.

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