Modulation of Neutrophil Responses

A Thin Line Between Friend and Foe

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ISBN: 978-94-6295-897-5

Printing of this thesis was financially supported by:
Mediphos Medical Supplies BV
AnaSpec, Inc.

Cover design and layout:
Giel van den Dungen

Printed by:
ProefschriftMaken ll www.proefschriftmaken.nl

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Modulatie van Neutrofiel Reacties

Een Dunne Lijn Tussen Vriend en Vijand

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 22 maart 2018 des middags te 12.45 uur

door

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geboren op 20 maart 1986 te Sint Oedenrode

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Prof. dr. Linde Meyaard

The research described in this thesis was performed at the Laboratory of Translational Immunology, Department of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands.

Studies in this thesis were financially supported by the Dutch Arthritis Foundation (Grant 12-2-406).

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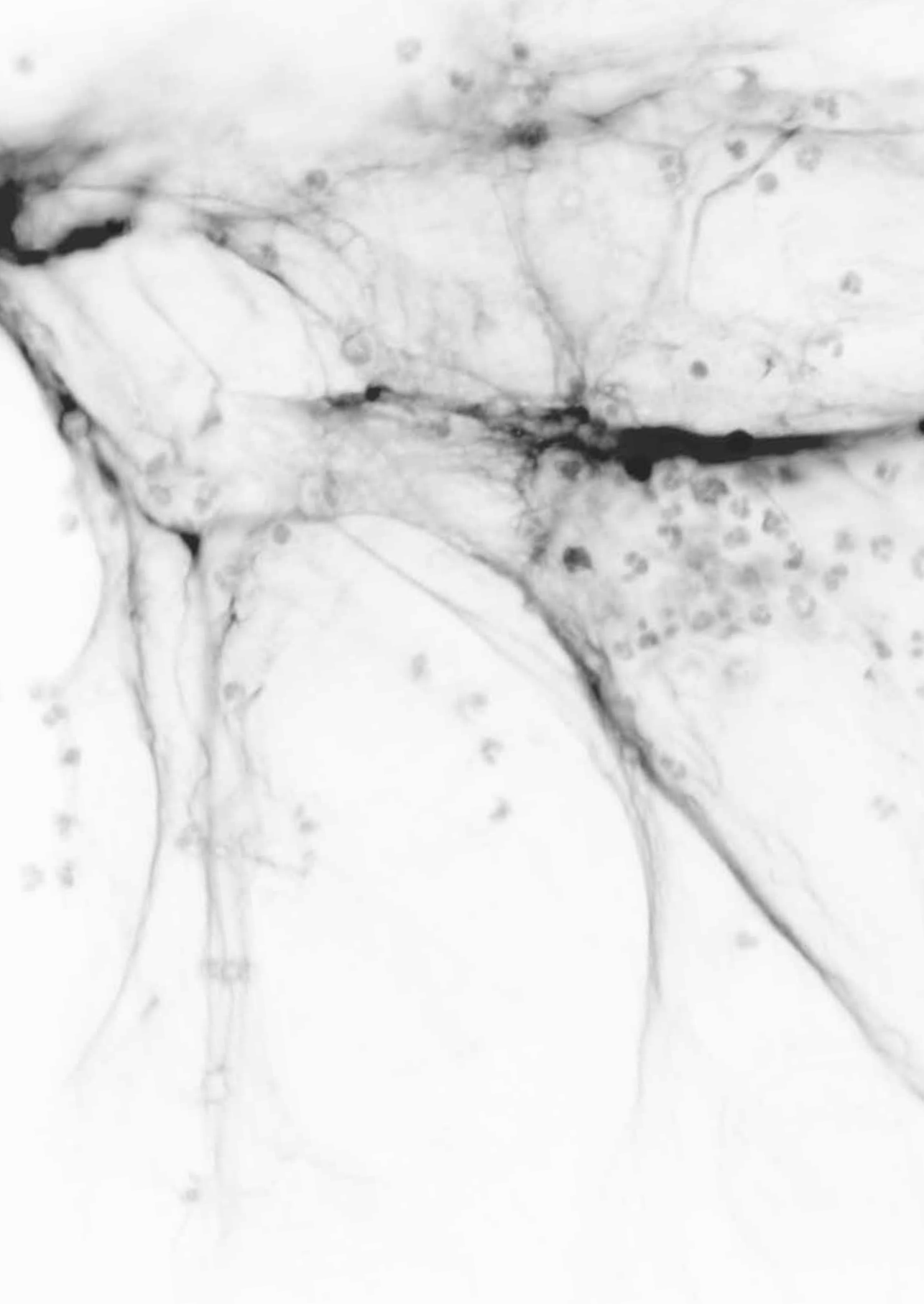

1 CHAPTER

GENERAL INTRODUCTION

The immune system is a complex network that protects the human body against malignant diseases and pathogenic infections. It distinguishes “self” from “non-self” and immediately goes into attack mode when invading microbes or tumor cells are recognized. Neutrophils, a type of leukocyte that belongs to the innate immune system, act as the first line of host defense against foreigners during acute infection. Neutrophils are crucial for clearance of the infection but prolonged neutrophil infiltration and activation contributes to tissue injury and organ failure[1-3]. This shows that immune responses mediated by neutrophils must be tightly regulated to protect the host from pathogen assaults without collateral damage and that our daily health critically depends on a well-balanced immune system.

THE LIFE OF A NEUTROPHIL

Neutrophils are formed within the bone marrow and migrate towards peripheral blood after maturation. Neutrophils are the most abundant circulating leukocytes (50-70%) and are present within spleen, liver and lung that most likely function as reservoir of mature neutrophils[1]. Circulating neutrophils are quiescent but become activated at the moment of infection or sterile inflammation. In affected tissue, pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) are present which activate tissue macrophages and mast cells to release tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and other chemokines. Endothelial cells near the site of infection respond to these signals through the expression of cellular adhesion molecules P- and E-selectin that capture neutrophils via binding to L-selectin and allow transendothelial migration[4]. Contact with endothelial cells as well as exposure to TNF-α, IL-1β, IL-8 and CXC chemokine ligand 2 (CXCL2) prime neutrophils into a “ready to fight” state and induce prolonged survival[4,5]. The chemotactic gradient from epithelial cells, subsequently, recruits neutrophils to inflamed tissue. Neutrophils express a broad package of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs) and RIG 1-like receptors (RLRs), which fully activate neutrophils upon binding to PAMPs and DAMPs[6]. In addition, Fc receptors (FcRs) as well as complement receptor 3 (CR3; also known as Mac-1) and CR4 recognize pathogens and necrotic cells that are opsonized by antibodies or complement activation product C3bi, respectively[7].

Neutrophils have an extensive arsenal necessary to eradicate the enemy, both intra- and extracellular. Firstly, neutrophils encapsulate pathogens in phagosomes, a process that is termed phagocytosis. Phagosomes fuse with preformed granules containing several antimicrobial peptides and reactive oxygen species (ROS) that kill the pathogen[8]. Phagocytosis is a fast and relatively clean way to eliminate microbes because it results in minimal tissue damage. Secondly, neutrophils secrete

antimicrobial compounds, like neutrophil elastase (NE), α-defensins and cathelicidins into the extracellular environment that kill pathogens by membrane permeabilization or inhibition of DNA and RNA biosynthesis. Degranulation of peptides is an active process but can also occur as a result of leakage from the phagosomes[9]. Finally, neutrophils are able to release NETs, filaments of DNA and histones decorated with antimicrobial peptides and enzymes such as myeloperoxidase (MPO), NE and LL-37, into the extracellular milieu to trap and kill microbes[10]. The exact mechanism that neutrophils choose to perform a proper attack is unknown, however one proposed mechanism is that neutrophils are able to sense the size of microbes and selectively release NETs in response to large pathogens whereas smaller pathogens are phagocytized[11].

Elimination of invaders is the main task of neutrophils, however they are also able to modulate the adaptive immune response. Neutrophils crosstalk with dendritic cells (DCs), T- and B-lymphocytes via secretion of cytokines or direct interaction. For example, lactoferrin, α-defensins and CC chemokine ligand 3 (CCL3) are chemoattractants that rapidly recruit DCs to sites of infection, whereas direct contact of DCs with neutrophils promotes maturation of DCs[12-14]. Furthermore, a particular subset of neutrophils facilitate Th1 and Th17 differentiation or secrete B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) that are required for B cell activation and survival[15,16]. Finally, a distinct neutrophil subset is characterized by a hypersegmented nucleus and $CD62L^{bright}/CD16^{dim}$ expression, which is able to suppress T-lymphocyte responses in severely injured patients and an acute inflammation human model[17].

When acute inflammation is over, proper removal of apoptotic and necrotic neutrophils and NET debris is essential and occurs via ingestion by macrophages and DCs or DNase I cleavage[18]. These cells in turn release tissue repair signals and anti-inflammatory cytokines like tumor growth factor beta (TGF-β) and IL-10 that abrogate further neutrophil recruitment[19]. Furthermore, intact neutrophils are able to re-enter the vasculature, a process that is called reverse migration and prevents abundant neutrophil presence in tissue that is recovering from inflammation[20]. Both processes are important in the resolution of inflammation.

THE MECHANISM OF NEUTROPHIL EXTRACELLULAR TRAP RELEASE

The formation of NETs is an active process that involves a multi-step mechanism. ROS, formed via MPO, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or mitochondria, play a central role in the induction of NETs. ROS trigger NE and MPO to translocate from the granules to the neutrophil nucleus promoting chromatin decondensation[21]. Simultaneously, ROS activate protein-arginine deiminase 4 (PAD4) that converts arginine to citrulline on histones promoting further

unfolding of chromatin[22]. As a consequence, the nuclear membrane disintegrates and the nuclear material is released into the cytosol where it is decorated with granular and cytosolic proteins. During the final stage, the plasma membrane disrupts and NETs are released into the extracellular environment (Fig. 1).

The original discovery of NETs arose from a study that stimulated neutrophils with phorbol myristate acetate (PMA), a chemical compound that activates protein kinase C (PKC) triggering many intracellular pathways in neutrophils[10,23]. NET release in response to PMA as well as other triggers, including immune complexes (ICs)[24,25], anti-LL-37[26], non-opsonized bacteria[27] and fungi[28], depends on NADPH oxidase-mediated ROS, supported by the studies on neutrophils from NADPH-deficient mice and patients with chronic granulomatous disease (CGD)[27,29,30]. The signaling mechanisms that promote NET release upstream of NADPH oxidase are poorly understood, however several studies have linked mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and protein kinase B (PKB) to it[31] (Fig. 1). In contrast, NADPH oxidase-independent NET release occurs as a response to ICs[32], ionomycin[33] and parasites[34]. These triggers are shown to

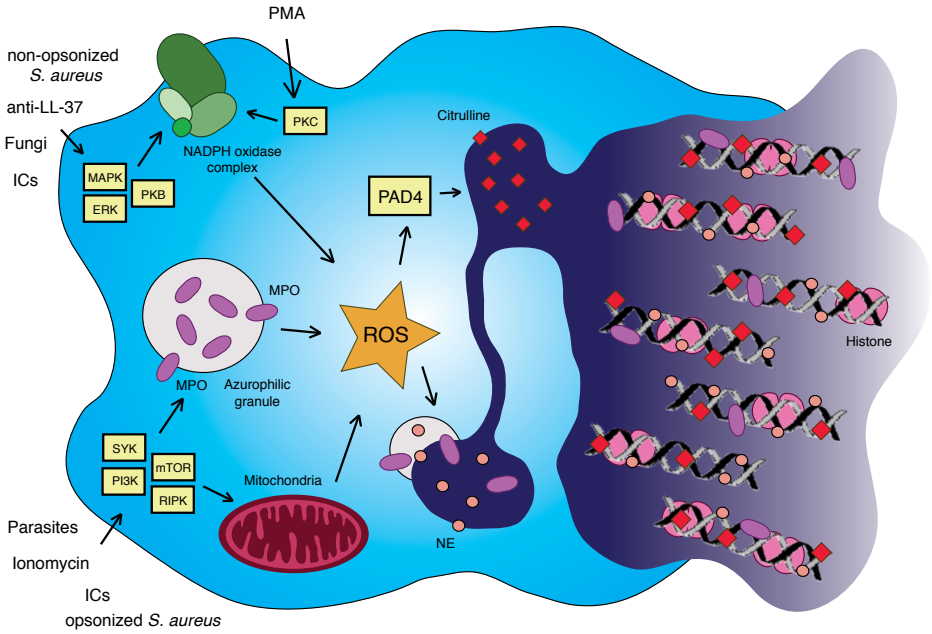

Figure 1 | **NET release can occur via different underlying signaling mechanisms**
Upon activation of the neutrophil, specific signaling molecules become activated, depending on the type of stimulus. PMA triggers PKC, whereas ICs, fungi, anti-LL-37 and non-opsonized *S. aureus* activate the MAPK/ERK/PKB pathway, which both result in NADPH oxidase-dependent ROS production. On the other hand, parasites, ionomycin, ICs and opsonized *S. aureus* have been shown to trigger SYK, PI3K, mTOR or RIPK that leads to MPO- or mitochondria-induced ROS production. ROS then trigger NE and MPO to translocate from their granules to the nucleus and activate PAD4 to covert arginine to citrulline on histones, which both promote chromatin decondensation. As a consequence, the nuclear membrane breaks and the nuclear material is released into the cytosol. During the final stage, the plasma membrane disrupts and NETs are released into the extracellular milieu.

regulate NET release via spleen tyrosine kinase (SYK), phosphoinositide 3-kinase (PI3K), mechanistic target of rapamycin (mTOR) or receptor-interacting serine/threonine-protein kinase (RIPK) and, most likely, induce PAD4 activity via MPO- and mitochondrial-mediated ROS[31,35] (Fig. 1). This suggests that: 1) the underlying signaling mechanisms of NET release depend on the stimulus and 2) multiple signaling mechanisms can lead to the release of NETs. More studies, preferably with physiological stimuli, are needed to determine the exact pathways that are essential in NET release.

NETS ARE INTRINSICALLY GOOD, OCCASIONALLY BAD BUT UGLY WHEN OUT OF CONTROL

NETs contribute to the antimicrobial strategy of neutrophils and trap bacteria, fungi, viruses and protozoa[36,37]. Moreover, antimicrobial proteases present in NETs kill these pathogens. The importance of NETs in protection against infection has been proven in *in vivo* experiments, which demonstrate that mice unable to release NETs have a higher susceptibility to various pathogens[38]. Although NETs are of great value for host defense, NET release must be tightly regulated to prevent pathology because incomplete clearance of NETs injures the host by inducing tissue damage. Antimicrobial peptides and histones present in NETs are highly cytotoxic and damage epithelial cells *in vitro* and keratinocytes *in vivo*[39,40]. Furthermore, NETs impair wound healing in diabetes by narrowing blood vessels or blocking of tissue repair signals, whereas wound healing is accelerated in NET-deficient mice[5,41].

The harmful influences of NETs also contribute to the development of many acute and chronic inflammatory disorders. Indeed, NETs play a role in the initiation and progression of cardiovascular diseases, such as atherosclerosis and thrombosis. In thrombosis, NETs promote the expression of von Willebrand factor (vWF) and P-selectin on the surface of venous endothelium to constitute a scaffold for platelets, red blood cells and clotting factor XII, which together enhance coagulation and initiate thrombus formation[42]. Interestingly, NET-induced coagulation is associated with cancer progression. NETs have strong adhesive properties, which allow them to bind tumor cells within the vasculature and contribute to metastasis. Moreover, mediators within NETs such as NE and matrix metalloproteinase 9 (MMP9) promote cancer growth and progression as well as angiogenesis[43].

The contribution of NETs to autoimmune diseases is often suggested and is mostly linked to the inflammatory response to autoantigens present in NETs. For example, patients with systemic lupus erythematosus (SLE) have increased levels of anti-ribonucleoprotein (RNP), anti-human neutrophil protein (HNP) and anti-dsDNA autoantibodies in their serum whereas high levels of autoantibodies against citrullinated peptides, β2 glycoprotein I and anti-neutrophil cytoplasmic antibodies

(ANCAs) are found in patients with rheumatoid arthritis (RA), antiphospholipid syndrome (APS) and small vessel vasculitis, respectively[44-46]. These autoantibodies trigger neutrophils to release NETs contributing to disease progression. In SLE, autoantibodies bind to NETs and form ICs, which activate plasmacytoid DCs (pDCs) to secrete interferon alpha (IFNα). On one hand, IFNα activates B-lymphocytes to increase to production of autoantibodies while on the other hand IFNα results in neutrophil activation leading to the release of more NETs[47,48]. The contribution of NETs to SLE has been supported by studies using a mouse model of lupus that show a decrease of disease severity when mitochondrial ROS is blocked and spontaneous NET release is suppressed[25].

In all probability, NETs mostly are harmful in the context of inflammatory diseases. Nevertheless, recent evidence demonstrates that NETs might serve some beneficial functions in sterile injury. In gout, a disease characterized by an acute sterile inflammatory reaction to MSU crystals, aggregated NETs interrupt the inflammatory circle by degrading chemokines and cytokines such as IL-1β via serine proteases[49]. Moreover, mice deficient in NET release develop exacerbated and chronic gout after MSU injection, which can be reduced by injection of aggregated NETs. This suggests that NETs contribute to multiple processes, either resolution or exacerbation, of disease development. Further research need to be performed to investigate the exact role of NETs in diseases and demonstrate their beneficial or detrimental characteristics.

THE ROLE OF INHIBITORY RECEPTORS IN THE REGULATION OF NET RELEASE

Ligation of various receptors triggers the release of NETs through distinct downstream signaling mechanisms[31]. However, the activation of neutrophils needs to be counterbalanced by immune suppression signals to avoid undesirable collateral damage or detrimental inflammation. Neutrophils express various inhibitory receptors that control immune responses and inflammatory processes. The majority of these inhibitory receptors signal through intracellular immunoreceptor tyrosine-based inhibition motifs (ITIM), which are defined by the conserved amino acid sequence: (I/V/L/S)xYxx(L/V/I), where x represents any amino acid[50]. Ligation of inhibitory receptors induces a conformational change that leads to phosphorylation of the tyrosine residues in the ITIMs by Src-family kinases. Phosphorylated tyrosine recruits Src homology region 2 domain-containing phosphatase-1 (SHP-1), SHP-2 or SH2 domain-containing inositol phosphatase (SHIP), which subsequently dephosphorylate various signaling proteins, including Src-family kinases, SYK and PI3K, and inhibit ongoing signaling of activating immunoreceptors[51,52]. Knockout

mice models have shown the importance of downstream phosphatases of inhibitory receptors in immune regulation. Indeed, neutrophils that lack SHP-1 or SHIP1 are hyper-adhesive and, even in absence of any stimulus, cause an inflammatory phenotype due to uncontrolled integrin signaling[53,54].

Although many ITIM-bearing receptors are known to regulate neutrophil effector functions (reviewed in[51,52,55]), only some of them are described to suppress NET release. For example, Sialic acid-binding Ig-like lectin-9 (Siglec-9) is an inhibitory receptor expressed on myeloid cells that recognizes sialic acids and high molecular weight hyaluronan (HMW-HA)[56,57]. Upon PMA stimulation, neutrophils release NETs, which can be abrogated by pretreatment of Siglec-9 with HMW-HA. The inhibition of NET release is counteracted by treatment with anti-Siglec-9 antibody that blocks the binding of HMW-HA to Siglec-9[57].

Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is a type I transmembrane glycoprotein and becomes activated upon high affinity binding with collagens[58]. LAIR-1 is expressed on the majority of peripheral blood mononuclear cells but absent on circulating quiescent neutrophils. At the moment that neutrophils become activated in blood or tissue, LAIR-1 is highly expressed[59]. In response to respiratory syncytial virus (RSV), neutrophils infiltrate the lungs and release NETs to control viral replication, while simultaneously immune injury of lung tissue is induced. Isolated airway-infiltrated neutrophils release NETs in *ex vivo* culture without any extra trigger and this is suppressed by ligation of LAIR-1 with agonistic antibodies[59]. This is consistent with a prior study where silencing of LAIR-1 mouse neutrophils show enhanced NET release[60].

IMMUNE MODULATION THROUGH SIGNAL INHIBITORY RECEPTOR ON LEUKOCYTES-1

Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) is a type I transmembrane receptor that belongs to the inhibitory immunoglobulin superfamily (IgSF) and its gene, VSTM1, is located in the leukocyte receptor complex (LRC) region of human chromosome 19q13.4[61]. SIRL-1 contains one extracellular IgV domain and two intracellular ITIMs. SIRL-1 is highly expressed on the plasma membrane of neutrophils, eosinophils and monocytes and activation of SIRL-1 results in the phosphorylation of tyrosine residues (Y206 and Y231) present in the ITIMs that recruit SHP-1 and SHP-2[61]. The physiological ligand of SIRL-1 is unidentified, however we have previously demonstrated that cross-linking of SIRL-1 with agonistic antibodies negatively regulates immune responses in monocytes and neutrophils. For example, SIRL-1 antibodies inhibit FcαR- and FcγR-mediated respiratory burst in monocytes and neutrophils[62]. Furthermore, when primary monocytes are stimulated with

lipopolysaccharide (LPS), lower amounts of TNF-α are produced by monocytes that express high level of SIRL-1 (SIRL-1^{high}) compared to SIRL-1^{low} monocytes[61]. Finally, SIRL-1 agonist antibodies suppress spontaneous and anti-LL-37 antibody-induced NET release in SLE and healthy donor neutrophils, respectively[26].

SIRL-1 has shown to suppress immune responses in monocytes and neutrophils, however the exact role of SIRL-1 in the modulation of the immune system is unknown. In our previous study, we have demonstrated that SIRL-1 is highly expressed on quiescent peripheral blood monocytes and neutrophils while SIRL-1 expression is reduced on activated inflammatory cells[62]. Although these data implicate that SIRL-1 provides a threshold for cell activation, identification of the SIRL-1 ligand will provide additional knowledge about the physiological role of SIRL-1. Moreover, given the significance of inhibitory receptors to protect the host from immunopathology, they are attractive targets for bacteria to exploit as an immune evasion strategy[63]. It is possible that bacteria secrete molecules that interact with SIRL-1 to inhibit innate immune cell responses and escape the immune system. Furthermore, we recently identified a single nucleotide polymorphism (SNP) in the promoter region of VSTM1 that results in abolished SIRL-1 expression in monocytes and is associated with atopic dermatitis (AD)[64]. This suggests that fluctuation of SIRL-1 expression on a specific cell-type indeed might have pathologic consequences. Additional research is necessary to understand the contribution of SIRL-1 to the immunological balance in health and disease.

RESEARCH QUESTION, GOAL AND SCOPE OF THIS THESIS

NET release is an efficient killing mechanism of neutrophils to fight invading pathogens, however uncontrolled NET release injures the host. The harmful potential of NETs in inflammation, makes them interesting targets for treatment of acute and chronic disorders. We aimed to better understand the role of NETs in health and disease and highlight potential NET specific therapeutic treatments, with focus on the inhibitory receptor SIRL-1. Currently described experimental approaches to quantify NET release are diverse but have their limitations and lack the possibility to study NET kinetics. In addition, many of these studies are based on NET release induced by the non-physiological stimulus PMA. **Chapter 2** describes a live imaging semi-automated approach to quantify NET release in response to physiological stimuli which allows us to study NET kinetics and the underlying signaling mechanisms of NET release. Subsequently, in **Chapter 3** we use this NET assay to investigate NET release in response to plasma of a large cohort of patients with SLE, SLE+APS and PAPS in relation to clinical and serological parameters. This provides us additional insight of the role of NETs in SLE and APS in relation

to disease activity and immunopathology.

Current and future research opens avenues for the development of potential therapeutic approaches to modulate neutrophil-mediated disease. **Chapter 4** provides an overview of neutrophil specific anti-inflammatory drugs that are already in clinical trials. Moreover, we discuss small molecule inhibitors and inhibitory receptors that show promising results in reducing neutrophil extracellular trap formation *in vitro* and *in vivo*.

SIRL-1 has been shown to suppress NET release in response to plasma and autoantibodies of SLE patients. To be a potential therapeutic target for pathogenic NETs, it is important to specifically modify NET release without affecting other neutrophil antimicrobial functions. In **Chapter 5** we investigate whether SIRL-1 is able to limit NET formation in response to other triggers and whether intracellular bacterial killing by neutrophils remains preserved upon SIRL-1 ligation. If so, it makes SIRL-1 a potential therapeutic target for treatment of NET-related diseases. However, to fully understand the role of SIRL-1 in immune regulation in health and disease, it is essential to study the interaction of the receptor with its ligand. **Chapter 6** describes an endogenous peptide that is able to bind and activate SIRL-1. In addition, bacteria may use inhibitory receptors as immune evasion strategy. We investigate whether secreted peptides from *Staphylococcus aureus*, closely related to these endogenous peptides, are able to activate SIRL-1.

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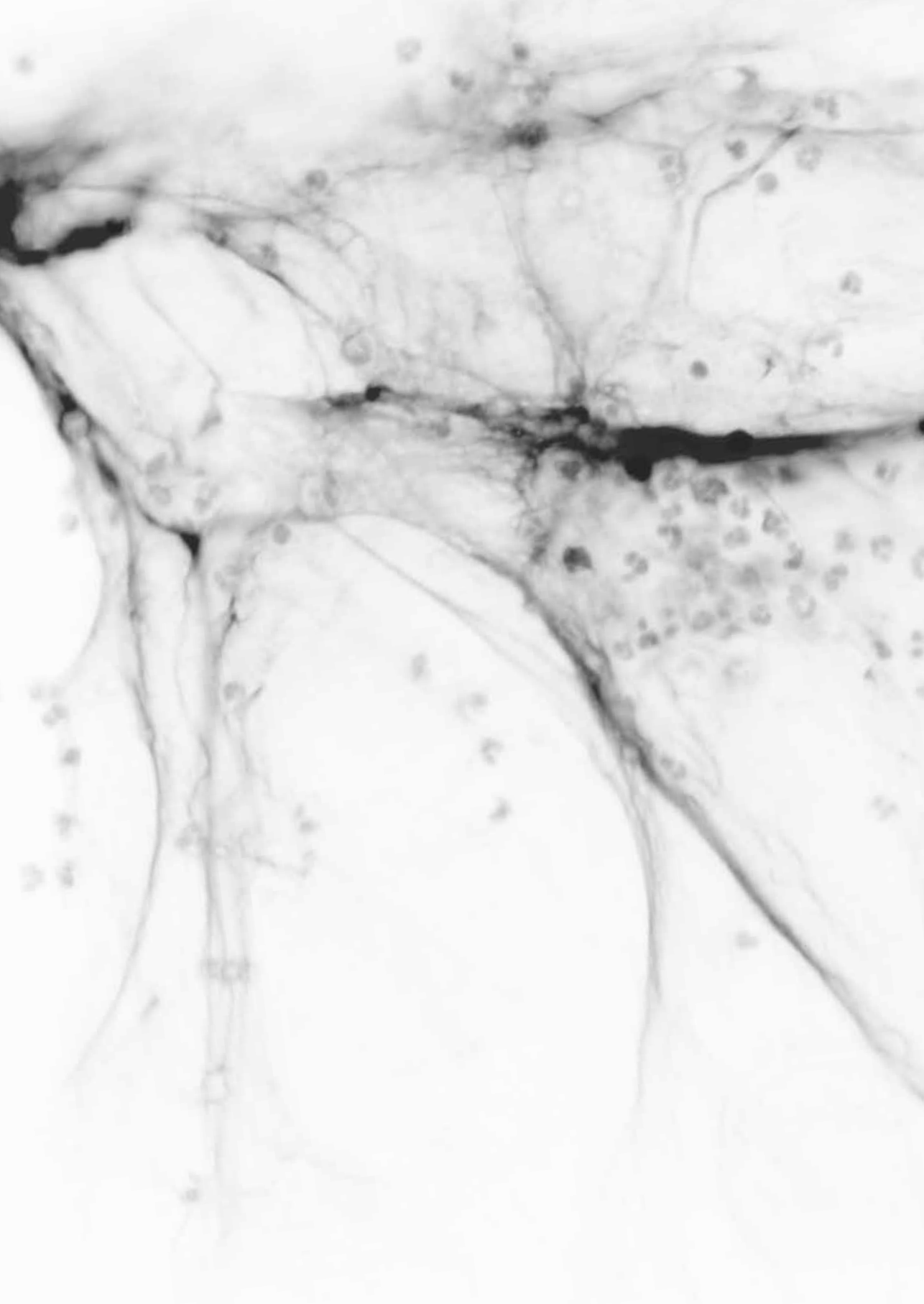

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CHAPTER

DIFFERENTIAL SIGNALING AND KINETICS OF NEUTROPHIL EXTRACELLULAR TRAP RELEASE REVEALED BY QUANTITATIVE LIVE IMAGING

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Scientific Reports. 2017 Jul 26;7(1):6529

ABSTRACT

A wide variety of microbial and inflammatory factors induce DNA release from neutrophils as neutrophil extracellular traps (NETs). Consensus on the kinetics and mechanism of NET release has been hindered by the lack of distinctive methods to specifically quantify NET release in time. Here, we validate and refine a semi-automatic live imaging approach for quantification of NET release. Importantly, our approach is able to correct for neutrophil input and distinguishes NET release from neutrophil death by other means, aspects that are lacking in many NET quantification methods. Real-time visualization shows that opsonized *S. aureus* rapidly induces cell death by toxins, while actual NET formation occurs after 90 minutes, similar to the kinetics of NET release by immune complexes and PMA. Inhibition of SYK, PI3K and mTORC2 attenuates NET release upon challenge with physiological stimuli but not with PMA. In contrast, neutrophils from chronic granulomatous disease patients show decreased NET release only in response to PMA. With this refined method, we conclude that NET release in primary human neutrophils is dependent on the SYK-PI3K-mTORC2 pathway and that PMA stimulation should be regarded as mechanistically distinct from NET formation induced by natural triggers.

INTRODUCTION

The discovery of neutrophil extracellular traps (NETs) has enriched our knowledge on the antimicrobial strategies that neutrophils use to fight invading pathogens. NETs are fibers of decondensed chromatin decorated with granular proteins and are released in the extracellular milieu to kill various pathogens[1-4]. Opposite to the beneficial role of NETs to innate immune defense, NET formation has been shown to contribute to inflammation in non-infectious diseases. The formation of NETs in tissue or inside the vasculature could lead to clot forming, metastasis or exposure of autoantigens and thus contribute to the pathogenesis of thrombosis[5], cancer[6] and autoimmune inflammatory diseases[7-10], respectively. In systemic lupus erythematosus (SLE), the presence of LL-37, human neutrophil peptide (HNP) and autoantibodies against these specific cellular components supports the formation of immune complexes (ICs), which trigger neutrophils to release NETs[11]. In addition, monosodium urate (MSU) crystals present in joints of gout patients have been described to abundantly induce NET release[12].

Many of the current reports on NET release are based on *in vitro* studies using the non-physiological stimulus phorbol myristate acetate (PMA). PMA is a potent stimulator of protein kinase C (PKC), which in turn activates the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex leading to abundant generation of reactive oxygen species (ROS)[13]. Although PMA has been used to study the biology of NETs in many publications, it is not a physiological stimulus and thus will differ from NET release induced by microbial and endogenous stimuli.

To experimentally address the release of NETs *in vitro*, in recent years many strategies have been developed. Microscopic techniques are most often used to determine and quantify NETs (reviewed by[14]). Microscopy is useful to visualize NET release and to detect specific NET components, but has its limitations. Non-automatic quantification of fixed time point microscopic experiments is rather inaccurate, because the results could easily be biased by the observer and it is impossible to correct for neutrophil input. Another technique combines microscopy with flow cytometry, also called multispectral imaging flow cytometry (MIFC), and quantifies NET release by visualization and side-scatter and is able to identify NET components by multiple fluorescence staining[15]. MIFC allows unbiased quantitative analysis, however it focuses on neutrophils in an early phase of NET release and may miss neutrophils that actually release NETs.

An important limitation that all these visualization techniques have in common is that they lack the possibility to study NET kinetics, on which there is currently no consensus. Rapid NET release has been shown in response to *Staphylococcus aureus* (*S. aureus*)[16], Fc receptor (FcR) activation[17,18], MSU[19], *Candida albicans* (*C. albicans*) hyphea[20], and *Leishmania*[21], while PMA[18], ICs[22] and *C. albicans* yeast[20]

trigger NET formation at later time points. Many of these studies were performed with fluorescence plate reader experiments using DNA binding dye's (i.e. PicoGreen or Sytox Green), however this will not distinguish DNA expelled as NETs from other forms of DNA release.

Live imaging approaches were shown to enable following single neutrophils and visualize NET release, which makes it the technique of choice to study neutrophil morphology and kinetics[23,24]. Recently, a novel semi-automated NET quantification has been described[25]. This technique is able to detect PMA-induced NETs based on the surface of Sytox Green staining. Because this procedure does not require extensive processing of cells, it has been shown to be reproducible and valid compared to manual counting. Here, we have used live imaging and a validated semi-automatic approach to quantify NET release in response to physiological stimuli in healthy donors (HDs) and in patients with chronic granulomatous disease (CGD). This allowed us to obtain novel information on the kinetics and underlying signaling pathways that result in NET release following non-inflammatory and inflammatory stimuli and it indicates that PMA is not a good model for any of those.

MATERIAL AND METHODS

Isolation of human neutrophils

Peripheral blood from HDs and from two CGD patients (twin boys) was collected in sodium-heparin tubes (Greiner Bio-One). All blood donors gave informed consent. Patients with CGD were recruited in the outpatient clinic of the department of Pediatric Immunology and Infectious Diseases of the University Medical Center Utrecht. Diagnosis of CGD was determined functionally by absence of respiratory burst upon stimulation in neutrophil (Phagoburst, BD Bioscience) and genetically by Sanger sequencing of the NCF1 gene showing a pathogenic homozygous mutation (delta GT). Neutrophils were isolated by Ficoll-Paque (GE Healthcare) density gradient centrifugation, after which erythrocytes were lysed in ammonium chloride buffer (155 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM EDTA in double-distilled H_2O; pH=7.2). Cells were resuspended in RPMI 1640 (Life Technologies) supplemented with 10% (v/v) heat-inactivated (HI) fetal bovine serum (FBS) (Biowest) and 50 U/ml Penicillin-Streptomycin (referred to as RPMI 10% hereafter). Purity of isolated neutrophils was analyzed using the CELL-DYN Emerald and was >88%. All experiments were performed in accordance with relevant guidelines and regulations approved by the Medical Ethical Committee of the University Medical Center Utrecht.

Bacterial culture and preparation

S. aureus Wood 46 was grown up to exponential phase at 37°C under aerobic

conditions in Todd-Hewitt Broth (THB) containing 1% (v/w) yeast extract. After quantification of bacteria by measuring OD_{600} (2×10^8 CFU with OD_{600}=0.4), bacteria were washed twice with chilled PBS and opsonized for 30 min at 37°C with 10% HI human pooled serum in PBS. In some experiments, bacteria were heat-killed (HK) for 60 min at 70°C before opsonization.

Chemical inhibitors

To study the underlying signaling NET pathway, several chemical inhibitors were used. Before stimulation, neutrophils were pre-treated for 30 min at 37°C in RPMI 10% with DMSO (vehicle control), 1 μM Wortmannin (PI3K inhibitor; Sigma-Aldrich), 1 μM R406 (SYK inhibitor; Selleckchem), 10 μM SB203580 (p38 MAPK inhibitor; Selleckchem), 10 μM SB202190 (p38 MAPK inhibitor; Santa Cruz Biotechnology) 10 μM MLN0128 (pan-mTORC inhibitor; Selleckchem), 10 μM BAPTA-AM (chelator of Ca^{2+}; Focus Biomolecules), 1 μM DPI (NADPH oxidase inhibitor; Sigma-Aldrich), 1 μM Rapamycin (mTORC1 inhibitor; Selleckchem), 1 μM Celastrol (NF-κB inhibitor; InvivoGen), 0.1 μM Bay-11-7082 (IκB phosphorylation inhibitor; InvivoGen), 0.1 μM GW5074 (cRaf1 kinase inhibitor; Santa Cruz Biotechnology), 0.1 μM U-73122 hydrate (PLC inhibitor; Sigma-Aldrich), 1 nM Bafilomycin A (vacuolar-type H^+-ATPase inhibitor; Sigma-Aldrich), 0.1 μM JNKi II 420128 (JNK inhibitor; Merck Millipore) or 0.1 μM Mytoquinone (mitochondrial ROS inhibitor; BIOTREND).

Preparation of ICs

Insoluble ICs were formed by using human serum albumin (HSA; Sanquin) and rabbit polyclonal anti-HSA IgG (Sigma Aldrich) as described previously[55]. Briefly, a mix of 5 μg HSA and 45 μg rabbit-anti-HSA antibody was made in a final volume of 50 μl PBS. After 1 h incubation at 37°C, insoluble ICs were formed and used for stimulation of neutrophils.

Microscopic live imaging NET assay

Neutrophils were incubated in RPMI 10% containing 20 μM Hoechst 33342 (AnaSpec, Inc.) for 30 min at 37°C, in the presence or absence of chemical inhibitors, and washed twice with RPMI 1640 (without phenol red) supplemented with 2% FBS, 50 U/ml Penicillin-Streptomycin, and 10 mM HEPES (referred to as RPMI-pr 2% hereafter). A total of 5×10^4 neutrophils were seeded on 0.001% poly-L-lysine (Sigma Aldrich) pre-coated wells of a clear bottom 96-wells plate (Ibidi) and challenged with 25 ng/ml PMA (Sigma Aldrich), 100 μg/ml MSU, 10 μg/ml ICs, opsonized *S. aureus* at a MOI of 10. All stimuli were resuspended in RPMI-pr 2% containing 4 nM Sytox Green (Life Technologies). NET release was recorded at 37°C and 5% CO_2 on the Pathway 855 bioimaging system (BD Biosciences) with a 10x objective during a period of 240 min. Every 30 min, a set of two images (Exc/Em: 350/461 nm (Hoechst) and 504/523 nm (Sytox Green)) was taken with an Orca high-resolution

CCD camera and four fields of view (each 415×317 µm) per condition were captured. The system was controlled by the AttoVision software (version 1.7/855). To make movies, images were taken every 2 min.

Semi-automatic NET quantification approach

This NET quantification approach is based on previously described methods[25,32]. For quantification of NETs, images were processed with Fiji software (version 2.0.0-rc-43/1.51d). Image stacks were created and converted in 8-bit grayscale. To count neutrophils, thresholding of t=0 Hoechst image was done with "Moments" logarithm followed by watershed segmentation to separate particles that touch. To count NETs, thresholding of Sytox Green stacks were performed with "Li" logarithm followed by watershed segmentation. The amount of Sytox Green+ neutrophils (35-68 µm^2) or NETs (>68 µm^2) were divided by the total number of neutrophils in t=0 to calculated percentages. For semi-automatic quantification analysis, three macros were created for: 1) making stacks, 2) counting neutrophils, and 3) counting Sytox Green+ neutrophils and NETs (Fig. S1).

Induction of neutrophil apoptosis and toxin-mediated cell death

Neutrophil apoptosis and toxin-mediated cell death was induced as described before[24]. Briefly, neutrophils were incubated with 20 ng/ml anti-human CD95 (Fas) antibody clone EOS9.1 (BioLegend) to induce apoptosis and 25 µg/ml toxins from *S. aureus* to induce cell death. Toxins were obtained by growing *S. aureus* Wood 46 overnight in THB containing 1% yeast. Bacteria were pelleted, supernatant was collected and sterilized with a 0.20 µm filter. Protein concentration was determined with BCA Protein Assay (Thermo Fisher Scientific).

Immunofluorescence staining of citrullinated histone H3

Neutrophils were resuspended in RPMI-pr 2% and a total of 5×10^5 neutrophils were seeded on 0.001% poly-L-lysine coated glass coverslips. Neutrophils were challenged as described before for 180 min at 37°C and 5% CO_2. Medium was gently discarded and neutrophils were fixed with 2% paraformaldehyde (Electron Microscopy Sciences) for 15 min at room temperature (RT). After blocking with 20% normal goat serum (Cell Signaling Technologies) in PBS containing 2% bovine serum albumin (BSA; Roche) for 30 min at RT, 0.25 µg/ml rabbit-anti-citrullinated histone H3 antibody (Abcam) in PBS/2% BSA was used for incubation overnight at 4°C. Next day, neutrophils were incubated with 2.5 µg/ml goat-anti-rabbit antibody conjugated with Alexa Fluor 594 (Life Technologies) for 120 min at RT, followed by 30 min of incubation at RT with 5 µM Hoechst 33342 in PBS to stain neutrophil nucleus. Coverslips were mounted in Fluoromount-G (Southern Biotech) and analyzed with Pathway 855 bioimaging system with a 20x objective.

For immunofluorescence staining of citrullinated histone H3 in the live imaging NET assay, neutrophils were incubated in RPMI-pr 2% containing Hoechst 33342 and 1x CellMask™ Deep Red Plasma membrane stain (Thermo Fisher Scientific) for 30 min at 37°C. After washing with RPMI-pr 2%, neutrophils were seeded and challenged with toxins, viable and HK opsonized *S. aureus*. All stimuli were resuspended in RPMI-pr 2% containing Sytox Green. After 180 min incubation at 37°C and 5% CO_2, 2 µg/ml rabbit-anti-citrullinated histone H3 antibody was added and incubated for 30 min. A mix of 0.67 µg/ml donkey-anti-rabbit (Life Technologies) and 0.67 µg/ml goat-anti-rabbit antibody, both conjugated with Alexa Fluor 594, was added and incubated for 15 min before NET release was recorded with a 40x objective. Sets of four images (Hoechst, Sytox Green, Exc/Em: 590/617 nm (AF594) and 649/666 nm (CellMask Deep Red)) were taken.

Statistical analysis

Data were analyzed in GraphPad Prism 6. The comparison of two samples was performed by two-tailed Mann-Whitney test and multiple comparisons were established by Kruskal-Wallis test. Each experiment was performed at least three times on independent occasions. $P<0.05$ was considered significant.

RESULTS

A semi-automatic quantification approach to analyze live imaging NET release

We performed a live imaging assay to monitor NET release over time that uses Hoechst stain at time point zero to control for neutrophil input and validated an earlier described analysis approach that specifically quantifies NET release[25]. To discriminate NETs from dying, Sytox Green permeable neutrophils (further referred as Sytox Green+ neutrophils), we assessed maximal surface of Sytox Green+ neutrophils and the minimal size of NETs. Using this approach we set thresholds for noise (35 µm^2), Sytox Green+ neutrophils (35-68 µm^2) and NETs (>68 µm^2). The use of Fiji macros ensured a semi-automatic quantification approach (Fig. S1).

To quantify NETs, the Sytox Green images were transferred to binary images and the size of Sytox Green+ neutrophils as well as the NETs was determined. Sytox Green+ neutrophils typically covered a surface of between 40 and 55 µm^2 while NETs covered a surface of over 68 µm^2 (Fig. 1A). In addition, NET surface increased over time because of diffusion while the size of Sytox Green+ neutrophils remained below 68 µm^2.

We used three separate approaches to confirm the differences between Sytox Green+ neutrophils and NETs. First, we counted all Sytox Green particles at time point 60 min and 240 min after stimulation with viable opsonized *S. aureus*. After

60 min, the majority of Sytox Green particles covered a surface between 0 and 40 μm^2 but remained below 68 μm^2. The surface of these Sytox Green particles increased (20-50 μm^2) after 240 min of stimulation. Moreover, NETs with a surface above 68 μm^2 appeared after 240 min of stimulation (Fig. S2). Second, with CellMask plasma membrane dye we secured that Sytox Green staining of Sytox Green+ neutrophils is intracellular while NETs are extracellular (Fig. 1B). Finally, citrullinated histone H3 was present only in the extracellular DNA while the Sytox Green+ neutrophil lacks citrullinated histone H3 (Fig. 1C). This confirms that the DNA was expelled in the form of NETs and that our NET surface-based threshold of >68 μm^2 is valid to genuine NETs.

We determined the number of neutrophils over time and showed similar amount of neutrophils at time point zero (t=0) and 240 min (Fig. S3). Subsequently, we used t=0 for correcting neutrophil input. Hoechst images were transferred to binary images and the number of neutrophils was determined (Fig. 1D). Likewise, with Sytox Green images at multiple time points, the number of Sytox Green+ neutrophils (in blue) and NETs (in red) was determined (Fig. 1E).

Upon exposure to viable opsonized *S. aureus*, permeabilization of the neutrophil plasma membrane occurred within 30 to 60 min where after Sytox Green entered the neutrophils. After 90 min, the neutrophil plasma membrane of part of the cells broke and DNA was released in the extracellular environment, while part of the cells stayed Sytox Green positive but did not release NETs (Fig. 1F and Supplemental video 1).

Quantification demonstrated that within the first 60 min of exposure to viable opsonized *S. aureus*, neutrophils became Sytox Green+ with ~70% being positive after 240 min (Fig. 1G). The release of NETs occurred after 90 min and ~12% of cells released NETs after 240 min of stimulation with viable opsonized *S. aureus* (Fig. 1H). Taken together, these data show that we have validated a surface-based semi-automatic analyzing approach which is able to specifically quantify physiologically-induced NET release over time and correct for cellular input.

Live imaging distinguishes NET release from toxin-induced cell death

Upon exposure to viable opsonized *S. aureus*, neutrophils either released NETs or only became permeable, becoming Sytox Green+ without expulsion of their DNA into extracellular space. Even after prolonged incubation, the Sytox Green+ neutrophils did not release NETs (Fig. S4 and Supplemental video 2), suggesting exposure to viable opsonized *S. aureus* results in two separate mechanisms of cell death. Since *S. aureus* toxins are known to induce cell death[24], we exposed neutrophils to toxins obtained from an overnight culture of *S. aureus* and monitored neutrophil fate over time. After exposure with toxins, neutrophils became permeable and Sytox Green+ (Fig. 2A). The lack of citrullinated histone H3 confirms that toxin-induced Sytox Green+ neutrophils are not NETs (Fig. 2B).

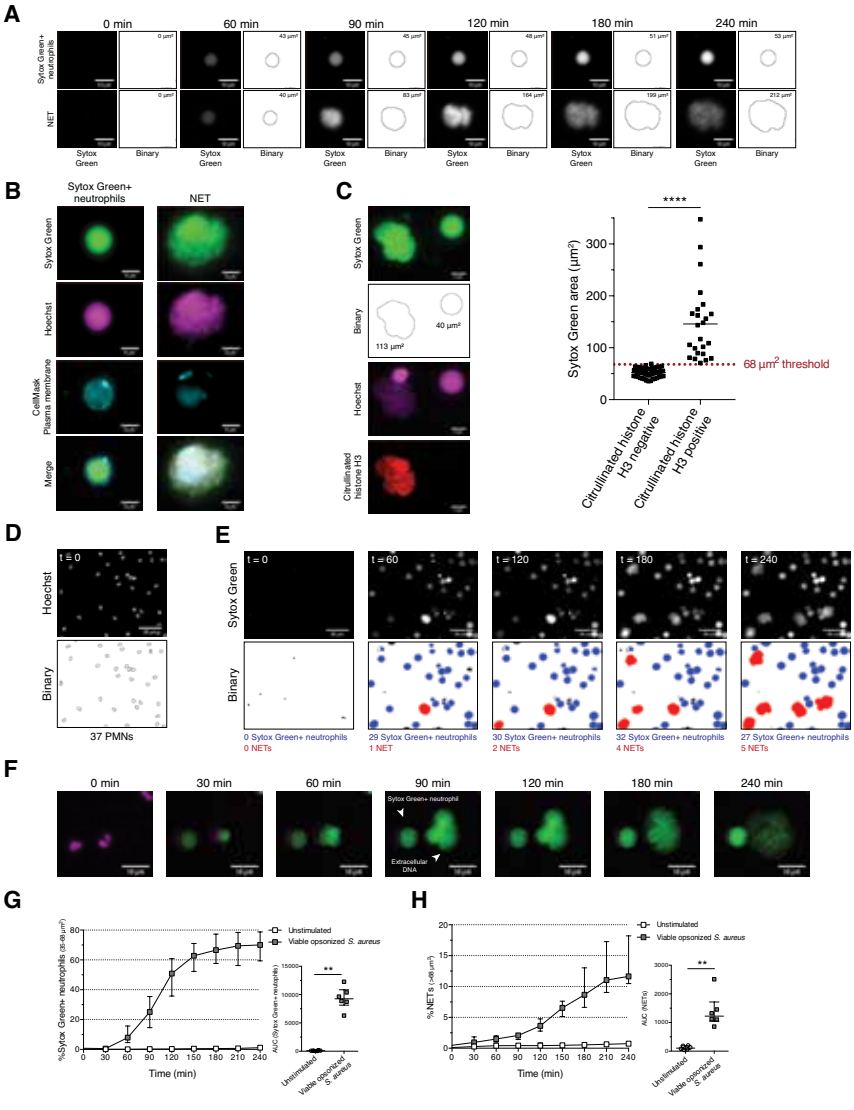

Figure 1 | **Surface-based analyzing approach to quantify NET release**
Neutrophils from HDs were stimulated with opsonized *S. aureus* and monitored over time for 4 hours using Hoechst 33342 and Sytox Green. (**A**) Sytox Green images from live imaging were transferred to binary images and the size of Sytox Green+ neutrophils (<53 µm²) and NETs (83-212 µm²) were measured. (**B**) Fluorescence microscopy using CellMask plasma membrane dye revealed that the DNA of Sytox Green+ neutrophils is intracellular and NETs are extracellular. (**C**) Citrullinated histone H3 is only present in Sytox Green particles with a surface above 68 µm², which confirms that these are NETs. Data from 3 independent experiments are presented. Statistical significance (****P<0.0001) was determined by two-tailed Mann-Whitney test. For quantification, the amount of neutrophils in t=0 (**D**), Sytox Green+ neutrophils (blue), and NETs (red) in multiple time points (**E**) were determined. (**F**) Indicated time points of live imaging (supplemental video 1; Hoechst 33342 in magenta and Sytox Green in green) showed the binding of Sytox Green to intracellular DNA (Sytox Green+ neutrophil) and extracellular DNA. For quantification, the amount of Sytox Green+ neutrophils or NETs were divided by the amount of neutrophils in t=0 and expressed as percentage of Sytox Green+ neutrophils (**G**) or NETs (**H**). Data from 5 independent experiments are presented as median ± interquartile range. Statistical significance (**P<0.01) was determined by two-tailed Mann-Whitney test. The images in **A**-**F** are representative of at least 3 experiments with neutrophils from different donors.

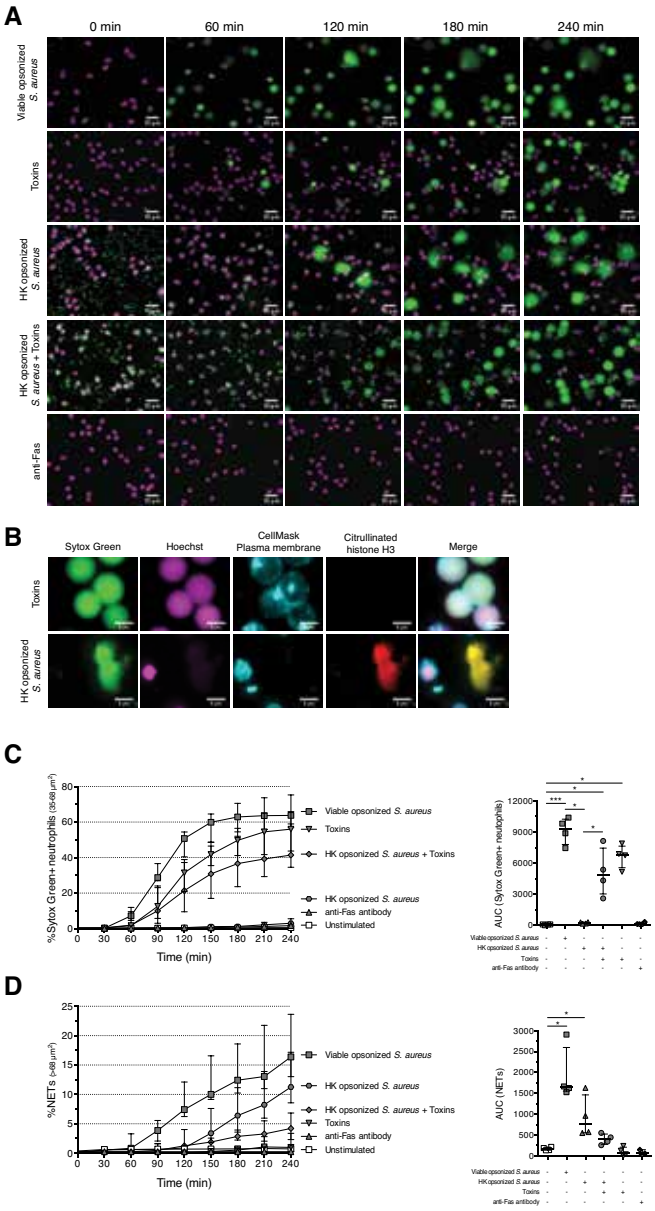

Figure 2 | **Distinguishing toxin-induced cell death and apoptosis from NET release**

Neutrophils from HDs were stimulated with anti-Fas antibody, toxins from overnight culture of *S. aureus* or viable and HK opsonized *S. aureus* and monitored over time for 4 hours. (**A**) Indicated time points from live imaging (supplemental video 3) showed that neutrophils release NETs in response to HK opsonized *S. aureus* while stimulation with toxins alone or a combination of toxins and HK opsonized *S. aureus* mainly resulted in Sytox Green+ neutrophils. Anti-Fas antibody neither induced Sytox Green+ neutrophils nor NET release. (**B**) Fluorescence microscopy using CellMask plasma membrane dye and anti-citrullinated histone H3 antibody confirms the presence of extracellular DNA in the form of NETs. Quantification of Sytox Green+ neutrophils (**C**) and NETs (**D**) of 2 independent experiments performed with 4 donors. The data are presented as median ± interquartile range. Statistical significance (**P*<0.05; ****P*<0.001) was determined by Kruskal-Wallis test. The images in **A** and **B** are representative of at least 3 experiments with neutrophils from different donors.

Our time-lapse NET quantification revealed that the first Sytox Green+ cells appeared after 40 minutes and ~58% of the cells being Sytox Green+ after 240 min without the release of NETs (Fig. 2C,D). Interestingly, not a single neutrophil became Sytox Green+ without also releasing citrullinated histone H3 positive NETs after stimulation with HK opsonized *S. aureus*, which cannot produce toxins (Fig. 2B,C,D and Supplemental video 3). To demonstrate the difference between HK and live opsonized *S. aureus*-induced NET release is because of toxin production, we supplemented HK opsonized *S. aureus* with toxins. The neutrophil response with HK opsonized *S. aureus* with exogenous toxins mimicked that of live opsonized *S. aureus*: neutrophils became Sytox Green+, but the majority of these cells did not produce NETs.

Thus, bacterial toxins are responsible for the permeabilization of the neutrophil plasma membrane after which Sytox Green enters the neutrophil. Our live imaging approach is able to distinguish NET release from toxin-induced cell death even when both processes are occurring in the same sample. Furthermore, upon stimulation with anti-Fas antibody to induce apoptosis, neither Sytox Green+ neutrophils nor NETs were present (Fig. 2A,C,D). This shows that we have developed an assay and method of analysis that distinguishes NETs from other cell death mechanisms.

MSU induces rapid NET release

Also in non-infectious conditions, neutrophils can be triggered by host factors to release NETs. The exposure of neutrophils to MSU or ICs results in abundant NET release[22,26]. PMA is a non-physiological stimulus often used for study of NETs *in vitro*[24]. Upon stimulation with MSU, ICs or PMA, neutrophils released NETs (Fig. 3A). NET release in response to ICs and PMA started around 90 min while MSU-induced NET release occurred already within 60 min, starting as soon as 30 min (Fig. 3B). The presence of citrullinated histone H3 in the extracellular DNA of neutrophils stimulated with PMA and MSU confirmed that the DNA was expelled in the form of NETs (Fig. 3C). This shows that NET kinetics is dependent on the type of stimulus, with MSU inducing rapid NET release.

PMA and physiological stimuli activate different pathways to induce NETs

To investigate the underlying pathway of NET release, we used chemical inhibitors to target specific downstream signaling molecules. Neutrophils were pre-treated with various inhibitors before exposure to live opsonized *S. aureus*, MSU, and PMA. We first determined the optimal concentration of the chemical inhibitors and chose the highest concentrations that did not induce Sytox Green signal (dying cells) in otherwise non-stimulated neutrophils (Fig. S5A).

The release of NETs in response to PMA was inhibited with 40-60% in the presence of DPI (NADPH oxidase inhibitor) and BAPTA-AM (chelator of Ca^{2+}) while treatment of neutrophils with Wortmannin (PI3K inhibitor), R406 (SYK inhibitor),

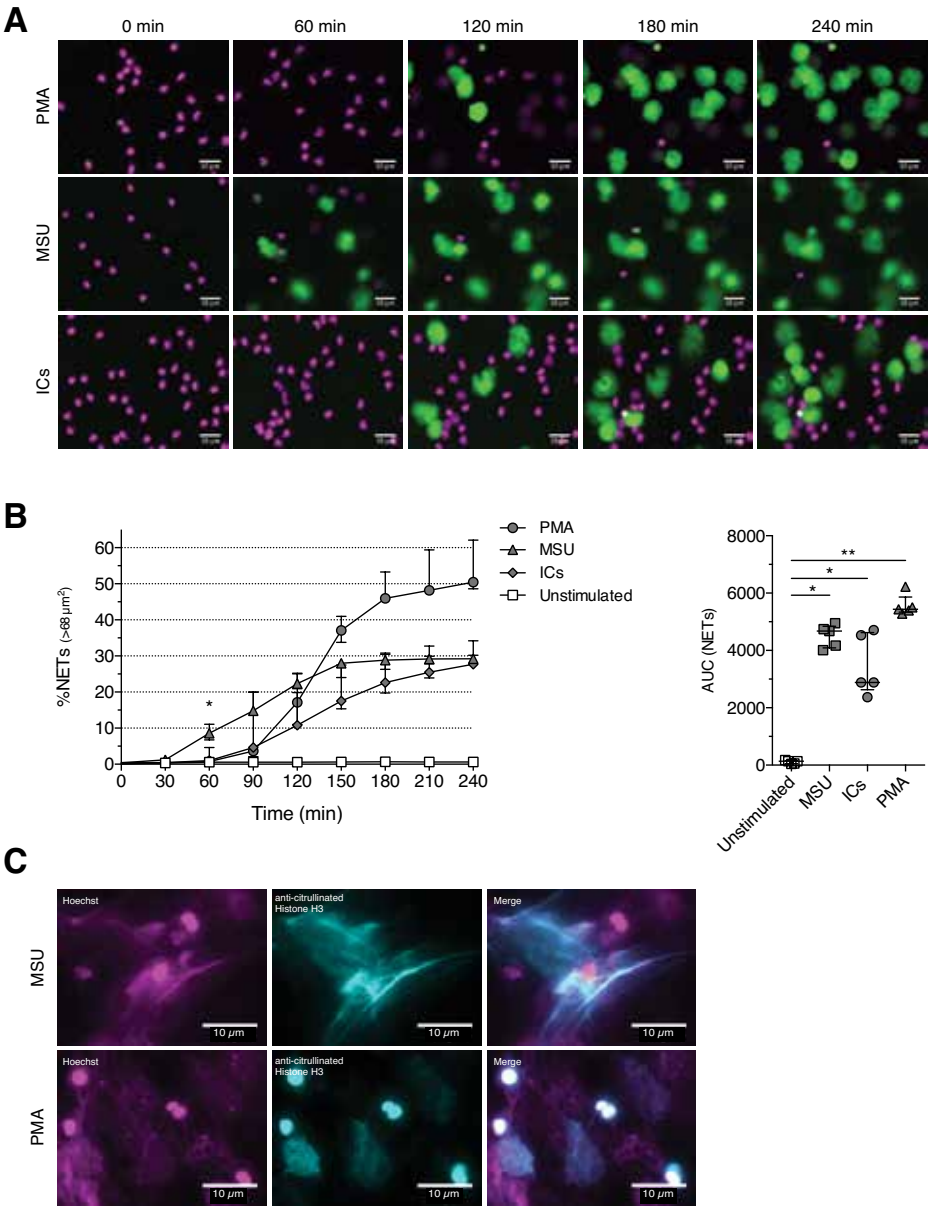

Figure 3 | **MSU induces rapid NET release**
Neutrophils from HDs were stimulated with MSU, ICs and PMA and monitored over time for 4 hours. (**A**) Indicated time points from live imaging showed MSU-induced NET release within 60 min while NET release in response to ICs and PMA occurred within 120 min. (**B**) Quantification of NETs of 5 independent experiments. The data are presented as median ± interquartile range and statistical significance (**P*<0.05; ***P*<0.01) was determined by Kruskal-Wallis test. (**C**) Fluorescence microscopic experiments revealed the presence of citrullinated histone H3 in the extracellular DNA after 180 min stimulation with MSU and PMA. The images in **A** and **C** are representative of at least 4 experiments with neutrophils from different donors.

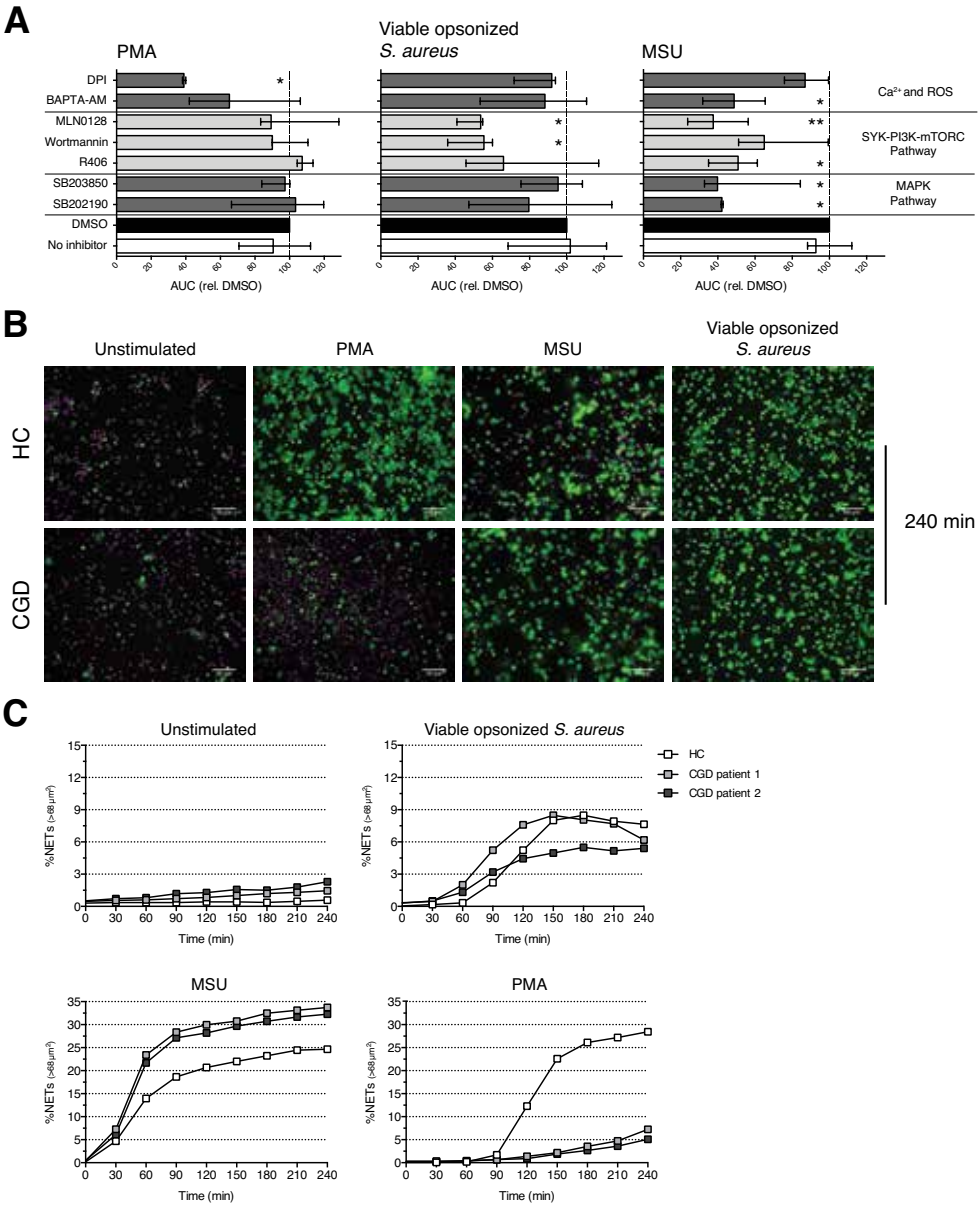

Figure 4 | **PI3K, SYK and mTORC2 are important players of the underlying signaling pathway of NET release**
Neutrophils from HDs were incubated with Wortmannin (PI3K inhibitor), R406 (SYK inhibitor), SB203580 and SB202190 (p38 MAPK inhibitors), MLN0128 (pan-mTORC inhibitor), BAPTA-AM (Chelator of Ca^{2+}), and DPI (NADPH oxidase inhibitor) before stimulation with MSU, PMA and viable opsonized *S. aureus*. (**A**) AUC of NET quantification of 3 independent experiments are presented as median ± interquartile range and statistical significance (**P*<0.05; ***P*<0.01) was determined by Kruskal-Wallis test. Neutrophils from one HC and two CGD patients were stimulated with MSU, PMA and opsonized *S. aureus* and monitored over time for 4 hours. (**B**) Merge images (Hoechst 33342 in magenta and Sytox Green in green) of time point 240 min showed decreased NET release of CGD neutrophils in response to PMA compared to HC neutrophils. (**C**) Quantification of NET release of 1 experiment with 1 HC and 2 CGD donors.

MLN0128 (pan-mTORC inhibitor), SB203580 and SB202190 (p38 MAPK inhibitors) did not affect NET release in response to PMA (Fig. 4A). To further study the role of NADPH oxidase in NET release, we used neutrophils from two male CGD patients (twins). These CGD patients contain a homozygous delta GT mutation in the NCF1 gene (gene encoding the p47phox protein; a component of NADPH oxidase), which leads to low levels of ROS production. PMA-induced NET release was abrogated in CGD neutrophils compared to neutrophils from a HD (Fig. 4B,C).

NET release induced by MSU and viable opsonized *S. aureus* was reduced with 40-60% when neutrophils were treated with Wortmannin, R406 and MLN0128 (Fig. 4A). Treatment of neutrophils with Rapamycin (mTORC1 inhibitor) did not attenuate NET release in response to these physiological stimuli (Fig. S5B). In addition, Celastrol (NF-κB inhibitor), Bay-11-7082 (IκB phosphorylation inhibitor), GW5074 (cRaf1 kinase inhibitor), U-73122 (PLC inhibitor), Bafilomycin A (vacuolar-type H^+-ATPase inhibitor), JNKi (JNK inhibitor) or Mytoquinone (mitochondrial ROS inhibitor) did not affect NET release (Fig. S5C). This suggests the importance of the SYK-PI3K-mTORC2 signaling pathway in NET release triggered by MSU and viable opsonized *S. aureus*, since the pan-mTORC inhibitor attenuated NET release and the mTORC1 inhibitor did not.

To prove a distinct underlying signaling mechanism in PMA-induced NET release compared to NET formation induced by MSU and viable opsonized *S. aureus*, we again used DPI treated neutrophils or neutrophils from CGD patients. Upon stimulation with MSU and viable opsonized *S. aureus*, DPI-treated neutrophils as well as CGD neutrophils did release NETs (Fig. 4A,B). Quantification revealed that NET release of CGD neutrophils is comparable or even increased compared to NET release from a HD (Fig. 4C). Although we only used one HD in this experiment, the release of NETs in response to PMA, live opsonized *S. aureus* and MSU of this HD is comparable with other HDs from previous experiments. Together, our data indicate that calcium and NADPH oxidase-induced ROS play an important role in PMA-induced NET release. In contrast, NET release in response to MSU and viable opsonized *S. aureus* acts via the SYK-PI3K-mTORC2 pathway and is independent of NADPH oxidase-mediated ROS.

DISCUSSION

NETs serve as an innate immune defense mechanism to protect the host against invading pathogens, while on the other hand uncontrolled NET release can contribute to disease development and progression. To visualize and quantify NET release *in vitro*, many experimental techniques are described, including fixed time point microscopy[27], live imaging[23,24,28,29], image-based flow cytometry[15], fluorescence

plate reader assay using DNA binding dye's[16,30,31] and ELISA based assays[30]. These techniques are useful for studying NET components, morphology and kinetics. However, it is important to be aware of the limitations of each of these techniques. In this study, we use a semi-automatic approach to quantify NET release in a live imaging assay, which is based on previous described methods[25,32]. Our approach was able to correct for neutrophil input and distinguishes NET release from other mechanisms of cell death, crucial aspects that are lacking in many NET quantification methods.

A unique mechanism of NET release in response to *S. aureus* was previously described: rapid (within minutes) and without breaching the neutrophil plasma membrane[16]. Consistent with this study, we previously have shown that, when using a fluorescence plate reader assay, Sytox Green fluorescence appears within 30 min when neutrophils were exposed to viable *S. aureus*[16,19]. We concluded at the time that neutrophils are able to rapidly release NETs in response to these stimuli. However, *S. aureus* secrete pore-forming toxins that contribute to bacterial virulence and are able to permeabilize neutrophils for DNA dyes that are normally cell membrane impermeable[33-35]. We now show that cytolytic toxins from viable *S. aureus* induce rapid permeabilization of the neutrophil plasma membrane allowing for Sytox Green influx and nuclear DNA staining without histone H3 citrullination. In strong contrast, actual NET release occurs at a later time point (Supplemental video 2). Indeed, HK *S. aureus* that cannot secrete toxins, are not able to permeabilize neutrophils and only trigger NET release. Without imaging, i.e. with a fluorescence plate reader, it is not possible to discriminate between nuclear DNA and NETs. Thus, we conclude that imaging is essential for measuring NET release.

Visualization of NET release in a live imaging assay shows that expelled DNA from the neutrophil diffuses around the neutrophil itself[23-25]. In contrast, fixed time point microscopic experiments show DNA structures that seem to be pulled apart and cover a large surface[25,36]. Similar structures of NETs were shown *in vivo* where blood flow and moving cells result in stretched NETs[37-39]. *In vitro*, this stretched morphology is induced by pipetting (Fig. S6). This shows that quantification of live imaging NET release is more accurate than quantification of NET release visualized with fixed time point microscopy.

Neutrophils rapidly kill pathogens by phagocytosis or by the secretion of proteases to protect the host against severe infection[40,41]. NETs have also been shown to eliminate microbes[1-4]. So far, it is not known which signal triggers the neutrophil to release their nuclear content in the extracellular milieu. The release of NETs could act as a cell death mechanism in which the neutrophil sacrifices itself to protect others. This suggests that NET release is a late response, which is induced when other antimicrobial strategies have failed. In addition, neutrophils sense microbe size and release NETs in response to large pathogens[42], arguing for an active

process that occurs directly after neutrophil contact with the microbe. In contrast with fluorescence plate reader data from our previous study[19], we here show that only MSU triggers rapid NET release while NET formation in response to opsonized *S. aureus*, PMA, and ICs occurs at a later phase. Others have also shown that PMA[18] and ICs[22] induce late NET release. Although these experiments were performed with a fluorescence plate reader assay and therefore unable to discriminate between bona fide NETs and permeable cells, we now confirm late phase NET release in response to PMA and ICs. Similarly, fluorescence plate reader experiments revealed that FcR activation[17,18], *Leishmania*[21], and *C. albicans* hyphea[20] induce rapid NET release while *C. albicans* yeast[20] triggers NET formation at a later time point. Our current data underline that these kinetics should be interpreted carefully and analyzed in an assay like ours, to determine the difference between cellular permeability and actual NET release.

There is no consensus of the requirement of ROS production for NET formation and current data on NET release suggests there are two differential signaling pathways that induce the release of NET. Direct stimulation of PKC, i.e. by PMA, provokes ROS-dependent NET formation, which diminishes in the presence of DPI. Similarly, DPI was published to attenuate NET release upon stimulation with ICs[22,43], anti-LL-37[44] and non-opsonized *S. aureus*[24]. Indeed, CGD neutrophils do not release NETs upon PMA[23,24,45-47] or non-opsonized *S. aureus*[24] stimulation. This demonstrates that some stimuli require NADPH-induced ROS to release NETs. On the contrary, we and others showed that DPI does not affect NET release when neutrophils are stimulated with MSU[19], ICs[48], uric acid[49], *Leishmania*[21], ionomycin[50] and opsonized *S. aureus*[16,19]. In addition, it was shown that CGD neutrophils are still able to release NETs when exposed to uric acid[49], which suggests the presence of a NADPH-oxidase independent NET signaling pathway. We now show that CGD neutrophils as well as DPI-treated neutrophils release NETs upon exposure of MSU and viable opsonized *S. aureus* while PMA-induced NET release is low. So far, data obtained with neutrophils from CGD patients confirm that the role of NADPH oxidase-dependent ROS in NET release is stimulus dependent. However, the discrepancies about the role of ROS in NET release in studies that use DPI, are challenging to interpret. We propose two distinct explanations: 1) The effect of DPI is concentration dependent and/or non-specific or; 2) inappropriate NET measuring techniques and quantification methods that lack experimental sensitivity are masking the true effect of DPI on NET release. Thus, the use of a live imaging assay that allows visualization and quantification of NET release and discriminates between actual NETs and other cell death mechanisms is essential for future NET studies.

A wide variety of cell surface receptors on neutrophils act via SYK and PI3K to induce neutrophil activation[51,52]. FcRs, Toll-like receptors and the P2Y6 receptor are amongst them and could be triggered upon exposure with ICs, opsonized

S. aureus and MSU[53], respectively. We show that the SYK-PI3K-mTORC2 pathway is involved in NET release upon physiological stimuli but not in PMA-induced NET release, which is consistent with previous data[22,54]. In addition, PMA provokes NET release that is morphologically different from other NETs (Supplemental video 4). This indicates that PMA-induced NETs should be regarded as mechanistically distinct from NET formation induced by natural triggers. Therefore we recommend to interpret statements from NET studies performed with PMA with care.

This study highlights the potency of a live imaging technique to study NET release. Using a validated semi-automatic quantification approach, we are able to correct for neutrophil input and distinguish between NETs and other cell death mechanisms, two aspects that increase the accuracy of NET quantification. At the same time, we reveal the limitations of fluorescence plate reader assays using cell-impermeable DNA binding dyes for measurement of NET release. The use of PMA to induce NET release should be avoided or interpreted with great care because it induces NET release completely different from all physiological stimuli.

ACKNOWLEDGEMENTS

The authors thank Florianne Hafkamp and Casper Schouls for the development of the ImageJ macros. The authors thank Dr. Nina van Sorge (Medical Microbiology, University Medical Centre Utrecht, Utrecht, the Netherlands) and Dr. Paul Naccache (Department of Microbiology-Infection and Immunology, Faculty of Medicine, Laval University, Quebec, Canada) for providing bacterial strains and MSU, respectively. The authors thank Tineke Kraaij, Dr. Onno Teng and Prof. Dr. Cees van Kooten (Department of Nephrology, Leiden University Medical Centre, Leiden, the Netherlands) for helpful discussions and sharing expertise. The authors thank Julia Drylewicz for performing statistical analysis (Laboratory of Translational Immunology, Department of Immunology, University Medical Centre Utrecht, Utrecht, The Netherlands).

This work was supported by the Dutch Arthritis Foundation (Grant 12-2-406). M.V. is supported by the Netherlands Organization for Scientific Research (NWO), division Earth and Life Sciences (ALW; Grant 863.14.016). L.M. is supported by a Vici grant (91815608) from Netherlands Organization for Scientific Research (NWO).

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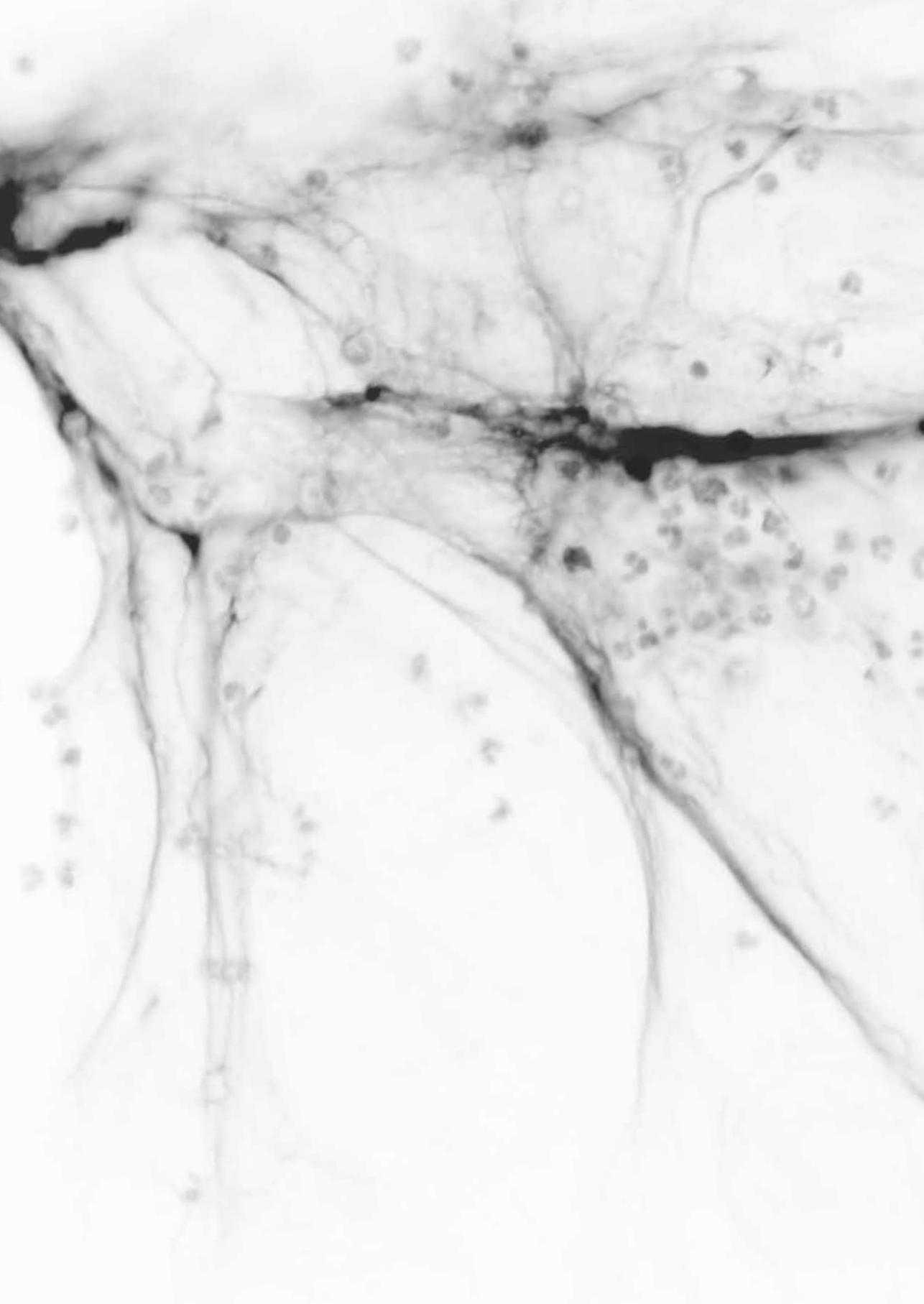
SUPPLEMENTARY INFORMATION

Supplemental figures and videos that accompanies this paper are at the Scientific Reports website (https://www.nature.com/articles/s41598-017-06901-w#MOESM5).



3 CHAPTER

NEUTROPHIL EXTRACELLULAR TRAP RELEASE IS ASSOCIATED WITH ANTINUCLEAR ANTIBODIES IN SYSTEMIC LUPUS ERYTHEMATOSUS AND ANTIPHOSPHOLIPID SYNDROME

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Manuscript accepted in Reumathology (Oxford)

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ABSTRACT

Objectives. Increased release of neutrophil extracellular traps (NETs) is implicated in the activation of plasmacytoid dendritic cells, vascular disease and thrombosis in systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS). However, studies comparing NET release between patients with SLE and APS are lacking. Here we evaluated plasma-induced NET release in a large cohort of patients with SLE, SLE+APS and primary APS (PAPS) in relation to clinical and serological parameters.

Methods. Neutrophils from healthy controls (HC) were exposed to plasma of heterologous HC (n=27), SLE (n=55), SLE+APS (n=38), or PAPS (n=28) patients and NET release was quantified by immunofluorescence. In a subset of SLE patients, NET release was assessed in longitudinal samples before and after a change in treatment.

Results. Plasma-induced NET release was increased in SLE and APS patients, with the highest NET release found in patients with SLE(±APS). Plasma of 60% of SLE, 61% of SLE+APS and 45% of PAPS patients induced NET release. NET release did not correlate with disease activity in SLE or APS. However, increased levels of antinuclear and anti-dsDNA autoantibodies were associated with increased NET release in SLE and APS. Only in SLE patients, elevated NET release and an increased number of low-density granulocytes were associated with a high interferon signature.

Conclusion. Increased NET release is associated with autoimmunity and inflammation in SLE and APS. Inhibition of NET release thus could be of potential benefit in a subset of patients with SLE and APS.

INTRODUCTION

Systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS) are overlapping autoimmune diseases that can occur separately or in the same patient. In SLE, immune complexes (IC) of autoantibodies are deposited into tissues leading to inflammation in several organs, including kidney, skin and joints. In APS, antiphospholipid antibodies (aPL) activate endothelial cells and trophoblasts resulting in thrombosis and pregnancy morbidity. APS is termed primary APS (PAPS) when no underlying disease such as SLE is present. In APS, most research has focused on the pro-thrombotic role of aPL. However, research over the last years indicates an important role for immune cells in the pathogenesis of (P)APS, often in a similar fashion as in SLE, although studies that compare immunopathology between SLE, SLE+APS and PAPS patients are scarce[1].

There is a growing interest in the role of neutrophils in rheumatic diseases[2]. Neutrophils act as a first line of defense against infectious invaders by, amongst other strategies, the release of neutrophil extracellular traps (NETs). NETs consist of decondensed chromatin decorated with neutrophil derived proteases and antimicrobial peptides, which trap and kill pathogens[3]. Neutrophils from SLE and PAPS patients are prone to release NETs spontaneously[4,5]. In addition, healthy neutrophils release NETs when stimulated *in vitro* with autoantibodies present in sera of SLE or APS patients[5,6]. Furthermore, DNase activity is decreased in SLE and APS resulting in increased NET exposure[2,7] and SLE and APS patients have increased numbers of circulating low-density granulocytes (LDG)[8,9], a subset of neutrophils prone to undergo NET formation. As a result, SLE and APS patients have elevated levels of NET remnants in the circulation[2,7] and NETs are present in affected tissues such as the skin or kidney in SLE or in aPL-induced thrombi[9,10].

Uncontrolled NET release triggers a pathological cascade of events relevant for the pathophysiology of SLE and APS. NETs induce tissue damage[2], activate the clotting system to promote thrombus formation[5], induce endothelial dysfunction[11] and represent a source of autoantigens[4]. Moreover, *in vitro*, NETs activate plasmacytoid dendritic cells (pDCs) to produce interferon alpha (IFNα)[4,9,12] which might explain the IFN signature in SLE and APS patients[13].

Until now, NET release has only been studied in SLE and APS separately in small-scale studies. The different methodologies to induce and quantify NET release hamper the comparison across studies. Recently we developed a novel NET assay which specifically measures NET release as it distinguishes NET release from other forms of neutrophil death while the automatic quantification avoids subjectivity[14]. Here, we employed our assay to investigate plasma-induced NET release in a large cohort of SLE, SLE+APS and PAPS patients in relation to clinical and serological parameters including the IFN signature.

MATERIAL AND METHODS

Study population

Patients were classified as either SLE or APS when fulfilling their respective classification criteria[21,22]. Patients that fulfilled both sets of criteria were classified as SLE+APS whereas APS patients that did not fulfill the classification criteria for SLE or another rheumatic disease were classified as PAPS. SLE, SLE+APS and PAPS patients and age/sex matched healthy controls (HC) were recruited from our outpatient clinic or in-house healthy donor service. None of the patients had evidence of an on-going infection. Within 2 hours of collection, whole blood was centrifuged for 10 minutes at room temperature at 1500 x g and plasma (from lithium-heparin vacutainers) or serum (from serum tubes) were stored at -80°C until further use. Disease activity was scored by SELENA-SLEDAI in patients with SLE and by the adjusted global antiphospholipid syndrome score (aGAPSS)[23] in patients with APS. Anti-dsDNA antibodies were measured by ELISA (Euroimmun), antinuclear antibodies were tested by indirect immunofluorescence on HEP-2000 cells at a serum dilution of 1:100. Results were reported as negative, weakly positive, positive or strongly positive. All study participants signed informed consent approved by the Medical Research Ethics Committee University Medical Center University Medical Center Utrecht review broad prior to the donation of blood in accordance with the declaration of Helsinki.

Isolation of human neutrophils

Neutrophils from healthy controls were isolated by Ficoll-Paque (GE Healthcare) density gradient centrifugation, after which erythrocytes were lysed in ammonium chloride buffer (155 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM EDTA in double-distilled H_2O; pH=7.2). Cells were resuspended in RPMI 1640 (Life Technologies) supplemented with 10% (v/v) heat-inactivated (HI) fetal bovine serum (FBS; Biowest) and 50 U/ml Penicillin-Streptomycin (Life Technologies).

Preparation of ICs

Insoluble ICs were formed by using human serum albumin (HSA; Sanquin) and rabbit polyclonal anti-HSA IgG (Sigma Aldrich) as described previously[14]. Briefly, a mix of 5 µg HSA and 45 µg rabbit-anti-HSA antibody was made in a final volume of 50 µl PBS. After 1 h incubation at 37°C, insoluble ICs were formed and used for stimulation of neutrophils in a concentration of 10 µg/ml.

Microscopic imaging of NET release

NET release by human neutrophils was analyzed by a quantitative live imaging NET assay as described before[14]. Briefly, nuclear DNA of neutrophils was stained with

Hoechst 33342 (AnaSpec Inc.) and neutrophils were challenged with 10 µg/ml ICs or 10% (v/v) plasma from HC and SLE patients in medium containing Sytox Green (Life Technologies). Autologous plasma was used as a negative control. Where indicated, neutrophils were pre-treated with 1 µM DPI for 30 min at 37°C. NETs were confirmed to be positive for citrullinated histone H3. NET release was recorded on the Pathway 855 bioimaging system (BD Biosciences) with a 10x objective. A set of two images (Hoechst and Sytox Green) was taken with an Orca high-resolution CCD camera and four fields of view per condition were captured. The system was controlled by the AttoVision software (version 1.7/855).

For fixed time point NET release, neutrophils were incubated in RPMI 1640 (without phenol red) supplemented with 2% FBS, 50 U/ml Pen-Strep and 10 mM HEPES (Life Technologies) containing 20 µM Hoechst 33342 for 30 min at 37°C. A total of $1x10^5$ neutrophils seeded in 0.001% poly-L-lysine (Sigma-Aldrich) pre-coated wells of a clear bottom 96-wells plate (Ibidi) and incubated with plasma from (heterologous, age-sex matched) HC, SLE, SLE+APS and PAPS patients for 3 hours and 45 min at 37°C and 5% CO_2. PMA was used as a positive control. Extracellular DNA was stained with 0.2 µM Sytox Green for 15 min at 37°C and fixed with 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 15 min at room temperature. Fixed NETs were imaged with a 10x objective. A set of two images (Hoechst and Sytox Green) was taken and twenty fields of view per condition were captured (Fig. 1A).

Semi-automatic NET quantification

Quantification of live imaging NET release was performed as described previously[14]. For automatic quantification of fixed time point NETs, a macro was created that contains the following steps: 1) Convert Sytox Green images in 8-bit grey scale images, 2) Thresholding with "Li" logarithm, 3) Watershed segmentation and 4) Analysis of NETs (>68 μm^2) (Fig. 1A). The quantification approach was controlled by Fiji software (version 2.0.0-rc-43/1.51d). NET area, the percentage of pixels per microscopic field of view that have been highlighted after thresholding, was log transformed and presented on the y-axes.

Quantification of the IFN signature

The IFN signature was quantified on isolated monocytes as previously described[13]. In brief, PBMCs were isolated by Ficoll density gradient separation. Subsequently, monocytes were isolated by CD14+ selection using the autoMACS pro (Miltenyi). RNA was extracted using the all-prep universal kit (Qiagen) and complementary DNA was generated using iScript (Biorad). qPCR using pre-designed primer/probe sets (Thermofisher) on the following type I IFN inducible genes: IFI44L, Serping1, IFITM1 and Ly6E and normalized to the expression of GUSB. Normalized gene-expression values were used to calculate a type I IFN score as previously described[24].

Identification of low-density granulocytes

Freshly isolated PBMCs were stained with CD14-APC-eF780 (eBioscience), CD45-PerCP (Biolegend) and CD16-V500 (BD). Samples were acquired on a LSR fortessa (BD). From the single cell population LDGs were defined as $CD45^{dim}$ cells with a high side scatter signal and negative for CD14 and reported as percentage of PBMCs.

Statistical analysis

The NET area of 20 different microscopic fields per well was averaged. The mean of log-transformed NET areas per plasma donor of four independent experiments was reported as the mean NET area. The J-statistic of the Youden index of the Receiver Operating Characteristic (ROC) curve of NET release in patients (SLE, SLE+APS and PAPS) as compared with HC was used to define a cut-off to stratify patients into high or low NET inducers. Differences between groups were tested two-sided by ANOVA and Tukey's post-test or *t*-test as appropriate (α=0.05) using SPSS (v22).

RESULTS

Validation of a high-throughput assay to measure plasma-induced NET release

Our live imaging assay to monitor NET release over time revealed NET release within 30 min after exposure to SLE plasma (Fig. 1B, videoS1). The presence of citrullinated histone H3 in the extracellular DNA confirmed the formation of NETs (Fig. 1C). To allow the measurement of >100 samples without a time difference between the first and last sample, we introduced a fixation step after 240 minutes, which did not affect the quantification of NETs (Fig. 1D). Pilot experiments with plasma and serum samples from SLE (n=15) and HC (n=8), showed increased NET induction by plasma from SLE patients as compared with HC. Although serum of both patients and controls had higher NET-induction capacity than plasma, no difference was observed between SLE and HC (Fig. 1E). In these pilot experiments, we observed a moderate (r=0.4375, *P*=0.002) correlation of NET release between independent experiments using different neutrophil donors (Fig. 1F).

Increased plasma-induced NET release in SLE, SLE+APS and PAPS

We next used plasma samples of HC (n=27), SLE (n=55), SLE+APS (n=38) and PAPS (n=28, Supplementary Table 1) patients to induce NET release in neutrophils of four HC donors in four independent experiments. Confirming our pilot experiments, the mean NET release of four independent experiments was higher using plasma from SLE and SLE+APS patients as compared with HC plasma (*P*<0.001), with a similar trend when using PAPS plasma ((*P*=0.14, Fisher's LSD: *P*=0.03) Fig. 1G,

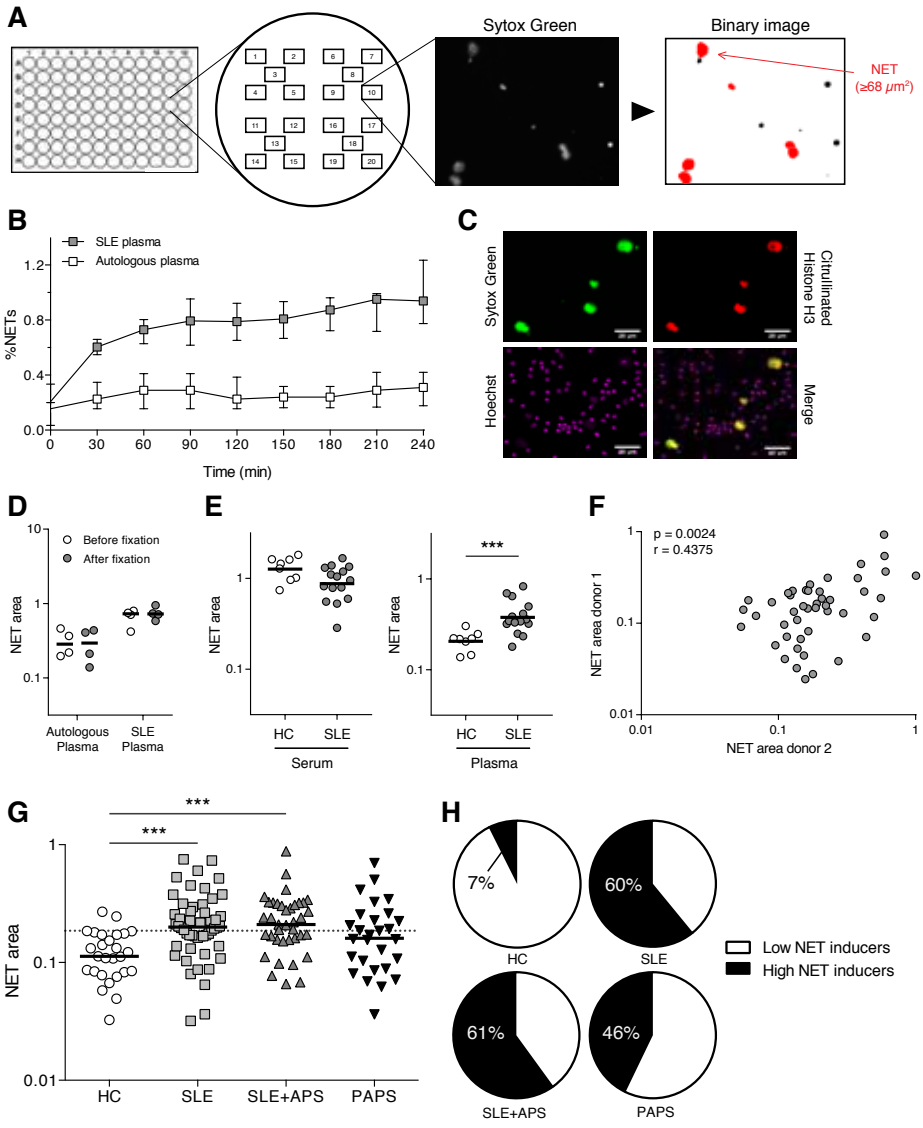

Figure 1 | NET release in response to plasma of SLE, SLE+APS and PAPS patients
(**A**) Experimental approach to measure NET release in a fixed time point. Twenty fields of view were captured per condition and Sytox Green images were used to analyse NET area. (**B**) Live imaging showed enhanced NET release of HC neutrophils when exposed to SLE plasma compared to autologous plasma. Data points represent median ± interquartile range of four independent experiments with four plasma samples each. (**C**) Citrullinated histone H3 staining confirmed the presence of actual NETs and (**D**) fixation after 4 hours did not affect NET quantification. In a pilot experiment, serum and plasma from HC (n=8) and SLE (n=15) patients was used to induce NET release in HC neutrophils. NET area of independent experiments with neutrophils from 3 different HC donors is presented. (**E**) Elevated NET release was shown in neutrophils exposed to plasma, not serum, from SLE patients compared with those exposed to plasma or serum from heterologous HC. (**F**) Plasma induced NET release correlated between independent experiments. (**G**) HC neutrophils exposed to heterologous plasma from SLE (n=55), SLE+APS (n=38) and PAPS (n=28) patients displayed increased NET release compared with those exposed to plasma from HC (n=27). NET area of independent experiments with neutrophils from 4 different HC donors is presented. (**H**) Prevalence of high NET release in patients with SLE, SLE+APS or PAPS. The data in **D**, **E** and **G** are presented as mean, ***P<0.001. The images in **C** are representative of at least three experiments with neutrophils from different donors.

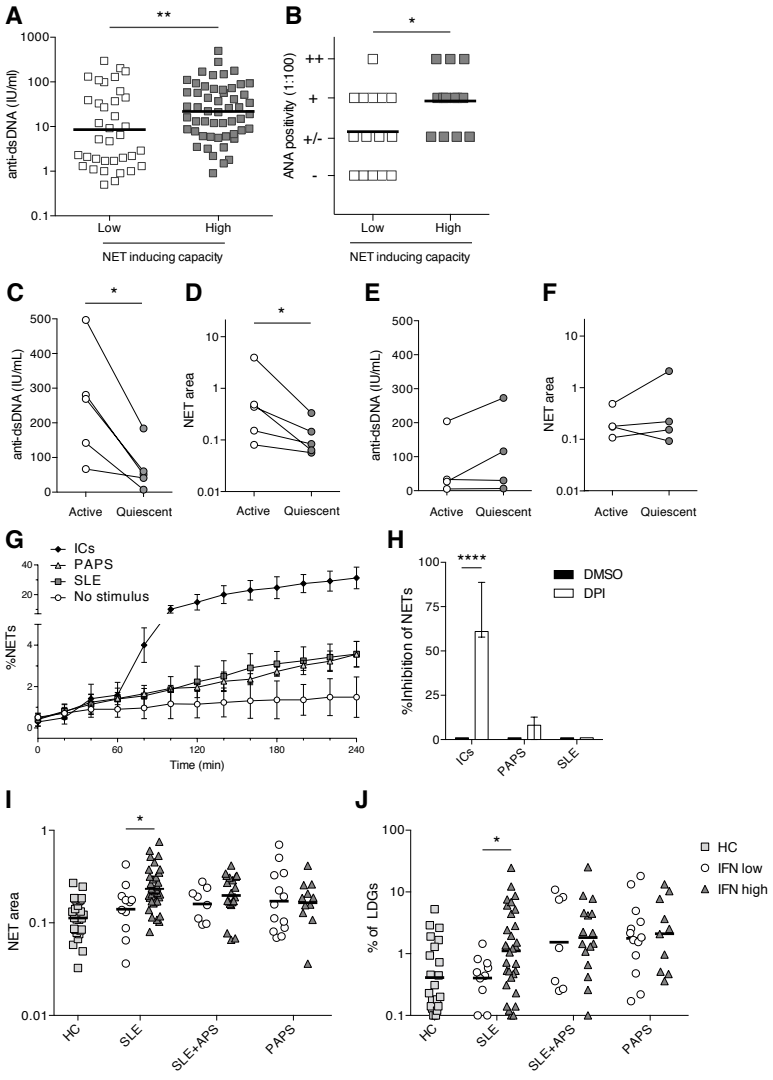

Figure 2 | **Plasma-induced NET release is associated with antinuclear antibody levels in plasma SLE and PAPS**
(**A**) SLE patients classified as high NET inducers contained elevated levels of anti-dsDNA antibodies, compared with those that are classified as low NET inducers. (**B**) PAPS patients classified as high NET inducers contained increased ANA staining intensities, compared with those that are classified as low NET inducers (- negative, +/- weak, + positive, ++ strongly positive). Longitudinal samples were collected from SLE patients at time of active disease as well as subsequent quiescent disease. (**C**) Anti-dsDNA antibody level and (**D**) NET area was increased in SLE patients with an active disease compared with those with a quiescent disease state. Patients with (**E**) stable or increasing anti-dsDNA antibodies between two time points (**F**) did not have a decrease in plasma-induced NET release. (**G**) NET release in response to IC was high compared to SLE and APS plasma-induced NET release. Data points represent mean ± SD of independent experiments with neutrophils of two HC donors and four plasma samples each. (**H**) DPI, NADPH oxidase inhibitor, supressed IC-induced NET release while SLE and PAPS plasma-induced NET release was independent of NADPH oxidase. The percentage of inhibition of NET release was calculated based on the area under the curve relative to neutrophils exposed to IC, PAPS or SLE plasma in the presence of DMSO. (**I**) Plasma from SLE patients classified as high IFN signature displayed elevated levels of NET release compared to those that were classified as low IFN signature. (**J**) Increased amount of LDGs were present in SLE IFN high patients compared with SLE IFN low patients. Differences of LDG amount between high and low IFN patients were not shown in SLE+APS and PAPS patients. The data in **A**, **B** and **G**-**J** are presented as mean, **P*<0.05, ***P*<0.01, ****P*<0.001.

Fig. S1). NET release did not differ among SLE, SLE+APS or PAPS patients (ANOVA, *P*=0.19). Setting a threshold by ROC-curve analysis (Fig. S2), plasma samples from 33/55 (60%) of SLE, 23/38 (61%) of SLE+APS and 13/28 (46%) of PAPS patients had high NET release (Fig. 1H), as compared with 2/27 (7%) of HC plasmas. Thus, our data show that plasma from the majority of SLE and APS patients induces NET release.

NET release did not correlate with clinical measures of disease activity, including SELENA-SLEDAI for SLE(±APS) patients (*P*=0.57, Fig. S3A) and the aGAPSS for (P)APS patients (*P*=0.88, Fig. S3B). Furthermore, there were no significant differences among clinical phenotypes including (active) lupus nephritis in SLE patients or APS patients with or without arterial or venous thrombosis or pregnancy morbidity. Also, NET release did not differ between patients treated with or without prednisolone, azathioprine, asprin or other immunosuppressants (*P*>0.05, data not shown).

Plasma-induced NET release is associated with antinuclear and anti-dsDNA antibodies

In vitro studies implicate NET release as a source of autoantigens eliciting the production of autoantibodies against nuclear components in SLE[5]. In line with these observations, SLE patients with high NET release had increased levels of anti-dsDNA antibodies compared to patients whose plasma induced low NET release (*P*=0.008, Fig. 2A). Likewise, PAPS patients with high NET release had elevated ANA staining intensities (*P*<0.05, Fig. 2B). In longitudinal samples, collected from SLE patients before and after a change in immunosuppressive therapy, a decline in anti-dsDNA antibodies (*P*=0.02, Fig. 2C) was paralleled by a decline in NET release (*P*=0.03, Fig. 2D, n=5), whereas patients with stable or increasing anti-dsDNA antibodies (Fig. 2E) between two time points did not have a decrease in plasma-induced NET release (*P*=0.48, n=4, Fig. 2F). Among SLE or APS patients, no specific association between the presence or absence of anti-β2 Glycoprotein I and anti-ribonucleoprotein (RNP) antibodies was observed.

NET kinetics is similar in SLE and PAPS

We previously showed that the kinetics of NET release differs between stimuli[14]. We observed no difference in the kinetics of plasma-induced NET release among high-inducing plasma samples of SLE or PAPS patients (Fig. 2G). In comparison to patient plasma, exposure of neutrophils to ICs induced abundant NETs, approximately 30% compared to 3% in patient plasma samples. DPI inhibits, amongst others, NADPH oxidase and NET release in response to ICs was inhibited by 60-70% in the presence of DPI while PAPS and SLE plasma-induced NET release was not inhibited in the presence of DPI (Fig. 2H).

NET release and LDGs are associated with the IFN signature in SLE

In vitro experiments implicate NETs and LDGs as a trigger for IFNα production by pDC[4,9], although no studies have explored NET release in relation to the presence or absence of the IFN signature. SLE patients with a high IFN signature (IFN-high) had higher NET release than patients with a low IFN signature (IFN-low) (P<0.01, Fig. 2I). Corroborating this finding we observed that IFN-high SLE patients had increased numbers of circulating LDGs (P<0.01, Fig. 2J). Interestingly, these associations were not seen in APS patients, neither in SLE+APS nor in PAPS (P>0.05).

DISCUSSION

Using a novel high throughput assay we show that plasma of SLE, SLE+APS and PAPS patients induces NET release, which is associated with antinuclear antibodies in PAPS and anti-dsDNA autoantibodies and the IFN signature in SLE patients. This study highlights the potential role of NET release in relation to autoimmunity and inflammation in SLE and APS and compares NET release in a large cohort of SLE and APS patients.

Previous studies have shown induction of NET release using serum of SLE or APS patients[6,15]. In small pilot studies, when comparing serum with plasma, serum induced a higher release of NETs than plasma, both in patients and HC, and no difference between HC and patients was observed. The generation of serum leads to platelet activation, which is a strong NET inducer[16] and is a likely cause for the higher NET release induced by serum samples. As a result, to avoid potential effects of platelet activation, we used patient plasma to trigger NET release in further experiments. Importantly, although similar trends were observed when using different neutrophil donors, the amount of NETs formed differed between neutrophil donors and therefore our results stress the need to use different neutrophil donors when studying NET release[16].

Besides the amount of NETs, the kinetics of NET release differ between stimuli[14]. We observed a rapid release of NETs (within 30 minutes) upon exposure to patient plasma in both SLE and PAPS patients. As the composition of NETs differs between stimuli[17], we speculate that the content of NETs could differ between SLE and APS, since NET release was differentially associated with the IFN signature in SLE and APS.

NET release in the context of SLE and APS has been mainly studied using purified antibodies or cytokines to trigger NET release, including anti-β2 Glycoprotein I, anti-RNP, anti-human neutrophil protein (HNP), anti-LL-37, anti-matrix metalloproteinase 9 antibodies and interleukin-18 and hyperacetylated microparticles[4-6,11,12,18-20]. As multiple factors present in patient plasma may induce NET release, it is unknown

which stimulus is responsible for the NET release in our assay, however the association of autoantibodies and the IFN signature with NET release suggest their involvement and this is clearly different in plasma from healthy individuals. Nevertheless NET release by purified factors should be interpreted with caution since the concentration and composition of these factors in patients' plasma might be different. Indeed, in our study NET release in response to ICs was much higher than in response to patient plasma. Moreover, IC-induced NET release is dependent on NAPDH oxidase, in contrast to plasma-induced NET release, although it is unknown whether the magnitude of NET release *in vitro* can be directly translated to *in vivo* situations.

Enhanced NET release is considered a major pathogenic factor linked to tissue damage, the IFN signature and other disease manifestations in both SLE and APS[4,5,10]. Consistent with this, we report increased NET release in patients with elevated autoantibodies or the IFN signature in SLE and APS. Treatment options that mitigate NET release could therefore be of added clinical value. Inhibition of NET release ameliorates mouse models of SLE and APS[10,21]. Several small inhibitory molecules reduce NET release *in vivo*[22] while hydroxychloroquine, a treatment for SLE, inhibits NET release *in vitro*[2]. We previously reported that triggering the inhibitory receptor SIRL-1 attenuates SLE plasma and autoantibody-induced NET release[6]. Our current results indicate that only a subset of patients, i.e. ~60% of SLE(±APS) and ~45% of PAPS patients would benefit from inhibiting NET release.

ACKNOWLEDGEMENTS

The authors thank Dr. Michiel van der Vlist and Dr. Inês Pascoal Martins Ramos, for helpful discussions.

This work was supported by the Dutch Arthritis Foundation (grant 12-2-406). L.M. was supported by a Vici grant (91815608) from Netherlands Organization for Scientific Research (NWO).

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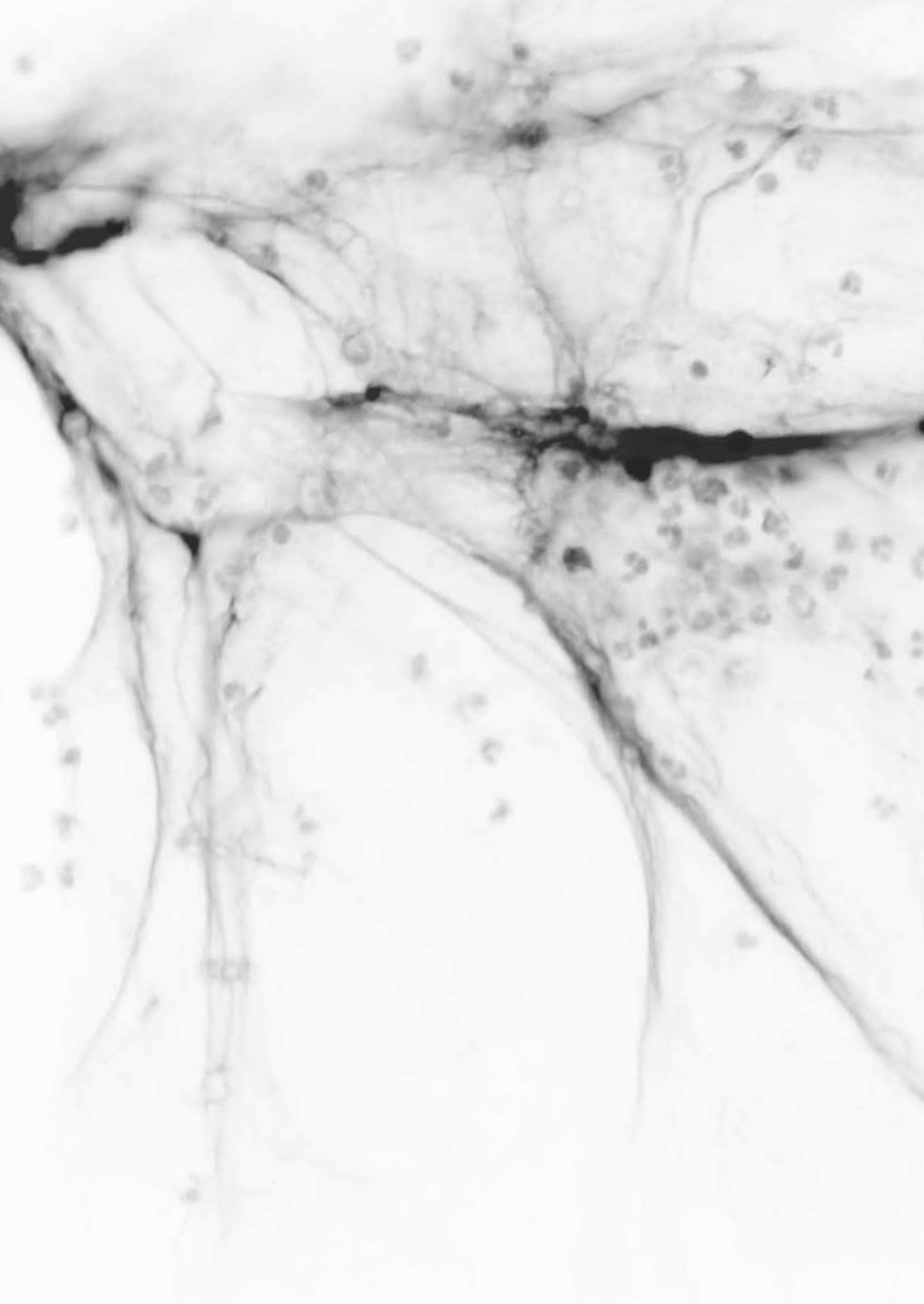
SUPPLEMENTARY INFORMATION

Supplemental figures, videos and table that accompanies this paper are at the Rheumatology (Oxford academic) website (https://academic.oup.com/rheumatology).



4 CHAPTER

FINE-TUNING NEUTROPHIL ACTIVATION: STRATEGIES AND CONSEQUENCES

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Immunology Letters. 2016 Oct; 178:3-9

ABSTRACT

In spite of their important role in host defense, neutrophils can also cause severe morbidity and mortality. Neutrophils have an extensive armory necessary to eradicate pathogens, but neutrophil infiltration and activation also induces major tissue injury associated with acute and chronic inflammatory disorders. Here, we review neutrophil antimicrobial functions and discuss their individual contribution to disease pathogenesis. Furthermore, we provide an overview of the anti-inflammatory drugs that can dampen neutrophil transmigration, elastase activity, and the production of reactive oxygen species, which are already in clinical trials. The discovery of potential inhibitors of the release of neutrophil extracellular trap is still in its infancy. Here, we discuss small molecule inhibitors and inhibitory receptors that show promising results in reducing neutrophil extracellular trap formation *in vitro* and *in vivo* and discuss the advantages and drawbacks of inhibiting the release of neutrophil extracellular traps as a therapeutic treatment. Specific suppression of neutrophil extracellular trap formation, preferably while other antimicrobial functions are preserved, would be an ideal approach to treat neutrophilic inflammation, since it prevents acute as well as chronic neutrophil-associated pathology.

INTRODUCTION

Neutrophils are central players of the innate immune system and constitute a first line of defense against invading pathogens. They exist in a resting state in the blood flow and become primed by bacterial products, cytokines, or chemokines, e.g. tumor necrosis factor alpha (TNF-α), granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-8, interferon-γ (IFNγ), and lipopolysaccharide (LPS)[1]. Primed neutrophils rapidly are recruited to the site of infection and use their extensive arsenal, including phagocytosis, secretion of proteases, production of reactive oxygen species (ROS), and the release of neutrophil extracellular traps (NETs), to ensnare and kill microbes[2,3] (Fig. 1). Neutrophil infiltration and activation also occurs in non-infectious inflammation. For instance, trauma or ischemia-reperfusion (IR) injury leads to necrosis of cells that release so-called damage-associated molecular pattern molecules (DAMPs), in the extracellular environment. DAMPs induce macrophages and other cells to release cytokines and chemokines, which amongst others activate neutrophils. These cells use the same strategies they use to fight microbes to discard necrotic cells.

Neutrophils are like kamikaze pilots: during and after elimination of the inflammatory stimulus, neutrophils undergo apoptosis to be taken up by macrophages to resolve the ongoing inflammatory response[4]. In addition, macrophages produce lipoxin A4 to inhibit further neutrophil infiltration and secrete IL-10 and transforming growth factor beta (TGF-β) to induce anti-inflammatory responses and thus avoid excessive tissue damage and initiate the healing process[4,5]. When the anti-inflammatory response fails, tissue breakdown products trigger the migration of more neutrophils to the site of inflammation. Moreover, neutrophils themselves produce IL-17, IL-8, TNF, and GM-CSF leading to further recruitment, activation and prolonged survival of neutrophils at the site of inflammation. In addition, neutrophils crosstalk with other immune cells, including macrophages, DCs, natural killer (NK) cells, and T and B cells, contributing to the initiation of the adaptive immune response[6]. Extensive neutrophil infiltration and activation contributes to tissue injury, including the loss of normal tissue architecture, which could result in organ failure[7] (Fig. 1). Furthermore, it is associated with increased IR injury and has been described in various lung diseases such as acute respiratory distress syndrome (ARDS)[8], cystic fibrosis (CF)[9], and chronic obstructive pulmonary disease (COPD)[10], as well as systemic autoimmune disorders, including systemic lupus erythematosus (SLE)[11] and rheumatoid arthritis (RA)[12].

Here, we discuss the importance of the tight regulation of neutrophil activity and describe the impact of uncontrolled neutrophil activity on the pathophysiology of disease. Effective therapeutic tools to reduce tissue damage by neutrophils are not available, however *in vitro*, *in vivo* and clinical studies with new various

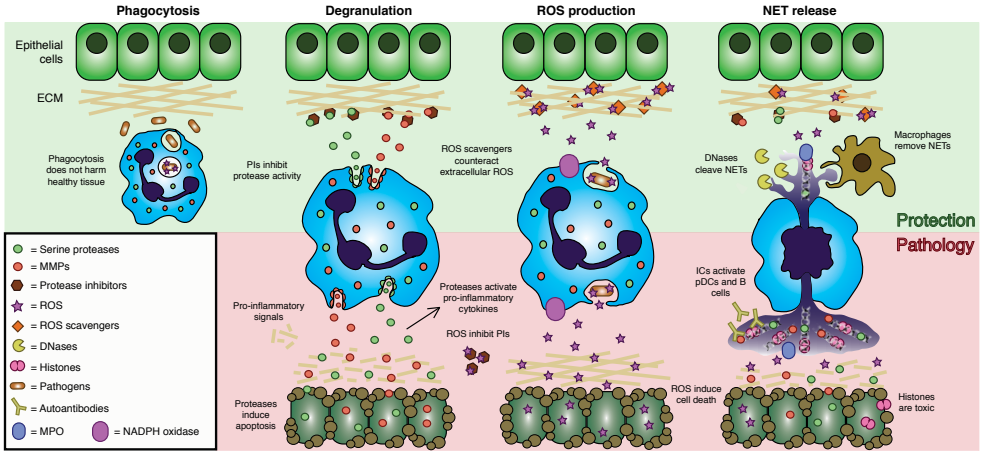

Figure 1 | **Neutrophil antimicrobial strategies in health and disease**
Upper part, the human body uses several mechanisms to counteract abundant proteases, extensive ROS or NETs. Lower part, uncontrolled neutrophil activation causes ECM degradation, healthy tissue cell death and exposure of autoantigens, which contribute to disease pathogenesis.

anti-inflammatory drugs show promising results in the suppression of neutrophil-dependent tissue damage. Complete suppression of neutrophil activity comes with a cost, since these cells are essential in host defense. The important questions to answer to optimize neutrophil treatment are: which effector mechanisms are most important for antimicrobial activity and which are most dangerous for the host?

NEUTROPHIL-MEDIATED PATHOLOGY AND POTENTIAL ANTI-INFLAMMATORY TREATMENTS

Blocking TEM as potential treatment in chronic inflammation

Blocking of transendothelial migration (TEM) may be an effective way of limiting neutrophil numbers in inflamed tissue. Interference with adhesion molecules on neutrophils or endothelial cells as well as with chemokines or chemokine receptors has been proposed as a therapeutic approach. Intercellular adhesion molecule-1 (ICAM-1) is expressed on the luminal side of endothelial cells and circulating neutrophils bind to this protein via β2-integrins and subsequently migrate through the endothelium to reach the site of inflammation[3]. In the past, *in vivo* studies have shown that small molecule ligands as well as neutralizing antibodies that block either a family of β2-integrins[13] or ICAM-1[14] can inhibit neutrophil infiltration and protect tissue from IR injury (Fig. 2, A1). Unfortunately, clinical trials based on this approach failed to reduce neutrophil infiltration in response to traumatic shock and IR injury[15,16].

Chemokines and chemokine receptors could also be targeted to interrupt

neutrophil infiltration in inflamed tissues (Fig. 2, A2). Indeed, SB-656933, an antagonist of the CXC chemokine receptor 2 (CXCR2), specifically inhibits the recruitment of neutrophils to the lungs of CF patients[17]. In addition, phase I and II clinical trials have shown that azithromycin, roflumilast, and MK-7123, antagonists of IL-8[18], acPGP[19], and CXCR2[20], respectively, reduce the level of neutrophilic inflammation and improve lung function in COPD patients. These results show that main clinical endpoints of these studies, including induced sputum neutrophil number, sputum inflammatory biomarker (neutrophil elastase (NE), myeloperoxidase (MPO), and free DNA), and forced expiratory volume in one second (FEV1) were reached. A third way of reducing neutrophils at the site of inflammation is to promote a process called reverse TEM (rTEM), i.e. the migration from extravascular tissue back into the vascular lumen[21] (Fig. 2, A3). Investigation of rTEM to prevent tissue damage is in the pre-clinical phase. Tanshirone IIA, a compound derived from a Chinese medicinal herb with profound anti-inflammatory effects, promotes rTEM of neutrophils in zebrafish and may provide a novel therapeutic approach to drive the resolution of inflammation[22]. A major drawback of interfering with transmigration to reduce the number of neutrophils at the site of inflammation is that this could lead to leukocyte adhesion deficiency (LAD)-like conditions. Neutrophils of LAD patients are not able to extravasate due to a complete absence of β2-integrin and therefore individuals are highly susceptible to infections, which could lead to life threatening situations[23].

Inhibition of NE restores airway inflammation

Neutrophils contain granules that fuse with the plasma membrane upon activation, releasing granular proteins and peptides into the extracellular environment. NE, proteinase 3 (PR3), cathepsin G (CatG), neutrophil serine protease 4 (NSP4), and a family of metalloproteinases (MMPs) are the main granular proteins and execute their antimicrobial effects by disrupting bacterial membranes, cleavage of cell wall peptidoglycans, or inhibiting microbial iron uptake[24]. To protect the host from potential damaging effects, endogenous protease inhibitors (PIs) counteract the activity of these proteases. Neutrophilic proteases and endogenous PIs are in balance in health, however a number of chronic lung diseases, including COPD and CF, are associated with insufficient amount of alpha-1-antitrypsin (AAT)[14] and tissue inhibitor of metalloproteinase[15], PIs of respectively NE and MMP-9, to mitigate damage. The resulting higher activity of these granular proteases leads to digestion of extracellular matrix (ECM) components, thereby disrupting the integrity of the barrier between epithelial and endothelial cells and destroying the elastic capacity of the lung tissue which may impact lung function[25]. Cleavage of the ECM in small fragments, in turn, stimulates the immune system and initiates a positive feedback loop of immune cell infiltration and tissue destruction[26]. Besides the effect of granular proteins on the ECM, recent data suggest that neutrophil proteases have caspase-like activity

and induce apoptosis at sites of inflammation[27,28]. Finally, recent data showed that neutrophil proteases could activate the IL-1 family cytokines, which then promote a strong pro-inflammatory response[29]. Thus, neutrophil proteases can directly induce cell death, cleave the ECM, and activate cytokines, which all intensify inflammation (Fig. 1).

Much attention has been given to the development of inhibitory agents that reduce the activity of NE because this granular protein is abundantly present in the lungs of CF and COPD patients and overwhelms the local antiprotease safeguard, thereby causing major tissue destruction[30]. In 2007, Griese et al.[31] performed a clinical study were they examined the effect of AAT inhalation on lung function, protease-antiprotease balance, and airway inflammation in CF patients. After 4 weeks of inhalation of AAT, protease-antiprotease balance was restored and a clear reduction of airway inflammation was observed but without an effect on lung function. In addition, an open-label pilot study showed that six weeks oral therapy with sildenafil, a phosphodiesterase inhibitor, decreased sputum elastase activity in adults with CF. This change in elastase is a sensitive measure of clinically important differences because it predicts subsequent lung function decline[32]. Additional analyses revealed that 75% of those who had a decrease in sputum elastase also had a decrease in sputum IL-8[33]. These results suggest that increasing the AAT level, and thus balancing elastase activity, contributes to diminished airway inflammation in CF and COPD patients, however again there is minimal improvement of lung function (Fig. 2, B4). Reduction of NE activity weakens the neutrophils innate ability to fight infection but it is unclear whether this results in recurrent bacterial infections in these patients. Recent *in vivo* studies observe that mice deficient for NE were less susceptible to infection, although the bacterial burden in organs of these mice were similar is in wild type[34]. Together, these results suggest that reduced activity of NE increases the tolerance of the host to infection by minimizing host tissue damage.

Decreased ROS activity shows clinical benefits in COPD patients

ROS, a collective noun for radicals ($O2^{\bullet-}$, OH^- and $OH^\bullet$) and non-radicals (H_2O_2 and HOCl) generated by enzymatic catalysis of O_2 through nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, are present in phagosomes and play a key role in killing phagocytized material[35]. Intra-phagolysosomal localization of ROS minimizes damage to host cells, however ROS can be spilled or produced in the extracellular milieu leading to major tissue damage. In healthy conditions, antioxidant enzymes such as glutathione peroxidase and catalase are expressed in the ECM of lung and airway tissue[36] and control the level of extracellular ROS to prevent further oxidation of molecules[37]. When this process fails and extracellular levels of ROS are elevated, healthy cells reduce their levels of ATP and accumulate intracellular Ca^{2+}. In addition, DNA is damaged and fatty acids, lipids, and proteins

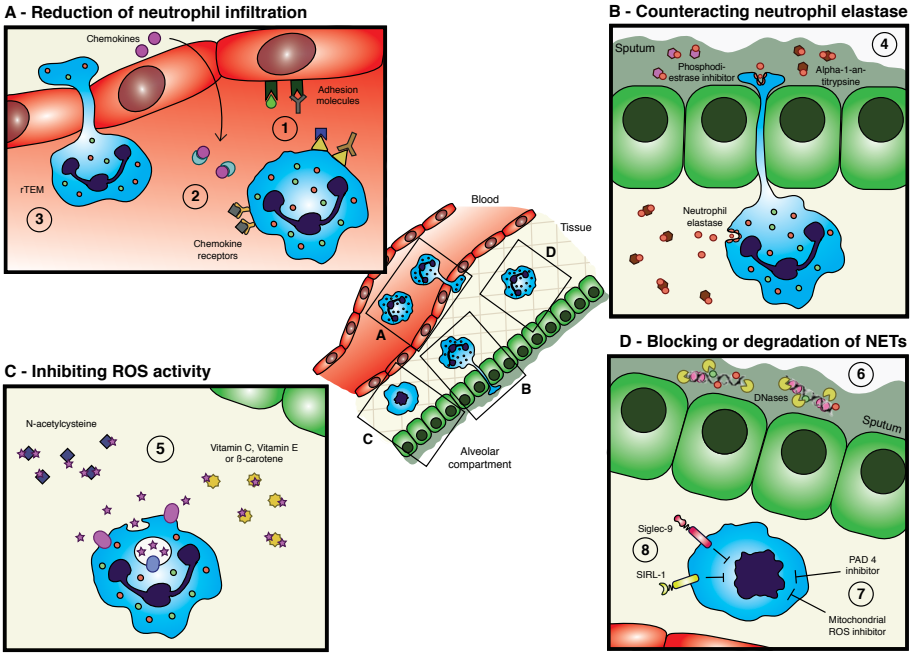

Figure 2 | **Current approaches to treat neutrophil-mediated diseases**
Several therapeutic strategies have been tested in pre-clinical and clinical studies. These studies are based on the inhibition of neutrophil infiltration (A1, A2, and A3), NE activity (B4), ROS activity (C5), and NETs (D6, D7, and D8).

are oxidized, all of which contribute to cell death and finally lead to endothelial dysfunction, thereby exacerbating disease severity[38,39]. ROS also inactivate AAT by oxidation of methionine in the reactive center of this PI, thereby amplifying elastase-dependent tissue damage[40] (Fig. 1).

The majority of potential ROS inhibitors and ROS scavengers are small molecules. Generally, these small molecules show promising results in reducing ROS activity *in vitro*[41,42] but show limited effectiveness *in vivo* due to a lack of specificity or an inability to reproduce their inhibitory effects in living animals[43]. N-acetylcysteine (NAC), a synthetic precursor of intracellular cysteine and glutathione (GSH), is widely used as an antioxidant to investigate the role of ROS in inducing apoptosis[44]. In a Wistar rat model of lung IR injury, intravenous administration of NAC after the end of the IR period resulted in improved lung histological scores and decreased MPO activity compared to the control group. These data suggest that NAC protects lung tissue against the damaging effects of ROS released after IR injury[45]. Targeting ROS with ROS scavengers is likely to be beneficial in the treatment of COPD. Indeed, clinical studies prove that oral administration of NAC rapidly reduces the oxidant burden in airway of stable COPD patients leading to clinical benefits[46,47]. In addition, supplementation with the antioxidants vitamin C, vitamin E, and β-carotene, has

been attempted in patients with COPD. A positive correlation between intake of these antioxidants and lung function was shown, which indicates that antioxidants have a role in protecting against oxidation-induced damage in COPD[48] (Fig. 2, C5). Nevertheless, scavenging ROS or inhibiting ROS production in neutrophils can be dangerous. Patients suffering from chronic granulomatous disease (CGD), caused by mutations in the genes encoding subunits of the NADPH oxidase complex, cannot effectively produce ROS and fail to kill ingested pathogens, resulting in severe infections[49]. Thus, a balance between the production of ROS and the administration of ROS scavengers must be maintained.

THE BIOLOGICAL RELEVANCE OF NET FORMATION

NETs as immune defense mechanism

Another strategy to fight against invading pathogens is the release of NETs, extracellular DNA composed of decondensed chromatin, histones, and antimicrobial proteins and peptides. NETs prevent the spread of infection and the highly localized concentration of azurophilic granular proteins, including NE, catG, PR3, defensins, and cathelicidin LL-37, in NETs kill bacteria[50-52], viruses[53], fungi[54,55], parasites[56], and protozoa[57]. Moreover, NETs also contain MPO, an enzyme that catalyze H_2O_2 to highly reactive HOCl and contributes to killing of microbes[50]. The exact underlying mechanism that leads to NET formation is still poorly understood because contradictory studies show that activation of several different pathways could lead to the release of NETs. Exposure of neutrophils to several bacterial genera[58], phorbol myristate acetate (PMA)[58,59], oxidized low-density lipoprotein (oxLDL)[60], monosodium urate (MSU) crystals[61] and *Leishmania amazonensis*[53] leads to mitogen-activated protein kinase (MAPK) signaling and NADPH oxidase activation, which finally induces NET release. On the other hand, *Staphylococcus aureus* (*S. aureus*)[51], MSU crystals[62], and immune complexes[63] have shown to induce NET release in a NADPH oxidase-independent manner which mainly relies on calcium influx or mitochondrial ROS. Similar as for NADPH oxidase, studies show that peptidyl arginine deiminase 4 (PAD4), NE or MPO are or are not important for the release of NETs.

The role of NETs in various diseases

To counteract NET activity and minimize damage to healthy tissue, the human body has developed different strategies; NETs can be removed by macrophages via phagocytosis[64] or can be cleaved by circulating DNase (Fig. 1). However, the effectiveness of DNase in protecting against tissue damage is unclear since DNA is cleaved but remaining granular proteins and histones are still present. NETs can damage epithelial cells via granular proteins, which induce major tissue damage and

pathology, as described above. In addition, histones are highly cytotoxic and are able to damage endothelium and alveolar wall epithelial cells leading to massive bleeding and edema in the lung[65-68]. In COPD and asthma, excessive NET formation in the upper airways of patients contributes to severity of airflow limitation and disease pathogenesis[69-71].

Besides the direct damage to healthy tissue, NETs contribute to the pathology of thrombosis. Platelets, erythrocytes and plasma proteins like fibrinogen, fibronectin and von Willebrand factor bind to NETs and form a stable clot in blood vessels that may block blood flow[72]. NETs also contribute to tumor progression in several ways: NETs inhibit apoptosis and induce proliferation of primary tumors and NETs also promote the development of metastases[73,74]. Additionally, NETs act as a source of autoantigens, which is thought to contribute to the development of several inflammatory and autoimmune diseases including RA and SLE[39] (Fig. 1). Indeed, a relation between NET release and disease was described in SLE[75,76]. Presumably, autoantibodies (autoAbs) bind to NETs and form immune complexes that are taken up by plasmacytoid DCs (pDCs) or B cells via Fc and Toll-like receptors. In B cells this leads to an increased production of autoAbs and in pDCs to the secretion of IFNα, which induces more NET release by neutrophils. Together, this is thought to contribute to the pathogenesis of SLE. Thus, NETs can both directly and indirectly contribute to the pathology of a large variety of diseases.

MANIPULATING NET RELEASE USING INHIBITORY AGENTS TO SUPPRESS COLLATERAL DAMAGE

Small inhibitory molecules reduce NET formation *in vivo*

The effectiveness of DNase in dissolving NETs is often shown *in vitro* as well as *in vivo*[50,77]. Even in clinical trials DNase is successfully used to diminish symptoms of cystic fibrosis[78] (Fig. 2, D6). However, DNase fails to remove histones and proteases, which still cause significant injury without being captured in NETs[79]. This suggests that DNase therapy provide less protection than prevention of NET formation itself by inhibiting important mediators of NET release, including NE, ROS, or PAD4. Preclinical evidence shows that spontaneous NET release in New Zealand mixed 2328 (NZM) model of murine lupus is dependent on the presence of mitochondrial ROS and PAD4 activity. Treatment with Cl-amidine, a PAD4 inhibitor, in these mice results in inhibition of *in vivo* NET formation and the reduction of circulating autoantibodies and complement[80] (Fig. 2, D7). In addition, MitoTEMPO, an inhibitor of mitochondrial ROS, blocks NET formation and reduces disease severity and type I IFN responses in a SLE mouse model[81] (Fig. 2, D7). These data suggest that inhibition of NET release, through reduction of mitochondrial ROS or PAD4 activity, mitigates the pathogenesis of SLE.

Besides the importance of ROS and PAD4, NE activity is essential in the release of NETs. Inhibition of NE activity via exogenous secretory leukocyte protease inhibitor (SLPI) reduces the release of NETs in response to PMA, TNF-α and *S. aureus* in human neutrophils *in vitro*. Moreover, SLPI-deficient mice having psoriasiform dermatitis are more efficient at generating NETs compared to psoriasiform dermatitis mice containing SLPI[82]. These data underline the importance of NE activity in NET release. Further experiments are necessary to prove that SLPI can be used to inhibit NET release *in vivo*. NETs are also present in sputum of CF and COPD patients and their presence correlates with the severity of airflow limitation[69,70]. In a clinical study, inhalation of DNase increased the amount of liquefied mucous-like structures of sputum of CF patients suggesting that NETs are responsible for the viscous gel-like structure of CF sputum[83].

Controlling the release of NETs via inhibitory receptors

In recent years, increasing numbers of inhibitory receptors have been identified on neutrophils that play a pivotal role in diverse aspects of neutrophil function[84]. The inhibitory signal of these receptors is transduced through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) located in the intracellular tail of the receptor. These ITIMs have the consensus sequence (I/V/L/S)xYxx(L/V/I) and recruit downstream phosphatases, such as Src homology region 2 domain-containing phosphatase-1 (SHP-1) and SHP-2, which lead to attenuation or abrogation of activation signals[85]. The activation state of immune cells is tightly controlled by immune inhibitory receptors. For example, leukocyte-associated Ig-like receptor-1 (LAIR-1) is not expressed on the surface of peripheral blood neutrophils of healthy donors, while stimulation of these neutrophils with granulocyte colony-stimulating factor (G-CSF), GM-CSF, TNF-α, and fMLP, a potent activator of neutrophils, significantly increases LAIR-1 expression on the membrane[86]. The increased expression of LAIR-1 during neutrophil activation implies an important role in regulation of neutrophil activation.

Sialic acid-binding Ig-like lectin-9 (Siglec-9) is an inhibitory receptor that is mainly present on neutrophils. Siglec-9 recognizes sialic acids on host immune cell surface glycans, suggesting that it may function in self-dampening of innate immune responses to prevent autoimmunity. *In vitro* studies demonstrate that Siglec-9 is able to reduce the level of ROS in neutrophils and diminish NET formation, which both lead to increased bacterial survival[87] (Fig. 2, D8). Mouse SiglecE, a functional analogue of human Siglec-9, shows similar dampening effects on neutrophils when it is activated. Reduced bacterial counts were recovered from peritoneal fluid, liver, and spleen of SiglecE-deficient mice intraperitoneally injected with *Streptococcus* bacteria compared to wild-type mice. This suggests that SiglecE is important in the termination of antimicrobial activity of neutrophils *in vivo*[88].

We have identified signal inhibitory receptor on leukocytes-1 (SIRL-1) as a member

of the transmembrane receptor Ig superfamily of immune inhibitory receptors and have shown that SIRL-1 is exclusively expressed on monocytes and granulocytes[89]. Recently, we have demonstrated that SIRL-1 specifically suppresses NET release in response to autoantibodies from SLE patients *in vitro* while full phagocytosis and intracellular bacterial killing capabilities are retained[62] (Fig. 2, D8). To be a viable therapeutic target, it is vital that inhibitory receptors suppress only one particular neutrophil function rather than shut down all neutrophil activities, since this would leave patients wide open for infections. SIRL-1 seems to fit the bill.

CONCLUDING REMARKS

The antimicrobial weaponry of neutrophils is necessary to combat pathogens, but may also induce collateral tissue damage. In health, the poor specificity of neutrophil armaments is counteracted in different ways creating a tightly regulated balance between neutrophil activity and inactivity. When this balance is disturbed, antimicrobial strategies also affect healthy cells leading to tissue destruction (Fig. 1). In this way, neutrophils contribute to the pathogenesis of chronic inflammatory and autoimmune disorders.

The medical need for neutrophil inhibitors is immense as is evident from the large number of human inflammatory diseases characterized by the presence of activated neutrophils in the affected tissues. We expect that effective neutrophil inhibitors would be a tremendous step forward in the treatment options for these inflammatory diseases. Neutrophil-specific treatment should be applied with caution. In acute inflammation, immediate inhibition of neutrophil infiltration and activation is necessary to spare healthy tissue from damage. The potential risk of infection could be reduced with prophylactic antibiotics. On the other hand, chronically inflamed tissue has already been damaged and should be treated differently. Inhibition of a particular neutrophil function could be beneficial and may allow restoration of tissue architecture.

Tipping the balance of neutrophil activity toward a more placid state may lead to increased risk for infections. Without additional antibiotic treatment, septic situations also observed in neutrophil-specific immunodeficiency diseases, such as neutropenia, LAD, CGD and SGD, could occur. Furthermore, neutrophils communicate with other immune cells and thereby contribute to the establishment and skewing of adaptive immune responses[90,91]. In addition, neutrophils are not a homogeneous population. Instead, in acute inflammation three subsets of neutrophils can be distinguished. Among these is a population characterized by a hypersegmented nucleus and $CD16^{bright}/CD62L^{dim}$ expression, which is able to suppress T-lymphocyte responses[92]. Moreover, neutrophils are important in wound healing. Secretion of LL-37[93] or

activation of β-catenin signaling in epithelial cells[94], likely via elastase-mediated cleavage of E-cadherin, increases epithelial cell proliferation and promotes wound closure. Finally, NETs are able to sequester and degrade cytokines, chemokines, and other signals that trigger inflammation[95].

Thus, blocking neutrophil infiltration or inhibiting neutrophil activity also will affect other immune responses. The damaging effects of NETs are multiple. Direct cytotoxicity to the host occurs through granular proteins and histones while the exposure of NET-associated autoantigens leads to self-reactive immune responses. Thus, inhibition of NET formation provides acute beneficial consequences for surrounding collateral tissue and lowers the possibility to develop chronic autoimmune diseases. Specific inhibition of the release of NETs is important in order to preserve intra-phagosomal antimicrobial killing provided by NE and ROS. Targeting inhibitory receptors to suppress NET formation opens up new avenues for the development of therapies for injurious neutrophilic inflammation. The discovery of novel inhibitory receptors will offer additional opportunities in the treatment of neutrophil-mediated diseases.

ACKNOWLEDGEMENTS

The authors thank Prof. dr. Erik Hack, Prof. dr. Leo Koenderman, Dr. Michiel van der Vlist and MSc Ruben Geerdink for critically reading and helpful suggestions on the manuscript.

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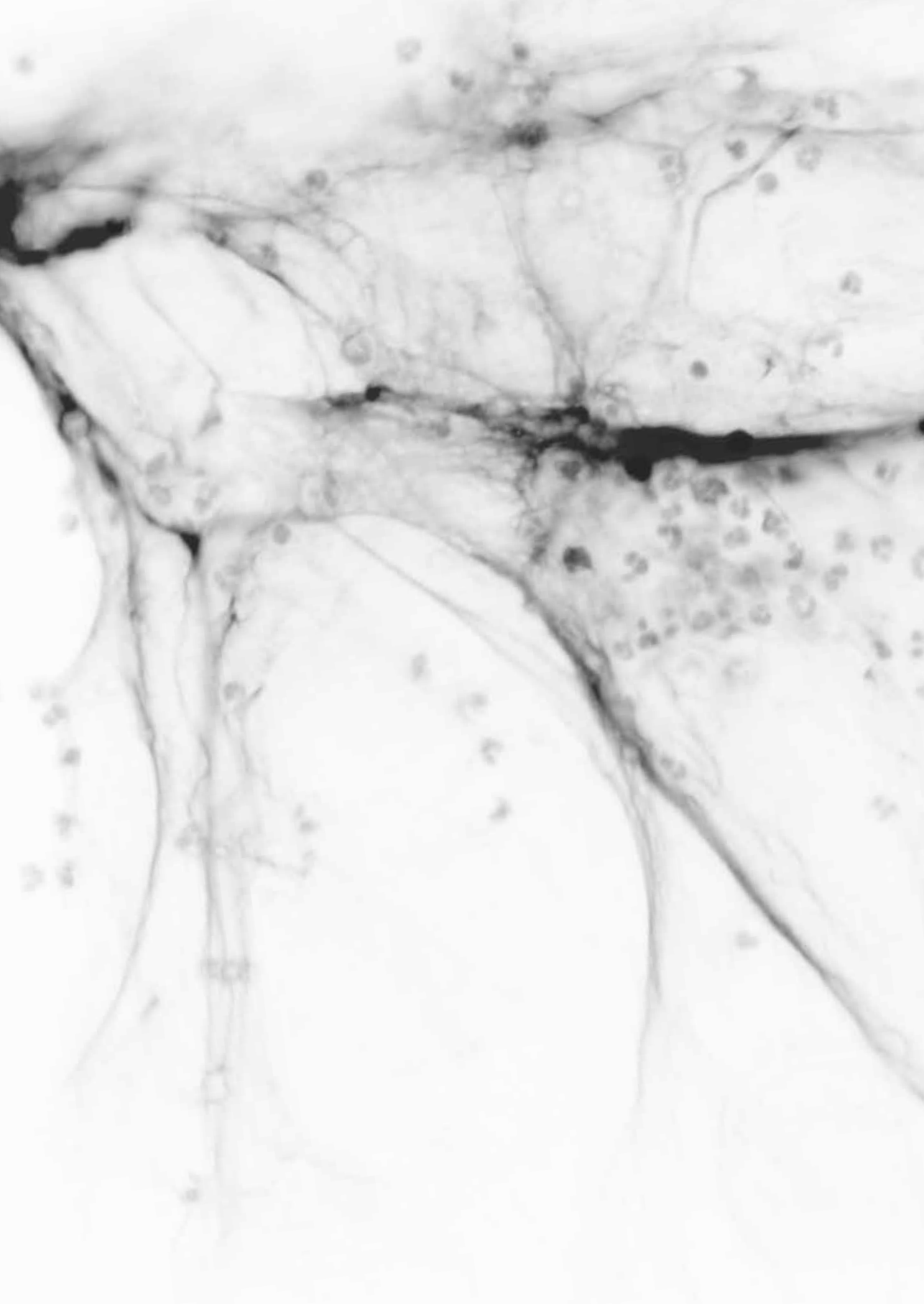

5

CHAPTER

SIGNAL INHIBITORY RECEPTOR ON LEUKOCYTES-1 LIMITS THE FORMATION OF NEUTROPHIL EXTRACELLULAR TRAPS, BUT PRESERVES INTRECELLULAR BACTERIAL KILLING

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Journal of Immunology. 2016 May 1;196(9):3686-94

ABSTRACT

In response to microbial invasion, neutrophils release neutrophil extracellular traps (NETs) to trap and kill extracellular microbes. Alternatively, NET formation can result in tissue damage in inflammatory conditions and may perpetuate autoimmune disease. Intervention strategies that are aimed at modifying pathogenic NET formation should ideally preserve other neutrophil antimicrobial functions. We now show that signal inhibitory receptor on leukocytes-1 (SIRL-1) attenuates NET release by human neutrophils in response to distinct triggers, including opsonized *Staphylococcus aureus* and inflammatory danger signals. NET release has different kinetics depending on the stimulus, and rapid NET formation is independent of NADPH oxidase activity. In line with this, we show that NET release and reactive oxygen species production upon challenge with opsonized *S. aureus* require different signaling events. Importantly, engagement of SIRL-1 does not affect bacterially induced production of reactive oxygen species, and intracellular bacterial killing by neutrophils remains intact. Thus, our studies define SIRL-1 as an intervention point of benefit to suppress NET formation in disease while preserving intracellular antimicrobial defense.

INTRODUCTION

Neutrophils are key effector cells in infection, inflammation, and tissue damage, making up the vast majority of circulating blood cells[1-5]. Neutrophils form neutrophil extracellular traps (NETs) in response to microbial invasion, and these are thought to prevent overwhelming infection[6-10]. NETs are extracellular structures composed of extruded DNA and decorated with histones and antimicrobial factors[6].

Initially, NETs were described as an antimicrobial strategy, but the tissue damaging potential of NETs has gained salient attention[11], with aberrant NET formation now being suggested to contribute extensively to the pathogenesis of sepsis[12], autoimmunity[13-16], vascular inflammation[17], and thrombosis[18-21]. The release of NETs within the circulation, as well as their interaction with platelets and red blood cells, has devastating pro-coagulant and pro-thrombotic consequences[18,19,22-24]. Histones directly cause epithelial and endothelial cell death[25,26], whereas release of NETs exposes self-antigens[27-29], possibly leading to induction and perpetuation of autoimmunity[16,30]. This is most evident in systemic lupus erythematosus (SLE), as immune complexes detected in SLE were found to trigger NET release[25,31,32].

In view of a suggested role for NETs in early innate immune defense, it seems dangerous to inhibit NET formation owing to the risk of increased microbial burden in settings of acute or chronic infection[33,34]. Therefore, the ideal NET inhibitor should be a neutrophil-specific, NET-inhibitory agent that preserves neutrophil respiratory burst, phagocytosis, intracellular bacterial killing, and other antimicrobial functions[35].

Signal inhibitory receptor on leukocytes-1 (SIRL-1) is a member of the transmembrane receptor Ig superfamily of immune inhibitory receptors, and it is exclusively expressed on myeloid cells, including neutrophils, eosinophils, and monocytes[36]. SIRL-1 contains two canonical ITIMs that are essential for its inhibitory function. Cross-linking of SIRL-1 limits the production of reactive oxygen species (ROS) by neutrophils following isolated cross-linking of Fc receptors (FcRs), whereas neutrophil phagocytosis is not affected[37]. Because the generation of oxidants has been reported to drive the release of NETs, targeting SIRL-1 represents a promising strategy to arrest NET formation and improve outcomes in settings where NETs cause harm.

Because we previously demonstrated that cross-linking of SIRL-1 suppresses the release of NETs in response to autoantibodies and plasma from SLE patients[38], we now investigated whether vital antimicrobial functions, other than NET formation, remain intact when SIRL-1 is cross-linked on the surface of neutrophils. Our present findings set the stage for SIRL-1 as an ideal therapeutic target to inhibit NET release in NET-mediated diseases.

MATERIAL AND METHODS

Reagents

Histopaque 1119, LPS (Salmonella typhosa), PMA, dichlorofluorescein diacetate (DCF), HRP, gentamicin, and poly-L-lysine were purchased from Sigma-Aldrich. Additional reagents included Ficoll (GE Healthcare), micrococcal nuclease (Worthington Biochemical), DNase I and PicoGreen (Invitrogen), Sytox Green, and Hoechst 33342 (Molecular Probes), diphenylene iodonium (DPI; Sigma-Aldrich), cytochalasin D (cytoD; Sigma-Aldrich), methyl-β-cyclodextrin (Sigma-Aldrich), PP2 (Sigma-Aldrich), wortmannin (Sigma-Aldrich), Ly294002 (Cell Signaling Technology), U0126 (Cell Signaling Technology), piceatannol (Sigma-Aldrich), Bay11-7082 (InvivoGen), celastrol (InvivoGen), and Amplex Red (Molecular Probes). Triclinic monosodium urate (MSU) crystals were synthesized and characterized as previously described by Naccache et al.[39].

Human neutrophil isolation

Human neutrophils were isolated from sodium-heparin anti-coagulated venous blood of healthy adults under protocols approved by the Medical Ethical Committee of the University Medical Center Utrecht. All donors gave informed consent. Neutrophil suspensions were prepared as previously described[38]. Unless stated otherwise, freshly purified cells were resuspended in RPMI 1640 (Life Technologies) supplemented with 2% (v/v) heat-inactivated (HI) FCS.

Culture of bacteria

Staphylococcus aureus Wood 46, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, and *Salmonella typhimurium* (all provided by Prof. J.A. van Strijp, Medical Microbiology, University Medical Center Utrecht, Utrecht, the Netherlands) were grown to exponential phase in Todd-Hewitt broth medium with aeration. Bacteria were quantified by measuring A_{600nm}. Bacteria were washed twice with PBS and opsonized for 30 min with 10% HI human pooled serum. In some experiments, bacteria were heat-killed for 60 min at 70°C before opsonization.

Determination of crystal phagocytosis by flow cytometry

Human neutrophils were incubated with 100 µg/ml MSU crystals at 37°C with gentle agitation for the indicated times. Uptake of crystals was determined by measuring the increase in side scatter by flow cytometry (FACS Calibur; BD Biosciences) and analyzed with FlowJo software (Tree Star, version 10.0.7r2). A threshold value for side scatter was determined in crystal-free samples, and MSU-challenged samples were evaluated for percentage of cells with higher side scatter than the threshold value.

Stimulation and inhibition of NET formation

The following stimuli were used at indicated concentrations: PMA at 25 ng/ml, MSU at 100 µg/ml, LPS at 1 µg/ml, opsonized and non-opsonized *S. aureus* at a multiplicity of infection (MOI) of 10, and anti-LL-37 Abs (Hycult Biotech) at 10 µg/ml. For inhibitor studies, neutrophils were pre-incubated with 10 µM DPI (an NADPH oxidase [Nox]-2 inhibitor), 10 µg/ml human IgG Fc fragments (Bethyl Laboratories), 20 µM cytoD (inhibitor of actin polymerization), 10 µM PP2 (Src kinase inhibitor), 20 µM wortmannin (PI3K inhibitor), 5 µM U0126 (ERK inhibitor), 20 µM piceatannol (Syk inhibitor), 5 µM methyl-β-cyclodextrin (lipid raft inhibitor), 5 µM Bay11-7082 (NLRP3 inflammasome inhibitor, IκB phosphorylation inhibitor), 5 µM celastrol (a triterpenoid compound), or DMSO (vehicle control) for 30 min before stimulation. Additionally, neutrophils were pre-incubated with 10 µg/ml isotype-matched control IgG or anti-SIRL-1 mAb 1A5, followed with 20 µg/ml goat anti-mouse $F(ab')_2$ fragments.

Analysis of NET formation by neutrophils

NET formation by human neutrophils was analyzed by fluorescence microscopy as described previously[38]. In short, a total of $0.5x10^6$ neutrophils were seeded on coated glass coverslips (0.001% poly-L-lysine) and challenged with the indicated stimuli for 30 and 180 min at 37°C. Cells were stained with Sytox Green (0.5 µM), gently washed, fixed with 4% paraformaldehyde, and stained with Hoechst 33342 (1 µM). Fixed cells were imaged with an Olympus IX71 wide-field inverted microscope with a UPlanSApo x20/0.75 air objective in Fluoromount-G (SouthernBiotech). Fluorescence was detected with a Photometrics EMCCD 1024x1024 pixel camera and softWoRx acquisition software.

For the quantification of NET formation, images were processed with ImageJ software (National Institutes of Health) as previously described[38,40]. Briefly, at least four fields of view (each 659x659 µm) per condition were captured. Contrast was adjusted to minimize background auto-fluorescence and a fluorescent threshold was set to result in positive staining only. The same contrast and threshold were applied to all images from all conditions within the experiment. To minimize differences in fluorescence, the same exposure times for excitation filters were applied between experiments. Typical exposure time for Sytox Green fluorescence (490/20, green channel) was 100 ms. Sytox-positive pixel counts were divided by the total number of pixels of thresholded 8-bit images using ImageJ software and expressed as the percentage of image area covered by positive fluorescence staining in each field of view.

For live cell imaging, neutrophils were allowed to settle for 30 min, and $1x10^5$ cells were seeded in each well of a black 96-well clear-bottom plate (Costar). Neutrophils were incubated in phenol red-free RPMI 1640 supplemented with 2% HI-FCS

and 10 mM HEPES or challenged with indicated stimuli in RPMI 1640 containing 2% HI-FCS and 10 mM HEPES and recorded at 37°C in 5% CO_2/95% air on the BD Pathway 855 bioimaging system with a 320 objective during a period of 180 min. NETs were detected with a mixture of cell-permeable (Hoechst 33342; 1 µM) and impermeable (Sytox Green; 2 nM) DNA fluorescent dyes. Every 2 min, a set of three images (phase contrast, blue and green fluorescence) was taken with a Hamamatsu Orca high-resolution CCD camera. The system was controlled by the BD AttoVision software (version 1.7/855). Individual frame overlays were prepared with ImageJ software.

To assess the kinetics of NET release, Sytox Green fluorescence was monitored in real time as described previously[41]. A total of $1x10^5$ neutrophils were resuspended in phenol red-free RPMI 1640 supplemented with 10 mM HEPES, 2% HI-FCS, and 1 µM Sytox Green and seeded into each well of a white 96-well plate. Sytox Green fluorescence (reflecting extracellular DNA) was measured every 5 min for the indicated times in a preheated fluorescence plate reader (Fluoroscan; Thermo Scientific) at 37°C with a filter setting of 480 nm (excitation)/520 nm (emission).

NET-DNA in neutrophil supernatants was quantified with a Pico-Green dsDNA detection kit as previously described[42]. After stimulation of neutrophils ($2x10^5$; in phenol red-free RPMI 1640, without FCS), the cells were incubated with micrococcal nuclease (500 mU/ml) for 15 min at room temperature to release NETs formed in response to stimulation. Nuclease activity was stopped with 5 mM EDTA. The supernatant was gently removed after centrifugation at 1200 rpm for 5 min. NET-DNA in cell-free supernatants was quantified with a PicoGreen dsDNA detection kit according to the manufacturer's instructions. Extracellular DNA was measured in a fluorescence plate reader (Fluoroscan; Thermo Scientific) with a filter setting of 480 nm (excitation)/520 nm (emission).

Immunostaining of NET components

Neutrophils were seeded on glass coverslips coated with 0.001% poly-L-lysine, allowed to settle, and challenged with opsonized *S. aureus* (MOI of 10) or left untreated for 10 min. Neutrophil elastase (NE) was immunostained as described elsewhere[38]. Briefly, cells were fixed with 4% PFA, permeabilized with 0.25% Triton X-100 in PBS, blocked (1% BSA and 0.1% Tween 20 in PBS), and incubated overnight with anti-NE Abs (sc-9518, Santa Cruz Biotechnology), which were detected with $F(ab')_2$ fragments of DyLight 594-coupled secondary Abs (Jackson ImmunoResearch Laboratories). For detection of DNA, Hoechst 33342 was used. Specimens were mounted in Fluoromount-G and analyzed with an UPlanSApo x20/0.75 air objective on a wide-field inverted microscope (IX71; Olympus).

Determination of ROS production

Extracellular ROS production was measured in real-time by chemifluorescence as previously described[37]. Alternatively, real-time intracellular generation of ROS was monitored in a DCF-based assay. Isolated neutrophils were allowed to settle (60 min, 37°C) and $1x10^5$ cells were preloaded for 20 min at 37°C with fluorescent probe DCF (10 µM). After incubation, cells were washed and carefully resuspended in RPMI 1640 supplemented with 2% HI-FCS in white 96-well plates. Fluorescence was measured every 5 min for the indicated times in a preheated fluorimeter at 37°C (Fluoroscan; Thermo Scientific) at 480 nm (excitation)/520 nm (emission).

Phagocytic and NET-mediated bacterial killing by neutrophils

Total killing of *S. aureus* was performed in the presence of 100 U/ml DNase I to prevent NET-mediated extracellular killing as previously described[37]. To inhibit Nox-2-dependent intracellular killing, we incubated selected wells with DPI (10 µM) for 30 min before adding the bacteria.

Phagocytic killing of *S. aureus*, *S. epidermidis*, *K. pneumoniae*, and *S. typhimurium* by neutrophils was measured in a gentamicin protection assay. Opsonized bacteria were added to the neutrophils at a MOI of 10. After 15 min, gentamicin was added to the medium at 100 µg/ml, followed by continued incubation for 20 min. Wells were then washed with PBS, the neutrophils were permeabilized with 0.1% Triton X-100 for 10 min, and bacterial counts were determined as described above.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (version 6.0). Data are presented as mean ± SD of independent experiments. A Student *t* test was used to compare two groups. For comparing more than two groups, nonparametric or parametric one-way ANOVA or Kruskal-Wallis test with Dunn post hoc testing was used where appropriate. A *P* value of ≤0.05 was considered to be statistically significant.

RESULTS

SIRL-1 regulates NET formation by human neutrophils only in response to specific signals

We have previously shown that ligation of SIRL-1 inhibits NET formation induced by autoantibodies[38]. We now addressed whether cross-linking of SIRL-1 altered NET formation in response to other stimuli by direct microscopic observation. Tissue deposition of MSU crystals causes a prevalent sterile inflammatory condition, called gout. Several studies have demonstrated that MSU crystals activate neutrophils

to form NETs[43,44]. As previously reported[38], SIRL-1 inhibited NET formation when neutrophils were exposed to anti-LL-37 autoantibodies (Fig. 1A), and likewise crystal-induced NET release was suppressed by cross-linking of SIRL-1 (Fig. 1B). As expected, no inhibition by SIRL-1 cross-linking was observed when NETs were induced with the potent protein kinase C activator PMA (Fig. 1B). Also, cross-linking of SIRL-1 did not inhibit the release of NETs when neutrophils were exposed to non-opsonized *S. aureus* or to the TLR4 agonist LPS (Fig. 1C). In contrast, in response to opsonized *S. aureus*, cross-linking of SIRL-1 on the surface of neutrophils reduced NET formation (Fig. 1C).

We previously published that SIRL-1 ligation does not affect phagocytic uptake of opsonized bacteria[37]. Similarly, cross-linking of SIRL-1 did not affect uptake of inflammatory MSU crystals by neutrophils as evidenced by flow cytometry in the side light-scattering properties (Fig. 1D). These findings suggest that the release of NETs in response to opsonized *S. aureus* can occur independently of phagocytosis. Indeed, pretreatment with an inhibitor of actin polymerization, cytoD, failed to cause significant inhibition of NET release (Fig. 1E), whereas control experiments confirmed that the uptake of opsonized bacteria is reduced in the presence of cytoD (data not shown). Inhibition of lipid raft formation by methyl-β-cyclodextrin also had no effect on the formation of NETs in response to opsonized *S. aureus*, further suggesting that NET formation does not require FcR-mediated phagocytosis of opsonized bacteria.

The selectivity for suppression of NET release through SIRL-1 may indicate that alternative cellular processes trigger NET formation, depending on the stimulus.

Opsonized *S. aureus* and MSU crystals stimulate a distinct form of rapid NET formation

NETs are extracellular lattices of DNA that can be visually determined (Fig. 1). The presence of extracellular DNA associated with NETs in the culture supernatant of activated neutrophils can be used as an alternative measure for NET formation. In a complementary strategy, we used PicoGreen to measure DNA release and found that treatment of neutrophils with LPS, *S. aureus*, MSU crystals, and PMA induced significant DNA release after 180 min in a concentration-dependent manner (Fig. 2A).

We assessed the kinetics of NET release by real-time quantification of DNA released in the supernatant with the cell non-permeable DNA-binding dye Sytox Green (Fig. 2B) as previously described[41]. In contrast to PMA-induced NET release, NETs in response to opsonized *S. aureus* and MSU crystals occurred earlier. Neutrophils form NETs in response to non-opsonized *S. aureus*, and opsonization of bacteria significantly accelerated *S. aureus*-induced NET release. Fluorescence microscopy images confirm that neutrophils challenged with *S. aureus* and MSU release NETs, and that opsonization of *S. aureus* enhanced NET formation (Fig. 2C).

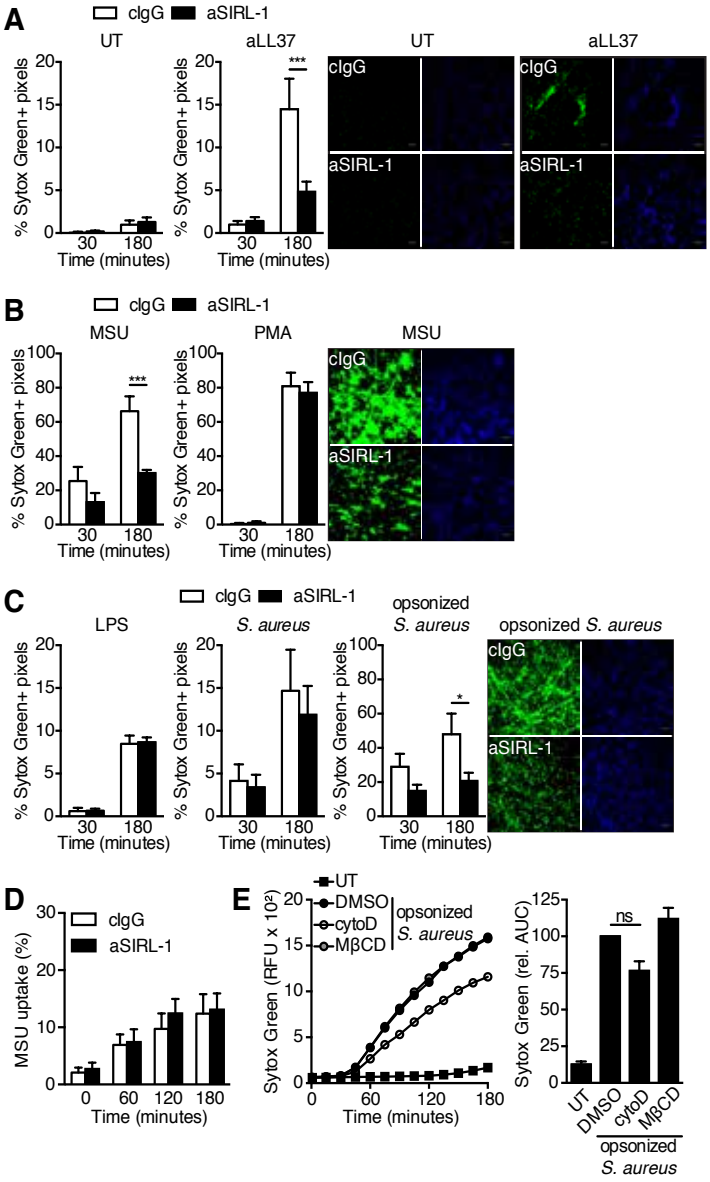

Figure 1 | SIRL-1 impairs NET formation to challenge with opsonized *S. aureus* and MSU crystals
(**A**-**C**) Neutrophils were either left untreated or challenged with anti-LL-37 mAb (**A**), MSU crystals or PMA (**B**), or *S. aureus* or LPS (**C**) for 30 or 180 min. Neutrophils were stained with the cell non-permeable DNA-binding dye Sytox Green (green) and the nuclear DNA-labelling dye Hoechst 33342 (blue). Representative images of neutrophils with or without cross-linking of SIRL-1 are shown. Scale bars, 50 µm. The densities of released DNA (i.e., the number of Sytox Green+ pixels divided by the total number of pixels x 100%) were determined after the indicated treatments. (**D**) Uptake of MSU was followed over time by flow cytometry, assessed by a change in side scatter. (**E**) Opsonized *S. aureus* induces NET formation in the presence of inhibitors of phagocytosis. Sytox Green was added to the medium to detect DNA and monitor real-time generation of NET release, the representative profiles of which are shown. The area under the curve (AUC) was calculated relative to cells exposed to *S. aureus* in the presence of DMSO. Data are presented as mean ± SD [(**A**-**C**) n=3, (**D**) n=4, (**E**) n=4]. **P*<0.05, ****P*<0.001 [(**A**-**C**) nonparametric one-way ANOVA, (**E**) Kruskal-Wallis test]. RFU, relative fluorescence unit.

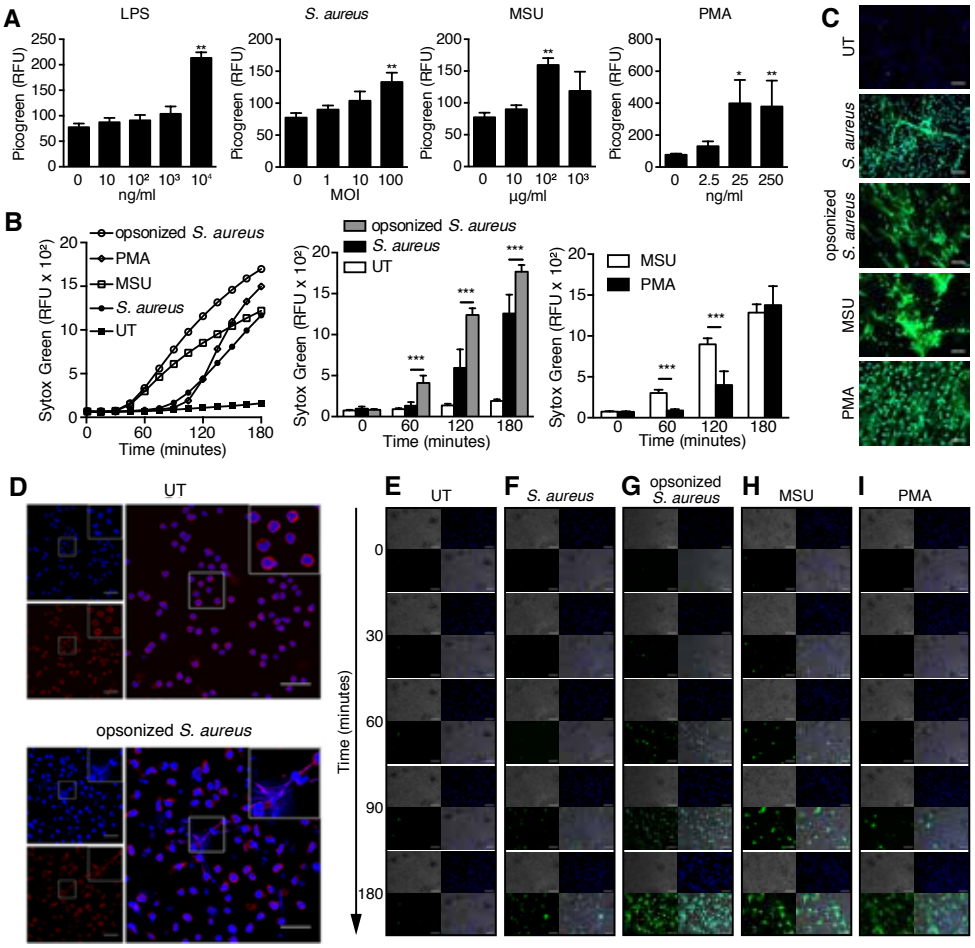

Figure 2 | Neutrophils rapidly release NETs when challenged with opsonized *S. aureus* and MSU crystals
Neutrophils were challenged with LPS, *S. aureus*, MSU, or PMA for 180 min. (**A**) NET-DNA quantification, assessed as PicoGreen fluorescence in the supernatant of neutrophils, exposed for 180 min to increasing amounts of the indicated stimuli, and presented as relative fluorescence units (RFU). (**B**) Real-time quantification of extracellular DNA (reflecting NETs), assessed as Sytox Green fluorescence. Representative fluorescence profiles are shown (RFU), and mean fluorescence intensities are depicted for each stimulus at the indicated time points. (**C**) Fluorescence microscopic images of NET release after neutrophils were challenged for 180 min. The presence of extracellular DNA is indicated by the green fluorescence, and the neutrophils were counterstained with Hoechst 33342 nuclear DNA stain (blue). Scale bars, 50 µm. (**D**) Immunostaining for NET components (blue, DNA; red, NE) after neutrophils were challenged with opsonized *S. aureus* for 10 min. The experiment was repeated three times with similar results. Scale bars, 50 µm. (**E-I**) Neutrophils were stained with a nuclear dye (blue), which stains live cells, and incubated in culture medium containing Sytox Green (green), after which cells were followed over time with live imaging. Depicted for each time point are phase contrast (top left panels), nuclear staining (top right panels), extracellular DNA staining (bottom left panels), and overlays of all three channels (bottom right panels). Scale bars, 50 µm. Results are depicted as mean ± SD [(**A**) n=4 (**B**) n=5]. Images are representative of at least three independent experiments (**C-I**). **P*<0.05, ***P*<0.01, ****P*<0.001 [(**A**) Kruskal-Wallis test, (**B**) parametric one-way ANOVA with Bonferroni's multiple comparisons post hoc test].

NE is a granular protein that associates with NETs. The presence of extracellular DNA that stains positively for NE is consistent with the process of NET formation. No NE was released when cells where left unstimulated (Fig. 2D). In contrast, after challenge with opsonized *S. aureus* for 10 min, neutrophils released extracellular DNA where NE co-localizes.

We activated neutrophils and monitored their release of NETs over time by live cell imaging (Fig. 2E). Upon exposure of neutrophils to non-opsonized *S. aureus*, the cells did not release DNA during the early phase (Fig. 2F). At later time points following stimulation, progressively more cells lost their condensed nuclear material and released NET-DNA, assessed as Sytox Green fluorescence. In contrast, neutrophils exposed to opsonized *S. aureus* rapidly released a high amount of NETs that increased with time (Fig. 2G). Similarly, inflammatory activation of neutrophils with MSU crystals caused robust NET formation that was detectable early after challenge (Fig. 2H). With similar kinetics as non-opsonized bacteria, stimulation with PMA resulted in an increase of Sytox Green fluorescence during the late phase after challenge. Ultimately, most neutrophils challenged for 180 min with PMA showed decondensed chromatin and formed NETs (Fig. 2I). Taken together, the time course analysis of NET release shows that the formation of NETs induced by *S. aureus* and PMA follows distinct kinetics, suggesting that distinct forms of NET release are at play.

Rapid NET release induced by opsonized *S. aureus* and MSU crystals does not require ROS production

Opsonized *S. aureus* and MSU crystals can activate neutrophils and significantly increase their intracellular ROS concentration[39,45]. To detect intracellular ROS produced by Nox-2, we used DCF, a fluorescent indicator of intracellular ROS. Pretreatment of neutrophils with DPI, a flavoprotein inhibitor of Nox-2, before challenge with opsonized *S. aureus* completely abolished the generation of ROS (Fig. 3A). In contrast, DPI failed to inhibit the release of NETs in response to opsonized *S. aureus* or MSU crystals, but it completely abrogated PMA-stimulated NET formation as previously reported[42] (Fig. 3B). Additionally, DPI had no effect on NET release induced by non-opsonized bacteria. This is in line with the relative absence of intracellular ROS generated after exposure to non-opsonized *S. aureus* (data not shown). Therefore, *S. aureus* triggers the release of NETs in a manner that does not depend on ROS production, distinct from PMA-induced Nox-2-dependent NET formation.

Activation of neutrophils by MSU crystals occurs in part through FcγRIIIB[46]. Blocking of FcγRs partially inhibited NET formation when neutrophils were challenged with opsonized bacteria or MSU crystals, but not in response to non-opsonized *S. aureus* (Fig. 3B), suggesting that ROS-independent NET release to these stimuli involves FcγR-mediated contact. The interaction of MSU crystals with

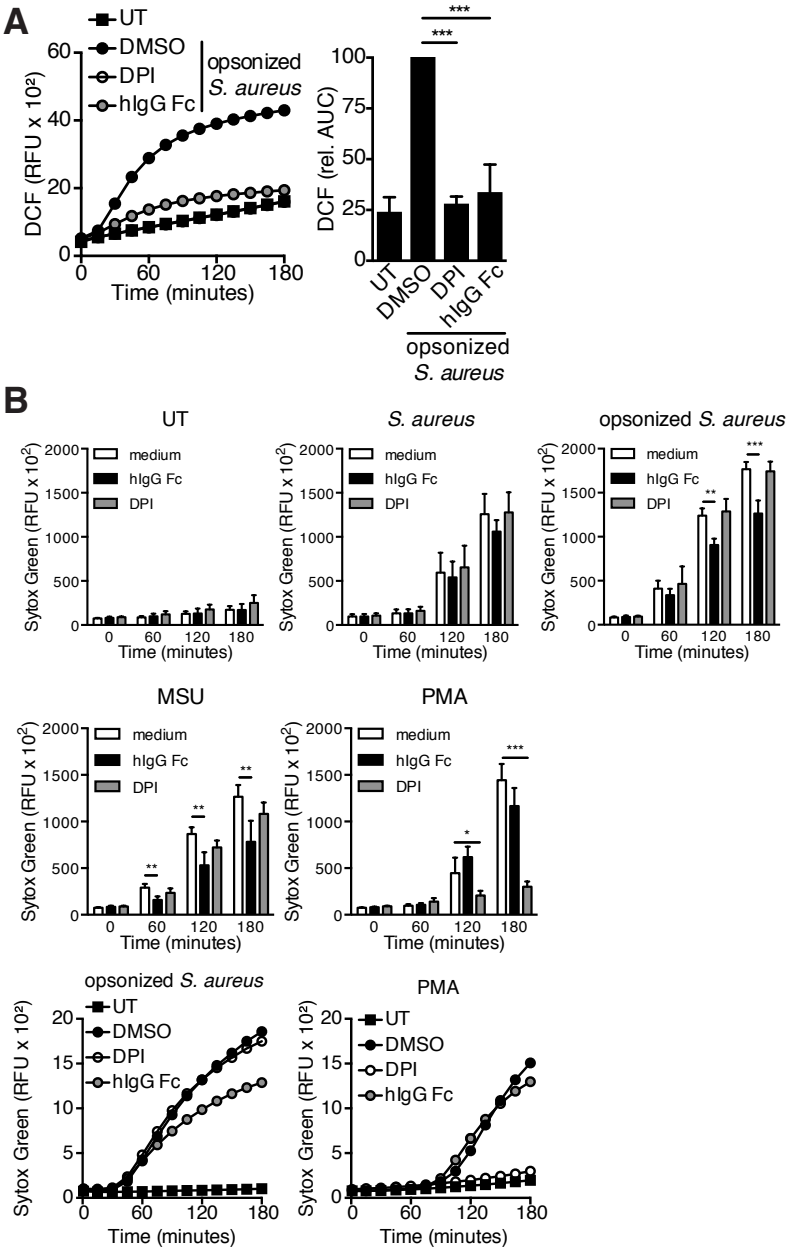

Figure 3 | NET release in response to opsonized *S. aureus* and MSU crystals involves FcγR-mediated contact, but does not require ROS production

(**A**) Neutrophils were exposed to opsonized *S. aureus* in the presence or absence of the Nox-2 inhibitor DPI, or human IgG Fc fragments. Real-time production of ROS was monitored by DCF fluorescence, the representative profiles of which are shown. The area under the curve (AUC) was calculated relative to cells incubated with opsonized *S. aureus* in the presence of DMSO. (**B**) Quantification of NET release during 3 h, assessed as Sytox Green fluorescence after challenge of neutrophils in the presence or absence of DPI, or human IgG Fc fragments. Data are depicted as mean ± SD [(**A**) n=3 (**B**) n=4]. **P*<0.05, ***P*<0.01, ****P*<0.001 (nonparametric one-way ANOVA). RFU, relative fluorescence unit.

FcγRIIIB is likely to be opportunistic in nature, because opsonization with IgGs is not a prerequisite[46]. In fact, it is highly unlikely that FcγRs provide a complete repertoire of the surface molecules with which MSU crystals and opsonized *S. aureus* interact. Indeed, we obtained only partial inhibition of NET formation in response to MSU crystals and opsonized bacteria with FcγR block.

NET release and ROS production upon challenge with opsonized *S. aureus* require distinct signaling events

We studied the contribution of FcR signaling to NET formation by using inhibitors of signaling molecules engaged by the ITAM-coupled FcγRs. Neutrophils were pretreated with inhibitors of Syk (piceatannol), Src (PP2), PI3K (wortmannin), or

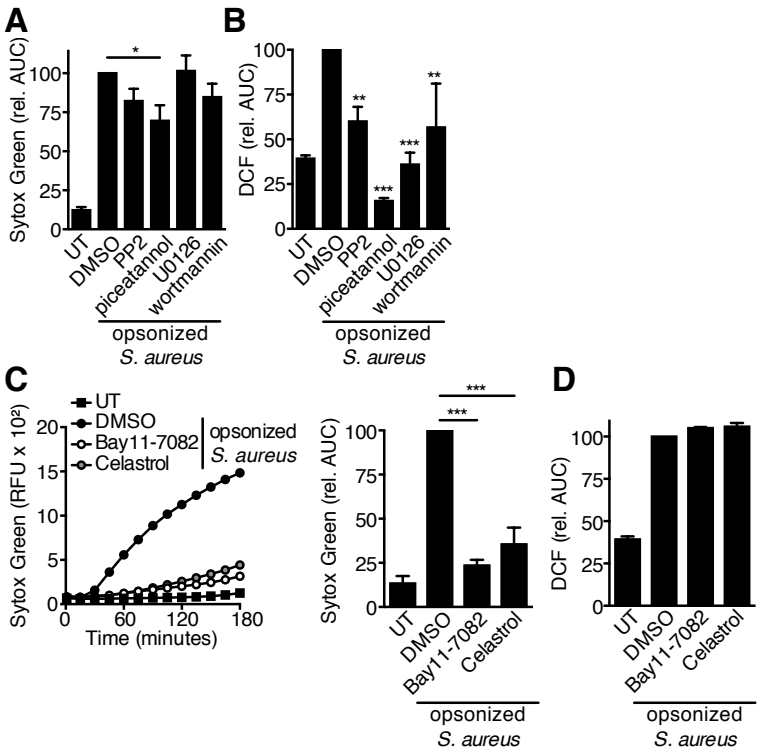

Figure 4 | NET release and ROS production upon challenge with opsonized *S. aureus* occur through distinct signaling pathways

Neutrophils were challenged with opsonized *S. aureus* for 3 h in the presence or absence of the indicated inhibitors. (**A**) Sytox Green was added to the medium to follow the release of NET-DNA in real time. (**B**) Neutrophils were incubated with opsonized *S. aureus* in the presence or absence of inhibitors, and the generation of ROS was monitored in a DCF-based assay. (**C**) Neutrophils were preincubated with Bay11-7082, Celastrol, or DMSO control before challenge with opsonized *S. aureus*. Representative profiles of *S. aureus*-induced NET-DNA release are shown. (**D**) *S. aureus*-triggered ROS generation in neutrophils treated with Bay11-7082 or Celastrol. The relative increase in fluorescence was calculated as the area under the curve (AUC) compared with that detected in DMSO-pretreated neutrophils incubated with opsonized *S. aureus*, and it is depicted in the figure as mean ± SD [(**A**) n=5, (**B**-**D**) n=3]. **P*<0.05, ***P*<0.01, ****P*<0.001 [(**A**) and (**B**) Kruskal-Wallis test, (**C**) non parametric one-way ANOVA]. RFU, relative fluorescence unit.

ERK1/2 (U0126) before exposure to opsonized *S. aureus*. Treatment of neutrophils with Syk inhibitors only partially inhibited NET formation in response to opsonized bacteria, whereas inhibition of Src, PI3K, and ERK1/2 had no effect (Fig. 4A). In contrast, blocking of Syk and ERK1/2 completely suppressed bacteria-induced ROS production (Fig. 4B). Treatment with inhibitors of Src and PI3K also resulted in diminished generation of ROS. These results suggest that *S. aureus*-induced receptor/Syk activation contributes to, but is not essential for, NET formation, whereas it is required for the generation of ROS.

The fact that Syk kinase-mediated signaling pathways play a minor role in the release of NETs in response to opsonized bacteria suggests that other intracellular signaling pathways are involved in NET release. Treatment with either Bay11-7082 or celastrol, inhibitors of IκBα phosphorylation and NF-κB, respectively, completely abolished rapid *S. aureus*-induced NET formation (Fig. 4C), whereas it had no effect on ROS production (Fig. 4D). Taken together, these results clearly highlight that differences exist in the requirement for signaling events involved in ROS production and NET formation after challenge with opsonized *S. aureus*.

SIRL-1 does not affect *S. aureus*-induced ROS production

In line with our previous findings[37], we observed an inhibitory effect of SIRL-1 on extracellular ROS when FcγRIIA (CD32) was triggered on neutrophils (Fig. 5A). Because neutrophil ROS production is an essential effector function involved in intracellular bacterial killing, we next aimed to evaluate whether ligation of SIRL-1 affects the generation of ROS in response to other stimuli. Activation of neutrophils through opsonized bacteria and MSU crystals is mediated by FcγRs[46]. Pretreatment with human IgG Fc fragments nearly completely abolished the intracellular generation of ROS (Fig. 3A). However, cross-linking of SIRL-1 on the surface of neutrophils had no effect on intracellular levels of ROS in response to opsonized *S. aureus* (Fig. 5B). Using $F(ab')_2$ fragments against SIRL-1, we excluded effects of the Fc part of the cross-linking Ab. Furthermore, ligation of SIRL-1 had no effect on extracellular ROS production by neutrophils exposed to MSU crystals (Fig. 5A). MSU crystals increase neutrophil intracellular ROS concentration in a dose-dependent manner (Fig. 5C). At none of the MSU concentrations tested did SIRL-1 ligation interfere with intracellular generation of ROS (Fig. 5D).

Defects in Nox-2-mediated ROS production enhance intracellular survival of *S. aureus*[47]. Also, *S. aureus* has been shown to be killed by NETs *in vitro*[6]. Therefore, we determined the effect of cross-linking SIRL-1 on total bacterial killing.

SIRL-1 signaling inhibits NET-mediated bacterial killing but preserves intracellular antimicrobial activity

Killing activity of neutrophils was determined on the basis of changes in the number

of viable bacteria over time (Supplemental Fig. 1). In the absence of both DNase and DPI, neutrophils can kill *S. aureus* by phagocytosis and through NET formation. Initially, efficient phagocytic killing was observed, and the presence of DNase had no effect, indicating that no NET-mediated killing occurred at 10 min. In contrast, 30 min after challenge with opsonized *S. aureus*, when neutrophils start to release NETs

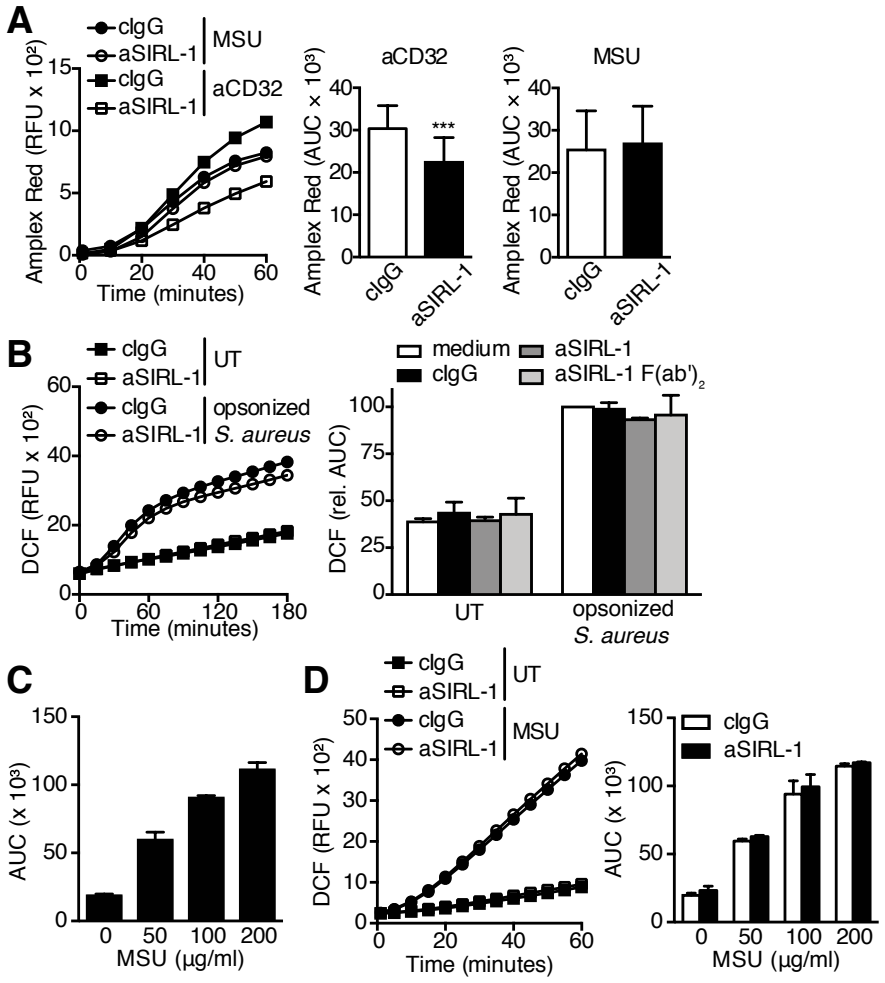

Figure 5 | **Engagement of SIRL-1 preserves the production of ROS by neutrophils upon phagocytosis of *S. aureus***
(**A**) Extracellular ROS was monitored in an Amplex Red-based assay, the representative profiles of which are shown. The area under the curve (AUC) was calculated and is represented as mean ± SD of independent experiments. SIRL-1 reduces CD32-mediated ROS production, whereas it fails to limit MSU-triggered generation of extracellular ROS. (**B**-**D**) Neutrophils were loaded with DCF and challenged with serum-opsonized *S. aureus* or MSU crystals in the presence or absence of anti-SIRL-1 mAb 1A5. Real-time production of intracellular ROS was followed by DCF fluorescence, the representative profiles of which are shown. The AUC was calculated relative to cells incubated with opsonized *S. aureus* and is depicted as mean ± SD [(**A**) aCD32, n=7, and MSU, n=8, (**B**) n=4, (**C**) n=3, (**D**) n=3]. ***P<0.001 (paired Student t test). RFU, relative fluorescence unit.

(Fig. 1C), phagocytic killing was diminished and most of the antimicrobial activity was mediated through NET formation. By 90 min, most neutrophils form NETs (Fig. 2G) and no changes in the number of viable bacteria were observed in the presence or absence of DPI (Supplemental Fig. 1).

We chose to use a 30 min incubation time in subsequent experiments, because at this time point neutrophils kill both by phagocytosis and through NET formation. Exposure of human neutrophils to opsonized *S. aureus* for 30 min in the presence of DNase I to dismantle NETs results in significantly increased total bacterial survival, which was not further enhanced by cross-linking of SIRL-1 (Fig. 6A). When human neutrophils were pretreated with DPI and then exposed to *S. aureus* for 30 min, total bacterial survival was also significantly increased compared with control cells. In this case, ligation of SIRL-1 further enhanced the inhibitory effect of DPI (Fig. 6A). This result is consistent with inhibition of NET formation by SIRL-1 and suggests that SIRL-1 specifically regulates cellular pathways required for extracellular NET-mediated, but not phagocytic, microbial killing. We incubated human neutrophils with various opsonized bacterial strains, including *S. aureus*, in an *in vitro* gentamicin protection assay. Treatment with DPI significantly increased intracellular survival of *S. aureus* (Fig. 6B), indicating that phagocytic bacterial killing depends on activity of Nox-2. In contrast, no difference in intracellular survival was detected with or without ligation of SIRL-1 (Fig. 6C). Thus, engagement of SIRL-1 on neutrophils inhibits extracellular bacterial killing while phagocytic killing is preserved.

DISCUSSION

Accumulating evidence supports that dysregulated NET formation can cause harm and perpetuate tissue damage in autoimmune disorders and other inflammatory conditions. Strategies that aim to limit the release of NETs are only now beginning to emerge[12,24,48]. We have previously proposed that targeting immune inhibitory receptors to arrest NET formation could be beneficial in the context of autoimmunity[38]. We showed that cross-linking of SIRL-1 suppresses the release of NETs in response to autoantibodies from SLE patients. Given the essential activity of neutrophils in innate immunity and their importance in preventing infections, therapeutic inhibitors of NET release should ideally preserve other neutrophil antimicrobial functions, such as ROS production and intracellular killing[35]. In this study, we show that SIRL-1 specifically controls NET formation, without compromising other important neutrophil antimicrobial functions, and advocate for the inhibition of NET release by cross-linking of SIRL-1. Recently, concern about experimental challenges in studying NET formation has been raised by others[49-51]. Detection of NETs by fluorescence microscopy still remains the most informative experimental approach. In the present

study, we visualized NETs as extracellular structures released by neutrophils that stain positive for DNA. Furthermore, we confirmed the true nature of observed NETs by co-staining the extracellular DNA with the granule protein NE, a specific marker of NET formation. Analysis of data obtained by fluorescence microscopy, however, remains challenging and difficult owing to different ways of expressing the extent of observed NET formation. In this study, we translated the microscopic observations into comparable semi-quantitative data with a standardized methodology previously described by others in the field[40,41]. We complemented our findings with additional experiments to assess NET formation, in which we quantify extracellular DNA by staining extracellular DNA with PicoGreen or Sytox Green. Although more quantitative

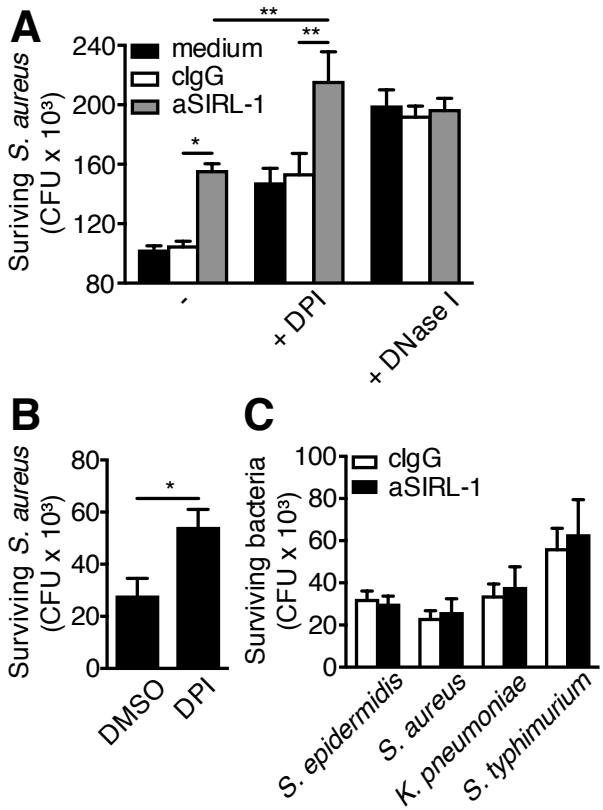

Figure 6 | Cross-linking of SIRL-1 inhibits NET-mediated bacterial killing but does not affect intracellular antimicrobial activity

(**A**) Total, intracellular (in the presence of DNase I), and extracellular NET-mediated (in the presence of DPI) killing of *S. aureus* by neutrophils was determined with (gray bars) or without (open bars) cross-linking of SIRL-1. (**B**) In a gentamicin-based assay, intracellular killing of *S. aureus* by neutrophil was determined with or without pretreatment with DPI. (**C**) Intracellular bacterial killing by neutrophils was determined as described for (**B**) with or without cross-linking of SIRL-1. Bars indicate mean number of recovered CFU ± SD [(**A**) n=7, (**B**) n=3]. **P*<0.05, ***P*<0.01 [(**A**) parametric one-way ANOVA with Bonferroni's multiple comparisons post hoc test, (**B**) paired Student *t* test].

and high throughput in nature, this parameter is less sensitive.

Consistent with our previous report[37], we show that cross-linking of SIRL-1 suppresses FcγRIIa-mediated ROS production in neutrophils (Fig. 5A). In contrast, in the present study, we show that SIRL-1 does not affect intracellular ROS production in neutrophils following challenge with opsonized *S. aureus*. It has been shown that TLR signaling cooperates with FcRs in the killing of intracellular bacteria by promoting assembly and thus activity of the Nox complex[52]. Most likely, signaling through SIRL-1 is not able to suppress synchronized FcR and TLR engagement and the resulting synergistic activation of Nox-2 in neutrophils.

Release of NETs after several hours has often been reported. This cellular process requires the generation of ROS[38,42,53]. However, ROS-mediated signaling is not the only way that NET release is triggered, as rapid NET formation (within minutes) was described, which is independent of oxidants[41,54]. Our present study shows that *S. aureus* triggers the formation of NETs through a mechanism that does not depend on ROS. Also, although NETs are formed in response to non-opsonized *S. aureus*, there is little, if any, generation of intracellular ROS (data not shown), suggesting little interplay between Nox-2 activity and NET formation. Following exposure to opsonized *S. aureus*, neutrophils produce large amounts of intracellular ROS. Activation of the kinase ERK has been implicated to be involved in ROS-dependent NET release[38,55]. Our data, however, show that inhibition of ERK does not suppress ROS-independent NET formation in response to opsonized *S. aureus*, whereas the ERK inhibitor U0126 completely abolished ROS production. Similarly, Src kinase and PI3K inhibitors did not block the release of NETs, but they inhibited the generation of ROS after exposure to opsonized *S. aureus*. Alternatively, NF-κB inhibitors Bay11-7082 and celastrol abrogated *S. aureus*-induced NET release, whereas ROS production was not affected in the presence of these inhibitors. Thus, distinct signaling events are responsible for the rapid release of NETs in response to *S. aureus*.

Celastrol and Bay 11-7082 are widely known for their potential to inhibit the transcription factor NF-κB[56,57]. Both compounds, however, have been shown to directly or indirectly modulate numerous cellular targets, including JAK kinase, ERK, and JNK[58,59]. Interestingly, celastrol was recently shown to act on activation of the kinase SYK, a very early signaling event during NET formation in response to serum IgG from SLE and RA patients[60]. Therefore, we suggest that celastrol and/or Bay 11-7082 could act on molecular targets upstream of NF-κB, rather than directly modulating NF-κB.

Possible therapeutic approaches to prevent NET formation and its damage to the host are needed. DNase has been used in animal models to remove NETs, and it is given as a therapeutic agent, for instance, in patients with cystic fibrosis. However, DNase might not be effective in removal of the cytotoxic mix of NET components, such as histones and NE. Indeed, others suggest that prevention of NET release

might provide more protection against the pathogenicity of NETs than removal of NETs. Anti-Mac-1 blocking Abs and protein arginine deiminase 4 (PAD4) inhibition have been proposed as strategies to arrest NET formation[12,24]. However, blocking Mac-1 is expected to resemble leukocyte adhesion deficiency in situations with a component of infection (such as sepsis). Although neutrophils that lack PAD4 remain capable of killing bacteria by means other than NET formation[12], PAD4 is expressed by many cell types, and the systemic consequences of PAD4 inhibition are not known. Also, the requirement for PAD4 is likely not common to all mechanisms of NET release[61,62], limiting the spectrum of NET-mediated disorders that could be targeted by PAD4 inhibition.

SIRL-1 is also expressed on the surface of monocytes and eosinophils. Thus, systemic effects of cross-linking SIRL-1 cannot be excluded, and it remains to be determined whether inhibiting NET formation by cross-linking SIRL-1 may improve outcomes in preclinical *in vivo* model systems. Nonetheless, this study highlights SIRL-1 as a target that is capable of suppressing the formation of NETs in response to autoantibodies, MSU crystals, and bacteria. Importantly, neutrophils retain their intracellular anti-bacterial activity when SIRL-1 is cross-linked on the surface of the cells. These findings warrant further exploration of SIRL-1 as a therapeutic target in settings where NETs harm.

ACKNOWLEDGEMENTS

We thank Prof. dr. Dirk Roos (Sanquin Blood Supply, Amsterdam, the Netherlands) for helpful comments and critical revision of the manuscript, and Prof. dr. Jos A. van Strijp (University Medical Center Utrecht, Utrecht, the Netherlands) for providing valuable reagents.

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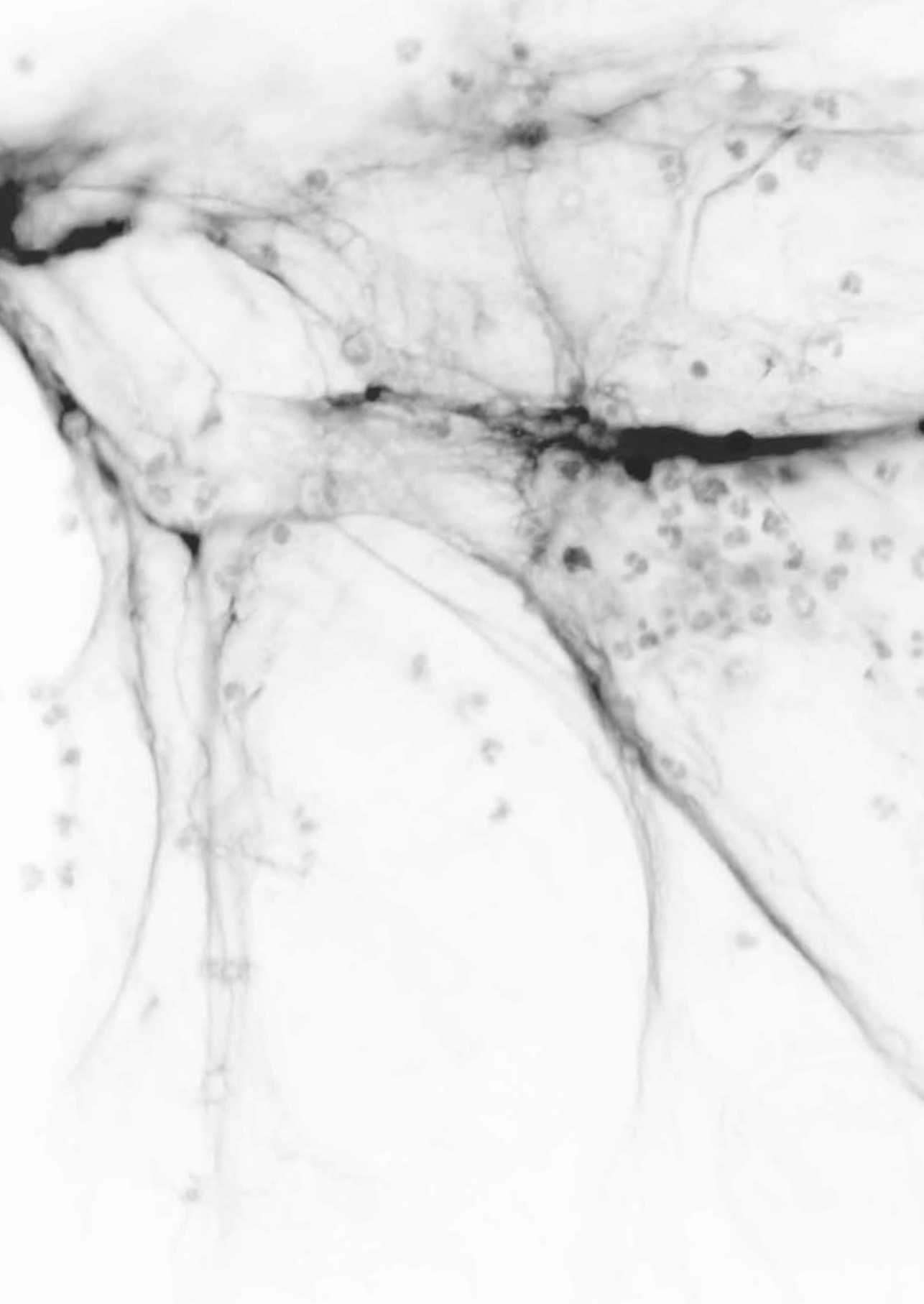
SUPPLEMENTARY INFORMATION

Supplemental figures that accompanies this paper are at the Journal of Immunology website (http://www.jimmunol.org/content/196/9/3686.long).



6 CHAPTER

BACTERIAL AND ENDOGENOUS AMPHIPATHIC α-HELICAL PEPTIDES ARE FUNCTIONAL LIGANDS FOR SIGNAL INHIBITORY RECEPTOR ON LEUKOCYTES-1

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Manuscript in preparation

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ABSTRACT

In host-pathogen interactions, both sides gain most from a mutually beneficial equilibrium. Inhibitory receptors protect the host from immunopathology, but also are attractive targets for bacteria to exploit as an immune evasion strategy. Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) is a negative regulator of myeloid cell function and dampens antimicrobial responses. So far, the natural ligand of SIRL-1 is undetermined. We found that supernatant from the human pathogen *Staphylococcus aureus* (*S. aureus*) can activate 2B4 NFAT-GFP reporter cells expressing a chimeric SIRL-1-CD3ζ protein. Screening the supernatant of 1,920 *S. aureus* (Nebraska Transposon Library), we identified phenol-soluble modulins (PSMs) as potential ligands. Indeed, *S. aureus* strains deleted for α-type PSMs, but not β-type PSMs were unable to activate SIRL-1-CD3ζ reporter cells, and activation was restored by introducing plasmid-encoded α-type PSMs. Importantly, synthetic PSMα3 peptide inhibited IgA-induced oxidative burst in a SIRL-1-dependent manner in monocyte-like cells. Human cathelicidin peptide LL-37 shows many characteristic similarities with *S. aureus* α-type PSMs. Synthetic LL-37 peptide induced GFP expression in SIRL-1-CD3ζ reporter cells, which could be blocked by anti-LL-37 antibody. Thus, this study identifies bacterial and human amphipathic α-helical peptides as ligands for SIRL-1. We propose that LL-37 restores immunological balance via SIRL-1 after immune activation, while α-type PSM-producing staphylococci exploit SIRL-1 as an immune evasion strategy to limit the microbial killing capabilities of immune cells.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is present as part of the healthy microbiota in about 25% of healthy individuals. However, when *S. aureus* breaches the outer physical barrier of the body, it can cause a multitude of diseases ranging from moderately soft tissue infections to life-threatening systemic diseases[1,2]. The pathogenicity of *S. aureus* strongly depends on a broad spectrum of virulence factors that challenge the effectiveness of the innate immune system[3,4]. The expression of many virulence factors is controlled by the accessory gene regulator (agr) locus consisting of the quorum-sensing system components (agrA, B, C and D) and the agr effector molecule RNAIII[5]. Although RNAIII is responsible for the regulation of multiple secreted and cell-surface associated virulence factors, agrA directly controls the expression of phenol-soluble modulins (PSMs)[5-8].

PSMs are amphipathic α-helical peptides mainly found in highly virulent species, such as methicillin-resistant *S. aureus* (MRSA). *S. aureus* expresses α-type PSMs (~20-25 amino acids (aa)), PSMα1-4 and δ-toxin, and β-type PSMs (~44 aa), PSMβ1 and PSMβ2[9]. In addition, the *Staphylococcus* cassette chromosome mec (SCCmec), present in MRSA, contains an embedded gene encoding for PSM-mec[10]. PSMs are produced in extraordinary high amounts by *S. aureus* and destroy membrane integrity by receptor-independent membrane attachment resulting in broad cytolytic activity towards host cells and concomitant tissue damage[9]. Recently, PSMα3 was shown to form cross-α amyloid-like fibers that enhance cytotoxicity and promotes biofilm stability[11,12]. In apparent contrast to immune evasion properties, PSMs activate formyl peptide receptor 2 (FPR2)[13] and toll-like receptor 2 (TLR2)[14] to initiate pro-inflammatory neutrophil responses in sub-cytolytic concentrations[13,15]. PSMs are also present in less pathogenic staphylococci to overcome the strongly varying physical-chemical environments of epithelial surfaces[16,17].

The dual function of PSMs, i.e. receptor-independent membrane perturbation and receptor-dependent immune regulation, is a feature that is shared by the host defense peptide LL-37[18]. LL-37 is an antimicrobial peptide that originates from the precursor human cathelicidin peptide (hCAP18) and is widely expressed in epithelial cells[19-21] and immune cells[22]. LL-37 shows biochemical, structural and functional similarities with α-type PSMs. LL-37 is also an amphipathic α-helical peptide that is secreted into the extracellular environment. It interacts with biological membranes of bacteria as well as eukaryotic cells and rapidly impairs membrane integrity and forms pores resulting in cytolysis[23,24]. In sub-cytolytic concentrations, LL-37 also regulates the immune system through binding to FPR2[25], P2X7 channel receptor[26] and macrophage integrin αMβ2 (Mac-1)[27] which promotes immune cell activation to clear pathogens. Finally, LL-37, like PSMα3, oligomerizes in α-helical conformation

and forms amyloid-like fibers that could be directly related to its cytotoxicity[28,29]. Thus, LL-37 is a highly effective antimicrobial weapon, but with a high potential for causing collateral damage to healthy cells.

Neutrophils and macrophages are key cells in immune defense against *S. aureus*. However, their activation needs to be counterbalanced by inhibitory receptors to control and resolve inflammatory processes. Signal inhibitory receptor on leukocytes-1 (SIRL-1) is an immune inhibitory receptor that belongs to the immunoglobulin superfamily (IgSF) of transmembrane receptors and is mainly expressed on granulocytes and monocytes[30]. SIRL-1 contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the intracellular domain that become phosphorylated upon receptor activation and recruit Src homology 2 domain-containing tyrosine phosphatases (SHP-1) and SHP-2[31]. SIRL-1 dampens the production of Fc receptor-mediated respiratory burst and opsonized *S. aureus*-induced neutrophil extracellular trap (NET) release[31-33]. So far the natural ligand of SIRL-1 is undetermined.

We here demonstrate that bacterial PSMα3 and human LL-37 are recognized by and activate SIRL-1 and propose that LL-37 interaction with SIRL-1 could represent a novel mechanism of immune regulation by antimicrobial peptides, whereas stimulation of SIRL-1 through PSMα3 could reveal a novel strategy of *S. aureus* to escape the immune system.

MATERIAL AND METHODS

Bacterial cultures and plasmid complementation

The bacterial strains and plasmids that were used in this study are described in supplementary table 1. *S. aureus* USA300 LAC, *S. aureus* USA400 MW2, *S. lugdunensis*, *S. epidermidis*, *S. capitis*, *S. warneri*, *S. hominis*, *S. caprae*, *Bacillus cereus* (*B. cereus*) and Group B *Streptococcus* COH1 were grown overnight in Tryptic Soy Broth (TSB; Sigma Aldrich) at 37°C with agitation. Growth curves were established by measuring optical density at 570 nm with the FLUOstar Optima (BMG Labtech). *Escherichia coli* (*E. coli*) was grown overnight in Lysogeny Broth (LB, Sigma Aldrich) at 37°C with agitation. Next day, bacterial cultures were centrifuged for 3 min at 2700 x g and supernatant was filtered through a 0.2 µm filter. Strains of the Nebraska Transposon Mutant Library (NTML) Screening Array were grown in 900 µl TSB supplemented with 5 µg/ml erythromycin in deep 96-well plates overnight at 37°C without agitation. Plasmid-harboring strains were grown in TSB supplemented with 25 µg/ml tetracycline overnight at 37°C with agitation.

cDNA constructs

Cloning of the extracellular part of human SIRL-1 cDNA into a retroviral pMX vector has been described before[30]. Tyrosines at position Y206 and Y231 were mutated to phenylalanines for generation of a signaling-deficient SIRL-1 receptor (SIRL-1 FF)[31]. To create chimeric reporter constructs, the extracellular domain of human SIRL-1 and human LAIR-1 were fused to the transmembrane and intracellular domain of human CD3ζ and cloned in the retroviral pMX puro vector[34].

Cell lines and transfectants

PLB-985 cells expressing human SIRL-1 or SIRL-1 FF and 2B4 NFAT-GFP reporter cells expressing SIRL-1-CD3ζ or LAIR-1-CD3ζ were generated as described previously[31,34]. All cells were maintained in RPMI 1640 (Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Biowest) and 50 U/ml Penicillin-Streptomycin (referred to as RPMI 10% hereafter).

To induce monocyte differentiation, PLB cells were seeded at a density of $2x10^5$ cells/ml in normal culture media supplemented with 50 nmol/L 1,25-dihydroxyvitamin D3 (Sigma Aldrich) for 3-4 days[35]. The differentiation state was verified by flow cytometry using mature monocyte markers, including CD11b (Beckman Coulter), CD14 (Sony), CD32 (StemCell Technologies), and CD89 (BD Bioscience). Expression of LAIR-1 and SIRL-1 was determined with PE-labeled anti-LAIR-1 (BD, clone DX26) and Alexa Fluor 647-labeled anti-SIRL antibodies, respectively. PE-labeled (BD) and Alexa Fluor 647-labeled (Biolegend) isotype-matched control IgG antibodies were used as negative control.

2B4 NFAT-GFP reporter cell assay

Reporter cell assay was performed with wt-CD3ζ, SIRL-1-CD3ζ or LAIR-1-CD3ζ 2B4 NFAT-GFP reporter cells and analyzed as described previously[34]. Briefly, 96-well MAXIsorp flat-bottom plates (Nunc) were coated overnight at 4°C with overnight bacterial supernatants or synthetic PSMs and LL-37 wt (AnaSpec, Inc.). Peptides PSMα3 wt, N'-formylated-PSMα3 wt, N'-formylated-δ-toxin and N'-formylated-PSMβ1 were synthesized by GenScript at 95% purity. Peptide PSMα3 F3A, PSMα3 G16A and PSMα3 K9P/F11P were kindly provided by Meytal Landau from Technion-Israel Institute of Technology, Israel. Mouse-anti-SIRL-1 mAb (clone 1A5; 10 µg/ml), mouse-anti-LAIR-1 mAb (clone 8A8; 10 µg/ml), Armenian hamster-anti-mouse-CD3 (clone 145-2C11; 10 µg/ml; BD) and human collagen I (5 µg/ml) were used as positive controls. After washings the wells with PBS, 0.5×10^5 cells in RPMI 10% were seeded to each well, and plates were incubated overnight at 37°C. Where indicated LL-37 coated wells were pre-incubated with mouse-anti-LL-37 mAb (clone 3D11; Hycult Biotech) for 60 min before adding reporter cells. After 2 hours of incubation, reporter cells were transferred to fresh wells and washed

twice with medium before overnight incubation at 37°C. Next day, GFP expression was analyzed by flow cytometry (FACS Fortessa; BD Bioscience) and analyzed with Flowjo software (version 10.0.7r2).

Reporter cell assay in response to human pooled serum (HPS; Sigma Aldrich) were performed differently. 0.5×10^5 cells were incubated in RPMI 10% containing different concentrations of HPS in U-bottom well plates (Nunc) for 120 min. Subsequently, cells were washed twice with RMPI 10% and transferred to a fresh U-bottom well plates before overnight incubation at 37°C. Next day, GFP expression was analyzed.

Determination of oxidative burst

Extracellular ROS production was measured as described before[31]. In short, wells were coated overnight at 4°C with 5 µg/ml IgA (Sigma Aldrich) alone or in combination with 5 µM PSMα3. Wells coated with a mix of IgA and 10 µg/ml anti-SIRL-1 were used as positive control for ROS inhibition. Monocyte-like differentiated PLB cells, stably transduced with SIRL-1 or SIRL-1 FF, were resuspended in HEPES buffer containing D(+)-glucose, BSA and $CaCl_2$. After washing the wells with PBS, $1x10^5$ cells were seeded to each well and 2x reaction buffer containing horseradish peroxidase and Amplex Red was added to the wells. Fluorescence (ex/em = 545 nm/590 nm) was measured every minute for the indicated time in a preheated Fluoroskan (Thermo Scientific) at 37°C.

Statistical analysis

Data were analyzed in GraphPad Prism software (version 6). Data were presented in median ± interquartile range and each experiment was performed at least three times in duplicate on independent occasions. The comparison of two samples was established by Kruskal-Wallis test and the comparison of three or more samples was established by a two-way ANOVA with Tukey's and Sudak's multiple comparisons test. $P<0.05$ was considered to be statically significant.

RESULTS

Staphylococci secrete a potential SIRL-1 ligand

We generated 2B4 NFAT-GFP reporter cells that express a chimeric protein consisting of the extracellular domain of human SIRL-1 and the intracellular domain of CD3ζ (SIRL-1-CD3ζ). LAIR-1-CD3ζ reporter cells, which express GFP upon collagen I binding[34] and wt-CD3ζ were used as negative control. Both, SIRL-1-CD3ζ and LAIR-1-CD3ζ reporter cells highly express their chimeric protein (Fig. 1A). To demonstrate proper functioning of our chimeric reporter system, cells were activated using

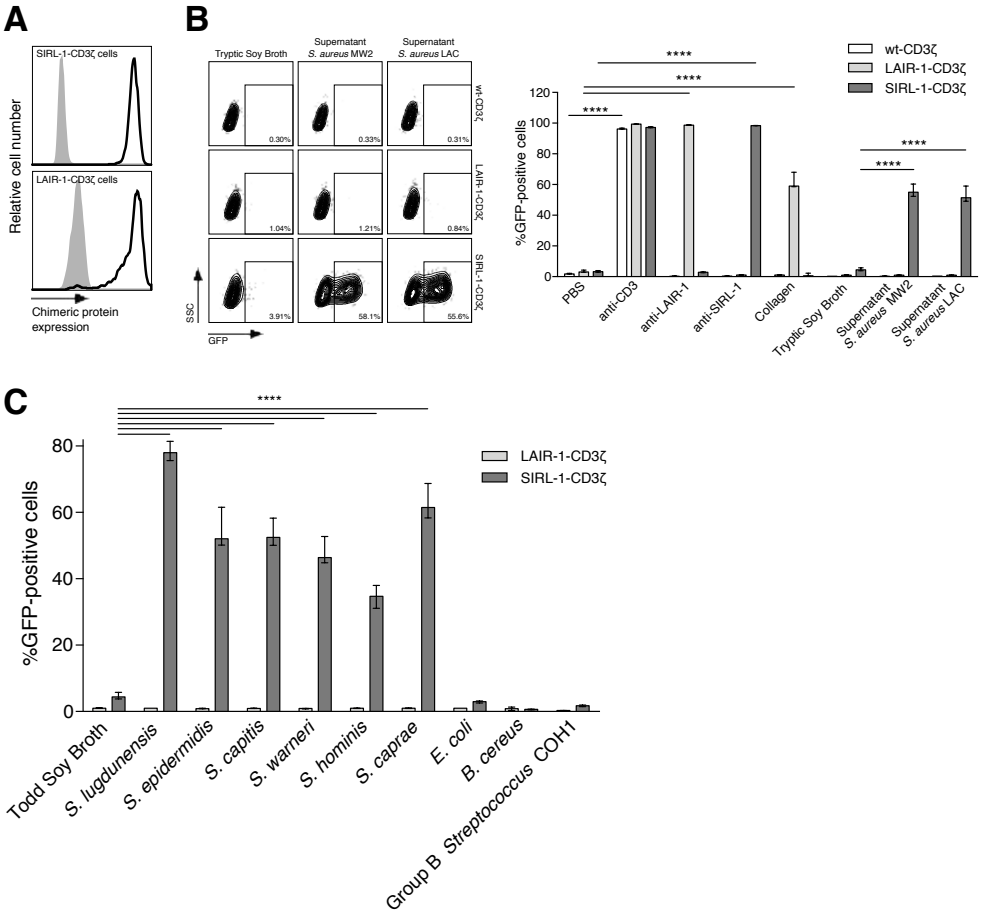

Figure 1 | **Supernatants of staphylococci induce GFP expression in SIRL-1-CD3ζ reporter cells**
(**A**) Surface expression of the chimeric proteins SIRL-1-CD3ζ and LAIR-1-CD3ζ on reporter cells (black lines). Gray peaks represent the isotype control antibodies. (**B**) Percentage of GFP expressing wt-CD3ζ, SIRL-1-CD3ζ and LAIR-1-CD3ζ reporter cells upon exposure to plate-bound overnight supernatant of *S. aureus* USA300 LAC and USA400 MW2. PBS and Tryptic Soy Broth were used as negative control, while anti-CD3, anti-LAIR, Collagen I, and anti-SIRL-1 were used as positive control. (**C**) GFP expression in SIRL-1-CD3ζ and LAIR-1-CD3ζ reporter cells upon exposure to supernatant of *S. lugdunensis*, *S. epidermidis*, *S.capitis*, *S. warneri*, *S. hominis*, *S. caprae*, *E. coli*, *B. cereus* and Group B *Streptococcus* COH1. Data represent median ± interquartile range of at least three independent experiments. Statistical significance (*****P*<0.0001) was determined by 2way ANOVA using Tukey's (**B**) and Sidak's (**C**) multiple comparison tests.

anti-CD3, anti-LAIR-1 and anti-SIRL-1 antibodies and induced up to ~100% GFP expression in wt-CD3ζ, LAIR-1-CD3ζ and SIRL-1-CD3ζ reporter cells, respectively. Receptor triggering of SIRL-1-CD3ζ reporter cells via plate-bound supernatant of overnight culture of *S. aureus* USA300 LAC and USA400 MW2, strains with high clinical importance and different genetic backgrounds, resulted in 55% and 58% GFP-expressing cells, respectively. This was comparable to the response of LAIR-1 reporter cells to the natural ligand collagen[34]. Neither of the supernatants induced

GFP expression in wt-CD3ζ and LAIR-1-CD3ζ reporter cells (Fig. 1B).

The conservation of the potential SIRL-1 ligand amongst staphylococci was tested with overnight supernatant of several staphylococcal species, including *S. lugdunensis*, *S. epidermidis*, *S. capitis*, *S. warneri*, *S. hominis* and *S. caprae*. Overnight supernatant of all tested staphylococci induced GFP expression in SIRL-1-CD3ζ reporter cells, whereas none of these supernatants induced GFP expression in LAIR-1-CD3ζ reporter cells (Fig. 1C). Interestingly, supernatant of *E. coli*, *B. cereus* and Group B *Streptococcus* COH1 did not induce GFP expression in SIRL-1-CD3ζ reporter cells, which suggests that a potential bacterial SIRL-1 ligand is broadly but specifically secreted amongst staphylococci.

The agr system regulates the secretion of a potential SIRL-1 ligand in *S. aureus*

To identify the bacterial SIRL-1 ligand we screened the supernatant of 1,920 single-gene transposon insertion mutants from the NTML for their ability to induce GFP in SIRL-1-CD3ζ reporter cells. We identified four Tn-insertion mutants that induced very low levels of GFP, comparable to negative control (TSB), three of them inside the agr operon: (Δ*agrA*, Δ*agrB*, and Δ*agrC*; red dots) and one inside the *sarA* gene encoding the upstream regulator of the agr operon (Δ*sarA*; green dot) (Fig. 2A). Other Tn-insertion mutants that induced low levels of GFP expression in SIRL-1-CD3ζ reporter cells contained a gene knockout that was not related to the *S. aureus* secretion system and since the ligand was secreted into the overnight supernatant, these TN-insertion mutants were not relevant for further investigation.

Upon retesting, exposure of SIRL-1-CD3ζ reporter cells to plate-bound supernatant of Δ*sarA*, Δ*agrA*, Δ*agrB* and Δ*agrC* NTML Tn-insertion mutants reproducibly resulted in reduced GFP expression compared to *S. aureus* wt (Fig. 2B), while bacterial growth was similar (Fig. 2C). These data confirm that the agr system regulates the secretion of a potential SIRL-1 ligand in *S. aureus*. NTML Tn-insertion mutants representing downstream effectors of the agr system, including Δ*spa*, the pentose phosphate pathway-responsive regulator (Δ*Rpir*), repressor of toxins (Δ*Rot*), endoribonuclease III (Δ*rnc*) and α-haemolysin precursor (Δ*hly*) or main staphylococcal protein secretion pathways, like twin-arginine translocation (Δ*Tat*), protein translocase subunit Y (Δ*secY*) and (Δ*secA*) did not result in reduced GFP expression in SIRL-1-CD3ζ reporter cells (Fig. 2D). This indicates that the SIRL-1 ligand in *S. aureus* is an agr-dependent factor but it is not one of the downstream effectors of the agr system that were tested here.

PSMα3 and δ-toxin induce SIRL-1-mediated signaling that depends on the α-helical structure

The PSM-encoding genes are strictly controlled by direct binding of agrA to the psm

operon promoters[8]. We tested independently generated PSM deletion mutants in *S. aureus* MW2 and LAC background[9], including a triple deletion mutant in PSMα1-4, PSMβ1-2 and hld (Δ*PSM*), PSM alpha (Δ*PSMα*), PSM beta (Δ*PSMβ*) and δ-toxin (Δ*hld*). Plate-bound overnight supernatant of *S. aureus* Δ*PSM* and Δ*PSMα* resulted in reduced GFP expression in SIRL-1-CD3ζ reporter cells, compared to GFP expression in response to *S. aureus* Δ*PSMβ*, Δ*hld* and *S. aureus* wt in both genetic backgrounds (Fig. 3A). Complementation of *S. aureus* Δ*PSM* with plasmid-encoded PSMα1-4 genes (pTXα1-4) and δ-toxin (pXT-*hld*) restored GFP expression in SIRL-1-CD3ζ reporter cells, while pTXβ1-2 (plasmid-encoded PSMβ1-2 genes) and pTXΔ16 (empty plasmid) did not restore GFP expression.

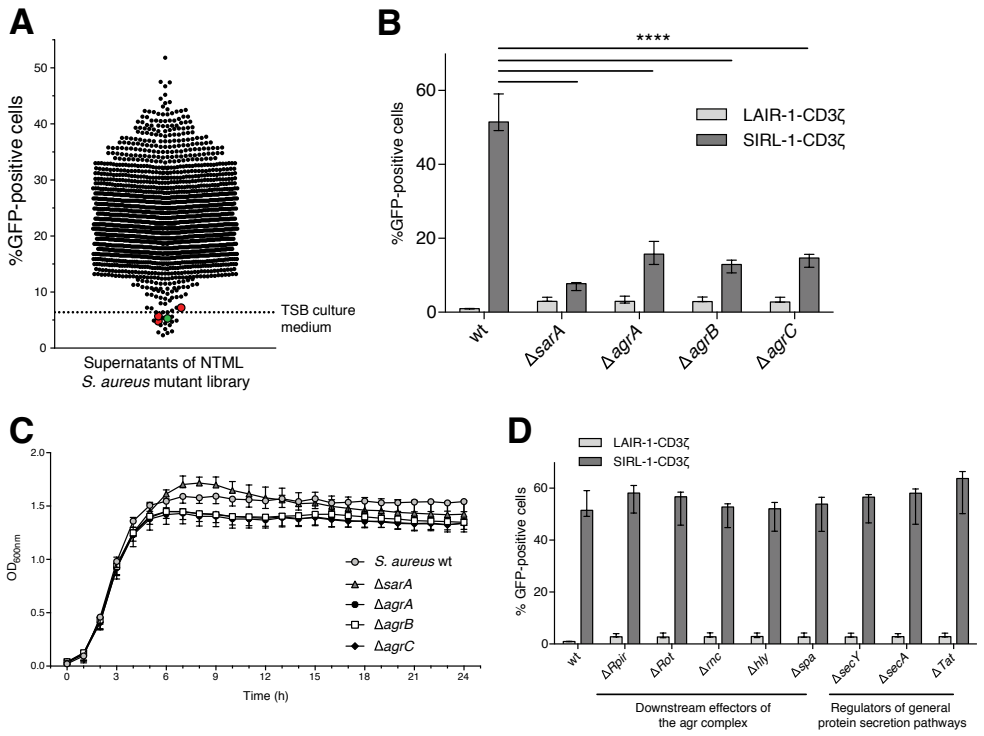

Figure 2 | The agr system regulates secretion of the potential SIRL-1 ligand in *S. aureus*
(**A**) Overnight supernatants of the NTML *S. aureus* mutant library that consists of 1,920 single-gene transposon insertion mutants were used for exposure to SIRL-1-CD3ζ reporter cells. Tn-insertion mutants of sarA (green dot) and the agr system (red dots) showed similar levels of GFP expression as Tryptic Soy Broth. Other Tn-insertion mutants that induced low levels of GFP expression in SIRL-1-CD3ζ reporter cells contained a gene knockout that was not related to the *S. aureus* secretion system. Data represent median of one screen performed in duplicate. (**B**) Percentage of GFP expressing SIRL-1-CD3ζ and LAIR-1-CD3ζ reporter cells upon retesting of Tn-insertion mutants of staphylococcal accessory regulator A (Δ*sarA*) and components of the agr system (Δ*agrA*, Δ*agrB*, and Δ*agrC*). (**C**) Growth curves of *S. aureus* wt and the Tn-insertion mutants Δ*sarA*, Δ*agrA*, Δ*agrB*, and Δ*agrC*. Data represent median ± range of one experiment performed in triplicate. (**D**) Percentage of GFP expressing SIRL-1-CD3ζ and LAIR-1-CD3ζ reporter cells upon exposure to supernatant of Tn-insertion mutants, including downstream effectors of the agr system (Δ*Rpir*, Δ*Rot*, Δ*rnc*, Δ*hly* and Δ*spa*) or main staphylococcal protein secretion pathways (Δ*Tat*, Δ*secY* and (Δ*secA*). Data in **B** and **D** represent median ± interquartile range of at least three independent experiments. Statistical significance (****P<0.0001) was determined by 2way ANOVA using Sidak's multiple comparison test.

We confirmed PSMα and δ-toxin as SIRL-1 ligands, using synthetic peptides that were formylated at the N-terminus to mimic bacterial protein synthesis. Both, N'-formyl-PSMα3 and N'-formyl-δ-toxin induced GFP expression in SIRL-1-CD3ζ reporter cells in a concentration-dependent manner, whereas N'-formyl-PSMβ1 did not induce GFP expression (Fig. 3C). Moreover, GFP expression did not depend on N'-formylation of the peptide, since non-formylated PSMα3 still induced GFP expression in SIRL-1-CD3ζ reporter cells (Fig. 3C). Of note, SIRL-1-CD3ζ reporter cell viability was unaffected when exposed to these concentrations of PSMs (data not shown).

PSMs are α-helical peptides and have been shown to form cross-α amyloid-like fibers[11]. To determine the importance of their tertiary structure to serve as SIRL-1 ligands, we used PSMα3 mutants that do not fibrillate (PSMα3 F3A) or do not form a α-helical structure (PSMα3 K9P/F11P)[11]. GFP expression in SIRL-1-CD3ζ reporter cells was reduced upon exposure of PSMα3 K9P/F11P, whereas PSMα3

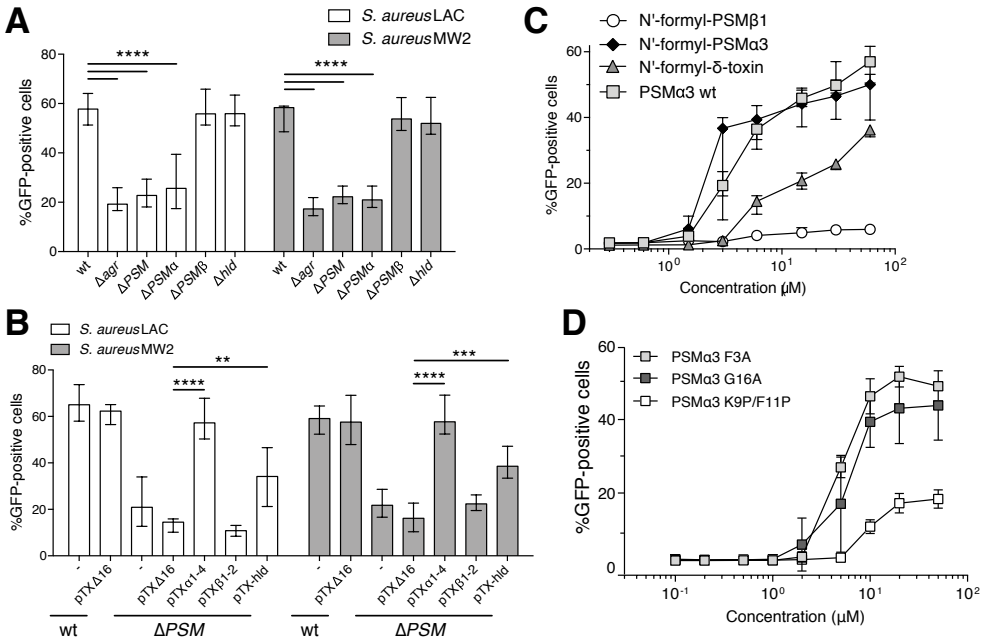

Figure 3 | PSMα3 and δ-toxin induce SIRL-1-mediated signaling
(**A**) Overnight supernatants of *S. aureus* LAC and MW2, including a triple deletion mutant in PSMα1-4, PSMβ1-2 and hld (Δ*PSM*), PSM alpha (Δ*PSMα*), PSM beta (Δ*PSMβ*) and δ-toxin (Δ*hld*) were used for exposure to SIRL-1-CD3ζ reporter cells. (**B**) Percentage of GFP expressing SIRL-1-CD3ζ reporter cells in response to *S. aureus* Δ*PSM* containing plasmids with PSMα1, α2, α3 and α4 genes (pTXα1-4), PSMβ1 and β2 genes (pTXβ1-2) or δ-toxin gene (pTX-*hld*). Empty plasmid (pTXΔ16) was used as negative control. (**C**) Plate-bound synthetic peptides of PSMα3 wt (non-formylated), N'-formyl-PSMα3, N'-formyl-PSMβ1 and N'-formyl-δ-toxin were exposed to SIRL-1-CD3ζ reporter cells in different concentrations. (**D**) Percentage GFP expressing SIRL-1-CD3ζ reporter cells upon exposure of PSMα3 G16A (non-related mutation), PSMα3 F3A (does not fibrillate), and PSMα3 K9P/F11P (does not form a α-helical structure) synthetic peptide mutants. Data represent median ± interquartile range of at least three independent experiments. Statistical significance (***P*<0.01, ****P*<0,001 and *****P*<0.0001) was determined by 2way ANOVA using Sidak's multiple comparison test.

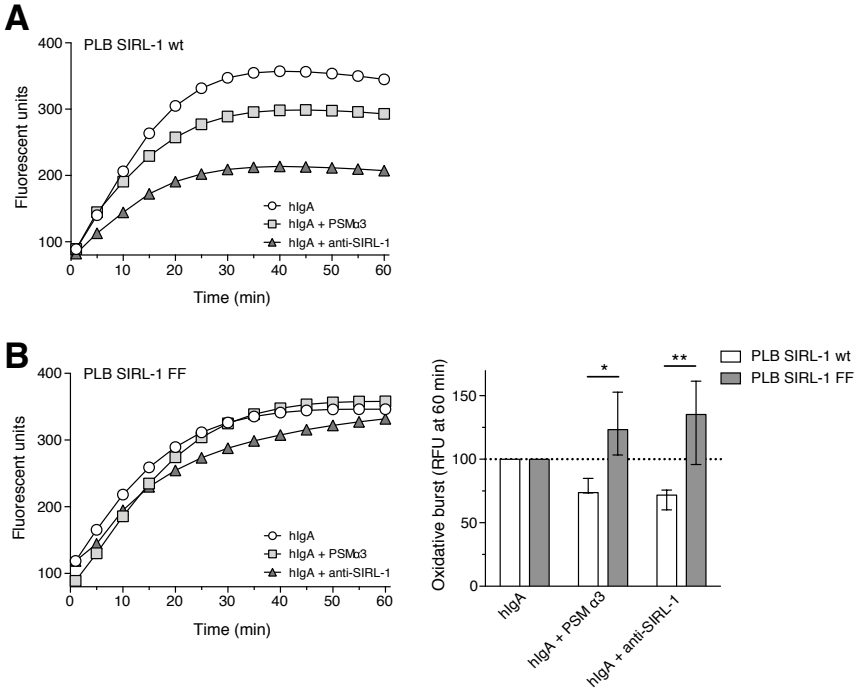

Figure 4 | PSMα3 inhibits FcαR-mediated oxidative burst via SIRL-1
(**A**) SIRL-1 transfected human myeloid PLB cells were differentiated towards monocyte-like cells and stimulated with plate-bound human IgA (hIgA) with or without concomitant stimulation of PSMα3. Anti-SIRL-1 antibody was used as positive control for inhibition of the oxidative burst. (**B**) Two tyrosines in the ITIMs of SIRL-1 were mutated to phenylalanines to generate signaling-deficient SIRL-1 receptor (SIRL-1 FF). Data were corrected for spontaneous ROS production. Data of ROS kinetics (**A** and **B** left graph) represent median of one representative experiment performed in triplicate. Fluorescent units at time point 60 min were used to calculate the inhibition relative to cells stimulated with hIgA (**B** right graph). Data represent median ± interquartile range of three independent experiments. Statistical significance (*P<0.05, **P<0.01) was determined by 2way ANOVA using Sidak's multiple comparison test.

F3A-induced GFP expression was comparable with PSMα3 wt (Fig. 3D). A non-related PSMα3 mutant G16A that does fibrillate and forms the α-helical structure was used as control and induced GFP expression in SIRL-1-CD3ζ reporter cells similar as non-formylated PSMα3 wt. Thus, these data indicate that PSMα3 and δ-toxin but not PSMβ are recognized by SIRL-1 and that activation depends on the α-helical structure, but not on amyloid formation, of PSMα3.

PSMα3 inhibits IgA-induced oxidative burst

SIRL-1 triggering inhibits human IgA (hIgA)-mediated ROS production[31]. To study whether PSMα3 can trigger SIRL-1-mediated inhibition, we used SIRL-1 transfected human myeloid PLB cells that express high levels of Fcα receptor (FcαR) upon differentiation towards monocyte-like cells. In these differentiated cells, hIgA triggering results in ROS production that can be inhibited when SIRL-1 is triggered by PSMα3 (Fig. 4A). PSMα3-induced inhibition of oxidative burst is dependent

on SIRL-1 intracellular signaling, since IgA-induced ROS production in PLB SIRL-1 FF cells, which lack functional ITIM motifs[30], could not be inhibited with PSMα3 (Fig. 4B). These data confirm a functional interaction of PSMα3 with SIRL-1 that dampens IgA-induced oxidative burst.

The human cathelicidin peptide LL-37 induces SIRL-1 signaling

The host defense peptide LL-37 shows structural and functional similarities to *S. aureus* PMSα3[18,25]. SIRL-1-CD3ζ reporter cells expressed GFP in a concentration-dependent manner upon exposure to LL-37, while LAIR-1-CD3ζ reporter cells did not respond (Fig. 5A). LL-37-induced GFP expression in SIRL-1-CD3ζ reporter cells was blocked with anti-LL-37 antibodies confirming specificity (Fig. 5B). LL-37 is present in human serum in concentrations that reach up to 1.18 μg/ml (= 0.26 μM)[36]. Therefore we exposed SIRL-1-CD3ζ reporter cells to human pooled serum (HPS), which resulted in ~55% GFP-expressing cells. Taken together, these data indicate that,although the concentration of LL-37 synthetic peptide we tested is higher than in HPS, the human amphipathic α-helical peptide LL-37 stimulates SIRL-1 and that this interaction potentially might occur in the circulation. However, it is difficult to compare synthetic LL-37 with HPS when coated on plastic because HPS contain most likely other components that activate SIRL-1.

DISCUSSION

The interaction of *S. aureus* with the human host occurs in two different ways: *S. aureus* can exhibit a commensal-like lifestyle on skin and moist squamous epithelium but can also behave as an invasive pathogen that induces life-threatening conditions[37]. How the immune system discriminates between commensals and pathogens is not clear. We previously showed that SIRL-1 activation dampens antimicrobial responses in neutrophils and monocytes[31-33]. Here we demonstrate that the bacterial and endogenous amphipathic α-helical peptides PSMα3 and LL-37 act as functional ligands for SIRL-1. We suggest that the interaction of PSMα3 with SIRL-1 is an immune evasion strategy for staphylococci, while LL-37 controls immunological balance in immune cells through interaction with SIRL-1.

LL-37 shows biochemical, structural and functional characteristics that are highly comparable with PSMα3. Pathogens are able to mimic host factors to modulate cellular activities of host immune cells, a phenomenon that is called molecular mimicry[38]. It is tempting to speculate that α-type PSMs were generated as a result of molecular mimicry of human LL-37. Interestingly, molecular mimicry of LL-37 has been described previously; the parasitic helminth *Fasciola hepatica* secretes the defense molecule fhHDM-1, which adopts a predominantly α-helical structure in

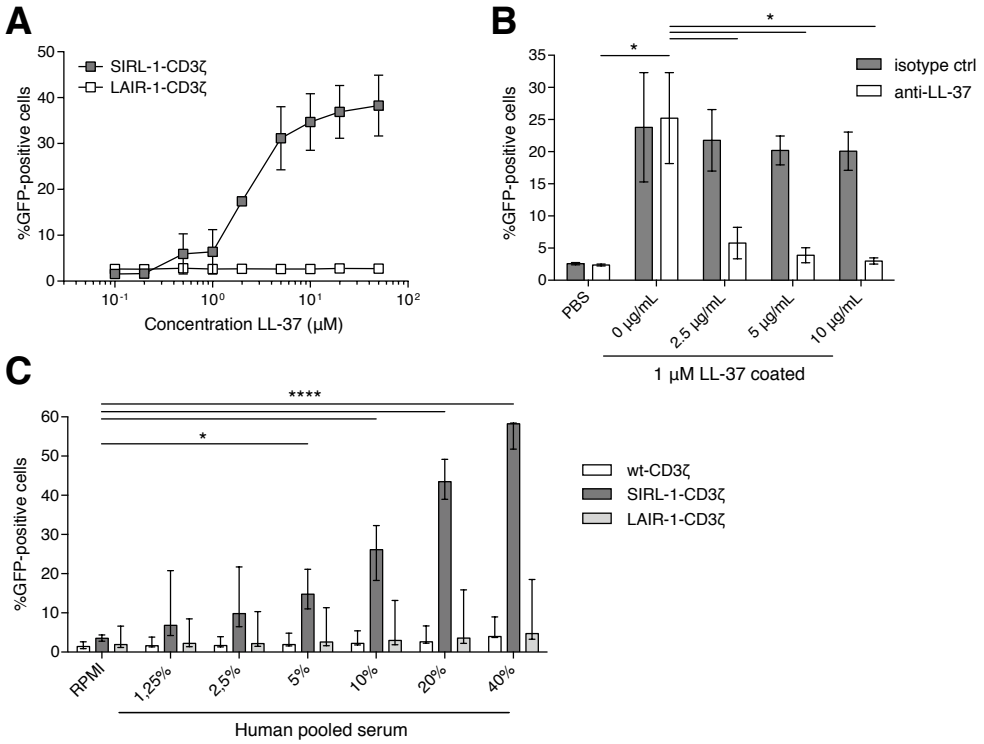

Figure 5 | **LL-37 induces SIRL-1 signaling**
(**A**) Plate-bound synthetic peptides of LL-37 were exposed to SIRL-1-CD3ζ and LAIR-1-CD3ζ reporter cells in different concentrations. (**B**) GFP expression in SIRL-1-CD3ζ reporter cells in response to 1 μM coated LL-37 was blocked with 2.5, 5 and 10 μg/ml anti-LL-37 antibody pre-incubation. Data represent median ± range of two independent experiments performed in duplicate. (**C**) GFP expressing SIRL-1-CD3ζ reporter cells in response to soluble human pooled serum (HPS). Data in **A** and **C** represent median ± interquartile range of three independent experiments. Statistical significance (**P*<0.05, *****P*<0.0001) was determined by 2way ANOVA using Sidak's multiple comparison test.

solution and exhibit characteristics similar to LL-37[39].

PSMs are broadly cytolytic against host cells and contribute to the development of biofilm[8,40]. The functional interaction of PSMα3 with SIRL-1 reveals an additional strategy for *S. aureus* to escape the immune system and potentially increase its infectively. In fact, the concept that bacteria express molecules that trigger inhibitory cell-surface receptors to manipulate signal transduction pathways in immune cells has been described previously[41]. For example, lipoteichoic acid present on the cell wall of *S. aureus* interacts with the ITIM-bearing paired Ig-like receptor B (PIR-B) in mice to suppress pro-inflammatory cytokine release by macrophages[42]. Similarly, anchored β-protein and sialylated capsular polysaccharide of Group B *Streptococcus* activate sialic acid-recognizing immunoglobulin superfamily lectin-5 (Siglec-5) and Siglec-9, respectively, reducing antimicrobial function of neutrophils and macrophages and promoting bacterial survival[43,44].

The host senses PSM-associated danger via FPR2, resulting in downstream

mitogen-activated protein kinases (MAPK) and extracellular signal-regulated protein kinase (ERK) signaling[45] and induction of pro-inflammatory immune responses[13]. Activation of SIRL-1 inhibits ERK phosphorylation[31]. Therefore, interaction of PSMα3 with SIRL-1 could interfere with FPR2-induced antimicrobial response. Future experiments aim to establish whether there is a direct connection between the downstream signaling pathway of SIRL-1 and FPR2.

We previously identified a functional single nucleotide polymorphism (SNP) in the promoter of VSTM1 (SIRL-1 gene), which leads to decreased SIRL-1 expression on monocytes and is associated with an increased risk of atopic dermatitis (AD)[46]. AD is a chronic disease that is characterized by increased inflammation in skin. Interestingly, skin colonization of *S. aureus* is a characteristic feature of AD. In addition, numerous members of the genus *Staphylococcus* are common colonizers of the human skin and some, such as *S. epidermidis* and *S. hominis*, are even found all over the body[47]. We here demonstrate that the overnight supernatant of many members of the genus *Staphylococcus* induce SIRL-1 activation. Although the supernatant of these staphylococci could contain other molecules that trigger SIRL-1, we suggest that α-type PSMs are secreted by these bacteria. Indeed, several staphylococci, from the aggressive human pathogens to less-pathogenic species secrete PSMs[48]. Additional experiments are necessary to establish whether PSMs from *S. aureus* and/or commensal-like staphylococci regulate host immune cell activity in skin.

LL-37 protects the host against invading pathogens at sites of inflammation and in wounds. Abundant presence of LL-37, however, is cytotoxic for eukaryotic cells[49]. Similarly, PSMs interact with the membrane of eukaryotic cells to induce cytolysis resulting in cell death, which is a powerful mechanism to abrogate host defense mechanisms. A likely possibility is that LL-37 and PSMα3 serve as a killing strategy which induces inflammation while simultaneously it suppresses innate immune cells via SIRL-1 to avoid abundant immune cell activity and healthy tissue damage. Besides its presence in inflamed tissue, LL-37 is present in human serum in relatively high concentrations[36]. The cytotoxic activity of both LL-37 and PSMs is inhibited in serum through binding to lipoproteins[50-53]. Although these complexes, composed of LL-37 or PSMs with lipoproteins, would not harm the host anymore, they might still function as negative regulators of immune cells in blood through engagement of SIRL-1. In addition, it is very likely that human serum contains other factors that activate SIRL-1. Futher investigation is necessary to determine additional SIRL-1 ligands in human serum.

Interaction of the peptides PSMα3 and LL-37 with SIRL-1 presents a novel mechanism of immune regulation by cytotoxic peptides and contributes to the understanding of the role of SIRL-1 in health and disease. The functional interaction of SIRL-1 with both microbial and endogenous amphipathic peptides thus may be an example of co-evolution of host and microorganism with mutual benefit.

ACKNOWLEDGEMENTS

The authors thank the Network on Antimicrobial Resistance in *S. aureus* (NARSA) for providing the Nebraska Transposon Mutant Library Screening Array, NR-48501. The authors thank BEI Resources for providing *S. lugdunensis* (strain M23590, HM-141) and *S. caprae* (strain C87, HM-246). The authors thanks Dr. Bas Surewaard (University of Calgary, Canada) for providing *S. aureus* LAC and MW2 knockouts and pTXΔ16 complementation plasmids. The authors thank Einav Tayeb-Fligelman and Dr. Meytal Landau (Technion-Israel Institute of Technology, Haifa, Israel) for providing the PSMα3 mutants synthetic peptides.

M.R., H.R. and L.M. are supported by a Vici grant (91815608) from the Netherlands Organisation for Scientific Research (NWO). M.L. is supported by the Dutch Arthritis Foundation (Grant 12-2-406). N.S. is supported by a VIDI grant (91713303) from the NWO.

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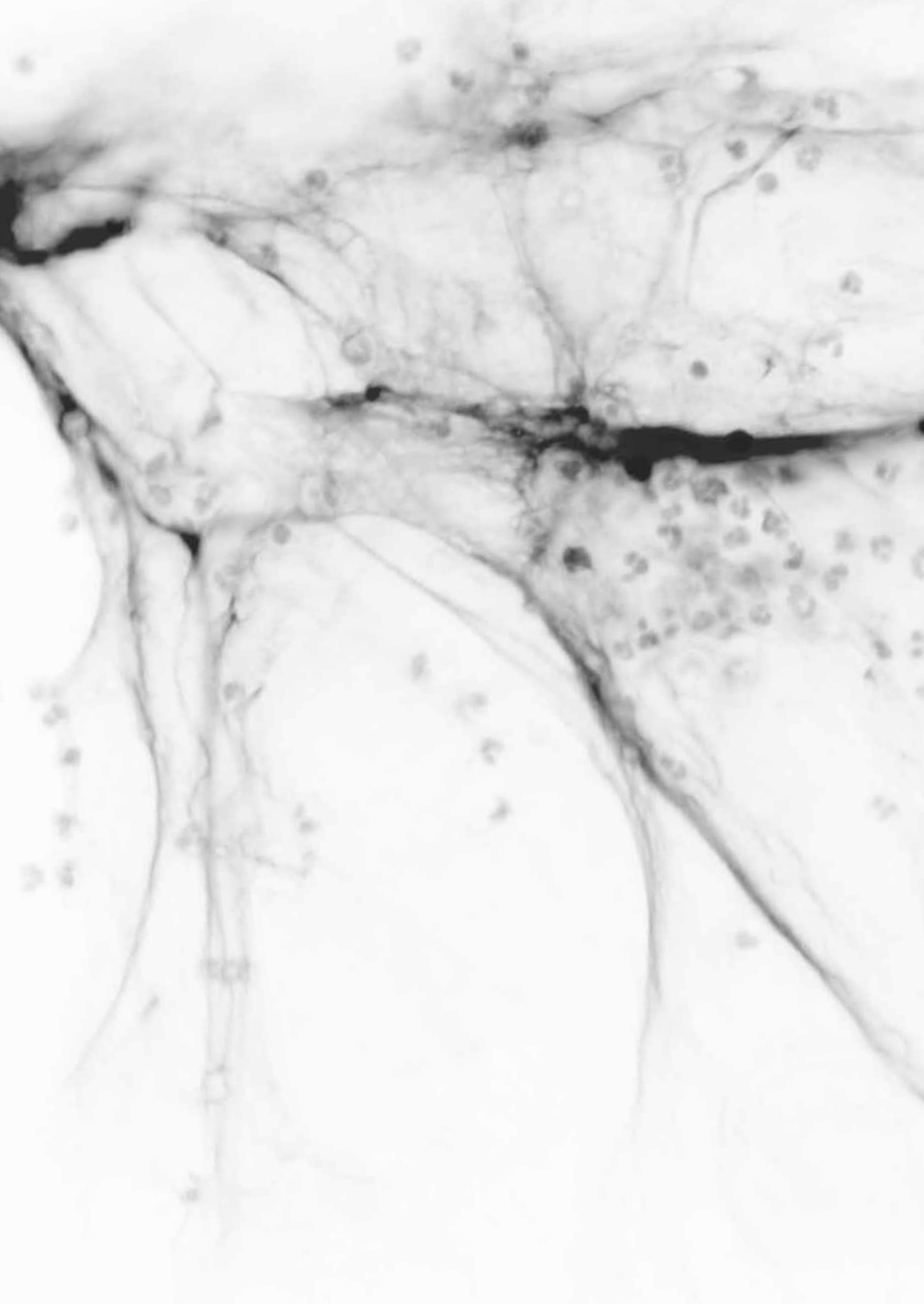
SUPPLEMENTARY INFORMATION

Supplementary Table 1

Bacterial strains	Source/References
S. aureus USA300 LAC wt	Wang et al.[9]
S. aureus USA300 LAC Δ*agr*	Wang et al.[9]
S. aureus USA300 LAC ΔPSM	Chatterjee et al.[54]
S. aureus USA300 LAC ΔPSMα1-4	Wang et al.[9]
S. aureus USA300 LAC ΔPSMβ1-2	Wang et al.[9]
S. aureus USA300 LAC Δ*hld*	Wang et al.[9]
S. aureus USA400 MW2 wt	Wang et al.[9]
S. aureus USA400 MW2 Δ*agr*	Wang et al.[9]
S. aureus USA400 MW2 ΔPSM	Chatterjee et al.[54]
S. aureus USA400 MW2 ΔPSMα1-4	Wang et al.[9]
S. aureus USA400 MW2 ΔPSMβ1-2	Wang et al.[9]
S. aureus USA400 MW2 Δ*hld*	Wang et al.[9]
S. lugdunensis M23590	BEI Resources, NIAID, NIH
S. epidermidis ATCC 49134	Own
S. capitis ATCC 35661	Own
S. warneri	Own
S. hominis	Own
S. caprae C87	BEI Resources, NIAID, NIH
E. coli DH5α	Own
B. cereus T2460	Own
Group B *Streptococcus* COH1	Own
Nebraska Transposon Mutant Library (NTML)	BEI Resources, NIAID, NIH

Plasmids	Description	Source/References
pTXΔ16	tetracycline (Tet) resistance, control plasmid	Wang et al.[9]
pTXα1-4	Tet resistance, PSMα1-4 genes constitutively expressed through xylose promoter	Wang et al.[9]
pTXβ1-2	Tet resistance, PSMβ1-2 genes constitutively expressed through xylose promoter	Dastgheyb et al.[55]
pTX-hld	Tet resistance, *hld* gene constitutively expressed through xylose promoter	Nakamura et al.[56]



7 CHAPTER

GENERAL DISCUSSION

THE IMPORTANCE OF NET RELEASE IN THE FIRST LINE OF DEFENSE AGAINST INVADING PATHOGENS

Since the discovery of NETs in 2004, the list of pathogens that trigger NET release is expanding[1]. NETs consist of extracellular fibers of decondensed chromatin that reach hundreds of nanometers in length to trap passing microbes. In addition, the antimicrobial peptides present in NETs efficiently eliminate these microbes *in vitro*[2], which demonstrate that NET release could be a valuable and fundamental weapon of neutrophils to fight against invading pathogens. Besides NETs, neutrophils use other antimicrobial strategies to attack different pathogens, however little is known about how neutrophils distinguish between these pathogens and what is the decisive trigger for a neutrophil to release NETs instead of phagocytizing the pathogen. Branzk and colleagues have shown that neutrophils sense microbe size and selectively release NETs in response to large *C. albicans* hyphae, whereas small *C. albicans* yeast are phagocytized into phagosomes[3]. The formation of phagosomes coincides with the fusion of azurophilic granules that contain antimicrobial compounds, such as neutrophil elastase (NE). The lack of phagosomes when large microorganisms cannot be phagocytized, allows NE to translocate to the nucleus inducing chromatin decondensation leading to NET release[3]. Furthermore, NE that is not present in phagosomes promotes degradation of actin cytoskeleton and blocks phagocytosis[4]. This indicates that NE, besides its own antimicrobial activity, plays an essential role in the choice of antimicrobial response of neutrophils against different microbes. However, it has been widely reported that also small bacteria induce NET release. Interestingly, many of these bacteria can survive the toxic environment of phagosomes and therefore it is likely that NET release also occurs against small microorganisms that escape phagosomal killing[5]. In fact, more virulent bacteria than non-virulent bacteria have been shown to induce NET release[6]. These data thus suggest that neutrophils first consider phagocytosis but immediately change their antimicrobial strategy and switch to NET release when microbes escape from phagosomal killing or when microbes are too large for uptake. In fact, this explains the extensive armory of neutrophils necessary to eradicate multiple types of pathogens. Broad weaponry increases the change of victory, even when one of the neutrophilic antimicrobial attacking strategies is abandoned.

Current data on the role of NET release in clearing pathogenic infection *in vivo* is contradictory. On the one hand, there are data that support NETs as part of the antimicrobial weaponry of neutrophils that contributes to the first line of defense against pathogenic infection. Indeed, in an *E. coli*-induced sepsis mouse model, NETs trap bacteria from the bloodstream and protect the host against bacterial dissemination[7], while reduced bacterial killing is demonstrated in NET-deficient

mice infected with group A *Streptococcus* (GAS)[8]. Furthermore, mice that are unable to release NETs are more susceptible to bacterial infection compared to wild type mice because they develop larger lesions after subcutaneously GAS injection. In a human CGD patient, the importance of NETs in the protection against invading *Aspergillus* is supported by restoration of NET release following gene therapy[9]. In contrast, more recent investigations demonstrate that abrogated NET release does not influence bacterial outgrowth. For example, NET-deficient mice are equally susceptible to bacteremia as wild type mice in polymicrobial sepsis[10] or *Burkholderia pseudomallei*-induced sepsis[11]. Likewise, neutrophils from people with Papillon-Lefèvre syndrome are incapable of releasing NETs while killing of *S. aureus* and *K. pneumonia* is comparable to healthy neutrophils[12].

So far, it is hard to determine the specific contribution of NETs in immune defense, since most antimicrobial peptides that are implicated in NET release are also essential for intracellular microbial killing of neutrophils. In addition, DNase treatment, which is often used to cleave NETs, also causes breakdown of biofilm and results in reduction of bacterial survival[13]. Further research is necessary to investigate the antimicrobial capacity of NETs and physiological conditions in which NETs are released. Based on the data described above and data from our experiments, we propose that NET release occurs in response to extremely high bacterial dose, for example in sepsis-like conditions. When bacterial concentration is too high, neutrophils overload their phagosomes resulting in phagosomal breakdown that finally results in NET release. However, a relatively low amount of pathogens are present in i.e. wounds and thus these pathogens will be killed through phagocytosis. Additional *in vivo* experiments that use different bacterial dose in different tissue have to be performed to investigate the importance of NETs in our daily protection from bacteria.

DISTINCT MECHANISMS OF NET RELEASE

NET release is a dynamic process including several phases that are characterized by morphological changes of the neutrophil. Therefore it is valuable to study the release of NETs over time. Our live imaging fluorescent microscopic approach quantifies NET release in response to physiological stimuli and simultaneously measures NET kinetics, distinguishes NET release from other cell death mechanisms and corrects for neutrophil input, aspects that are lacking in other experimental approaches (**Chapter 2**). With the use of this technique, we have demonstrated that opsonized *S. aureus*-induced NET release starts around 90 min. In contrast, we (**Chapter 5**) and others previously showed that, when using a fluorescence plate reader assay, Sytox Green fluorescence appears within

30 min when neutrophil are exposed to viable *S. aureus*[14,15]. We now conclude that this early Sytox Green fluorescence is due to Sytox Green+ neutrophils induced by cytolytic toxins from viable *S. aureus*[16].

NET release can occur via distinct mechanisms, including suicidal and vital NET release. Suicidal NET release implies a cell death mechanism by which neutrophils sacrifice themselves, while vital NET release would allow neutrophils to release their DNA and maintain bacterial killing via phagocytosis[17]. Pilsczek and coworkers have shown that vital NET release occurs in response to *S. aureus* and triggers neutrophils to extrude vesicles with nuclear DNA into the extracellular environment that then rupture and release NETs[15]. Vital NET release generates cytoplasts (cell without nucleus) that crawl and phagocytize bacteria[18]. In contrast to these findings, in our live imaging experiments the plasma membrane of neutrophils completely ruptures when NETs are released in response to opsonized *S. aureus*, suggesting that opsonized *S. aureus* induces suicidal NET release. However, since the frequency of vital NET release is low, we might have missed vital NET release.

The neutrophil stimulus determines the activation of the underlying signaling pathway of NET release in which the production of ROS is essential. Others and we have demonstrated that PMA, IC, anti-LL-37, and non-opsonized *S. aureus* induce NET release in a NADPH oxidase-dependent manner[19-22] presumably initiated by the upstream signaling molecules MAPK, ERK and PKB[23]. In contrast, NET release independent of NADPH oxidase is induced by MSU, IC, uric acid, *Leishmania*, and opsonized *S. aureus*[14,15,24-26] via activation of SYK, PI3K, mTOR or RIPK[23,27]. NADPH oxidase-independent NET release is most likely mediated by ROS that is produced by MPO or mitochondria[23,27]. A recent study established a distinct signaling cascade of NET release that includes the cell-cycle pathway and demonstrated that mitogens, lamins and cyclin-dependent kinases 4 and 6 (CDK4/6) are essential regulators of NET release[28]. Mature neutrophils do not divide, but these data indicate that neutrophils reactivate the cell-cycle pathway to release NETs. Based on these data, we conclude that NET release is an active process that is initiated via multiple signaling pathways depending on the stimulus.

Although NET formation has also been shown *in vivo*, it is important to note that neutrophils react differently in *in vitro* settings. Indeed, neutrophils that adhere to plastic surfaces *in vitro* immediately initiate a certain level of ROS compared to suspension neutrophils[29]. As described above, ROS is an essential player in the downstream signaling pathway of NET release and this would suggest that NET release occurs at the moment neutrophils adhere to the bottom of the wells. Still, in our live imaging experiments non-stimulated neutrophils did not show Sytox Green positivity or NET release, which indicates that the background level of NET release is low and that *in vitro* live imaging is a reliable technique to study NET release.

THE PATHOGENIC ROLE OF NET RELEASE IN SLE AND APS

Besides their potential role in the defense against infection, NETs contribute to the development and progression of chronic autoimmune diseases, such as SLE and APS. Both diseases are characterized by an increased level of NETs in circulation[30,31], which is most likely the result of decreased DNase I activity[30,31] as well as neutrophils that are more prone to release NETs[32,33]. Increased NET release implicates a possible role in the pathogenesis of SLE, which is confirmed by different SLE mouse models that show reduced disease severity *in vivo* when NET release is suppressed[20,34]. In addition, NETs are present in biopsies of affected tissue in skin and kidney of SLE patients[35]. Although these data indicate that NETs induce damage and injury to the host in SLE, it is not completely known which NET-dependent factors contribute to actual tissue damage. Granular proteases, present in NETs, cause damage to healthy tissue, however the human body uses several mechanisms to counteract the activity of these antimicrobial proteases (**Chapter 4**). Probably the most severe tissue damage will be caused by extracellular histones, since these interact with the membrane of epithelial and endothelial cells and alter its permeability to induce cytolysis and apoptosis[36]. Furthermore, histones activate TLR2 and TLR4 that subsequently trigger the release of pro-inflammatory cytokines by immune cells, which results in acute sterile inflammation *in vivo*[37]. Mice lacking TLR2 and TLR4 expression are protected for a histone-mediated inflammatory response. From these data, we suggest that NET-associated antimicrobial compounds induce a minimal amount of healthy tissue damage, since the human body has developed counteracting mechanisms to protect itself from these cytotoxic compounds. However, abundant exposure to extracellular histones will provoke immune responses that lead to sterile inflammation.

NETs can be a source of autoantigens that induce auto-inflammatory responses leading to the production of autoantibodies. Indeed, NETs activate pDCs in a TLR9-dependent manner, which then produce IFNα which triggers the generation of autoantibodies by B-lymphocytes or activates neutrophils to release more NETs[32,38,39]. Furthermore, a distinct subset of neutrophils, called LDGs, has been described in SLE and APS[35,40]. LDGs are more prone to release NET and display an enhanced pro-inflammatory profile with increased expression of IFNα, which contributes to the persistence of chronic inflammatory conditions[35,41]. Interestingly, in our experiments, increased NET release is associated with a type I IFN signature and enlarged number of LDGs in SLE, but not APS. We thus speculate that specific triggers in SLE activate LDGs to secrete IFNα and that these triggers are absent in APS. In addition, autoantibodies against ribonucleoprotein (RNP) induce NETs containing oxidized mitochondrial DNA, which activates the expression of IFNα in myeloid cells through activation of the STING pathway[20]. To our knowledge, anti-RNP autoantibodies are

only described in plasma of SLE patients and thus could be the trigger that induces the increased type I IFN signature in SLE. To clarify that, future studies should focus on NET release and IFNα secretion in response anti-RNP autoantibodies.

NET release of HC neutrophils in response to plasma of SLE and APS patients is increased compared to heterologous HC plasma-induced NET release. This suggests that plasma of SLE and APS contain specific factors that initiate NET release. We here demonstrate that increased NET release is associated with elevated levels of antinuclear and anti-dsDNA antibodies in SLE and APS patients, respectively (**Chapter 3**). We and others previously showed that autoantibodies, which are present in plasma of SLE and APS patients, are able to induce NET release[21,32,33,38,42,43], assuming that autoantibodies trigger neutrophils to release NETs. On a contrary, elevated levels of autoantibodies present in serum of SLE and APS patients could be due to increased NET release, which would suggest that the presence of autoantibodies is a consequence of NET release. What is cause and consequence is hard to distinguish and actually both statements could be true and would support the presence of a pathogenic loop in SLE.

SIRL-1 AS A POTENTIAL THERAPEUTIC TARGET TO SPECIFICALLY MODULATE NET RELEASE

Besides SLE and APS, NETs are present in other autoimmune diseases as well as cardiovascular diseases and cancer. However, it is unclear whether the progression and development of these diseases indeed rely on NET release. A recent study showed that NET release promotes the development and progression of liver metastases *in vivo* which is abrogated when mice are treated with DNase I or PAD4 inhibitors to abolish NET release[44]. In a thrombotic mouse model, NET-deficient mice have remarkably reduced thrombus formation compared to mice that are able to release NETs. Infusion of neutrophils rescues thrombus formation in wild type mice demonstrating that NET release is crucial for pathological venous thrombosis[45]. In a contrary, NET-deficient mice and wild type mice develop similar disease severity in an autoantibody-mediated mouse model of arthritis[46]. Taken together, these data indicate that NET release may contribute to the development and progression of some disease models in mice, however additional research is necessary to study the physiological consequence of NET release in human disease. Based on these data, we speculate that the release of NETs is an obnoxious consequence of the disease that contributes to additional complications, rather then the actual trigger that causes the disease. However, therapeutic treatments that reduce NET release could be beneficial in both scenarios and therefore it is important to continue development of NET-specific therapies.

Although the discovery of potential NET inhibitors is still in its infancy, various small molecule inhibitors and inhibitory receptors demonstrated promising results in reducing NET release *in vitro* and *in vivo* (**Chapter 4**). We previously showed that SIRL-1 is able to suppress NET release in response to plasma of SLE patients[21] and we here demonstrate that the intracellular bacterial killing of neutrophils remains preserved when SIRL-1 is triggered (**Chapter 5**). This suggests that SIRL-1 provides a potential therapeutic target to break the pathogenic loop in SLE without reducing neutrophil-mediated immune defense. As described above, the underlying signaling pathway of NET release depends on the stimulus of the neutrophil. MSU crystals, pro-inflammatory triggers present in the joints of gout patients, activate neutrophils via multiple mechanisms including FcγRIII and CD11b[47] and the P2Y6 receptor[48]. Likewise, opsonized *S. aureus*-induced NET release depends on Fc receptors, TLR2 and complement factor 3. We here confirm that MSU- and opsonized *S. aureus*-induced NET release depends on FcγRs, since human IgG Fc fragments partly reduce NET release. FcRs are ITAM-containing receptors that recruit Src-family kinases and SYK upon activation[49]. Others showed that SYK is an essential player in FcR-mediated neutrophil activation[19,50], which is consistent with our findings that NET release in response to MSU and opsonized *S. aureus* is regulated via the SYK-PI3K-mTORC pathway (**Chapter 2**). Of note, we demonstrate that SYK and PI3K are also important players in opsonized *S. aureus*-induced respiratory burst. This implicates that NET release and ROS production, induced by opsonized *S. aureus*, follows a similar signaling cascade, however SIRL-1 limits NET release but not respiratory burst in response to opsonized *S. aureus*. This indicates that SIRL-1 signaling acts downstream of PI3K affecting a signaling pathway, which specifically leads to NET release but not respiratory burst. Moreover, this also explains why SIRL-1 is unable to inhibit PMA-induced NET release, since this does not include the PI3K pathway.

The inability of SIRL-1 to suppress the respiratory burst in response to MSU and opsonized *S. aureus* is in contrast with our previous study that reported an inhibited FcγRIIa-mediated respiratory burst after SIRL-1 activation in primary neutrophils[51]. Fc receptors directly trigger ROS production via SYK- and PI3K-dependent mechanisms[52] (Fig. 1). Exposure of MSU and opsonized *S. aureus* to neutrophils trigger the respiratory burst via, amongst others, toll-like receptors (TLRs) that initiate downstream signaling via myD88 and activate the p38, JNK and NF-κB pathways[53]. In addition, signals from TLRs prime neutrophils that lead to a more robust activation of the NADPH oxidase complex[54]. We thus suggest that direct signaling via SYK and PI3K can be inhibited via SIRL-1 triggering, while additional signaling pathways triggered by TLRs are not affected by SIRL-1 (Fig. 1). At the same time, this marks the downside of SIRL-1 as a possible therapeutic treatment for NET-associated diseases because SIRL-1 will probably not suppress NET release that is initiated via

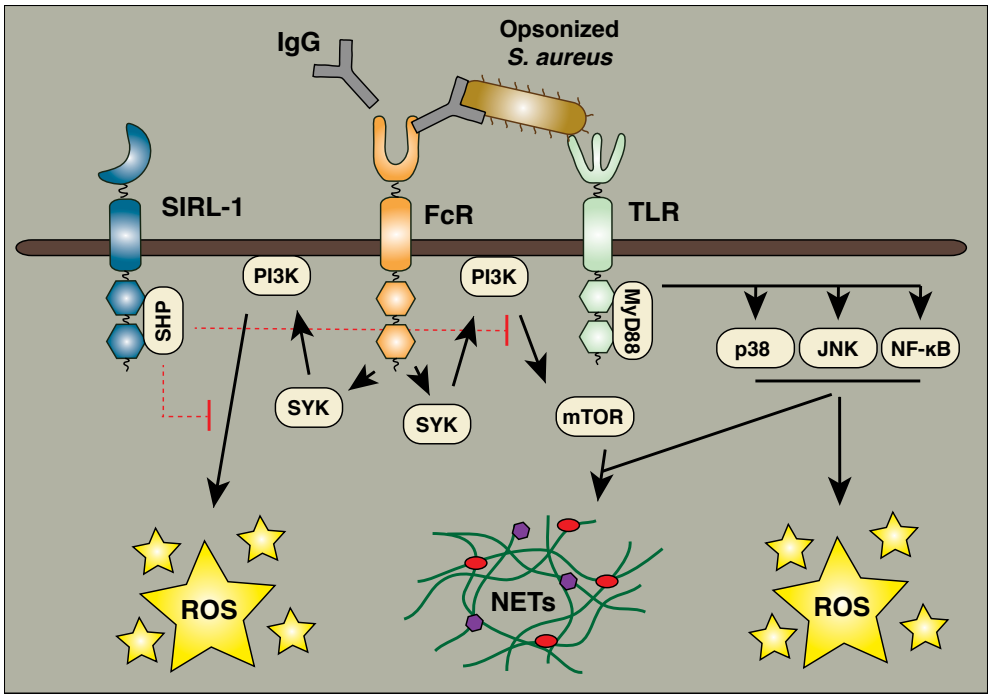


Figure 1 | **Downstream signaling of SIRL-1**
Upon activation of FcγRIIa with purified antibodies, innate immune cells (i.e. neutrophils and monocytes) produce ROS via a SYK and PI3K dependent manner. SIRL-1 signaling acts downstream of PI3K and suppress respiratory burst. When neutrophils are exposed to opsonized-*S. aureus*, FcRs and TLRs are triggered which results in the production of ROS and the release of NETs. SIRL-1 signaling acts on the SYK-PI3K pathways and suppresses opsonized-*S. aureus*-induced NET release while it is unable to suppress respiratory burst in response to opsonized-*S. aureus*.

signaling downstream pathways that are distinct from SYK and PI3K.

Given the role of SIRL-1 as a specific suppressor of NET release and the fact that LDGs are potent NET releasers in SLE and APS, we wonder whether SIRL-1 is able to modulate NET release in LDGs, which could benefit SLE and APS patients. We showed that SIRL-1 is expressed on the plasma membrane of LDGs (data not shown), however further experiments should determine whether activation of SIRL-1 suppresses NET release in LDGs. SIRL-1 is expressed on neutrophils as well as monocytes and eosinophils[55] and thus lacks the specificity to make it an ideal NET inhibitor. Indeed, expression of SIRL-1 on multiple cell types could lead to systemic effects *in vivo* when treating patients with SIRL-1 agonistic antibodies. Moreover, it is unknown whether inhibiting NET release via SIRL-1 may improves outcome in preclinical models. Thus, further investigation is necessary to evaluate the true potential of SIRL-1 to modulate NET release *in vivo*, which faces the hurdle of the absence of SIRL-1 expression in mice.

SIRL-1 INTERACTS WITH SHORT AMPHIPATHIC α-HELICAL PEPTIDES AND CONTROLS IMMUNE CELL ACTIVATION

We have characterized SIRL-1 as a negative regulator of immune responses in monocytes and neutrophils but, so far, the physiological mechanism of activation of SIRL-1 was unidentified. We here demonstrate that SIRL-1 interacts with the human cathelicidin peptide LL-37, which reveals novel insight in the physiological role of SIRL-1 in controlling immunological balance (**Chapter 6**). LL-37 is originally characterized in wounds to eliminate invading bacteria, fungi and viruses[56], however nowadays LL-37 has also been shown to interact with receptors to induce pro-inflammatory responses in innate immune cells[57]. Abundant LL-37 induces damage to host cells and therefore it is essential that LL-37 activity and LL-37-dependent pro-inflammatory signaling is counterbalanced to avoid healthy tissue injury. We here propose that LL-37 interaction with SIRL-1 functions as a negative feedback to suppress innate immune cells in tissue and to avoid abundant immune cell activity and healthy tissue damage (Fig. 2A). Furthermore, lipoproteins bind with LL-37 in tissue and blood to inhibit its cytolytic activity[58,59]. We therefore hypothesize that the lipoprotein-LL-37 complex interacts with SIRL-1 and functions as negative feedback to inform immune cells that danger has disappeared (Fig. 2B). The physiological relevance of SIRL-1 interaction with LL-37 may be deduced from the expression profile of SIRL-1. SIRL-1 is highly expressed on neutrophils, monocytes and eosinophils in circulation but becomes downregulated upon cell activation[51]. This implicates that SIRL-1 provides a threshold for activation of quiescent cells in circulation or for newly recruited cells to an inflammatory site.

Bacteria express molecules on their membrane or secrete them into the extracellular environment to trigger inhibitory-cell surface receptors and thereby manipulate signal transduction pathways in immune cells leading to increased bacterial survival[60]. We here demonstrate that *S. aureus* secretes PSMα3 that interacts with SIRL-1 to suppress the respiratory burst and thus propose that *S. aureus* exploits SIRL-1 to suppress antimicrobial responses and thereby evades elimination by immune cells (Fig. 2C). Like LL-37, PSMα3 has been shown to elicit other functions on human immune cells, which are primarily regulated through interaction with FPR2[61,62]. FPR2 is a pro-inflammatory receptor that initiates an immune response upon binding with PSMα3 and thus senses highly pathogenic Staphylococci, a mechanism developed by immune cells to recognize and counteract Staphylococci-associated danger. From the *S. aureus* point of view, interaction of PSMα3 with SIRL-1 represents an ingenious strategy to inhibit immune cell activation and thus escape immune cell killing. On the other hand, similar to LL-37, PSMs interact with lipoproteins present in tissue and blood, which neutralize their cytotoxic activity. From the host point of view, this PSM-lipoprotein complex could

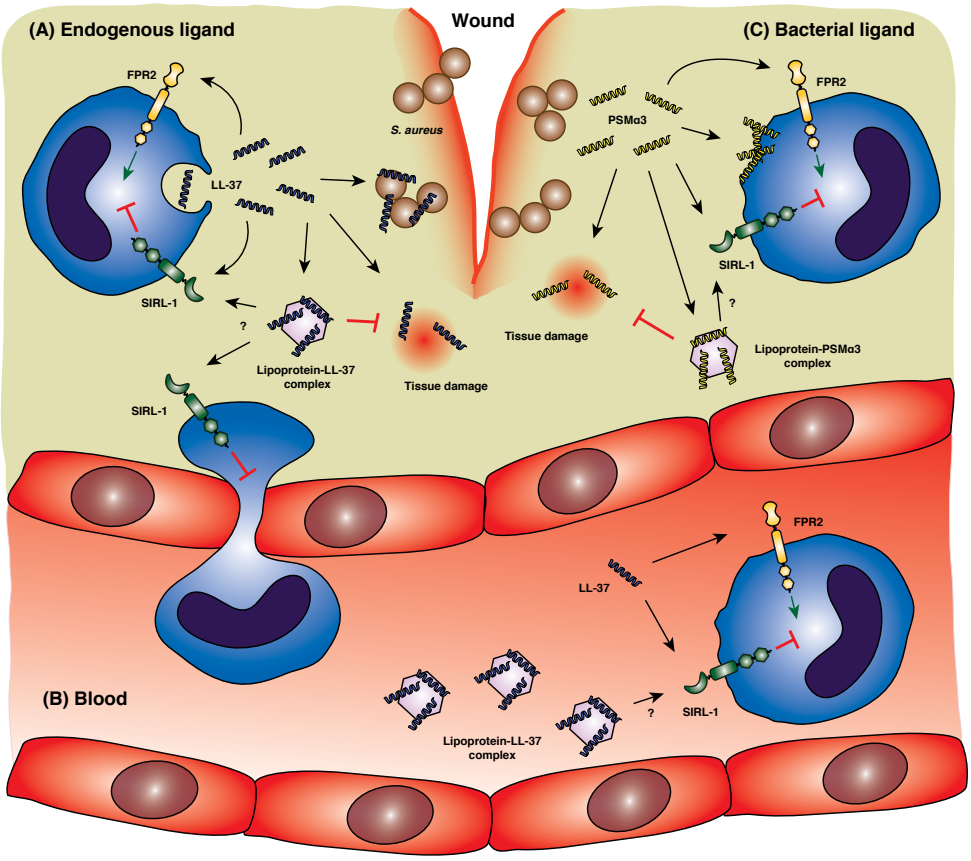


Figure 2 | **The role of PSMs and LL-37 in the regulation of innate immune responses**
(A) When bacteria invade the human body, innate immune cells (i.e. neutrophils or monocytes) become activated and release LL-37 to kill the intruder. Interaction of LL- 37 with FPR2, a pro-inflammatory receptor, leads to additional immune cell activation. However, LL-37 is cytotoxic and abundant exposure of LL-37 leads to healthy tissue damage. Therefore, LL-37 interacts with SIRL-1 to inhibit responses of already activated immune cells or newly recruited cells. Furthermore, interaction of LL-37 with lipoprotein neutralizes the cytotoxic activity of LL-37 and thus this complex could bind to SIRL-1 and function as negative feedback. (B) Free LL-37 as well as lipoprotein-LL-37 complex are present in blood and thus could provides a threshold for activation of quiescent cells in circulation. (C) However, when *S. aureus* invades the human body, it secretes PSM as virulence factors. PSMs induce tissue inflammation and are able to induce cytolysis of immune cells by binding in the plasma membrane. In addition, PSMs interact with SIRL-1 to suppress innate immune cell antimicrobial responses. The human body counteracts the activity by the expression of FPR2 that interacts with PSMs and thus senses Staphylococci-associated danger. Furthermore, lipoproteins interact with PSMs to neutralize their cytotoxicity and possibly serve as a SIRL-1 ligand.

again be a mechanism of negative feedback to inform immune cells that danger is over (Fig. 2C).

Cathelicidins are produced by a wide variety of species, including insects, reptiles and mammals and although these peptides are sequentially diverse, they share function, structure and mechanism[63,64]. This indicates that cathelicidins are most likely present for million of years and remained conserved during evolution. Bacteria have been designed to be adaptable to survive challenging environments. Molecular

mimicry is a powerful tool used by bacteria and other pathogens to modulate cellular activities of the host[65]. Based on the similarities of LL-37 and PSMs and the fact that LL-37 is present in the majority of animals, we speculate that PSMs of Staphylococci are generated as a result of molecular mimicry of LL-37. Similar to LL-37, PSMs interact with membranes of bacterial and eukaryotic cells to induce cytolysis that leads to cell death, which is a powerful mechanism to kill the opponent[66]. In addition, α-type PSMs interact with SIRL-1 to suppress antimicrobial activity of immune cells, which increases the survival rate of Staphylococci. Interestingly, molecular mimicry of LL-37 has been described before in parasitic helminthes. A peptide secreted by *Fasciola hepatica* (fhHDM-1) adopts a predominantly α-helical structure in solution and exhibit similar characteristics as LL-37[67]. Of note, eosinophils play an important role in the defense against helminthes and SIRL-1 is highly expressed on eosinophils[68]. Further investigation should determine whether fhHDM-1 molecules from helminthes interact with SIRL-1 on eosinophils to limit the microbial killing capabilities of immune cells.

A novel soluble protein, VSTM1-v2, is described to be a splice variant of SIRL-1 that lacks the transmembrane domain. Although the exact mechanism is unknown, VSTM1-v2 promotes differentiation and activation of Th17 cells, which suggests that VSTM1-v2 represents a secreted protein with cytokine activities that acts on a counter-receptor on T-lymphocytes[69]. Besides its cytokine activity, we suggest that VSTM1-v2 could act in wounds as a strategy for immune cells to compete with PSM-SIRL-1 interaction and thus VSTM1-v2 could function as a prevention mechanism against immune suppression by Staphylococci. On the other hand, VSTM1-v2 could interact with LL-37 in wounds to compete with LL-37-SIRL-1 interaction and thus function as an activation mechanism for immune cells at the moment it is necessary to eradicate the invading pathogen. As an example of such a mechanism, we reported earlier that LAIR-2 is a soluble receptor that, similar to LAIR-1, contains high affinity for collagens[70]. LAIR-2 prevents binding of LAIR-1 to collagen, which suggests that LAIR-2 has an immune regulatory function. Further research into the expression of VSTM1-v2 in immune cells at the moment of infection and into the possible role of VSTM1-v2 to prevent the interaction of SIRL-1 with PSMα3 and LL-37 is needed.

CONCLUDING REMARKS

In this thesis, we focused on the release of NETs by neutrophils and investigated their role in the defense against invading microbes and their contribution to disease pathogenesis. We conclude that NETs may be of more importance in autoimmunity than in innate immune defense. NETs have not been convincingly demonstrated to be the actual trigger that contributes to disease development and

progression. However, in most diseases it seems that NET release is an obnoxious consequence of the disease that contributes to additional complications. Actually, this still makes NETs interesting targets for the development of novel therapeutic approaches. Reduced NET release will contribute to a more immunosuppressive environment leading to decreased chronic inflammation. SIRL-1 is a promising target to suppress NET release, however SIRL-1 triggering specifically affects FcR-mediated responses. Other inhibitory receptors suppress NET release initiated via distinct activating signaling mechanisms. Thus, therapeutic treatment that combines multiple inhibitory receptors to suppress NET release will probably be more potent as a therapy for NET-associated diseases. Identification of the SIRL-1 endogenous and bacterial ligand contributes to our understanding of the physiological role of SIRL-1 in immune regulation. Simultaneously, it opens a new avenue to investigate SIRL-1 in bacterial infection and inflammatory diseases.

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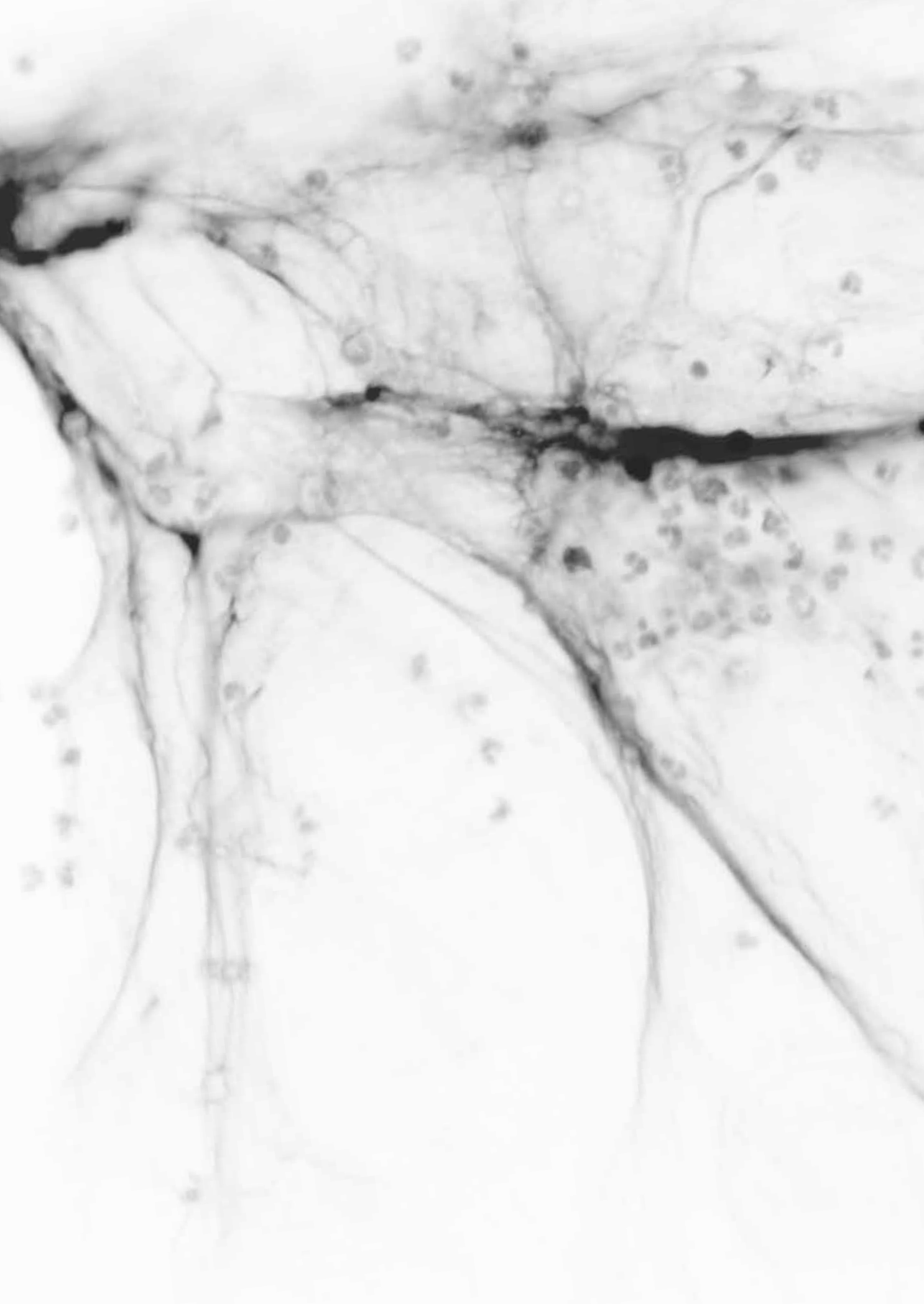


APPENDIX

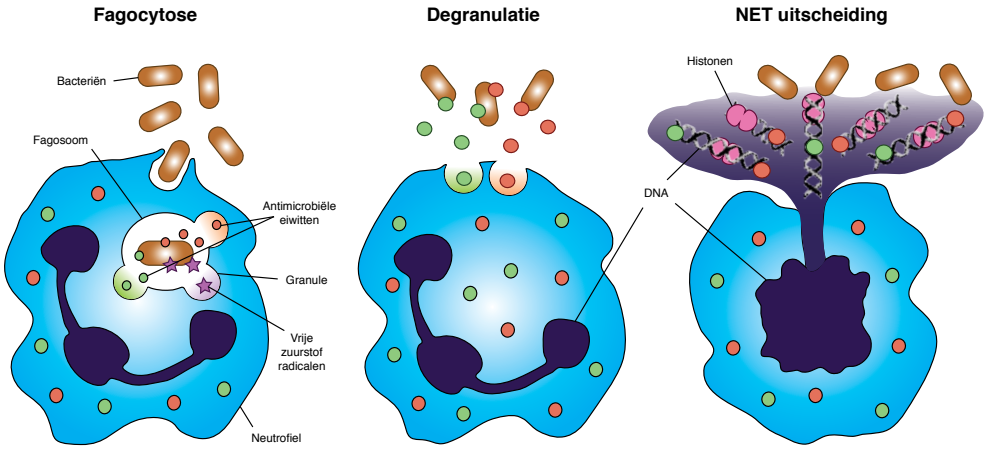
NEDERLANDSE SAMENVATTING

Het immuunsysteem is een complex netwerk dat mede bestaat uit witte bloedcellen (leukocyten) die het menselijk lichaam beschermen tegen ziekteverwekkers en kanker. Het immuunsysteem kan “eigen” van “niet eigen” onderscheiden en zal pathogenen, zoals bacteriën, virussen en schimmels, of kankercellen meteen vernietigen wanneer ze worden herkend. Er zijn verschillende soorten leukocyten met ieder een eigen functie. Een neutrofiel is een leukocyt die behoort tot het aangeboren immuunsysteem en het menselijk lichaam beschermt tegen indringers. Neutrofielen hebben een belangrijke rol bij de aanval en de opruiming van binnendringende pathogenen, maar tegelijkertijd leidt aanhoudende activatie van neutrofielen tot schade aan gezond weefsel. Daarom moet een immuunreactie door neutrofielen goed gereguleerd worden om het menselijk lichaam te beschermen tegen pathogenen zonder bijkomende weefselschade. Onze dagelijkse gezondheid hangt dus sterk af van een goed gebalanceerd immuunsysteem.

De neutrofiel

Neutrofielen worden gevormd in het beenmerg en migreren naar het bloed op het moment dat ze volledig ontwikkeld zijn. Neutrofielen zijn de meest voorkomende leukocyten in het bloed (~$5x10^9$ neutrofielen per liter bloed = 50-70% van alle leukocyten) en bevinden zich daar in een niet geactiveerde staat. Op het moment van infectie of steriele ontsteking komen er verschillende pro-inflammatoire (ontstekingsbevorderende) moleculen vrij die de neutrofielen in staat van alertheid brengen waardoor ze infiltreren in het geïnfecteerde of beschadigde weefsel. Uiteindelijk worden deze neutrofielen maximaal geactiveerd en vernietigen ze de pathogenen of het necrotisch (doodgaand) weefsel.

Neutrofielen beschikken over een uitgebreid wapenarsenaal dat ze gebruiken om de vijand te elimineren (Figuur 1). Allereerst zijn neutrofielen in staat om pathogenen ‘op te eten’ (fagocyteren). Bij dit proces worden pathogenen omsloten in fagosomen die vervolgens fuseren met granulen. Deze granulen beschikken over antimicrobiële eiwitten en vrije zuurstof radicalen die de pathogenen doden. Fagocytose is een snelle en relatief schone manier van eliminatie, omdat het plaatsvindt in de neutrofiel (intracellulair) waardoor er minimale schade is aan omliggend gezond weefsel. Daarnaast kan een neutrofiel zijn antimicrobiële eiwitten uitscheiden in de extracellulaire omgeving, dit proces wordt degranulatie genoemd. Tenslotte is de neutrofiel in staat zijn desoxyribonucleïnezuur (DNA) naar buiten te spugen wat zich vervolgens verspreidt in web-achtige structuren (zie kaft van dit proefschrift). Deze structuren van DNA worden “Neutrofiel Extracellular Traps” (NETs) genoemd en hebben de eigenschap dat pathogenen er in blijven kleven. De antimicrobiële eiwitten die aanwezig zijn in de NETs zorgen voor vernietiging van de



Figuur 1 | **Het wapenarsenaal van de neutrofiel**
Neutrofielen kunnen bacteriën en andere pathogenen fagocyteren, waarbij de pathogenen worden omsloten in een fagosoom dat vervolgens fuseert met granulen. Deze granulen beschikken over antimicrobiële eiwitten of vrije zuurstof radicalen die de pathogenen doden. Verder kan een neutrofiel ook zijn antimicrobiële eiwitten uitscheiden in de extracellulaire omgeving om pathogenen te doden. Dit proces wordt degranulatie genoemd. Tenslotte kunnen neutrofielen hun DNA naar buiten spugen wat zich verspreidt in een web-achtige structuur. Deze structuren worden NETs genoemd en bevatten antimicrobiële eiwitten om de pathogenen te doden.

pathogenen. Het exacte mechanisme dat de neutrofiel gebruikt om te kiezen welke aanvalstechniek hij gaat hanteren, is tot op heden nog onbekend. Recent onderzoek laat zien dat een neutrofiel mogelijk het formaat van pathogenen detecteert en specifiek NETs uitscheid als reactie op grote pathogenen terwijl kleinere pathogenen worden gefagocyteerd.

De goede en slechte eigenschappen van NETs

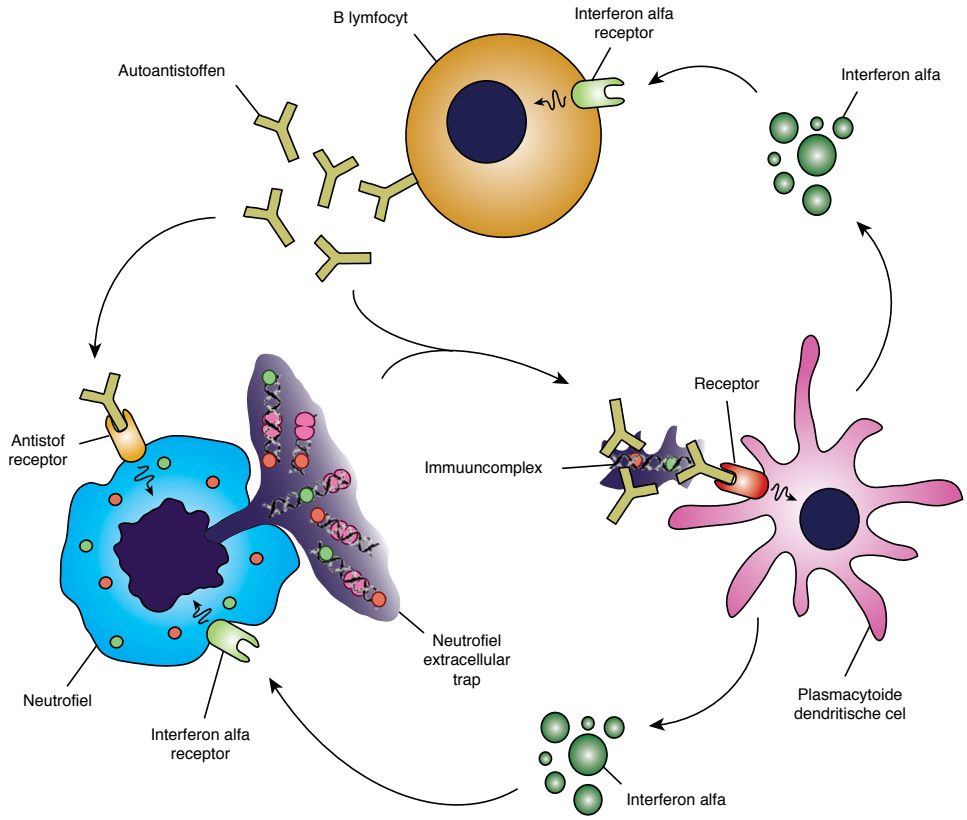
In 2004 werden NETs voor het eerst beschreven na de blootstelling van neutrofielen aan bacteriën. Later kwam men erachter dat NETs ook worden uitgescheiden als reactie op virussen en schimmels maar ook na blootstelling aan cytokines (moleculen die het immuunsysteem reguleren) en zelfs chemische stoffen. Hoewel *in vitro* (Latijnse benaming voor in een reageerbuis) experimenten laten zien dat NETs in staat zijn om pathogenen te doden, is de exacte rol van NETs *in vivo* (Latijnse benaming voor in een lichaam) nog niet helemaal duidelijk. Experimenten met NET-knock-out muizen (muizen waarvan de neutrofielen geen NETs uitscheiden) geven tegenstrijdige resultaten. Enerzijds zijn NET-knock-out muizen meer gevoelig voor bacteriële infecties, wat ervoor pleit dat NETs een belangrijke antimicrobiële rol spelen. Andere experimenten laten zien dat neutrofielen van NET-knock-out muizen prima in staat zijn om bacteriën te elimineren en infecties te voorkomen. Dit suggereert dus dat NETs helemaal niet nodig zijn voor de bescherming tegen pathogenen. Vervolg onderzoek is noodzakelijk om de exacte rol van NETs als antimicrobieel verdedigingsmechanisme *in vivo* te bepalen.

Het is van enorm belang dat de regulatie van NETs gecontroleerd verloopt, omdat er bij een overmaat aan NETs of een te lange blootstelling van NETs aan lichaamscellen schade ontstaat aan gezond weefsel. De antimicrobiële eiwitten en histonen (eiwitten waar het DNA omheen is gewonden) in NETs zijn erg toxisch voor gezonde lichaamscellen. Daarnaast is er aangetoond dat NETs zorgen voor vertraging van wondgenezing. Dit komt mede doordat de NETs bloedvaten versperren en daardoor een blokkade vormen voor moleculen die belangrijk zijn voor weefselherstel. Bovendien blijken NETs een belangrijke rol te spelen in de ontwikkeling van trombose. De aanwezigheid van NETs in bloedvaten zorgt voor samenklontering van bloedplaatjes, rode bloedcellen en eiwitten die een functie hebben bij bloedstolling. Deze klonten (genaamd trombus) blokkeren de bloedstroom en verminderen bloedtoevoer waardoor weefsel afsterft. Wanneer dit gebeurt in het hart of de hersenen is een hartinfarct of beroerte het gevolg. De vorming van trombus worden ook geassocieerd met de ontwikkeling en uitzaaiing van kanker. Wanneer kankercellen los komen uit de originele tumor en zich in het bloedvat bevinden, blijven ze kleven in de NET-geïnduceerde trombus en zal er op die plek een uitzaaiing van de tumor ontstaan.

Naast de bijdrage van NETs aan bovenstaande ziektes, is duidelijk dat NETs een belangrijke rol spelen in veel auto-immuunziektes: reumatoïde artritis (RA), systemische lupus erythematodes (SLE), antifosfolipiden syndroom (APS) en vasculitis. Deze ziektes worden gekenmerkt door grote hoeveelheden van een bepaald soort antistoffen in het bloed van de patiënt. Normaal beschermen antistoffen het lichaam tegen ziekteverwekkers van buitenaf, maar de antistoffen in patiënten met auto-immuunziektes herkennen lichaamseigen moleculen en worden daarom auto-antistoffen genoemd. Uitgebreide studies in SLE patiënten hebben aangetoond dat deze auto-antistoffen neutrofielen activeren waarna NETs worden uitgescheiden. Vervolgens binden de auto-antistoffen aan de NETs en vormen immuuncomplexen (ICs). Deze ICs activeren een andere soort leukocyt, genaamd plasmacytoide dendritische cel (pDC), wat resulteert in de productie van de pro-inflammatoire cytokine interferon alfa (IFNα). Daaropvolgend stimuleert IFNα enerzijds de productie van meer auto-antistoffen terwijl IFNα anderzijds neutrofielen stimuleert om meer NETs uit te scheiden (Figuur 2). Het immuunsysteem in SLE patiënten wordt dus continu geactiveerd wat leidt tot ontstekingen overal in het lichaam, zoals huid, gewrichten, nieren en hersenen. Tot op heden is het onmogelijk om SLE patiënten te genezen maar wij en vele andere wetenschappers denken dat het blokkeren van NET uitscheiding door neutrofielen een mogelijke therapie kan zijn voor patiënten met SLE of ander auto-immuunziektes. *In vivo* experimenten in SLE muis modellen geven veelbelovende resultaten en laten zien dat het blokkeren van NET uitscheiding leidt tot vermindering van ernst van de ziekte. Vervolgonderzoek is noodzakelijk om dit ook in de mens aan te tonen.

Inhibitoire receptoren op neutrofielen reguleren NET uitscheiding

Op het oppervlak van neutrofielen bevinden zich veel verschillende eiwitten (receptoren) die binden aan moleculen uit de omgeving, zoals eiwitten van pathogenen, eiwitten van lichaamscellen of cytokines. De binding van deze moleculen (genaamd liganden) aan receptoren is specifiek, zoals een sleutel in een slot, en is een vorm van communicatie tussen de omgeving en de neutrofiel. Op het moment dat een ligand bindt aan een activerende receptor van de neutrofiel, zal er een uitgebreide intracellulaire signaleringscascade worden gestimuleerd. Dit zal uiteindelijk leiden tot de activatie van de neutrofiel. Onderzoek heeft uitgewezen dat de binding van liganden aan verschillende soorten activerende receptoren van neutrofielen leidt tot de uitscheiding van NETs.



Figuur 2 | Continue activatie van het immuunsysteem in SLE patiënten
Grote hoeveelheden auto-antistoffen in het bloed van SLE patiënten activeren neutrofielen om NETs uit te scheiden. Auto-antistoffen die binden aan NETs vormen immuuncomplexen. Deze immuuncomplexen activeren plasmacytoide dendritische cellen die vervolgens interferon alfa gaan produceren. Interferon alfa zal enerzijds neutrofielen activeren om meer NETs uit te scheiden terwijl anderzijds interferon alfa B lymfocyten activeert om meer auto-antistoffen te maken.

Naast activerende receptoren heeft de neutrofiel ook inhibitoire receptoren. Binding van liganden aan deze inhibitoire receptoren zal uiteindelijk leiden tot remming van een immuunreactie. Deze inhibitoire receptoren zijn dus belangrijk voor de balans van neutrofiel activatie en zorgen er voor dat er geen ongewenste weefselschade ontstaat door langdurig geactiveerde neutrofielen. Wij doen onderzoek naar de functie van één van de inhibitoire receptoren, genaamd "Signal Inhibitory Receptor on Leukocytes-1" (SIRL-1). Eerder onderzoek van ons heeft aangetoond dat SIRL-1 in staat is om de productie van vrije zuurstof radicalen te remmen. Daarnaast hebben we aangetoond dat, wanneer we neutrofielen stimuleren met auto-antistoffen van SLE patiënten, neutrofielen NETs uitscheiden en dat uitscheiding van NETs wordt verminderd door SIRL-1. SIRL-1 is niet de enige inhibitoire receptor die NET uitscheiding vermindert, ook andere inhibitoire receptoren zoals Sialic Acid-Binding Ig-Like Lectin-9 (Siglec-9) en Leukocyte-Associated Immunoglobulin-Like Receptor-1 (LAIR-1) remmen de uitscheiding van NETs. Dit geeft aan dat inhibitoire receptoren belangrijk zijn bij het tegengaan van overvloedige uitscheiding van NETs en dus zijn inhibitoire receptoren een mogelijk richtpunt voor therapeutische behandelingen van NET-gerelateerde ziektes, zoals SLE.

Doel van het onderzoek beschreven in dit proefschrift

Zoals hierboven staat beschreven, hebben NETs goede en slechte eigenschappen. NETs dragen bij aan de bescherming van het menselijk lichaam tegen bacteriële infecties terwijl NETs ook een rol spelen bij de ontwikkeling van verschillende ziektes. We spreken dus over een spreekwoordelijke "dunne lijn tussen vriend en vijand". In dit proefschrift streven we ernaar om meer te weten te komen over de exacte functies van NETs in gezonde en zieke situaties. Verder onderzoeken we of NETs een mogelijk doelwit zijn voor therapeutische behandelingen, daarbij bestuderen we of de inhibitoire receptor SIRL-1 te gebruiken is als therapie.

Hoofdstuk 2: Visualisatie en kwantificatie van NET uitscheiding

Sinds de ontdekking van NETs zijn er verschillende technieken beschreven om NETs te bestuderen, echter hebben deze experimentele methodes hun beperkingen. In **Hoofdstuk 2** wordt een nieuwe methode beschreven om, met behulp van microscopie, NET kinetiek (NET uitscheiding door de tijd heen) te visualiseren en kwantificeren. We laten zien dat onze techniek onderscheid maakt tussen NET uitscheiding en andere manieren van celdood, iets wat andere methodes vaak niet kunnen. Bovendien tonen we aan dat, afhankelijk van de stimulus waaraan de neutrofiel wordt blootgesteld, NETs snel of laat uitgescheiden worden. Veel studies gebruiken de chemische stof phorbol myristate acetaat (PMA) om NET uitscheiding te stimuleren bij neutrofielen. Hoewel PMA een krachtige stimulator is, tonen wij aan dat de NET kinetiek en de intracellulaire signaalcascade als reactie op PMA

verschilt van fysiologische stimuli zoals bacteriën, ICs en uraatkristallen (aanwezig in de gewrichten van jicht patiënten). Uit deze ontdekkingen concluderen we dus dat PMA niet moet worden gebruikt voor het bestuderen van NETs omdat dit dan op een compleet andere manier gebeurt dan bij fysiologische stimuli.

Hoofdstuk 3: Een associatie tussen NET uitscheiding en auto-antistoffen in SLE en APS
NET uitscheiding wordt gestimuleerd door auto-antistoffen van SLE en APS patiënten. Deze auto-antistoffen worden geïsoleerd uit het bloed van patiënten en vervolgens blootgesteld aan neutrofielen. Echter, in het bloed van SLE en APS patiënten zitten naast auto-antistoffen nog vele andere moleculen die mogelijk NET uitscheiding reguleren. Verder worden SLE en APS altijd los van elkaar onderzocht in kleinschalige studies terwijl het overlappende auto-immuunziektes zijn die gezamenlijk in een patiënt kunnen optreden. In **Hoofdstuk 3** wordt in een grootschalige studie onderzocht wat het effect is op gezonde neutrofielen na blootstelling aan bloedplasma van patiënten met SLE, APS of SLE+APS. We tonen aan dat de uitscheiding van NETs is verhoogd na blootstelling aan bloedplasma van SLE, APS en SLE+APS ten opzichte van gezond bloedplasma. Verder is ook de hoeveelheid auto-antistoffen gemeten in het bloedplasma van SLE en APS patiënten. We laten zien dat er een correlatie is tussen NET uitscheiding en de hoeveelheid auto-antistoffen in SLE en APS, dus meer auto-antistoffen komt overeen met verhoogde NET uitscheiding. Uit onze studie concluderen we dat naast geïsoleerde auto-antistoffen ook bloedplasma zelf in staat is om NET uitscheiding te stimuleren en dat de auto-antistoffen in het bloedplasma daar waarschijnlijk een grote rol bij spelen in zowel SLE als APS patiënten.

Hoofdstuk 4: Het reguleren van neutrofiel activatie, de strategieën en gevolgen
Hoofdstuk 4 bestaat uit een literatuurstudie die beschrijft welke ziektes worden gekenmerkt door overvloedige activatie van neutrofielen en omschrijft de resultaten van verschillende klinische studies waarin het effect van neutrofielen wordt getest na toedienen van anti-ontstekingsmedicijnen. Echter, het onderdrukken van neutrofielen is gevaarlijk en daarom beschrijven we de aandoeningen die ontstaan in ziektes waarbij patiënten geen of verminderende neutrofiel activiteit hebben.

De ontdekking van medicijnen die NET uitscheiding remmen, staat tot op heden nog in de kinderschoenen, maar er worden al enkele niet volledig ontwikkelde behandelingen beschreven die veelbelovende resultaten laten zien. We bespreken de voor- en nadelen van deze behandelingen.

Hoofdstuk 5: SIRL-1 remt NET uitscheiding en laat andere neutrofiel functies intact
Ons eerdere onderzoek heeft aangetoond dat het uitscheiden van NETs plaatsvindt na blootstelling van neutrofielen aan auto-antistoffen van SLE patiënten en dat

NET uitscheiding wordt verminderd door SIRL-1. SIRL-1 is dus in staat om NET uitscheiding te remmen. In **Hoofdstuk 5** tonen we aan dat SIRL-1 NET uitscheiding ook remt na blootstelling aan andere stimuli, zoals bacteriën en uraatkristallen, terwijl NET uitscheiding niet wordt geremd door SIRL-1 na blootstelling aan PMA. Hieruit concluderen we dat SIRL-1 invloed heeft op bepaalde, maar niet alle, intracellulaire signaalcascades die betrokken zijn bij NET uitscheiding.

Het remmen van neutrofiel activiteit is gevaarlijk, dus willen wij specifiek NET uitscheiding remmen terwijl andere neutrofiel functies behouden blijven. Hier laten we zien dat het vermogen van de neutrofiel om bacteriën te doden niet verandert door remming van NET uitscheiding met SIRL-1. We concluderen dus dat SIRL-1 een mogelijke therapeutische behandeling zou kunnen zijn voor NET-gerelateerde ziektes zoals SLE, omdat het specifiek NETs remt terwijl de neutrofiel nog in staat is om bacteriën te doden en dus het lichaam te beschermen tegen infecties.

Hoofdstuk 6: Het ligand van SIRL-1

SIRL-1 is een inhibitoire receptor en zoals hierboven staat beschreven heeft elke receptor een ligand, een molecuul dat specifiek bindt aan de receptor. Binding van het ligand aan SIRL-1 zorgt ervoor dat SIRL-1 'aanstaat' wat uiteindelijk leidt tot remming van de neutrofiel. Tot op heden was het ligand van SIRL-1 nog onbekend maar in **Hoofdstuk 6** worden twee moleculen beschreven die SIRL-1 kunnen aanzetten. Het eerste molecuul wordt uitgescheiden door *Staphylococcus aureus* (een bepaalde bacterie soort). Hieruit concluderen we dat deze bacterie een strategie heeft ontwikkeld om neutrofielen te remmen via SIRL-1 en daardoor een mogelijkheid creëert om te ontsnappen aan neutrofielen. Het tweede molecuul dat SIRL-1 aanzet, wordt uitgescheiden door neutrofielen en andere leukocyten die aanwezig zijn in het bloed van het menselijk lichaam. We concluderen hieruit dat het immuunsysteem een manier heeft ontwikkeld om zichzelf te remmen en dus overvloedige activatie van neutrofielen tegen gaat.

Conclusie

In dit proefschrift hebben we ons gefocust op NET uitscheiding door neutrofielen en hebben we de rol van NETs in gezonde en zieke situaties onderzocht. We concluderen dat NETs belangrijker zijn in de ontwikkeling van auto-immuunziektes dan in de bescherming tegen pathogenen. Echter, de kennis is nog niet overtuigend dat NETs daadwerkelijk de oorzaak zijn van de ontwikkeling en progressie van ziektes. Sterker nog, in de meeste gevallen lijkt NET uitscheiding een onaangenaam gevolg te zijn van een ziekte wat uiteindelijk bijdraagt aan extra complicaties. Toch maakt dat NET uitscheiding niet minder interessant als mogelijk richtpunt voor therapeutische behandeling.

Verminderde NET uitscheiding leidt tot verlaagde auto-immuniteit en dus

afnemende chronische ontsteking. SIRL-1 zou een mogelijke rol kunnen spelen in nieuwe therapeutische behandelingen om NET uitscheiding te remmen, alleen is SIRL-1 niet in staat om elke signaalcascade, die leidt tot NET uitscheiding, te remmen. Meerdere inhibitoire receptoren laten zien NET uitscheiding te remmen en daarom zou het kunnen dat een therapie, die gebruik maakt van een combinatie van inhibitoire receptoren, krachtiger is. Deze gedachte opent nieuwe wegen voor de behandeling van toekomstige NET-geassocieerde ziektes.

DANKWOORD

Trots! Dat is het woord wat in me opkomt als ik mijn proefschrift in handen heb. Een geweldig resultaat na 4,5 jaar onderzoek. Ik realiseer me dat de vele samenwerkingen met collega's en de steun van het thuisfront bijdragen aan de totstandkoming van dit proefschrift. Graag wil ik daar iedereen voor bedanken.

Allereerst Linde! Bedankt voor de mogelijkheid dat ik mijn promotieonderzoek in jouw lab uit heb mogen voeren. Het was een leuke en leerzame tijd. Ik besef dat ik de laatste jaren veel heb bijgeleerd en dat jij daar een belangrijke rol in hebt gespeeld. Met veel plezier en interesse heb ik in je onderzoeksgroep gewerkt en zo zal ik er altijd over terugdenken. Samen met Louis weet je een leuke en goede groep mensen samen te stellen die hard vóór en met elkaar willen werken. Hopelijk blijft deze succesformule nog lang voortbestaan.

A special thanks to the people with whom I closely collaborated. Louis Bont, voor de waardevolle input in mijn project en de leuke momenten tijdens werkoverleg en groepsuitjes. Margreet Westerlaken, voor de intensieve samenwerkingen en de leuke momenten die we op het lab hebben beleefd. Je bent een fijne collega. Luuk van den Hoogen, voor het SLE en APS cohort en je waardevolle bijdrage aan deze studie. Ik heb met heel veel plezier met je samengewerkt. Michiel van der Vlist, voor je kritische maar zeer waardevolle bijdrage aan de NET live imaging studie. Ik heb erg veel respect voor je en denk graag terug aan de muzikale momenten op het lab. Nina van Sorge, voor de waardevolle input in de SIRL-1 ligand studie en voor alle bacterie-gerelateerde informatie die je me gegeven hebt. Kristof Van Avondt, voor de hulp en de goede samenwerkingen tijdens de eerste maanden van mijn promotieonderzoek. Cor Breukel, voor het moleculaire kloneringswerk dat je gedaan hebt om SIRL-1 transgene muizen te genereren. Margot Linssen, voor het assisteren en uitvoeren van de *in vivo* SIRL-1 transgene muis proeven. David Egan, for the valuable tips and trics about live imaging. Wim de Lau, voor de hulp en de informatie die noodzakelijk waren voor de zoektocht naar het ligand van SIRL-1. Joris van Montfrans, voor het aanleveren van bloed van CGD patiënten. Teck Low, for the mass spectrometry experiments that you performed to find the SIRL-1 ligand. Jan vd Linden, voor de onmogelijke taak om mij kennis te laten maken met de BD Pathway.

Sincere acknowledgements for the people who helped me in- and outside the lab and gave me materials and protocols to perform experiments. Sjef Verbeek, Paul Naccache, Rob van Dalen, Nienke ter Haar, Nadia Vazirpanah, Ciara Angiolilli, Alsya Affandi, Sandra Cardoso, Erinke van Grinsven, Maaike Nederend, Lei Houben,

Deon Kanters, Tieneke Kraaij, Lieneke Jongeneel, Julia Drylewicz, Gerrit Spierenburg, Sigrid Otto, Jeroen van Velzen, Pien van der Burght, Henk Aanstoot, Saskia ter Braak and Yvonne Schemkes.
Many thanks to all the people that gave me valuable advices and with whom I had fun scientific discussions: Leo Koenderman, Kiki Tesselaar, José Borghans, Nienke Vrisekoop, Niels Bovenschen, Kris Reedquist, Tim Radstake, Ruth Fritsch-Stork, Joel van Roon, Albert Heck and Jos van Strijp.

Thanks to all the members of the LTI. I will remember all the nice little talks in the corridor and next to the coffee machine. Daarnaast wil ik ook de mensen uit onze OIO kamer bedanken voor alle gezelligheid over de jaren: Francesca, Tessa, Eefje, Sofie, Ruben, Fleurieke, Annemieke, Selma, Kuldeep, Inês, Maarten en Maarten.

Of course I am grateful to all the current and former members of the Meyaard/ Bont group. Thanks to you, I had a great time in Utrecht. I will never forget the nice and funny moments (i.e. joking around, singing and “Indian” dancing) that we have experienced during lab work, group meetings, retreat and conferences. Louis, Marieke, Michiel, Inês, Kuldeep, Doron, Matevž, Sjanna, Tiago, Ruben, Helen, Margreet, Susi, Thijs, Amie, Marije, Marloes, Sjamir, Kristof, Barbara, Sepide, Sjors, Amelia, Anita, Sanne, Steven, Carien, Kim, Frederique, Florianne, Floor, Lara, Jorn and Wouter, I will miss you…Amígos!!

Florianne, graag wil ik je bedanken voor je bijdrage aan mijn promotieonderzoek. Ik ben met heel veel plezier je begeleider geweest. Met deze inzet en enthousiasme gaat het helemaal goed komen met je wetenschappelijke carrière.

A special thanks goes to my paranymphs, Matevž and Giel, for the all things that they arranged to complete my PhD. Matevž, when I think about the funny and crazy moments that we experienced during lab work, a smile appears on my face. I have a new friend and hopefully that will stay forever. Giel, ik ben enorm blij met de cover en lay-out van mijn proefschrift en dit heb ik volledig te danken aan jou.

Bovendien wil ik mijn familie en vrienden enorm bedanken voor hun belangstelling en gezelligheid de afgelopen jaren. Pap, Mam, Roy en Kristi bedankt voor de steun en vertrouwen die jullie me hebben gegeven. Lieve Selly, heel erg bedankt voor jou begrip, steun en liefde. Een hele dikke kus en knuffel voor jou.

CURRICULUM VITAE

Maarten van der Linden werd geboren op 20 maart 1986 in Sint Oedenrode. Na het behalen van zijn HAVO diploma aan het Zwijsen College in Veghel is hij in september 2005 begonnen met de studie biologie en medisch laboratoriumonderzoek aan de Avans Hogeschool in Breda. Tijdens zijn bachelor voerde hij een onderzoeksstage uit aan the University of Tampere, Institute of Medical Technology, Biochemistry of Cell Signaling in Tampere, Finland onder begeleiding van Dr. Herma Renkema. Zijn afstudeerstage volgde hij bij Schering-Plough afdeling Molecular Pharmacology in Oss onder begeleiding van Toon van der Doelen. In september 2009 is Maarten gestart met de studie Medical Biology aan de Radboud Universiteit Nijmegen. Tijdens zijn master voerde hij een onderzoeksstage uit aan het Radboud Universitair Medisch Centrum afdeling Pathologie in Nijmegen onder begeleiding van Dr. William Leenders en Dr. Ilse Roodink. Zijn afstudeerstage volgde hij bij Philips research afdeling Molecular Diagniostics in Eindhoven onder begeleiding van Dr. Kalyan Dulla. In april 2013 begon Maarten als promovendus bij de afdeling Laboratory of Translational Immunology van het Universitair Medisch Centrum Utrecht onder begeleiding van Prof. Dr. Linde Meyaard. De resultaten van zijn onderzoek staan beschreven in dit proefschrift.

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*,# Authors contributed equally to the work