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## Host modulation of excessive innate immune responses

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# Host modulation of excessive innate immune responses

*By*

Johannes Westman

2016



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DOCTORAL DISSERTATION

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To be defended on 22th of January 2016 at 9 AM in Segerfalksalen, Wallenberg  
Neuroscience Center, Lund, Sweden.

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About the front cover:

Scanning electron microscopy of a lung from a mouse injected with mixture of extracellular histones and p33. The picture was taken by Matthias Mörgelin.

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*To my family and loved ones,*



# Preface

I have a memory from when I was a child, not more than 7 years old. I was in the hallway in our house when I asked my mother why the skin on my arm was itching. She answered that there maybe was a bug or insect on me, too small for my eyes to see. At first, I felt disgusted and so I went to the bathroom to wash my arm. Later in life I have come back to this memory as an early sign that I always have been interested in what happens at a microscopic level. During school, biology was my favorite subject, maybe even though I didn't realize it at that time. I especially enjoyed 'white' biology (cell biology and microbiology) in contrast to 'green' biology (botanic and ecology). I moved to Lund from Uppsala in 2007. During my undergraduate studies at Lund University I have been fortunate to focus on my favorite subject biomedicine, and during my years as a PhD student, my interest in the human body and its immune system has grown even deeper. I realize that I have almost spent a decade here. Today when I submit my thesis, I feel that I have finished what I started.

This thesis concerns part of the innate immunity, but far from everything is covered. I decided early to not write traditional review chapters on every type of immune cell and every innate immune mechanism. Instead, I have put focus on a few segments of innate immunity that I think is important for understanding my research. I found it enjoyable to write, and I hope you find it enjoyable to read.



Lund, 30th of November 2015



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## Original papers

- Paper I      **Johannes Westman**, Finja C Hansen, Anders I Olin, Matthias Mörgelin, Arthur Schmidtchen, Heiko Herwald.  
p33 (gC1q receptor) prevents cell damage by blocking the cytolytic activity of antimicrobial peptides.  
Journal of Immunology. 2013 Dec 1;191(11):5714-21.
- Paper II      **Johannes Westman**, Emanuel Smeds, Linda Johansson, Matthias Mörgelin, Anders I Olin, Erik Malmström, Adam Linder, Heiko Herwald. Treatment with p33 Curtails Morbidity and Mortality in a Histone-Induced Murine Shock Model.  
Journal of Innate Immunity. 2014;6(6):819-30.
- Paper III      **Johannes Westman**, Praveen Papareddy, Madelene W Dahlgren, Bhavya Chakrakodi, Anna Norrby-Teglund, Emanuel Smeds, Adam Linder, Matthias Mörgelin, Bengt Johansson-Lindbom, Arne Egesten, Heiko Herwald.  
Extracellular histones induce chemokine production in whole blood *ex vivo* and leukocyte recruitment *in vivo*.  
PLoS Pathogens. 2015. In press.
- Paper IV      Sonja Oehmcke, **Johannes Westman**, Johan Malmström, Matthias Mörgelin, Anders I Olin, Bernd Kreikemeyer, Heiko Herwald. A novel role for pro-coagulant microvesicles in the early host defense against *streptococcus pyogenes*.  
PLoS Pathogens. 2013;9(8):e1003529.

## Papers not included

Daniel Svensson, **Johannes Westman**, Claes Wickström, Daniel Jönsson, Heiko Herwald, Bengt-Olof Nilsson.

Human endogenous peptide p33 inhibits detrimental effects of LL-37 on osteoblast viability.

Journal of Periodontal Research. 2015 Feb;50(1):80-8.

# Abbreviations

aa, amino acid  
AMP, antimicrobial peptide  
ATP, adenosine triphosphate  
bp, basepair  
CCL2, chemokine (C-C motif) ligand 2  
CCL3, chemokine (C-C motif) ligand 3  
CCL7, chemokine (C-C motif) ligand 7  
CCL20, chemokine (C-C motif) ligand 20  
CTH, calf thymus histones  
CXCL9, chemokine (C-X-C motif) ligand 9  
CXCL10, chemokine (C-X-C motif) ligand 10  
CXCR3, chemokine (C-X-C motif) receptor 3  
kDa, kilodalton  
DAMP, damage-associated molecular pattern  
DNA, deoxyribonucleic acid  
DNase, deoxyribonuclease  
dsDNA, double stranded DNA  
EA.hy926, endothelial somatic cell hybrid  
ELISA, enzyme-linked immunosorbent assay  
HDP, host-defense peptide  
HKH20, HMWK-related antimicrobial peptide  
HMWK, high-molecular-weight kininogen  
HUVEC, human umbilical vein endothelial cell

ICU, intensive care unit  
IFN- $\gamma$ , interferon gamma  
IL, interleukin  
LL-37, cathelicidin-related antimicrobial peptide  
LPS, lipopolysaccharide  
MD-2, myeloid differentiation factor 2  
MG-63, osteoblast-like sarcoma cell line  
MV, microvesicle  
NAT26, HMWK-related antimicrobial peptide  
NET, neutrophil extracellular trap  
NF $\kappa$ B, nuclear factor kappa-light-chain-enhancer of activated B cells  
nM, nanomolar  
NOD, nucleotide-binding oligomerization domain receptor  
PAMP, pathogen-associated molecular pattern  
PBMC, peripheral blood mononuclear cell  
PRR, pattern recognition receptor  
PS, phosphatidylserine  
SLE, systemic lupus erythematosus  
*S. pyogenes*, *streptococcus pyogenes*  
TLR, toll-like receptor  
TNF- $\alpha$ , tumor necrosis factor alpha  
TF, tissue factor

# Abstract

A fundamental role of the innate immune system is to elicit a proper response against both invading pathogens and tissue damage or danger signals. Although the innate immune system often is successful in eliminating danger, an excessive host-response can result in severe tissue injury. Factors that contribute to the elimination of the microbe are the same factors that cause tissue injury. Therefore, innate immune response has to be carefully balanced to avoid excessive inflammation with systemic consequences. The first part of this thesis is focused on molecular interactions between host-defense peptides and p33 (globular C1q receptor), a protein that can modulate cell injury. We show *in vitro* how recombinant p33 binds and neutralizes cytotoxic effects of the host-defense peptides human beta defensin 3 and LL-37. Moreover, treatment with p33 was also shown to modulate the sepsis-like pathogenesis caused by extracellular histones and rescue mice in a histone-induced shock model. Extracellular histones are released into tissue and circulation after cell necrosis or neutrophil extracellular trap formation. They are found in patients with severe inflammatory diseases and contribute to disease progression. However, for the first time, we have shown that extracellular histones contribute to resolving local inflammation. Low concentrations of extracellular histones contribute to innate immunity by inducing chemokine production and leukocyte recruitment which might be beneficial for the host. The final part of this thesis describes a novel defense mechanism of microvesicles against the gram-positive bacterium *Streptococcus pyogenes*. We show that procoagulant microvesicles are released after stimulation of peripheral blood mononuclear cells with M1 protein from *S. pyogenes*. These microvesicles bind to the surface of bacteria, induce clotting and thereby prevent bacterial dissemination *in vivo*. In conclusion, this thesis describes new mechanisms involved in host defense as well as mechanisms involved in the modulation of excessive immune responses.

# Populärvetenskaplig sammanfattning

**M**änniskans kropp är koloniserad av ett stort antal svampar, virus och bakterier. I själva verket har vi tio gånger fler bakterier i oss än mänskliga celler. Koloniseringen sker utan sjukdomssymptom och de ofarliga bakterierna bildar tillsammans vår normalflora. Tyvärr finns det också några få sorters bakterier som utgör ett hot, dessa kallar vi för patogener. Normalflorabakterierna skyddar oss dels genom att producera vitaminer som vi kan tillgodoräkna oss, men främst genom att täcka yta på hud och i mag-tarmkanal. På så sätt förhindrar de möjligheten för patogener att orsaka infektion.

Under evolutionen har djur och bakterier utvecklats tillsammans. De har därför haft miljoner år på sig att anpassa sig till varandra. Under denna tid har människokroppen utvecklats olika system för att, vid behov, försvara sig mot bakterieinfektioner. Dessa system kallar vi för vårt immunförsvar. Immunförsvaret kan delas upp i två separata delar, medfött immunförsvar och förvärvat immunförsvar. Det medfödda immunförsvaret är ospecifikt, men agerar snabbt och kraftfullt ifall det skulle stöta på en främmande struktur (bakterie, virus eller svamp) på fel plats. Det förvärvade immunförsvaret är långsamt och det kan ta upp emot en vecka innan det aktiveras. Det förvärvade immunförsvaret måste först få signaler från det medfödda immunförsvaret att en patogen har påträffats, för att sen tillverka ett specifikt motvape mot just denna patogen.

Denna avhandling handlar enbart om det medfödda immunförsvaret och dess reglering. Att immunförsvaret är bra på att bekämpa infektioner vet vi. Det fungerar näst intill felfritt och de flesta försök av bakterier att invadera vår kropp går omärkta förbi. Däremot är det oerhört viktigt att immunförsvaret inte reagerar för starkt på infektionen då det skulle orsaka olika typer av vävnadsskada. Därför måste det kraftfulla medfödda immunförsvaret vara hårt reglerat när det angriper bakterierna. Immunförsvaret kan liknas vid en militär försvarsmakt som utkämpar ett krig bland sin egen civilbefolkning. Utan ett reglerat immunförsvar skulle militären utöver att eliminera fienden, skada både civilbefolkningen och byggnader. Många dödsfall orsakade av bakterieinfektioner sker inte av bakterien i sig, utan av immunförsvarets överdrivna reaktion mot bakterierna. I många fall



kan inflammationsresponsen mot infektionen övergå från en lokal till en systemisk inflammation (hela kroppen). Detta tillstånd efterföljs ofta av blodtrycksfall, cirkulationsproblem, organsvikt och slutligen dödsfall.

Min första delarbete beskriver hur ett protein som vi kallar p33 styr delar av immunförsvaret att eliminera bakterier utan att orsaka skada på kroppens egna celler. Vid en infektion frisätter hudceller och olika immunceller korta positivt laddade peptider (antimikrobiella peptider) med bakteriedödande effekt. Dessa försvarspeptider är utvecklade för att snabbt binda till bakterien och slå sönder bakteriens skal. Finns det för mycket av försvarspeptiderna kan de tappa sin specificitet till bakterier och därmed också börja förstöra kroppens egna celler. Vi beskriver hur ett negativt laddat cellyteprotein kallat p33 (eller gC1q bindande protein) kan binda upp och hämma försvarspeptiderna så att de inte förstör kroppens egna celler. (Westman et al 2013, The Journal of Immunology).

I mitt andra delarbete använder vi oss av en chockmodell där möss utsätts för en injektion av extracellulära histoner. Detta är en modell där vi efterliknar histonernas patologiska effekt i en sepsis, men utan att vi behöver använda oss av levande bakterier. Histoner finns vanligtvis i cellkärnan, men vid infektion/inflammation läcker histonerna ut från immunceller ut i blodbanan där de orsakar skada. Vi upptäckte att p33 kunde binda till och hämma histonerna som orsakade chocken. Vi tillverkade och renade fram p33 och använde det som en behandling av mössen. I gruppen av möss som fick den sepsisliknande chocken dog alla inom 30 minuter. I gruppen av möss som behandlades med p33 överlevde 50 %. (Westman et al 2014, Journal of Innate Immunity).

Mitt tredje delarbete har precis accepterats i tidskriften PLoS Pathogens. I detta manuskript visar vi hur låga koncentrationer av extracellulära histoner kan påskynda inflammationen i en lokal infektion. Histonerna kan binda till en receptor som kallas TLR4 på en viss sorts immunceller som vanligtvis känner igen bakterier. Resultatet av aktiveringen är att nya immunceller rekryteras till den lokala skadan. Därmed fungerar extracellulära histonerna som viktiga immunostimulerande adjuvanter. (Westman et al 2015, PLoS Pathogens, i tryck)

I avhandlingens sista delarbete byter vi fokus från p33 och extracellulära histoner och tar oss in i mikrovesiklarnas värld. Mikrovesiklar är små sfärer omslutna av ett cellmembran som lämnar kroppens celler vid olika typer av cellaktivering. Vesiklarna omsluts av samma cellmembran och innehåller liknande molekyler

som cellen vesikeln frisattes ifrån. Däremot om cellen har varit i kontakt med bakterier kan vesikeln innehålla ett förändrat proteininnehåll både inuti och på ytan av vesikeln. I detta arbete visar vi att blodceller som varit i kontakt med ett bakteriellt protein (M protein) från *Streptococcus pyogenes*, frisätter mikrovesiklar med egenskaper att binda till bakterier och starta koagulationssystemet. Genom att binda till bakterieytan och starta koagulationen så förhindras bakteriens förmåga att sprida sig (Oehmcke et al 2013, PloS Pathogens).



# CHAPTER 1

## *Introduction to the subject*

### **Infection and inflammation**

**B**acteria colonize humans at all time and the human body contains ten times more bacterial cells than human cells. Most of these bacteria are harmless and rarely cause disease; in fact, they might even be essential for our survival. These non-pathogenic bacteria are called ‘commensals’.

They grow in symbiosis with our body for the benefit of both parts. However, at certain stages of dysfunctional immunity, all commensals can shift into ‘opportunistic pathogens’ and cause disease. Only a few of thousands of bacterial strains are harmful, these are termed ‘pathogens’. The possibility for pathogenic bacteria to invade the human host is limited by our immune system. During evolution, the immune system has co-evolved with bacteria and developed an arsenal of different defense mechanisms to fight of invading pathogens. While most bacteria are limited by the immune system, others can evade the immune system and invade host tissues which results in disease. The effectiveness of the host defense as well as the virulence of the pathogen determines the outcome of the infection. Our immune systems response to an infection may cause inflammation. Inflammation can easily be observed through a mixture of five symptoms that characterizes it: *redness, swelling, heat, pain and loss of tissue function*. Aside from infection inflammation can also be initiated in sterile environments, at events such as various types of tissue injury, hypoxia, burns, or exposure to sterile irritants. The inflammatory response is terminated when the infection is cleared and the damaged tissues are repaired.

### **The immune system**

The immune system can be sectioned into the innate immune system and the adaptive (acquired) immune system. The innate immune system is the first line of defense against any invading pathogen and it is present in both invertebrates and vertebrates. It is a complex network of molecular and cellular systems with the drive of detecting and eliminating pathogens and sterile tissue damage. It is spread throughout the body: in intestines, skin, tissue, airways, mouth, even in tears.

Briefly described, the innate response consists of several dynamic defense systems including anatomical barriers, antimicrobial peptides and proteins, pattern recognition receptors, cytokine producing cells, phagocytic and granular cells, complement system and coagulation system. It is highly unspecific and is activated regardless of a prior exposure of the infectious agent. The adaptive immune system is slow and more specific. It is not involved in the first recognition of a pathogen, but rather involved in elimination of pathogens in a late phase of infection. It is dependent on primary pathogen recognition by the innate immune system. After recognition, an antigen specific immunity develops, but it takes up to a week before the adaptive immune system starts to fight of a new intruder. Hence, once exposed, the adaptive system rests silently with a memory, ready to wake up if the same pathogen tries to invade a second time.

# CHAPTER 2

## *Purpose of this thesis*

In this thesis, I aimed to further characterize how the host modulates an overproduction of danger signals. Danger signals (including antimicrobial proteins, host-defense peptides and DAMPs) form a key part of the innate response to invading pathogens as well as to sterile injury. However, very little is known about the modulation of danger signals. During infection or tissue injury, the host needs to maintain a limited and local release of danger signals to resolve the situation without causing further harm to the host. Without a limitation of danger signals, the host would suffer from an over-activation of the immune system and life-threatening conditions may be reached.

- The first part of this thesis covers a new mechanism for modulation of excessive innate immune responses. We describe how p33 (gC1q receptor) protects host cells from cell lysis induced by AMPs and we also show how p33 can be used to modulate pathogenesis caused by extracellular histones *in vivo*.
- In the second part, we demonstrate that extracellular histones are more than just pathological bystanders during necrosis and NETosis. Instead, we show that extracellular histones in low concentrations are important mediators of the innate immune response by directly interacting with TLR4/MD-2 on monocytes, inducing chemokine production which leads to a recruitment of leukocytes to the site of inflammation.
- In the last part, we describe a novel defense mechanism for microvesicles. We show how pro-coagulant microvesicles are a part of innate immunity since they bind to the surface of *S. pyogenes* and induce clotting which prevents bacterial dissemination.



# CHAPTER 3

## *How the host responds to danger*

### **Pattern recognition**

**T**he innate immune system recognizes bacteria through germ line encoded recognition receptors called Pattern recognition receptors (PRRs). There are several types of PRRs including Toll-like receptors, RIG-I-like receptors, NOD-like receptors and C-type lectin receptors. PRRs are expressed on various immune cells including macrophages, dendritic cells, specific types of T-cells, B-cells and on epithelial cells [1]. PRRs all share some common characteristics. First of all, they recognize and bind conserved microbial epitopes, so called pathogen associated molecular patterns (PAMPs), leading to intracellular signaling cascades that quickly induce the expression of a broad range of inflammatory proteins [1, 2]. Different PRRs recognize different PAMPs. They are constitutively expressed in the plasma membrane of host cells, or intracellular in endolysosomes or in the cytoplasm. Further, PRRs are highly conserved among species and are found in all organisms from plants and fruit flies to large mammals.

One of the major types of PRRs are the toll-like receptors (TLRs). They are composed of leucine-rich repeats in the ligand-binding domain and a cytoplasmic Toll/IL-1 receptor domain that interacts with adaptor molecules such as MyD88 [1-3]. TLRs can be found either in the plasma membrane (TLR1, 2, 4, 5 6) or intracellular in endosomes (TLR3, 7, 8, 9) [1]. TLRs sense structures from bacteria, viruses, fungi and mycoplasma [2]. Different TLRs have affinity for different microbial or host-derived patterns. For example, TLR4 is the common receptor for LPS from gram-negative bacteria [2-4], while TLR2 recognizes several ligands, including peptidoglycan, lipotechoic acid and M protein from the gram-positive pathogen *Streptococcus pyogenes* [4, 5]. Further, TLR7, 8 and 9 has been described to be the receptor for nucleic acids [1]. Signaling through TLRs induces the activation of the transcription factor NFκB which drives the transcription of several genes involved in inflammation, cell survival and cell proliferation [2, 3, 6]. Activation of most TLRs leads to the synthesis of



inflammatory cytokines and chemokines which is an essential part of the third paper concerning extracellular histones.

### The danger model

Earlier, pattern recognition in the innate immune system was described to aim for a discrimination of "self" and "non-self" [3]. Scientists believed that if a protein were self-produced, it would be completely tolerated by the immune system. This description was based on early observations that specific surface receptors specifically bound to a bacterial or viral component and initiated an immune response. Even though this model gave the field a practical diversion, it failed to describe the whole story of pattern recognition. PRRs also recognize host-derived components if they are to be found at the wrong location, for example after tissue damage, when intranuclear proteins are released after necrosis [7, 8]. To address a part of this problem, 'the danger model' was proposed by Polly Matzinger in 1994 [8, 9]. The danger model suggests that the innate immune system's main concern is to respond to danger signals rather than only foreign microbial patterns [9]. The danger model groups endogenous damage/danger signals (alarmins) and exogenous PAMPs together as damage associated molecular patterns (DAMPs). This model argues that PAMPs and DAMPs convey a similar message and elicit similar responses [7-9].

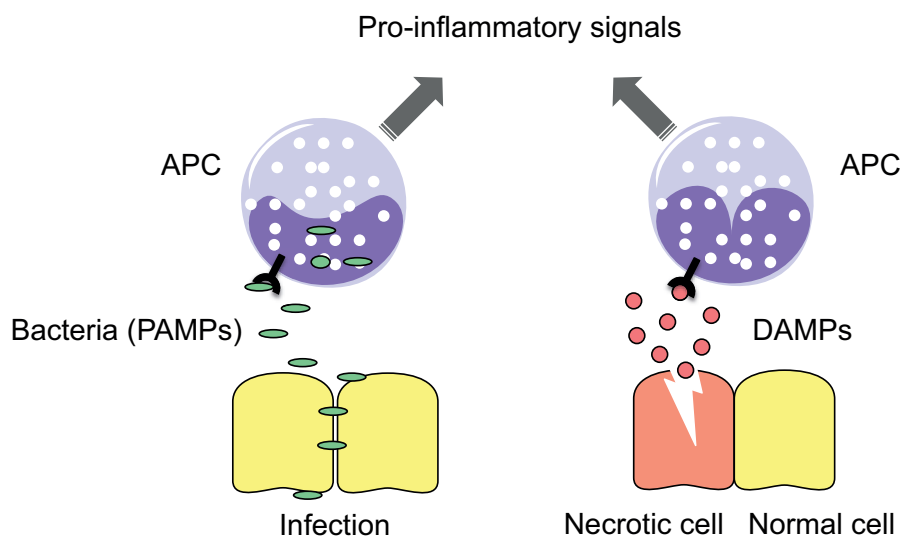


Figure 1. The danger model suggest that innate immunity respond to danger signals as well as bacterial patterns.

### **Damage associated molecular patterns (DAMPs) and alarmins**

During trauma or damage-induced responses, host-derived molecules such as DNA, ATP, DNA-binding proteins, reactive oxygen species and heat-shock proteins will cause inflammation [6-8]. These molecules are termed DAMPs. DAMPs are intracellular proteins released into the surrounding environment from necrotic cells where they can bind and activate immune cells expressing pattern recognition receptors [10]. Endogenous DAMPs therefore induce a similar response as foreign PAMPs. DAMPs are structurally diverse and unrelated host-derived proteins that can recruit and activate antigen-presenting cells. To be classed as DAMPs, they need certain features. They are often highly cationic which may influence their potency as immune enhancing mediators and also often results in an antimicrobial activity of the DAMP. They differ from cytokines since they are stored and quickly released as a response to infection or inflammation [11]. Further, they have chemotactic activity to other immune cells and exert immune enhancing activity *in vivo*. DAMPs can recruit and activate cells by directly binding to G-protein coupled receptors that mediate cell recruitment, or indirectly by inducing cytokine/chemokine production in other leukocytes [11]. The danger model tells us that PRRs recognize bacterial compounds as well as DAMPs [2, 8, 9]. On a macroscopic level, tissue destruction is a major contributor of DAMPs release. Tissue inflammation can be induced in several ways, such as through mechanical forces, excessive heat, cold, chemicals, poisons, radiation and hypoxia. [6]. Although it is known that tissue destruction leads to inflammation with similar symptoms as from an infection, the insight that the mechanisms were so similar was new. On a microscopic level, DAMPs are released into the extracellular environment upon necrosis, NET release or through active secretion using the endoplasmic reticulum-Golgi secretion system [11].

### **Host-defense peptides**

Host-defense peptides (also referred to as antimicrobial peptides) are 10-50 aa long front-line effector peptides found in all complex living organisms. They were first studied due to their broad antimicrobial activity against bacteria. In *in vitro* and *in vivo* models, HDPs are effective killers of both gram-positive and gram-negative bacteria [12, 13], but they also exert activity against some viruses and fungi. HDPs are greatly expressed in the skin by epithelial cells and neutrophils. Also, paneth cells in the small intestine express HDPs. Other HDPs are released as small derivatives after proteolytic cleavage of polypeptides such as coagulation factors and complement proteins [14-17]. While some HDPs are continuously expressed, others are induced by inflammatory stimuli [18-21].

HDPs are folded into different structures including  $\alpha$  helices,  $\beta$  sheets, extended helices and loops [22]. They have a high content of positively charged aa lysine and arginine and have hydrophobic and hydrophilic regions [12, 13]. The combination of cationicity and hydrophobicity facilitates cell membrane binding and pore-forming properties of HDPs. There are fundamental differences between microbial and mammalian cells including membrane composition, transmembrane potential, lipid composition and presence of cholesterol [23, 24]. Characteristics like these determine HDPs discrimination between eukaryotic and bacterial cells. Thus, this specificity leads to that HDPs selectively eradicate pathogens without causing excessive tissue damage. However, during hyperinflammatory disorders, the production and release of HDPs can be so extreme that they lose their specificity for bacterial membranes and start to induce host cell damage [24-30]. HDPs are upregulated and contribute to the pathogenesis in several inflammatory diseases including psoriasis, rosacea and severe sepsis [20, 31-34].

Further, HDP binding to lipid membranes may induce conformational changes in structure, depending on the lipid membrane [23, 34]. Linear peptides may take helical structures and monomeric peptides can organize in oligomers which may affect their selectivity for bacterial and mammalian membranes. The exact killing mechanism of most HDPs is still somewhat unclear. Most HDPs destabilize bacterial membranes either by direct pore formation or detergent-like effects, while others are translocated into the bacteria where they interfere with protein synthesis or metabolic pathways [33].

Lately, HDPs have been shown to exert direct immunomodulatory functions on the innate and the adaptive immune system, respectively. This is the reason for changing their name from antimicrobial peptides to host-defense peptides. Some HDPs have immunostimulatory properties while others have immunomodulatory activities. During gram-negative bacterial infections, immunostimulatory LPS may be released in free form or through microvesicles, causing massive inflammatory reactions through binding of TLR4. Some HDPs can bind and neutralize LPS directly [15, 35, 36], while other HDPs bind PRRs and thereby prevent receptor activation [37]. Other immunomodulatory effects include HDP-induced recruitment of immune cells [32, 38], macrophage and dendritic cell differentiation [39, 40]. HDPs can also modulate wound repair [41] and cause NET formation [42].

# CHAPTER 4

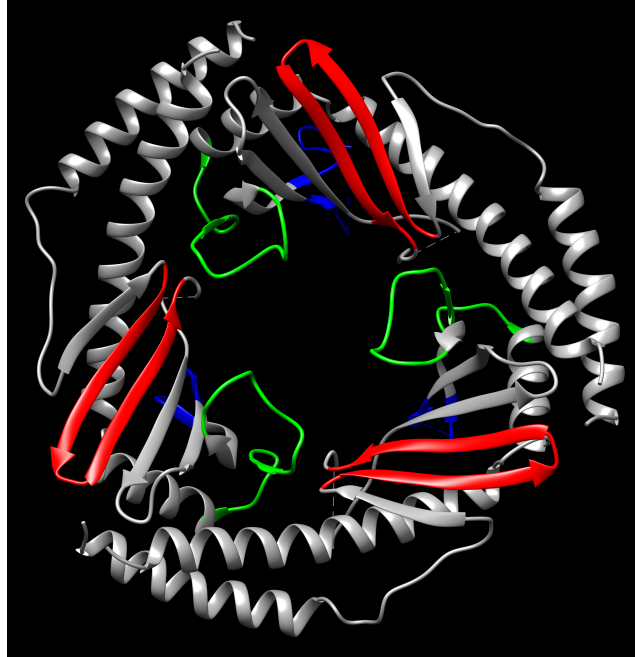
## *p33, a modulator of danger signals*

### **p33 – A protein with many names and many functions**

**P**33 (also known as globular C1q receptor, C1q binding-protein or p32) is a 33 kDa, negatively charged protein ubiquitously expressed by several cell types. The protein was first discovered and described in 1994. It was originally reported to be a receptor for the globular heads of C1q, a central protein in the complement activation cascade [43]. Although there are no signaling events occurring when p33 binds C1q, and therefore the terminology has changed into p33 or C1q binding-protein. Instead, when p33 bound C1q, complement activation was prevented [44]. The affinity between C1q and p33 is moderate (240 +/- 10 nM) [45, 46]. This opened up the question that maybe there were more important ligands for p33 than C1q. Since then several ligands have been described for p33, both endogenous and bacterial and viral proteins [26, 46-50]. Three p33 molecules form together a trimer during non-reducing conditions [26]. The p33 homotrimer is shaped as a doughnut-shaped ring (see figure 1) and can interact with up to three ligands simultaneously [26].

### p33 expression

Cells expressing p33 include endothelial cells, neutrophils, lymphocytes, keratinocytes and platelets [26, 44, 46, 51]. The protein is synthesized as a 282 aa precursor protein with a signaling motif for the mitochondria. After protein translation, the pro-protein is cleaved into two fragments between amino acids 73 and 74. The mature form (74-282) is highly cationic with an isoelectric point of 4.15 [26, 46]. The cellular localization of the mature form of p33 has been debated [45]. Several observations have been made of membrane bound p33 [26, 44, 52], but it is



*Figure 2. p33 homotrimer with AMP-binding regions in green, blue and red.*

not known how p33 is bound to the cell surface. Dedio et al reported that p33 is mainly expressed in the mitochondria of cells and they argue that the function of p33 needs to be reconsidered [45]. In my first paper, I observed that p33 was expressed in the endothelial lining of blood vessels in the lungs of mice. The endothelial cell line EA.hy926 was also positively stained for p33, and we also found p33 expressed in the cell membrane of HUVECs.

In my second paper, I found that when we injected recombinant p33 intravenously into mice, it disappeared from the circulation within 2 hours. The p33 staining in mice was still observed for up to a week, indicating that even though p33 disappears from the circulation, it is still present in the animal. Our theory is that p33 quickly gets bound to vessel walls and organs, although this has not been confirmed [50]. Our finding is in contrast to other studies reporting that p33 is circulating in the blood stream [53]. One explanation could be that circulating p33 measured in these studies are bound to platelets or microvesicles [51]. p33 is ubiquitously expressed in cells, but can also be upregulated from different inflammatory stimuli including LPS, TNF- $\alpha$  and IFN- $\gamma$  which indicates important

functions in immunity [44]. Also, stimulation of MG-63 cells (osteoblast-like) with LL-37 leads to an upregulation of p33 [54].

### **p33 ligands**

p33 has several ligands due to its negative charged surface. Besides C1q, the best described ligands for p33 are fibrinogen [55], high-molecular-weight kininogen (HMWK) [46], vitronectin [56, 57] and coagulation factor XII [58]. Both HMWK and factor XII are central in the contact system (intrinsic pathway of coagulation) and p33 can with these two proteins initiate contact system activation at cellular surfaces [59]. HMWK consists of 6 different domains with different properties. During contact system activation, HMWK is proteolytically cleaved by kallikrein which leads to the release of these domains. While domain 4 contains bradykinin, a potent vasodilator [60], domain 3 (NAT26) and domain 5 (HKH20) contain cationic peptides classed as AMPs [17, 46]. Since HMWK bound p33 through the cationic domain 5 (HKH20) and a synthetic version of HKH20 was described to have antimicrobial activity [61], we speculated that maybe p33 could bind other AMPs than HKH20. This initiated my first paper in *Journal of Immunology* describing how p33 can interact with different AMPs, and thereby prevent cell damage by blocking the cytolytic activity of AMPs. Interestingly, p33 can bind up to three AMPs simultaneously. Other ligands described for p33 is Protein A from *Staphylococcus aureus*. Protein A binds to p33 on blood platelets which mediates bacterial adhesion to the platelets [47]. Other bacteria that can interact with p33 are *Bacillus cereus* and *Listeria monocytogenes* and these interactions may be important for bacterial invasion [62, 63]. There are also viral and parasite ligands for p33. Both Hepatitis C and B virus interacts with p33 [64-66] while the malaria parasite plasmodium falciparum uses p33 as a receptor for the vascular endothelium [67].

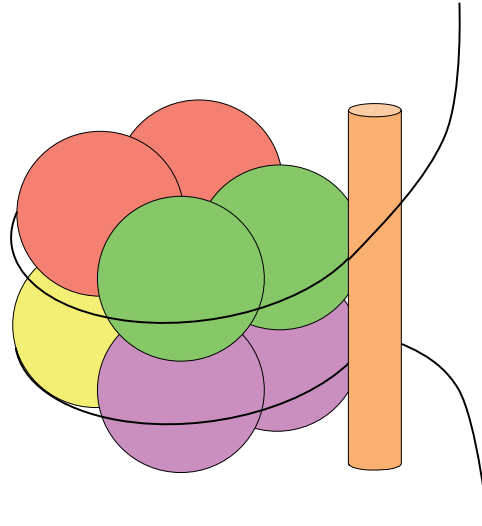


# CHAPTER 5

## *The role of histones as danger signals*

### **Histone structure and nomenclature**

**L**ately histones have gained a great amount of attention in the field of innate immunity and infectious diseases. Under normal conditions histones are located inside the nucleus of every nucleated cell in the human body. Histones bind tightly to DNA to organize our genes in nucleosomes. There are five different subclasses of histones divided into two groups: core histones (H2A, H2B, H3, H4) and linker histones (H1 and H5). Each nucleosome consists of one H3-H4 tetramer and two H2A-H2B dimers with 147 bp of dsDNA wrapped around it. Several nucleosomes are organized into chromatin structures by a single molecule of H1 or (H5 in erythrocytes). The core histones share high structure similarity although they differ in their cationic amino acids. Histone H1, H2A and H2B are rich in lysine (K) while H3 and H4 are arginine (R) rich. Further, histones are highly conserved throughout vertebrates and invertebrates since they are involved in important events such as cell division and gene transcription. Observations that extracellular histones could kill bacteria were made already 1942 [68], and a decade later Hirsch et al reported that arginine-rich histones were highly antimicrobial against various species of bacteria [69]. It has also been shown that antimicrobial activity from histones is important for immunity in invertebrates since they lack a proper adaptive immune system [70]. Although the activity of histones is little affected by pH, the antibacterial activity of histones is lost in high salt conditions [69].



*Figure 3. The nucleosome with histone subclasses in different colors.*



### Histone release

During inflammation histones are released either passively through necrosis into the extranuclear and extracellular space or actively through formation of neutrophil extracellular traps (NETs). The major contributors to extracellular histones are neutrophils undergoing NETosis. NETosis is an active but terminal event where neutrophils extrude their nuclear content together with cytosolic granular proteins into the extracellular space. This occurs when neutrophils are stimulated with certain foreign or endogenous molecules and it is a defense mechanism against invading pathogens. The content of NETs include sticky DNA that is bound together with antimicrobial peptides and proteins such as myeloperoxidase, LL-37, lactoferrin and histones. NETs bind and capture bacteria thus preventing bacterial spreading [71]. However, it is still debated whether NETs are bactericidal or just bacteriostatic. Some pathogens express DNases which enables the bacteria to disseminate the NET and thereby survive inside the host. Also, endogenous DNases will eventually degrade the NETs leading to a release of extracellular histones into the surroundings. Several reports have described that NETs can contribute to disease progression. For example, NETs in diabetic patients impair wound healing [72], and NETs can contribute to tumor progression and thrombosis [73, 74]. When histones are released from necrosis or NETosis they affect the host in several ways and today extracellular histones are classified as classical damage-associated molecular patterns (DAMPs) or alarmins.

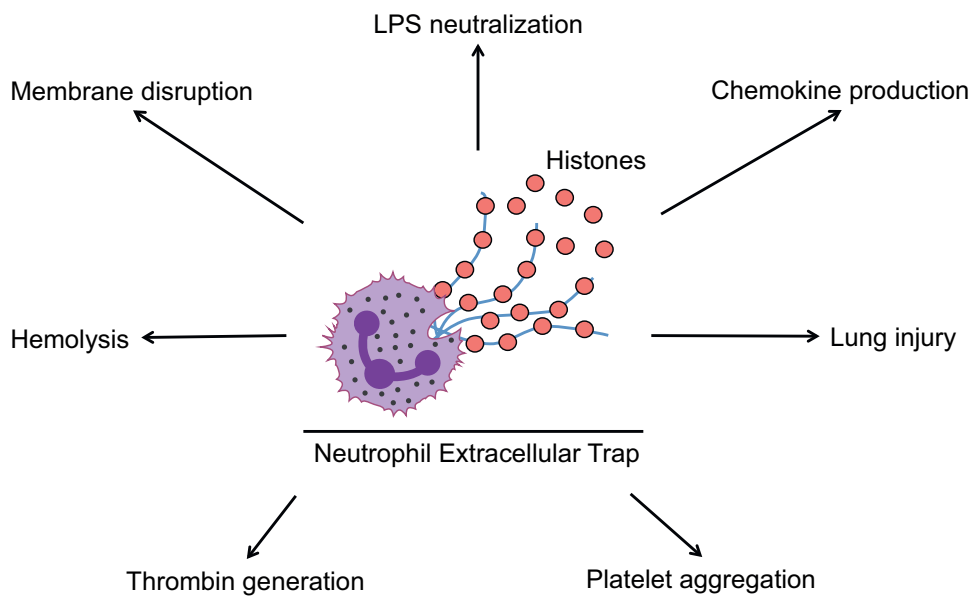
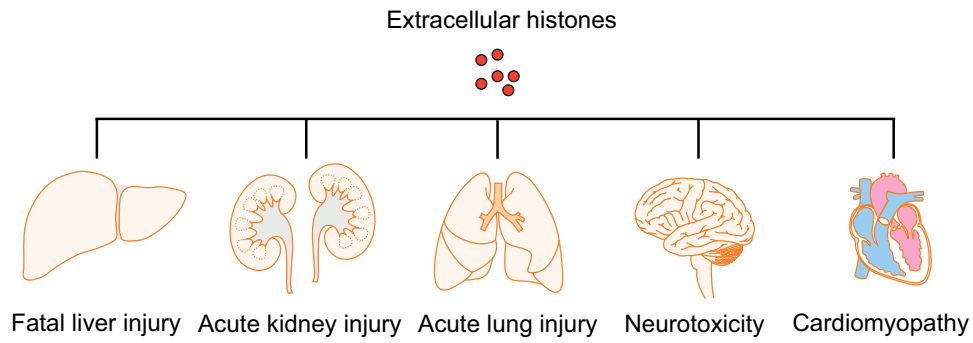


Figure 3. Histone release in NETs and its role in disease.

### **Extracellular histones in disease**

Extracellular histones are found in the circulation in a variety of diseases including severe sepsis, malaria, cancer, systemic lupus erythematosus (SLE), chronic obstructive pulmonary disease, cystic fibrosis and lung trauma. They have a great potential as biomarkers of severe inflammation due to their release in NETs or during cell necrosis. In 2009 Xu et al published a field-opening article in *Nature Medicine* describing the pathogenicity of extracellular histones in a LPS-mouse model. The LPS model is a commonly model used for severe sepsis. They injected mice with a lethal dose of LPS and then treated the mice by administrating an inhibitory antibody against Histone H4. They further showed that extracellular histones induced endothelial dysfunction and organ failure in these mice [75]. This clearly brought light to the role of extracellular histones in systemic inflammation. Thus, a new research branch opened up and during the next five years several papers were published describing how extracellular histones contribute to disease progression [50, 75-89]. The same group later published that extracellular histones were mediators of death through TLR2 and TLR4 dependent mechanisms [86]. The same year, extracellular histones were reported to cause thrombin generation, thrombocytopenia, fatal liver injury and kidney injury in mice [85, 87-89]. Further, the malaria parasite *Plasmodium falciparum* was shown to release histones into the blood stream which contributes to the pathogenesis of malaria [90]. Histones were also reported to activate the NLRP3 inflammasome [82, 84]. Finally, articles have described histones to have a role in neurotoxicity [83], platelet activation [91], phosphatidylserine (PS) exposure [77], erythrocyte aggregation [77] and cardiomyopathy [76]. Histones are released in complexes with DNA in nucleosomes before each complex is degraded. Histone/DNA complexes are highly immunogenic [92] and both DNA antibodies and histone antibodies are found in patients with SLE [93, 94]. Taken together, researchers have shown a role for extracellular histones to both sterile and infectious diseases, and the take home message has been that extracellular histones are ugly players causing damage wherever they are. Two review articles summarizing this in more detail were published in 2014 [78, 79].

Clearly, histones were proven to be a good target for a drug candidate. Already several inhibitors of extracellular histones, both endogenous and exogenous have been reported. First of all, activated protein C was shown to degrade histones, thereby preventing endothelial dysfunction [75]. Further, plasma albumin binds and inhibits histone-induced platelet aggregation [81]. Recombinant thrombomodulin [80] and now p33 [50] have also been reported to bind and neutralize the pathogenesis caused by extracellular histones *in vivo* and *in vitro*.



*Figure 4. Overview of extracellular histone pathology.*

However, in all publications that report the pathogenicity of extracellular histones extremely high doses (up to 75 mg/kg) were used which one could argue is not of physiological relevance. In my third manuscript, we investigated the role of lower levels of extracellular histones and found out that presence of extracellular histones induced leukocyte recruitment to the site of injection which may be beneficial for the host. Still more studies are needed to better characterize the role of extracellular histones under normal hemostasis and in disease.

# CHAPTER 6

## *Membrane vesicles in innate immunity*

Activated cells release various kinds of membrane vesicles into the extracellular environment. The most commonly described are exosomes, microvesicles (MVs) and apoptotic bodies. Exosomes (50-100 nm) are synthesized inside the cells and exported through degranulation of multivesicular bodies [95]. MVs varies in size from 50 nm up to 1000 nm. They are larger than exosomes, but smaller than apoptotic bodies, and are formed through membrane blebbing (trigocytosis) of eukaryotic cells [95]. MV release is induced by cell stress, injury or activation from pathogens or endogenous signals. Interestingly, MVs are also released from different bacterial species [96]. The surface of eukaryotic MVs is characterized by a higher content of the anionic lipid phosphatidylserine (PS) together with surface proteins from the cell of origin [97]. In resting cells is PS mainly positioned in the inner membrane of cell membranes, but during membrane blebbing the composition of the membrane lipids is changed and a high number of PS lipids are exposed in the outer membrane of the microvesicles. MVs cargo consist of proteins, RNA, mRNA, microRNA and DNA from the cell of origin and MVs can fuse with other cells and thereby transport molecules through horizontal transfer without exposing molecules to the extracellular environment. All blood cells, including endothelial cells, erythrocytes and platelets release MVs into the circulation. The features and composition of MVs depend on the activation of the origin cells. They can contribute to disease by horizontal transfer of molecules or by initiating coagulation. There is a potential role for MVs as biomarkers in different diseases. MVs with certain phenotypes are released from cancer cells, and MVs are elevated in sepsis patients, although their elevation predicts a positive outcome of the disease [98, 99].

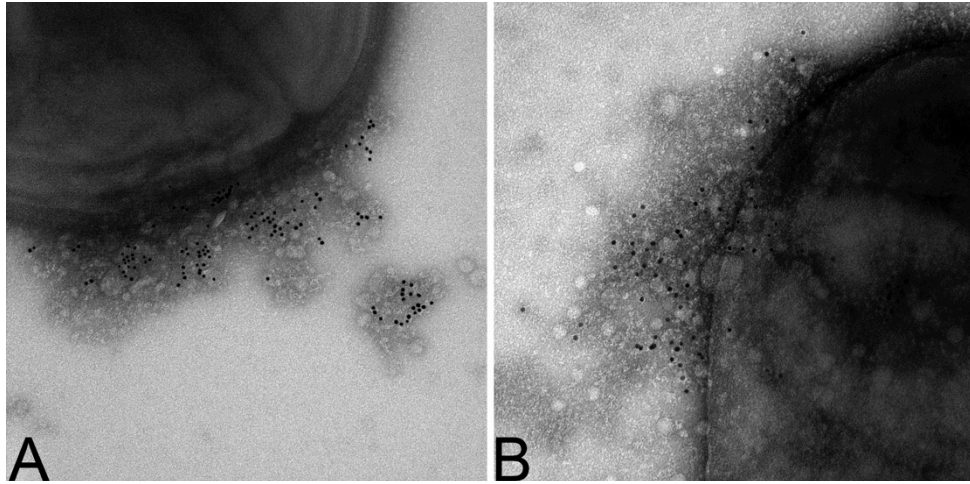


Figure 5. Microvesicles bound to the surface of bacteria. Monocytic microvesicles are stained with gold-labeled tissue factor (A) or gold-labeled antibodies against Annexin V (B).

In 2013, Oehmcke et al reported that soluble M protein from *S. pyogenes* triggered MV-release from PBMCs [100]. These MVs were pro-coagulant in two ways when compared to MVs from non-stimulated PBMCs. First, they expressed tissue factor in their membrane which could activate the extrinsic pathway of coagulation. Secondly, they exposed the negatively charged lipid PS in their outer membrane, leading to activation of the contact system. In my fourth paper, we describe how pro-coagulant MVs bind to *S. pyogenes*, induce the coagulation system and thus prevent bacterial dissemination in mice infected with *S. pyogenes*. MV binding to bacteria was facilitated through fibrinogen and M1 protein on the surface of the bacteria.

# CHAPTER 7

## *Present investigation*

### **Paper I**

p33 (gC1q Receptor) Prevents Cell Damage by Blocking the Cytolytic Activity of Antimicrobial Peptides

### **Background**

Antimicrobial peptides (AMPs/HDPs) represent a part of the innate immune system that encounters the first line of defense against invading microorganisms. In low concentrations, AMPs exert broad antibacterial activity by forming pores in the bacterial membrane due to their cationic charge. However, the specificity for bacteria is concentration dependent and when released in higher concentrations these AMPs can become toxic to eukaryotic cells. During hyper-inflammatory conditions, the systemic levels of AMPs are elevated and this has been shown to correlate with circulatory derangements. Since some of the host defense mechanisms selectivity for foreign pathogens cannot be guaranteed, evolution has armored cells with molecules to modulate these self-toxic effects. p33 is a negatively charged multi-ligand protein that is expressed on the surface as well as intracellular on various cell types such as endothelial, neutrophils, lymphocytes and platelets. It was originally identified as a receptor for complement protein C1q, but the binding of p33 to C1q is only of moderate affinity, and other ligands with higher affinity have been described. Here we describe the function of p33 to bind and neutralize cytotoxic AMPs at the host cell surface and thereby prevents the host from AMP-induced host cell damage.

### **Aims**

- To describe the binding of p33 to AMPs
- To further describe the expression of p33 *in vivo*
- To investigate the protective effect of p33 on antimicrobial peptides *in vitro*

## **Results and conclusions**

In this study, we show for the first time a defense mechanism against the cytotoxic activity of antimicrobial peptides. p33 bound several AMPs with high affinity and reduced their lytic activity against endothelial cells and erythrocytes. The native state of p33 is a homotrimer of 95.5 kDa, and we showed with electron microscopy that each p33-monomer could bind one AMP. Further, we mapped the AMP binding site on p33 using a competitive ELISA. The AMP binding site was mapped to amino acids 115-135, 185-205, 213-233 on p33. A 3D-model of these binding sites on p33 revealed that the three major binding epitopes were in close proximity. Immunohistological examinations showed that p33 was located in the membrane of endothelial EA.hy 926 cells as well as in the endothelial lining of blood vessels in mice lungs while no p33 was detected in neither healthy nor sepsis plasma. Therefore we speculated that the AMP-neutralizing effect of p33 was concentrated to the host cell surface rather than in the circulation. With electron microscopy we showed that at moderate concentrations, AMPs co-localized with p33 on the endothelial membrane and the cell membrane was intact. At higher concentrations, the AMPs attached to the endothelial membrane without binding p33, and this interaction led to membrane damage and blebbing. These data suggest that p33 has an important regulatory function in the early immune response to infection by preventing otherwise self-destructing activity of AMPs against host cells.

## **Paper II**

Treatment with p33 Curtails Morbidity and Mortality in a Histone-Induced Murine Shock Model

### **Background**

During recent years, the role of extracellular histones has been described to play a role in the pathology in several disease conditions such as severe infectious diseases, cancer, SLE, malaria and lung trauma. In healthy cells, histones are located in the nucleus where they are involved in chromatin packaging and gene transcription. The release of histones into the extracellular space is result of cell necrosis but histone release has also been observed during more controlled processes such as NET formation and apoptosis. In 2009, Xu et al presented the first study in *Nature Medicine* on the role of extracellular histones in disease [75]. They demonstrated an impressive infection model where mice were rescued from LPS induced sepsis only by administration of an anti-Histone H4 antibody. Since then, several publications have strengthened the hypothesis that histones can contribute to several of the complications seen in sepsis including the induction of inflammatory reactions, thrombocytopenia, thrombin generation and host-cell

necrosis. Taken together, these data suggest that histones are interesting targets for drug development. Histones and antimicrobial peptides have several features in common. For instance, they are highly cationic, rich in either lysine or arginine, exert antibacterial activity and are cytotoxic towards host cells. Thus, we decided to investigate if p33 which effectively neutralized AMPs, also could prevent histone-induced pathology *in vitro* and *in vivo*.

### **Aims**

- To investigate the release of extracellular histones in subjects with deep tissue infections and septic shock
- To describe the interaction between p33 to histones
- To investigate the protective effect of recombinant p33 on histones *in vitro* and *in vivo*
- To apply recombinant p33 as a therapeutic agent in a histone-induced murine shock model

### **Results and Discussion**

As described in the *Background*, we based this study on the toxicity of extracellular histones demonstrated by Xu et al 2009 [75]. We started by describing the presence of extracellular histones in patients with deep tissue destruction (cellulitis) caused by *S. pyogenes*. Healthy tissue sections showed intact nuclei with no or little extracellular histone H4 while cellulitis sections showed large areas without cell integrity where dissemination of extracellular histone H4 and extracellular DNA was observed. Further, histone H4 levels were examined in the circulation of patients with septic shock using mass spectrometry. Histone H4 was detected in all septic shock-patients while no histone H4 was detected in the healthy controls. Several experiments included data to show how recombinant p33 could bind and neutralize the pathologic features of extracellular histones. A mix of calf thymus histones (CTHs) containing all subclasses (H1, H2A, H2B, H3 and H4) was used. CTHs induced platelet aggregation, erythrocyte hemolysis and lung damage in mice, all which was inhibited when CTHs were pre-indubated with recombinant p33. Interestingly, a lethal dose of CTHs caused death in mice within 30 minutes due to severe thrombosis. However, when the mice were co-administrated with p33, all animals survived the first 2.5 h and after 7 days, 50 % of the population were fully recovered. Together, our data show that p33 is a potential drug candidate against diseases with documented histone associated pathology.



### **Paper III**

Extracellular Histones induce Chemokine Production in Whole blood *ex vivo* and Leukocyte Recruitment *in vivo*

#### **Background**

The main focus of the innate immune system is the rapid and rather broad and to some extent unspecific response to infections or tissue injury. Immune cells express PRRs specialized in identifying foreign patterns called PAMPs. However, also danger signals or DAMPs are detected by these PRRs. DAMPs differ from PAMPs by being host-derived, normally found intracellular or intranuclear in eukaryotic cells. Once cells are exposed to stress or trauma, these endogenous molecules leak out and contribute to inflammation. One major group of DAMPs consists of extracellular histones. As mentioned in previous articles, high concentrations of extracellular histones contribute to sepsis pathology in several steps. However, in non-toxic concentrations, we speculate that extracellular histones could act beneficial for the host. Previous studies have shown that cytokine production (IL-6, TNF- $\alpha$ , IL1- $\beta$ ) occur when mice are injected intravenously with calf thymus histones, and that the cytokine production is decreased in TLR2 and TLR4 knockout mice. However, no one has investigated the interaction of histones with PRRs in detail. Also, we speculated that there could be other cytokines/chemokines induced by histone.

#### **Aims**

- To screen for new cytokines induced by extracellular histones
- To investigate which immune cells that are crucial for histone-induced chemokine production
- To describe the interaction between extracellular histones and TLR4
- To show how histones can induce leukocyte recruitment *in vivo* in non-toxic concentrations

#### **Results and Discussion**

In this study, we describe how low levels of extracellular histones induce chemokine production, mainly CXCL9 and CXCL10, and leukocyte recruitment to the site of inflammation by interacting with TLR4/MD-2 on monocytes. The study was initiated by using heparinized blood as starting material. Plasma from blood

stimulated with histones was incubated on a membrane containing antibodies against more than 100 cytokines, chemokines and growth factors. Several cytokines/chemokines were upregulated, such as IL-6, IL-8, TNF- $\alpha$ , but we also found chemokines that never had been reported to be upregulated after stimulation with extracellular histones. These include CXCL9, CXCL10, CCL2, CCL3, CCL7 and CCL20. We confirmed this by injecting CTHs intravenously into mice, and used a similar membrane but for mice plasma. Here we found mainly CXCL10 but also CXCL9 to be upregulated. Therefore we decided to focus on CXCL10 for the rest of the study. CXCL10 is a potent chemokine that attracts cells expressing CXCR3 receptor. This receptor is found on several cell types, including activated T-cells, monocytes/macrophages, neutrophils, eosinophils and NK-cells. Further characterization revealed that histones signals through TLR4/MD-2 on monocytes. Histones were injected subcutaneously into an air pouch in wild-type and TLR4 knockout mice. The next day, CXCL10 levels and migrating cells were analyzed in the air pouch. CXCL10 production as well as the leukocyte recruitment was lost in TLR4 knockout mice. It was mainly monocytes and neutrophils that had migrated into the air pouch. Finally, we investigated sections from patients with necrotizing fasciitis caused by *S. pyogenes*, since we know these sections contain high levels of extracellular histones. In addition to Histone H4, we also found high staining of CXCL10 in these sections. At several positions, CXCL10 and Histone H4 were found co-localized, indicating a potential correlation. Also, CXCL10 was found highly up-regulated in the plasma from sepsis patients at the ICU compared to patients with milder infections or healthy controls. Together, these findings demonstrate a dual role of extracellular histones. They may have beneficial function locally at lower concentrations. But as previously described, during hyper inflammatory conditions when homeostasis is lost, high levels of histones contribute to pathology.

## **Paper IV**

A Novel Role for Pro-Coagulant Microvesicles in the Early Host Defense against *Streptococcus pyogenes*

### **Background**

The coagulation system has recently been described as a host defense against invading pathogens. Several coagulation factors can bind and prevent bacterial dissemination and enzymatic cleavage of coagulation factors often releases cationic AMPs. Another recently described part of the host defense are

microvesicles. MVs are small lipid bilayered vesicles in 100-1000 nm in diameter. They are released from various cell types through membrane blebbing in a  $\text{Ca}^{2+}$  dependent mechanism. MVs are often studied as biomarkers for different disease conditions as they are released from cells undergoing stress, and their cargo originates from the cells that they are released from. A major human pathogen is the gram-positive bacterium *S. pyogenes*. Infections with the bacteria can reach from self-limiting pharyngitis to invasive and life threatening necrotizing fasciitis and severe sepsis. *S. pyogenes* strains are classified by the virulence factor known as M protein. M proteins are found both surface bound and soluble due to cleavage by host and pathogen-derived enzymes. M protein induces vascular leakage, tissue factor expression and cytokine production in human monocytes and endothelial cells through activation of TLR2. In this report, we show that when we stimulate whole blood or PBMCs with soluble M protein, monocytes release tissue factor (TF) expressing MVs that binds to *S. pyogenes* and prevents bacterial dissemination.

### **Aims**

- To investigate the pro-coagulant activity of MVs
- To describe the interaction between pro-coagulant MVs, linker proteins and *S. pyogenes*
- To show how MVs prevent bacterial dissemination *in vitro* and *in vivo*

### **Results and conclusions**

In this study, we show how pro-coagulant MVs bind to M1 protein on the surface of *S. pyogenes*. This binding works as an opsonization of bacteria for the coagulation system since the bound MVs express TF. When mice infected with *S. pyogenes* were treated with pro-coagulant MVs, bacterial dissemination was decreased and survival was improved. TF is upregulated on the surface of monocytes, macrophages and endothelial cells in response to infection and when we stimulate blood with M1 protein from *S. pyogenes* these pro-coagulant MVs are released from those cells into the circulation. Throughout the study, we used MVs from non-stimulated cells as control (control MVs). Interestingly, MVs are the main reservoir of blood borne TF. Further, we showed that fibrinogen is essential for MV-induced opsonization. Plasma proteins were necessary for pro-coagulant MVs to bind to *S. pyogenes*, and when plasma deficient in fibrinogen was used the effect was lost. Surface plasmon resonance studies showed that fibrinogen binds better to pro-coagulant MVs than control MVs. This is explained by an upregulation of fibrinogen-binding sites on the pro-coagulant MVs.

Fibrinogen-binding integrins CD18 and CD11b were highly upregulated. Further, antimicrobial proteins such as lysozyme and neutrophil defensin 1 was enriched in pro-coagulant MVs. Pro-coagulant MVs are significant increased in patients suffering from streptococcal sepsis, and this is also observed in an invasive mouse model. Finally, pro-coagulant MVs or control MVs were injected in mice infected with *S. pyogenes*. Treatment with pro-coagulant MVs prolonged survival and decreased mortality rate, indicating that pro-coagulant MVs prevents bacterial dissemination and thereby are a part of the early host defense to an infection.



# CHAPTER 8

## *Conclusions and future perspectives*

Hopefully, this thesis will broaden the perspective of the definitions of antimicrobial peptides (AMPs), host-defense peptides (HDPs) and damage-associated molecular patterns (DAMPs). In my thesis I have intentionally used a mixture of abbreviations for these molecules. I have done this because they largely share properties. Many DAMPs has antimicrobial activity and many AMPs can activate PRRs. HDPs are both bactericidal and immunomodulatory. However, I must of course clarify that this does not apply to all AMPs, HDPS and DAMPs. p33 is a molecule that we showed was able to prevent cell damage by binding and blocking cytolytic AMPs. When we first started this study, it was not clear to us how potent AMPs were in effecting other parts of the innate immune system, such as inducing cytokine production and directly act chemotactic for immune cells. If I could have change anything in my publications, I would have extended my AMP-p33 study and looked into how p33 could modulate chemotactic and inflammatory properties of certain AMPs.

Sine the first publication describing the pathological effects of extracellular histones, scientist has seen them as key mediators in inflammatory diseases such as severe sepsis. Although I agree on that they can cause these effects, I am not convinced that the physiological concentrations used in these studies are physiological relevant. Therefore I hope my work on how extracellular histones directly bind TLR4/MD-2 on monocytes and induce chemokine production will bring new light into this field. I am convinced that extracellular histones act as DAMPs on the immune system. However, there are no publications describing if extracellular histones directly are chemotactic. This would be needed to 100 % class them as DAMPs.

In the nearest future, I will hopefully continue investigating the role of extracellular histones as DAMPs and adjuvants for the innate immune system and how bacteria can interfere with them. For example, there are certain virulence factors of *S. pyogenes* that can neutralize DAMPs.

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