University of Veterinary Medicine Hannover
Department of Physiological Chemistry

The role of the transcription factor HIF-1α in the formation of antimicrobial phagocyte extracellular traps

THESIS
Submitted in partial fulfilment of the requirements for the degree

DOCTOR OF PHILOSOPHY
(PhD)

awarded by the University of Veterinary Medicine Hannover

by

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Braunschweig

Hannover, Germany 2014
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Date of final exam: 05.11.2014

Parts of the thesis have been published previously in:

Sponsorship:
DFG grant KO 3552/4-1
DAAD fellowship to Lena Völlger
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List of abbreviations

AMP          antimicrobial peptide
ARNT         aryl hydrocarbon receptor nuclear translocator
ATRA         all-trans retinoic acid
BMMCs        bone marrow-derived mast cells
BSA          bovine serum albumin
C57BL/6      C57 black 6, common inbred strain of laboratory mouse
Ca^{2+}      calcium
Cl-amidine   chloramidine, PAD4 inhibitor
CBP          CREB-binding protein
CFU          colony forming units
CGD          chronic granulomatous disease
CRAMP        cathelicidin-related antimicrobial peptide
CREB         cAMP response element-binding protein
CoCl_{2}     cobalt chloride
Cu^{2+}      copper
DAPI         4′,6-Diamidino-2-phenylindol
DFO          desferrioxamine
DMSO         dimethyl sulfoxide
DNA          deoxyribonucleic acid
dsDNA        double-stranded DNA
DPI          diphenyleneiodonium chloride
ET           extracellular traps
E. coli      escherichia coli
EPO          erythropoietin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FIH</td>
<td>factor inhibiting HIF</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAS</td>
<td>group A streptococci</td>
</tr>
<tr>
<td>GO</td>
<td>glucose oxidase</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia inducible factor</td>
</tr>
<tr>
<td>HIF-1</td>
<td>hypoxia inducible factor 1</td>
</tr>
<tr>
<td>HIF-1$\alpha$</td>
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</tr>
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<tr>
<td>HIF-3</td>
<td>hypoxia inducible factor 3</td>
</tr>
<tr>
<td>HL-60</td>
<td>human promyelocytic leukemia cells</td>
</tr>
<tr>
<td>HMC-1</td>
<td>human mast cells</td>
</tr>
<tr>
<td>HRE</td>
<td>hypoxia responsive element</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL-8</td>
<td>interleukin-8</td>
</tr>
<tr>
<td>IMDM</td>
<td>iscove’s modified dulbecco’s medium</td>
</tr>
<tr>
<td>IPAS</td>
<td>inhibitory PAS domain protein</td>
</tr>
<tr>
<td>LL-37 (hCAP 18)</td>
<td>cathelicidin, human cationic antimicrobial protein</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>magnesium</td>
</tr>
<tr>
<td>MC</td>
<td>mast cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>MCETs</td>
<td>mast cell extracellular traps</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>Min</td>
<td>minutes</td>
</tr>
<tr>
<td>MN</td>
<td>micrococcal nuclease</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>m-RNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MRSA</td>
<td>methicillin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NE</td>
<td>neutrophil elastase</td>
</tr>
<tr>
<td>NETs</td>
<td>neutrophil extracellular traps</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>superoxide</td>
</tr>
<tr>
<td>PAD4</td>
<td>peptidylarginine deiminase 4</td>
</tr>
<tr>
<td>PAS</td>
<td>Per/Arnt/Sim</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PETs</td>
<td>phagocyte extracellular traps</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PHD</td>
<td>prolyl hydroxylases</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear neutrophils</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute Medium</td>
</tr>
<tr>
<td>rpm</td>
<td>Rounds per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Staphylococcus Aureus</td>
</tr>
<tr>
<td>S. flexneri</td>
<td>Shigella Flexneri</td>
</tr>
<tr>
<td>S. pneumonia</td>
<td>Streptococcus Pneumoniae</td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>Streptococcus Pyogenes</td>
</tr>
<tr>
<td>shRNA</td>
<td>Small Hairpin RNA / Short Hairpin RNA</td>
</tr>
<tr>
<td>si-RNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor β</td>
</tr>
<tr>
<td>THB</td>
<td>Todd Hewitt Broth</td>
</tr>
<tr>
<td>TNF α</td>
<td>Tumor Necrosis Factor α</td>
</tr>
<tr>
<td>TPEN</td>
<td>N,N,N',N'-Tetrakis(2-Pyridylmethyl)ethylenediamine</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>vHL</td>
<td>Von Hippel-Lindau Complex</td>
</tr>
<tr>
<td>wt</td>
<td>Wild Type</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>Zinc</td>
</tr>
<tr>
<td>µg</td>
<td>Micro Gram</td>
</tr>
<tr>
<td>µm</td>
<td>Micro Meter</td>
</tr>
<tr>
<td>µM</td>
<td>Micro Molar</td>
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</tbody>
</table>
Summary

Phagocyte extracellular trap (PET) formation has recently been described as a novel host innate immune defense of phagocytes. PETs consist of a DNA backbone with associated histones, proteases and antimicrobial peptides. Those PETs provide a matrix to entrap and kill microbes. They were discovered in neutrophils for the first time and named as neutrophil extracellular traps (NET). Nevertheless, besides neutrophils also mast cells, eosinophils and macrophages/monocytes are able to release extracellular traps. Knowledge is emerging regarding the cellular processes that precede the formation of PETs. However, the regulatory mechanisms which mediate PET formation are still rarely understood. Therefore, the overall aim of this study is to evaluate the role of the transcription factor hypoxia inducible factor 1α (HIF-1α) in the formation of PETs. Neutrophils and mast cells were chosen as representative PET forming cells within this study. HIF-1α is a major regulator of energy homeostasis and cellular adaptation to low oxygen stress. Further, there is increasing evidence, that HIF-1α is activated upon exposure to microbial pathogens and supports the bactericidal activity of phagocytes. The well-known mechanisms in the post-translational regulation of HIF-1α levels as well as its short half-life might therefore characterize HIF-1α as an attractive pharmacological target. HIF-1α agonists that are designed to activate bactericidal mechanisms of host immune cells could conceivably be used alongside conventional antibiotics, and are predicted to function effectively against drug-resistant bacteria.

At first, the role of HIF-1α in the antimicrobial activities of mast cells (MCs) was investigated. Therefore, the new pharmacological agent AKB-4924 was used to inhibit prolyl hydroxylases involved in the HIF-1α degradation pathway and to subsequently increase HIF-1α protein levels. Enhancement of HIF-1α activity with AKB-4924 resulted in increased antimicrobial activity in human and murine MCs. Importantly, mast cell extracellular trap (MCET) formation was induced by treatment of mast cells with AKB-4924. Interestingly, inhibition of phagocytosis by using cytochalasin D in the presence or absence of AKB-4924 did not affect the antimicro-
brial activity of the human mast cell line HMC-1 cells, confirming that an extracellular bactericidal activity is mediated by HIF-1α. However, blocking the formation of reactive oxygen species (ROS) resulted in inhibited antimicrobial activity of HMC-1 cells. Additionally, bone marrow derived mast cells (BMMCs) isolated from HIF-1α deficient mice showed a significantly reduced antimicrobial effect against *S. aureus* compared to control BMMCs. Importantly, also the AKB-4924 mediated MCET formation was absent in those cells. Therefore, the obtained results indicate that the transcription factor HIF-1α is a key regulator of the extracellular antimicrobial activity and the formation of PETs in mast cells.

The second aim was to establish cell culture conditions using a surrogate neutrophil cell line (differentiated HL-60 leukocytes) to mimic primary neutrophils. Upon chemical differentiation of HL-60 cells with DMSO or all-trans retinoic acid, HL-60 cells do not exert similar antibacterial activities compared to blood-derived neutrophils. The development of neutrophil characteristics is therefore insufficient. Thus, we conclude that differentiated HL-60 cells are of limited value to replace primary cells in *in vitro* experiments to investigate antimicrobial activity and PET formation. Additionally, standardized culture protocols to analyze PET formation in primary neutrophils do not exist so far. Therefore, the most appropriate cell density and duration of PET induction experiments was evaluated using primary blood derived neutrophils. Best results in PET induction and visualization were obtained with primary blood derived neutrophils at a concentration of 2x10^6 cells /ml and a stimulation time of 3 hours. These conditions have thus been chosen for the following experiments.

The last aim was the evaluation of the role of iron chelating HIF-1α agonist in PET formation by neutrophils. Within this study it was shown that the iron chelating agent Desferrioxamine (DFO) boosts the formation of PETs in human and bovine primary blood derived neutrophils. Iron supplementation abolished that effect. Other HIF 1α-agonists showed a similar phenotype. Thus, it can be hypothesized that HIF-1α might regulate key factors involved in PET formation and stabilization e.g. PAD4, ROS, elastase or LL-37. This was proven experimentally by biochemically blocking respective enzyme function. In conclusion, these data have shown that HIF-1α might play an important role in the formation of PETs in neutrophils as well as mast cells. This knowledge will help to design drugs that could modulate innate immune cell functions against infections.
Lena Völlger; Die Rolle des Transkriptionsfaktors HIF-1α bei der Bildung von antimikrobiellen "phagocyte extracellular traps".

Zusammenfassung

*Phagocyte extracellular trap* (PET) - Bildung wurde unlängst als ein neuartiger Mechanismus von Phagozyten zur Verteidigung gegen Infektionen beschrieben. Das Grundgerüst der PETs besteht aus DNA, die mit Histonen, Proteasen und antimikrobiellen Peptiden assoziiert ist. PETs ermöglichen das extrazelluläre Einfangen und Abtöten von mikrobiellen Erregern und wurden als erstes in neutrophilen Zellen entdeckt. Mittlerweile ist jedoch bekannt, dass neben Neutrophilen auch Mastzellen, Eosinophile und Makrophagen sowie Monozyten in der Lage sind PETs zu bilden. Mit der Zeit wird immer mehr über die zellulären Prozesse bekannt, die bei der PET-Bildung eine wichtige Rolle spielen. Die regulatorischen Mechanismen hinter der PET-Bildung sind jedoch noch kaum verstanden und ihre Entschlüsselung bedarf weiterer Forschung. Deshalb war das übergreifende Ziel dieser Arbeit, die Rolle des Transkriptionsfaktors Hypoxie-induzierter Faktor 1α (HIF-1α) in der Bildung von PETs zu untersuchen. Im Rahmen dieser Studie, wurden Neutrophile und Mastzellen als repräsentative, PET-bildende Zellen ausgewählt. HIF-1α spielt bei der Regulation des Energiehaushaltes sowie der Anpassung an Bedingungen mit reduziertem Sauerstoffgehalt in Zellen eine wichtige Rolle. Zudem gibt es weitere Hinweise, dass HIF-1α durch Kontakt mit mikrobiellen Erregern aktiviert wird und die bakterizide Wirkung von Phagozyten unterstützt. Die kurze Halbwertszeit und die gut verstandenen Mechanismen der post-translationalen Regulation von HIF-1α machen den Transkriptionsfaktor zu einem attraktiven pharmakologischen Ansatzpunkt. HIF-1α Agonisten werden gezielt entwickelt, um die bakterizide Wirkung von Immunzellen zu unterstützen und zusätzlich zu konventionellen Antibiotika, vor allem aber bei Infektionen mit arzneimittelresistenten Bakterien, eingesetzt zu werden.

Zuallererst, sollte die Rolle von HIF-1α in der antimikrobiellen Wirkung von Mastzellen untersucht werden. Der neue Wirkstoff AKB-4924 wurde dabei zur pharmakologischen Inhibierung von Prolyl-Hydroxylasen eingesetzt, die im Abbauprozess von HIF-1α eine wichtige Rolle spielen. Eine Steigerung der HIF-1α Aktivität mittels AKB-4924 führte zu einer erhöhten antimikrobiellen Wirkung in humanen sowie in
murinen Mastzellen. Wichtig ist, dass dabei auch die Bildung von PET in Mastzellen (MCET) induziert wurde. Interessanterweise beeinflusste die Inhibition der Phagozytose mittels Cytochalasin D, auch in Gegenwart von AKB-4924, die antimikrobielle Wirkung von humanen Mastzellen (HMC-1 Zellen) in keiner Weise. Dieses Ergebnis bestätigt, dass HIF-1α eine extrazelluläre antimikrobielle Wirkung auslöst. Wird jedoch die Bildung von reaktiven Sauerstoffspezies (ROS) behindert, ist auch eine verringerte antimikrobielle Aktivität in HMC-1 Zellen zu beobachten. Zusätzlich konnte gezeigt werden, dass knochenmarksabstammende Mastzellen (BMMCs), isoliert aus HIF-1α defizienten Mäusen, im Vergleich zu Kontroll-BMMCs, eine signifikant reduzierte antimikrobielle Wirkung gegen S. aureus aufweisen. Zudem konnten dieselben Zellen, als Antwort auf eine Stimulation mit AKB-4924, keine MCETs ausbilden. Diese Ergebnisse weisen darauf hin, dass der Transkriptionsfaktor HIF-1α eine wichtige regulatorische Rolle bei der extrazellulären antimikrobiellen Wirkung und der Bildung von PETs in Mastzellen spielt.


Das abschließende Ziel dieser Studie war es, die Rolle von eisenchelatbildenden HIF-1α Agonisten bei der Bildung von PETs in Neutrophilen zu untersuchen. Dabei
konnte gezeigt werden, dass der Eisenchelator Desferrioxamine (DFO) die Bildung von PETs in humanen sowie bovinen primären, blutabstammenden Neutrophilen fördert. Eine ergänzende Zugabe von Eisenionen verhindert jedoch die DFO induzierte PET-Bildung. Weitere HIF-1α Agonisten wiesen zudem einen identischen Effekt auf. Daher kann angenommen werden, dass HIF-1α eine Schlüsselrolle in der PET-Bildung spielt und z.B. PAD4, ROS, Elastase oder LL-37 reguliert. Hinweise darauf konnten bereits experimentell gewonnen werden, indem die Funktion der entsprechenden Enzyme biochemisch inhibiert wurde.

Zusammenfassend zeigen die erzielten Ergebnisse, dass HIF-1α eine wichtige Rolle bei der Bildung von PETs in Neutrophilen und Mastzellen spielen könnte. Mit diesem Wissen können Arzneistoffe entwickelt werden, welche die antimikrobielle Wirkung von Immunzellen gegen auftretende Infektionen unterstützen.
1 Introduction

Phagocyte extracellular traps (PETs) have recently been described as a novel host innate immune defense of phagocytes (von Köckritz-Blickwede and Nizet, 2009). Their backbone structure consists of nuclear or mitochondrial DNA and is associated with histones, antimicrobial peptides and cell-specific proteases. PETs thereby provide a matrix to entrap and kill microbes. PETs (Figure 1.1) were initially discovered in neutrophils [Brinkmann et al., 2004], but later on it has been shown that besides neutrophils also other cells like mast cells [von Köckritz-Blickwede et al., 2008], eosinophils [Yousefi et al., 2009] and macrophages/monocytes [Chow et al., 2010] are able to release extracellular traps. Knowledge is emerging regarding the cellular processes in activated phagocytes that precede the formation of PETs. However, the regulatory processes involved in PET formation are still unclear. Therefore, the aim of this study is to evaluate the role of the transcription factor hypoxia inducible factor 1 (HIF-1α) in the formation of PETs. The short half-life and well-understood mechanisms for post-translational regulation of HIF-1α levels, lead to emerging discussions on HIF-1α as an attractive pharmacological target to fine-tune immune cell functions for the treatment of different diseases. Therefore, HIF-1α agonists that are designed to activate bactericidal mechanisms in host immune cells could conceivably be used alongside conventional antibiotics against drug-resistant bacteria such as methicillin-resistant Staphylococcus aureus (MRSA) [Nizet and Johnson, 2009].

Figure 1.1: Immunofluorescent micrograph of phagocyte extracellular traps (green), formed by neutrophils. The nuclei are stained in blue.
1.1 **Neutrophils and mast cells as key players of the innate immune system**

The human body has two different ways to fight against infections. The adaptive immune system and the innate immune system. The adaptive immune system needs several days before it is able to defend the body against pathogens, but is however highly specific and effective [Mayer, 2007]. The innate immune system is thought to be the evolutionary older defense strategy [Kimbrell and Beutler, 2001], is nonspecific and is often called the first line of defense [Zipfel et al., 2007]. Also, physical borders like the skin and epithelial cells with moving cilia or chemical barriers like mucus belong to the innate immune system. It is found in animals as well as in plants and provides immediate defense against infections [Litman et al., 2005]. Besides those physical borders also cellular compartments like the complement system or specialized white blood cells like for example neutrophils or mast cells belong to the innate immune system [Segal, 2005]. Those cells are able to destroy invading pathogens by intracellular killing machinery, so called phagocytosis, and prevent pathogens from spreading in the host.

Mast cells were first described in 1878 by Paul Ehrlich and are characterized by their large granules. They are derived from progenitor cells within the bone marrow that migrate into the peripheral blood and subsequently into vascularized tissue before they undergo their final maturation. Mature mast cells are then found in tissues at the interface towards the external environment such as mucosa of the respiratory and gastrointestinal tract or the skin [Abraham and Malaviya, 1997]. The granules contain heparin and histamine among other proteins that are released after allergen contact. They are therefore well known to play a role in allergic reactions like asthma and anaphylaxis [Puxeddu et al., 2003]. Mast cells also participate in a wide variety of important biological functions e.g. immune defense against parasites, tissue remodeling [Henz, 2008] and wound healing, as well as fibrosis, autoimmune diseases and in tumors [Puxeddu et al., 2003]. Upon stimulation, mast cells release antimicrobial peptides [Di Nardo et al., 2003], inflammatory mediators, proteases and cytokines and chemokines that recruit neutrophils to the site of infection [Zhang et al., 1992; Abraham and Malaviya, 1997].
Neutrophils, also called polymorphonuclear neutrophils (PMN), are the most abundant type of white blood cells and therefore have an essential part in the innate immune system. They represent approximately 50% to 60% of all leukocytes and are one of the first cells which are present at the side of infection [Nathan, 2006; Brinkmann and Zychlinsky, 2007]. The frontline function of neutrophils in the innate immune defense has been classically understood to reflect a variety of potent intracellular antimicrobial mechanisms that require phagocytic uptake of the bacteria. Neutrophils internalize and kill different pathogens by phagosome formation in which hydrolytic enzymes and reactive oxygen species (ROS) are secreted. Additionally, they are also able to release different antimicrobial agents e.g. granule proteins, proteases and antimicrobial peptides similar as mast cells and thereby mediate immunomodulatory functions.

Interestingly, both cell types, neutrophils and mast cells, have been shown to additionally exhibit an extracellular antimicrobial activity by the formation of phagocyte extracellular traps, which will be explained in the following chapter.

1.2 Phagocyte extracellular traps (PETs)

Phagocyte extracellular traps (PETs) consist of nuclear or mitochondrial [Yousefi et al., 2009] DNA fibers as a backbone with associated antimicrobial peptides, histones, and cell-specific proteases [Brinkmann et al., 2004; Fuchs et al., 2007] and thereby provide a matrix to entrap (Figure 1.2.1) and occasionally kill certain microbes [Li et al., 2010]. PET formation was first described in a landmark study in 2004 by Brinkmann et al. In the beginning, PET formation was thought to be a unique mechanism restricted to neutrophils [Brinkmann et al., 2004] but already a few years later, von Köckritz-Blickwede et al., (2008) showed that also mast cells are able to release mast cell extracellular traps with antimicrobial functions. Nowadays there is evidence that PETs can also be formed by other immune cells e.g. eosinophils [Yousefi et al., 2009], macrophages-monocytes [Chow et al., 2010] and fibrocytes [Kisseleva et al., 2011].
In the case where cells form PETs, they are undergoing a programmed cell death. This process is morphologically distinct from other classical cell death processes including apoptosis and necrosis, and hence was dubbed “ETosis” [Wartha and Henriques-Normark, 2008]. During ETosis, the nuclei lose their shape, the euchromatin homogenizes, granule membranes as well as the nuclear membrane disintegrate, which allows both components to associate [Fuchs et al., 2007]. Finally, those mixed components are released as fibers into the extracellular space. If this cell death program is specifically performed by neutrophils, it is called NETosis [Brinkmann et al., 2004; Steinberg and Grinstein, 2007]. During NETosis DNA fibers with different associated proteins, the neutrophil extracellular traps (NETs) are released.

As mentioned above, PETs have been shown to be able to entrap and kill several bacterial pathogens and thereby prevent their spreading within the infected host. However, some bacteria have evolved a number of mechanisms to avoid killing by extracellular traps. As an example, it has been described that members of the Streptococcus family are able to reduce PET formation by secreting proteases which cleave neutrophil activating agents like interleukin-8 (IL-8) [Zinkernagel et al., 2008], by secretion of nucleases to degrade the DNA backbone of PETs [Buchanan et al., 2006] and by gaining a resistance against antimicrobial components of the PETs e.g. the host peptide LL-37 [Lauth et al., 2009]. Table 1 shows examples of several bacterial mechanisms used to avoid entrapment or killing by extracellular traps.
Table 1: Bacterial mechanisms to avoid entrapment and/or killing by PETs [adapted, von Köckritz-Blickwede and Nizet, 2009]

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Evasion Strategy</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus pneumonia</td>
<td>Preventing entrapment within NETs by capsule (serotype 1, 2, 4, 9 V) and D-alanylated lipoteichoic acids (LTA)</td>
<td>Wartha et al., 2007</td>
</tr>
<tr>
<td>Streptococcus pneumonia</td>
<td>NET-degradation by DNase EndA</td>
<td>Beiter et al., 2006</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>NET degradation by DNase Sda1/2</td>
<td>Buchanan et al., 2006</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>Resistance against LL-37-mediated killing within NETs/MCETs by surface M1 protein</td>
<td>Lauth et al., 2009</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>Reduction of NET production by IL-8 protease SpyCEP</td>
<td>Zinkernagel et al., 2008</td>
</tr>
<tr>
<td>Streptococcus suis</td>
<td>NET degradation by nuclease SsnA</td>
<td>de Buhr et al., 2014</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>Suppression of NETs by sialic acid engagement of Siglec receptors and consequent inhibitory signaling</td>
<td>Carlin et al., 2009</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>Resistance against NET-killing by surface lipooligosaccharides</td>
<td>Hong et al., 2009</td>
</tr>
<tr>
<td>Aeromonas hydrophila</td>
<td>NET degradation by nuclease</td>
<td>Brogden et al., 2012</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Change of surface charge by D-alanylation of lipoteichoic acid</td>
<td>Kraus et al., 2008</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>NET degradation by nuclease nuc (MN)</td>
<td>Berends et al., 2010</td>
</tr>
<tr>
<td>Vibrio cholera</td>
<td>NET degradation by nuclease Xds/Dns</td>
<td>Seper et al., 2013</td>
</tr>
</tbody>
</table>
1.3 Mechanisms of PET formation

The cellular processes which lead to the formation of PETs are not fully known yet. Different inducing factors like IL-8, lipopolysaccharide (LPS), interferon (IFN) α/γ, phorbol myristate acetate (PMA), hydrogen peroxide (H$_2$O$_2$) or several bacteria or fungi can induce PET formation [von Köckritz-Blickwede et al., 2010; von Köckritz-Blickwede and Nizet, 2009]. Figure 1.3.1 shows a schematic model of the process of NET formation.

![Schematic figure of the process of NET-formation](image)

Figure 1.3.1: Schematic figure of the process of NET-formation [adapted, von Köckritz-Blickwede and Nizet, 2009]. NET formation is induced due to pathogens themselves or several agents like PMA, IL-8, LPS or IFNα/γ + C5a. Activation of NADPH oxidases lead to the formation of reactive oxygen species. The nuclear membrane disrupts, PAD4 mediates the decondensation of chromatin and nuclear components mix with the cytoplasmic content of the cell. In the end the dead cell releases granular and nuclear particles and the extracellular traps are formed. After induction of PET development, pathogens get entrapped within those structures and thus are prevented from spreading within the host as well as kept in an area of high concentration of antimicrobial agents and in the end get killed.

After initiation of ROS formation by e.g. activation of NADPH oxidases, the neutrophil nuclear membrane dissolves and chromatin is subsequently decondensated by peptidylarginine deiminase 4 (PAD4). The disrupted nuclear membrane allows...
nuclear components to mix with the cytoplasmic content and granular proteins of the cell [Fuchs et al., 2007]. Finally those mixed components of nuclear and cytoplasm are released into the extracellular space and extracellular traps are formed.

After PET formation, pathogens get entrapped within the NETs structures and are prevented from spreading within the host. Areas with high concentrations of antimicrobial agents in close proximity to the NETs further support the bacterial clearance. However, the exact killing process is still unknown.

1.3.1 Reactive oxygen species (ROS) formation

Evidence implicates that the production of ROS is an essential signal leading to the elaboration of PETs [von Köckritz-Blickwede and Nizet, 2009]. Therefore, the most frequently used pathway to induce PET formation is triggered by PMA, a protein kinase C (PKC) activator [Tahara et al., 2009]. PKC, in turn, activates the NADPH oxidase complex [Dekker et al., 2000] that produces superoxide anions which serve as a starting product for ROS that also constitute to the oxidative burst. NADPH oxidase activation by the above-mentioned stimuli leads to the formation of ROS, which initiates PET formation. ROS are oxygen containing, chemically reactive molecules like oxygen ions themselves (superoxide (O$_2^-$)) and peroxides (H$_2$O$_2$). They are naturally formed within the mitochondria as a byproduct in the normal cell metabolism and play important roles for cell signaling processes and cell homeostasis. Moreover, immune cells such as phagocytes produce and store ROS in so called granules, to fight against invading pathogens. Table 2 shows examples of several properties of ROS.
Table 2: Properties of exemplary reactive oxygen species [adapted, Hampton et al., 1998]

<table>
<thead>
<tr>
<th>ROS</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide</td>
<td>Mild oxidant and reductant with limited biological activity; reduces some iron complexes to enable hydroxyl radical production by the Fenton reaction; inactivates iron/sulfur proteins and releases iron; limited membrane permeability</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>Oxidizing agent; reacts slowly with reducing agents such as thiols; reacts with reduced iron and copper salts to generate hydroxyl radicals; reacts with heme proteins and peroxidases to initiate radical reactions and lipid peroxidation; membrane permeable</td>
</tr>
<tr>
<td>Singlet oxygen</td>
<td>Electronically excited state of oxygen; reacts with a number of biological molecules, including membrane lipids to initiate peroxidation</td>
</tr>
<tr>
<td>Hydroxyl radical</td>
<td>Extremely reactive with most biological molecules; causes DNA modification and strand breaks, enzyme inactivation, lipid peroxidation; very short range of action; generates secondary radicals, e.g., from bicarbonate, chloride</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>Reacts very rapidly with superoxide to give peroxynitrite; reaction with oxygen favored at high oxygen tension; forms complexes with heme proteins; inactivates iron/sulfur centers; forms nitrosothiols</td>
</tr>
</tbody>
</table>

The impact of impaired ROS formation on bacterial clearance in the human body can be easily observed in chronic granulomatous disease (CGD) patients. Affected individuals have mutations within the NADPH oxidase which lead to an impaired or completely absent oxidative burst resulting in recurrent and often life threatening infections [Smith and Curnutte, 1991]. However, the CGD phenotype can be linked to a NADPH oxidase malfunction. Neutrophils from healthy donors tested in anaerobic environments [Mandell, 1974], or in the presence of the NADPH oxidase inhibitor diphenyleneiodonium (DPI), are also impaired in their ability to kill bacteria, similar to
those of CGD patients [Mandell and Hook 1969; Ellis et al., 1988; Hampton and Winterbourn, 1995; Keshari et al., 2013]. Since the oxidative burst is impaired in CGD patients, Fuchs et al., tested whether NET formation is also impaired in affected individuals. The authors found that PMA treated neutrophils from each tested individual CGD patient are unable to generate ROS [Fuchs et al., 2007]. Furthermore, PMA or S. aureus activated neutrophils from CGD patients lack the ability to form NETs. Neutrophils also did not show the characteristic morphological changes that occur during NET formation, such as breakdown of the nuclear envelope and the subsequent mixing of nuclear components with the cytoplasmic content. Additionally, the authors were able to show that indeed a mutation within the NADPH oxidase caused the failure to generate ROS. Neutrophils from CGD patients were able to form NETs, which were similar to those released by neutrophils from healthy donors, when they were stimulated with glucose oxidase (GO) [Fuchs et al., 2007]. Glucose oxidase was used to generate hydrogen peroxide exogenously and downstream of NADPH oxidase. These data concluded that NADPH oxidase is a key enzyme in PET formation and was confirmed by several other authors [Keshari et al., 2013; Hakkim et al., 2011; Reimijisen et al., 2011, Palmer et al., 2012; Parker et al., 2012].

1.3.2 Peptidylarginine deiminase 4 (PAD4)

Another key biochemical marker of PET formation appears to be the deimination of arginine residues into citrulline residues in histones, a post-translational modification catalyzed by peptidylarginine deiminase (PAD4), which facilitates chromatin decondensation and thereby the formation of PETs [Wang et al., 2004; Wang et al., 2009]. Since the DNA is normally tightly packed around histones, chromatin decondensation is necessary to allow the disassembly of histones and DNA and to enable those DNA fibers to be released into the extracellular space.

Peptidylarginine deiminase 4 (PAD4), also known as PADI4 or PADV is a homodimer that functions as a transcriptional co-regulator and was first identified in human HL-60 leukemia cells [Nakashima et al., 1999]. It belongs to a protein family called amidino-transferase superfamily [Shirai et al., 2001] which, under conditions when calcium ions are present, is known to convert arginine to citrulline post-translationally. The modification from a positively charged arginine to a neutral citrulline is thought to
induce protein unfolding [Tarcsa et al., 1996; Vossenaar et al., 2003]. PAD4 for instance, citrullinates the histones H2A, H3 and H4 which leads to histone hypercitrullination [Wang et al., 2009]. It is highly expressed in peripheral blood neutrophils and localized within the nucleus [Nakashima et al., 2002; Su et al., 2004]. Until now, five different human PAD isoenzymes have been identified [Vossenaar et al., 2003] and each enzyme displays tissue-specific expression patterns. PAD1 is located in the skin epidermis where it citrullinates keratins and filaggrins. In brain and muscle tissues, myelin basic proteins are citrullinated by PAD2. Hair follicles contain PAD3 and trichohyalin is citrullinated. PAD4 was identified in granulocytes, monocytes and macrophages. Last but not least, PAD6 has been found in oocytes and embryonic stem cells [Liu et al., 2011]. The PAD4 homodimer is characterized by a unique inserted nuclear localization sequence within the PAD isoenzymes [Nakashima et al., 2002; Arita et al., 2004]. In 2011, Liu et al. found that the dimerization of PAD4 is essential for its full enzyme activity [Liu et al., 2011]. PAD4 function is able to be inhibited by a chemical substance called chloramidine [Chumanevich et al., 2011; Li et al., 2010]. If chloramidine is present, the conversion of arginine to citrulline residues cannot take place anymore and histone hypercitrullination as well as subsequent chromatin decondensation is impeded. In 2010, Andrade et al. could show that PAD4 function can also be inhibited by PAD4 autocitrullination. Furthermore, they defined Arg-372 and -374 as potential autocitrullination targets that inactivate PAD4 enzyme function as well as change the protein structure. As already mentioned above, Wang et al. recently found out that PAD4 mediated histone hypercitrullination regulates the unfolding of chromatin structures during the formation of NETs [Wang et al., 2009]. However, the regulatory mechanisms behind this phenomenon are still unclear. Furthermore, it was shown that neutrophils derived from PAD4 deficient mice are not able to form NETs after stimulation with chemokines or incubation with bacteria, and are therefore also lacking the bacterial killing of e.g. S. flexneri by NETs [Li et al., 2010]. To assess the susceptibility to infections of PAD4 deficient mice in vivo, the authors used a mouse infectious disease model of necrotizing fasciitis. They showed that PAD4 deficient mice are more susceptible to bacterial infections with group A streptococci (GAS) type M1 than PAD4 wt mice.

Summarizing this part, evidence about the mechanisms associated with formation of PETs is increasing and PAD4 as well as NADPH oxidase have been identified as two
key enzymes involved in this process. However, very little is known about the regulatory processes especially on the transcriptional level. Here, the role of the transcription factor HIF-1α will be in focus.

1.4 Hypoxia inducible factor 1 (HIF-1)

Hypoxia inducible factor 1 (HIF-1) is a heterodimeric transcription factor complex consisting of two helix loop helix subunits: the oxygen regulated α-subunit and a constitutively expressed β-translocator (ARNT) [Zinkernagel et al., 2007; Zaremba and Malech, 2005; Semenza, 2004]. Figure 1.4.1 displays a schematic model of HIF-1, its co-activator p300, and further indicates DNA binding of HIF-1 at its specific binding site.

![Figure 1.4.1: Schematic figure of HIF-1 and selected target genes [adapted, Ozer and Bruick, 2007]](image)

HIF-1α protein stability is tightly regulated by the cellular oxygen concentration. It accumulates under hypoxic conditions and is quickly degraded by ubiquitination and subsequent proteasomal degradation under normoxia [Zaremba and Malech, 2005]. Figure 1.4.2 displays a schematic overview on the regulation of HIF-1α under conditions of normal oxygen concentration as well as under hypoxic conditions.
Figure 1.4.2: Schematic figure of HIF-1α regulation under normoxic and hypoxic conditions. “(A) During normoxia, O₂-dependent proline hydroxylases modify HIF-1α proline residues 402 and 564. Asparagine 803 is hydroxylated by FIH, which decreases HIF-1α interaction with the p300/CBP transcriptional coactivators. The hydroxylated prolines are recognized by vHL, a component of an ubiquitin ligase complex that ubiquitinates (Ub) HIF-1α and thereby targets it for proteasomal degradation. (B) During hypoxia and/or bacterial infection, when proline hydroxylases are not active, HIF-1α regulates transcription at HREs by accumulating and binding to HIF-1β and p300/CBP, which results in transcription of hypoxia-inducible genes involved in angiogenesis, glucose transport and metabolism, erythropoiesis, inflammation, apoptosis, and cellular stress. EPO, erythropoietin.” [Zaremba and Malech, 2005].
The degradation of HIF-1α under normoxic conditions is regulated by oxygen- and iron-dependent prolyl hydroxylases (PHDs) [Zinkernagel et al., 2007]. A component of the ubiquitin (Ub) ligase complex, the von Hippel-Lindau (vHL) complex, recognizes the hydroxylation and targets HIF-1α for proteasomal degradation. Another step of the O₂-dependent regulation represents the hydroxylation of an asparagine residue by factor inhibiting HIF-1α (FIH). FIH blocks the interaction of HIF-1α with p300/CBP (CREB-binding protein) transcriptional coactivator proteins, which leads to a decreased transcription of HIF-1α regulated genes under normoxic conditions [Zaremba and Malech, 2005]. Under hypoxia, HIF-1α immediately accumulates due to interruption of its degradation pathway by inhibition of the oxygen- and iron-dependent hydroxylation. The heterodimeric transcription factor complex is then formed and is able to interact with its coactivators which leads to binding of HIF-1 to specific binding sites, the so called hypoxia responsive elements (HREs) [Nizet and Johnson, 2009; Zinkernagel et al., 2007; Peyssonnaux et al., 2005; Zaremba and Malech, 2005]. HIF-1α binding regulates the transcription of target genes which encode for example for erythropoietin, glucose transporters, glycolytic enzymes, antimicrobial factors and the angiogenic factor VEGF [Zinkernagel et al., 2007; Zaremba and Malech, 2005].

HIF-2α is a protein which is structurally and functionally related to HIF-1α [Semenza, 2004] and is able to heterodimerize with HIF-1β as well [Tian et al., 1997]. Both heterodimers (HIF-1α:HIF-1β and HIF-2α:HIF-1β) are able to bind to HREs with partially overlapping but also with different target genes [Hu et al., 2003; Sowter, 2003]. Thus, depending on the cell type and genes, gene expression can be induced or suppressed by HIF-1α [Kelly et al., 2003]. Gu et al. have identified a third protein, called HIF-3α in 1998, but so far its role is not well known, except for the inhibitory PAS (Per/Arnt/Sim) domain protein, IPAS. IPAS is a splice variant of HIF-3α and is able to inhibit HIF-1α activity by binding to the transcription factor subunit and therefore inhibiting the dimerization of HIF-1α and ARNT [Makino et al., 2001, 2002]. Table 3 shows some developmental and physiological functions of HIF-1.
HIF-1α also appears to be a key mediator during inflammatory processes [Walmsley et al., 2008]. During a bacterial infection, HIF1-α is both stabilized and able to direct an immune response against the pathogen. In vivo studies have shown that HIF-1α can function in a way to aid the skin epithelium in its barrier function against bacterial invasion and that bacterial proliferation was controlled by the HIF-1α pathway [Peyssonnaux et al., 2008, 2005]. Peyssonnaux et al. also hypothesized that during LPS-induced sepsis, HIF-1α may ‘play an important role in mediating the inflammatory responses’ [Peyssonnaux et al., 2007]. Additionally, in 2005, the same authors showed that accumulation of HIF-1α induced by hypoxia initiates the increased production of nitric oxide (NO) and tumor necrosis factor alpha (TNF α). These substances control the bacterial proliferation and induce the inflammatory response as well as stabilize HIF-1α itself. Furthermore, in 2008, Weidemann and Johnson described that HIF-1α knock-out-mice showed a high embryonic lethality. They
concluded that HIF-1α also plays a critical role in physiological function and not only in adaptation to hypoxic conditions. The iron chelators deferoxamine (DFO) or cobalt chloride (CoCl₂) can induce the DNA binding activity and gene expression of HIF-1α [Wang and Semenza, 1993]. Besides hypoxia, DFO and CoCl₂ induce the HIF-1α transactivation domain function [Kallio et al., 1998; Jiang et al., 1997; Pugh et al., 1997]. Thus HIF-1α expression and activity may be regulated by a common mechanism [Semenza, 2004].

1.4.1 HIF-1 in innate immune cells

Peyssonnaux and colleagues demonstrated in 2005 for the first time that HIF-1α expression regulates the antibacterial capacity of phagocytes. Using conditional gene targeting, the authors examined the contribution of HIF-1α to myeloid cell innate immune function, focusing on neutrophils and macrophages. HIF-1α was induced by different bacterial infections including *S. aureus* and *S. pyogenes*, even under normoxia, and regulated the production of key immune effector molecules. Although the full spectrum of HIF-1α downstream targets remains to be determined, the expression of a number of molecular effectors of host defense, including cathelicidin-related antimicrobial peptide (CRAMP), TNF-α and the granule proteases cathepsin G and elastase, significantly correlated with HIF-1α levels [Peyssonnaux et al., 2005]. They further stated that mice that lack HIF-1α in their myeloid cell lineage showed decreased bactericidal activity and were not able to restrict a systemic spread of an infection from its initial tissue. Stabilization of HIF-1α through deletion of the regulatory vHL tumor-suppressor protein in contrast, supported the production of defense factors and improved bactericidal activity of myeloid cells [Peyssonnaux et al., 2005].

Regarding neutrophils in particular, Cramer et al. [Cramer et al., 2003] showed that HIF-1α deficiency reduces motility, cell aggregation, invasiveness, bacterial killing and ATP levels (~40%) in HIF-1α null PMNs. This indicates that HIF-1α is required for the maintenance of the intracellular energy homeostasis in neutrophils. Furthermore, it was found that hypoxia is able to enhance bactericidal activities of human polymorphonuclear leucocytes [Peyssonnaux et al., 2005; Walmsley et al., 2006; Wang and Liu, 2009]. The authors exposed healthy volunteers to an oxygen concentration of 12% for 2h and afterwards assessed neutrophils functions. They detected
increased chemotaxis, phagocytosis and respiratory burst [Wang and Liu, 2009]. A second study by the same group showed that moderate exercise performed under 12% oxygen enhanced neutrophil phagocytosis and promoted apoptosis [Wang and Chiu, 2009]. This goes in line with the findings from Mecklenburgh et al. in 2002. They showed that neutrophils are protected from apoptosis via a mechanism dependent on prolyl hydroxylase domain/hypoxia-inducible factor 1α (PHD/HIF-1α) if kept under hypoxic conditions [Mecklenburgh et al., 2002]. Additionally, PHD3 was identified as a selective regulator of neutrophil hypoxic survival [Walmsley et al., 2011].

Due to the short half-life and well-understood mechanisms for post-translational regulation of HIF-levels, HIF is an attractive pharmacological target to fine-tune immune cell functions for the treatment of different diseases. HIF-1α agonists that are designed to activate bactericidal mechanisms of host immune cells could conceivably be used alongside conventional antibiotics, and are predicted to function effectively against drug-resistant bacteria such as methicillin-resistant Staphylococcus aureus (MRSA) [Zinkernagel et al., 2007; Nizet and Johnson, 2009].

In accordance with this hypothesis, a recent study demonstrated that the HIF-1α agonist L-Mimosine significantly delayed progression of S. aureus abscesses in a mouse subcutaneous challenge model. The authors showed that L-Mimosine can boost the capacity of human phagocytes and whole blood to kill S. aureus in a dose-dependent fashion and thereby reduce the size of S. aureus-induced skin lesions [Zinkernagel et al., 2008]. This provided the first proof of principle for a novel approach to the treatment of bacterial infections by pharmacologically augmenting HIF-mediated host phagocytic functions. However, the detailed mechanisms behind this HIF-mediated bactericidal activity of phagocytes are still not entirely clear.

1.5 Aims

Knowledge is beginning to emerge regarding the cellular processes that precede the formation of PETs by activated phagocytes. However, the transcriptional regulatory processes involved in PET formation are still unclear. It is also unknown whether HIF-1α is involved in PET formation.
Therefore, the overall aim of this study is to evaluate the role of HIF-1α in the formation of PETs. Two different cells will be in focus; neutrophils and mast cells as representative PET forming cells. Specific aims are:

Aim 1: Evaluation of role of HIF-1α in PET formation by mast cells

Aim 2: Improvement of neutrophil culture conditions to characterize NET formation

Aim 3: Evaluation of role of HIF-1α in PET formation by neutrophils

Aim 1: Evaluation of role of HIF-1α in PET formation by mast cells.

There are well established cell culture conditions for permanent mast cell lines as well as murine primary bone-marrow-derived mast cells available to induce and visualize PETs. Those cell culture conditions also allow working with genetically modified mast cells. Thus, as a first step the role of HIF-1 will be analyzed in permanent and primary mast cells including HIF-deficient cells.

Aim 2: Improvement of neutrophil culture conditions to characterize NET formation.

In contrast, for neutrophils there is no standardized protocol existing that allows the usage of a permanent neutrophil cell line that is efficiently generating NETs. Primary neutrophils cannot be cultured longer than 6-12h due to their short life span. Thus, the primary aim was to establish cell culture conditions using a surrogate PMN cell line (differentiated HL-60 leukocytes), that is capable of performing like primary neutrophils and which would also facilitate experiments using genetically modified surrogate PMNs. Another aim is to determine the optimal time period and cell density to best induce and visualize NET formation in primary neutrophils.

Aim 3: Evaluation of the role of iron chelating HIF-1α agonist in PET formation by neutrophils.

Afterwards, NET formation induced by different well-known HIF-1α agonists by means of iron chelating agonists will be revealed. Last but not least the role of putative HIF-targets for NET formation is elucidated: The influence of ROS, PAD4, neutrophil elastase (NE) and LL-37 on NET formation will be studied in more detail.
This new knowledge will have significant implications for understanding the HIF-1α mediated immune response as a novel target against drug-resistant bacterial infections.
Material and Methods

The Material and Methods part will be described for each publication independently.

2.1 Material and methods for chapter 3.1:

A novel role for the transcription factor HIF-1α in the formation of mast cell extracellular traps

2.2 Material and methods for chapter 3.2:

Characterization of the antimicrobial activities of HL-60 cells as an alternative model to study neutrophil functions

2.2.1 Bacterial strains and growth conditions

For bacterial killing assays, *S. aureus* Newman was used; for NET induction assays, a nuclease-deficient derivative of *Staphylococcus aureus* USA 300 LAC (*S. aureus* AH1787) was used (Berends *et al*., 2010). The absence of bacterial nuclease activity ensured to capture total NET formation without interference with NET degradation. Bacteria were grown in brain heart infusion (BHI) medium at 37°C shaking. An overnight culture was diluted 1:100 into fresh medium and grown to mid-logarithmic phase (OD$_{600}$=0.5). Bacteria were then harvested by centrifugation, suspended in PBS and adjusted to the desired concentration by optical density at 600 nm. Further dilutions were prepared in cell culture medium.

2.2.2 HL-60 cells

HL-60 cells were received from the “Deutsche Sammlung für Zellkulturen” (DSMZ, DSMZ-No.: ACC-3).

2.2.3 Cultivation and differentiation of HL-60 cells

The myeloid leukemia cell line HL-60 was propagated in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from PAA). To induce a neutrophil-like phenotype, cells were treated with either 1.25% DMSO for 3 days, 1.25% DMSO for 4 days or 1 µM retinoic acid for 4 days, reaching a maximum cell count of $1 \times 10^6$ cells/ml. For experiments, differentiated cells were collected by centrifugation for 10 minutes at 118 x g, washed once with PBS and finally adjusted to a density of $2 \times 10^6$ cells/ml in RPMI 1640 supplemented with 2% nuclease-free FBS (heat-inactivated at 70°C). HL-60 cells cultured and differentiated according to this protocol will further be referred to as nHL-60.
2.2.4 Isolation of human blood-derived neutrophils

Human primary blood-derived neutrophils were isolated from fresh heparinized blood of healthy donors described by (von Köckritz-Blickwedel et al., 2010). 25 ml blood was slowly layered on top of 25 ml Polymorphprep™ (Axis-Shield PoC) in a 50 ml Falcon tube, close to the flame and taking care to avoid mixing. The sample was centrifuged at 500 x g for 30 min at room temperature with brake off. In the meantime one 8 mm glass cover slide per well was placed into a 48 well non-treated suspension culture plate. For a better attachment of the cells on the cover slide, the glass slides were coated with 50 µl of 0.01% Poly-L-lysine (# P4707, Sigma) for 10-30 minutes at room temperature (RT). The wells were washed twice with 200 µl 1x PBS (# H15-001, PAA) to remove the excessive Poly-L-lysine. After completion of the centrifugation step, the remaining plasma and mononuclear cells were sucked off with a transfer pipette and discarded. Figure 2.2.4.1 displays a schematic of the density gradient after centrifugation.

![Diagram of density gradient after centrifugation with separated cell populations.](image)

The PMN layer (5-10 ml) was removed with a new transfer pipette and added to a fresh 50 ml Falcon tube. The tube was filled up to 50 ml with 1x PBS and centrifuged at 500 x g for 10 min with brake on. After the spin, the supernatant was discarded and the cell pellet was resuspended with 5 ml of molecular grade water (# 3255.1,
Roth) for 5 sec to lyse red blood cells. The falcon was filled up to 50 ml with 1x PBS and centrifuged at 500 x g for 10 min with brake on. The above step was repeated if the pellet was still pink. RPMI 1640 (# E15-848, phenol red free, PAA) was used as cultivation medium. After the final wash, the supernatant was discarded and the pellet was resuspended in 1000 µl RPMI 1640 by pipetting up and down. The cells were counted to determine the required amount (PMN/ml). Therefore tryphan blue (#15250 061, Invitrogen) was used to discriminate between living and dead cells. Neutrophils were adjusted to a density of 2 x 10⁶ cells/ml in RPMI 1640 supplemented with 2% nuclease-free FBS [Chow et al., 2010].

2.2.5 Bacterial killing assay

To determine the bactericidal activity of nHL-60 cells or neutrophils, cells were co-incubated with bacteria at a multiplicity of infection (MOI) of 2 in a final volume of 500 µl in 48-well non-treated cell culture plates. All incubations were carried out at 37°C and 5% CO₂ in a humidified incubator. Prior to infection, cells were pre-stimulated for 20 minutes with phorbol 12-myristate 13-acetate (PMA; 25 nM), with or without addition of cytochalasin D (10 µg/ml, Sigma) to block phagocytosis. Control cells received the vehicle (DMSO) in the same dilution. Bacteria were then added to the cells, the plates were centrifuged for 5 minutes at 472 x g and incubated for 30 minutes. Cells were lysed by addition of 50 µl of 0.25% Triton X-100 in PBS and serial dilutions were plated on Todd-Hewitt agar plates for viable count. All conditions were analyzed in duplicate. Results were expressed as surviving bacteria compared to bacterial growth under the same conditions in the absence of neutrophils.

2.2.6 NET induction assay

The capacity of nHL-60 cells and blood-derived neutrophils to form NETs was assessed after stimulation with PMA and S. aureus AH1787. Cells were seeded on 8-mm cover slips coated with poly-L-lysine, stimulated with 25 nM PMA and/or bacteria at a MOI of 2 as indicated and centrifuged for 5 minutes at 472 x g. The plates were then incubated at 37°C and 5% CO₂ in a humidified incubator for 1, 2, 3 or 4 hours. Cells were fixed by addition of paraformaldehyde (PFA) in PBS to a final
concentration of 4% PFA. For all conditions, preparations were performed in duplicate.

2.2.7 NET visualization and quantification

Fixed cells were washed three times with PBS and permeabilized and blocked with 2% BSA in 0.2% Triton X-100/PBS for 45 minutes at room temperature. Incubation with a mouse monoclonal anti-H2A-H2B-DNA complex (clone PL2-6 (Losman et al., 1992), 0.5 µg/ml in 2% BSA in 0.2% Triton X-100/PBS) was carried out overnight at 4°C, followed by washing (3 x with PBS) and subsequent incubation with an AlexaFluor488-labelled anti-mouse antibody for 45 minutes at room temperature. After washing, slides were mounted in ProlongGold antifade including DAPI and analyzed by confocal fluorescence microscope using a Leica DMI6000CS confocal microscope with a HCXPLAPO 40 × 0.75 - 1.25 oil objective. Preparations with an isotype control antibody were used for setting adjustment. For each preparation, three randomly selected images were acquired and used for quantification of NET producing cells. Data were expressed as percentages of NET-forming cells in relation to the total number of cells. The mean value derived from \( n = 6 \) images for each condition per experiment was used for statistical analysis.

2.2.8 Myeloperoxidase (MPO) detection assay

The cells were treated in the same way as for the NET induction assay. The visualization and quantification differed only in the used antibodies. For the myeloperoxidase detection a polyclonal rabbit anti myeloperoxidase antibody (Dako firma) in 2% BSA in 0.2% Triton X-100/PBS was used. The secondary antibody was an Alexa-Fluor 488-labelled anti-rabbit antibody (1:1000; Invitrogen). All other steps were identical compared to the NET visualization and quantification.

2.2.9 Statistical analysis

The average values derived from independent experiments performed in duplicate were used for statistical analysis and are depicted as mean and standard error of the
mean (SEM). Comparisons between stimulated and non-stimulated cells of equally
differentiated cells were performed by paired one-tailed t-test as indicated. Compari-
sions between differently differentiated cells or different cell types were performed as
unpaired one-tailed t-test. In both cases, differences with P<0.05 were considered
statistically significant.
2.3 Material and methods for chapter 3.3:

Influence of cell density on phorbol 12-myristate 13-acetate (PMA) mediated NET-formation

2.3.1 Isolation of human blood derived neutrophils

Human primary blood-derived neutrophils were isolated from freshly heparinized blood of healthy donors as previously described [von Köckritz-Blickwede et al., 2010]. For the performed in vitro NET assays, the neutrophils were seeded on poly-L-lysine-coated (0.01%, Sigma) cover slides in 24-well plates at a concentration of 2x10⁴, 5x10⁴, 1x10⁵ or 5x10⁵ cells/well (250µl/well). RPMI 1640 without phenol red (PAA) was used for cultivation of the cells at 37°C and 5% CO₂. The cells were either stimulated with 25 nM PMA (InvivoGen Corp., San Diego, CA, US) or RPMI medium alone for 1, 2, 3 and 4h. After incubation, the cells were fixed by adding PFA (#15710 Electron Microscopy Science) at a final concentration of 4% for 15 min at room temperature. Subsequently the samples have been immune stained as described below.

2.3.2 Visualization of NETs

Fixed cells were washed three times with PBS, blocked and permeabilized with 2% BSA in 0.2% Triton X-100/PBS for 45 min at room temperature. Incubation with a 1:5000 dilution of a mouse monoclonal anti-Histone1-DNA complex (Millipore MAB3864, 2.2 mg/ml in 2% BSA in 0.2% Triton X-100/PBS) was carried out overnight at 4°C. Samples were washed with PBS and subsequently incubated with an Alexa-Fluor-488-labelled goat-anti-mouse antibody (1:1000; Invitrogen) for 45 min at room temperature. After washing, slides were mounted in ProlongGold® antifade with DAPI (Invitrogen) and analyzed by confocal fluorescence microscope using a Leica TCS SP5 confocal microscope with a HCX PL APO 40x 0.75-1.25 oil immersion objective. Settings were adjusted with control preparations using an isotype control antibody. For each preparation, three randomly selected images were acquired and used for quantification of NETs. The mean value derived from n = 6 images for each condition per experiment was used for statistical analysis.
2.3.3 NET quantification

To quantify the percentage of cells releasing NETs, the software ImageJ was used. The quantification took place by counting the cells which released NET structures and are associated with a NET within the image (Figure 3.3.1e-h). The green numbers represent cells that were counted as NET negative; the red numbers show cells that were counted as NET positive. Due to the previous immunostaining, the NETs were displayed in green, nuclei and nuclear DNA in blue.

2.3.4 Statistical analysis

Data were analyzed using Excel 2010 (Microsoft) and GraphPad Prism 5.0 (GraphPad Software). Due to donor-specific variations in spontaneous NET-release, each experiment was performed with neutrophils derived from a minimum of three independent healthy blood donors. For each preparation, a minimum of six randomly selected images were acquired per slide and used for quantification of NET-producing cells. Data are expressed as percentages of NET-forming cells in relation to the total number of cells visualized with DAPI to stain the nuclei. Differences were analyzed by using a one-tailed Student’s t-test. The significance is indicated as *p<0.05; **p<0.005 and ***p<0.001.
2.4 Material and methods for chapter 3.4:

Iron chelating agents Desferrioxamine and L-Mimosine stimulate formation of neutrophil extracellular traps (NETs)

2.4.1 Bacterial strains

S. aureus strain LAC (pulsed-field type USA300), a community-acquired CA-MRSA strain (Voyich et al., 2005), was used for entrapment studies as previously described (Berends et al., 2010).

2.4.2 Microscopy to visualize the effect of DFO and L-Mimosine on NET formation

Human neutrophils were isolated from fresh heparinized blood by density centrifugation at 500 x g using Polymorphprep™ (Axis-Shield PoC). Neutrophils have been seeded on cover slides covered with 0.01% Poly-L-lysine (# P4707, Sigma). 5×10^5 cells in 250 µl RPMI 1640 (# E15-848, phenol red free, PAA) were seeded per well in a 24-well-plate. The cells were either stimulated with 25 nM PMA (InvivoGen Corp., San Diego, CA, US), 300 µM DFO (D9533, Sigma) or 300 µM L-Mimosine (M0235, Sigma) for 3h at 37°C with 5% CO₂. After incubation, the cells were fixed by adding PFA (#15710 Electron Microscopy Science) at a final concentration of 4% for 15 min at room temperature and kept at 4°C until subsequent immunostaining.

2.4.3 Immunostaining of H2A-H2B-DNA complex for NET visualization

Fixed cells were washed three times with PBS, blocked and permeabilized with 2% BSA PBS + 0.2% Triton X-100 for 45 min at room temperature. Incubation with a mouse monoclonal anti-H2A-H2B-DNA complex (clone PL2-6, 0.5 µg/ml) in 2% BSA PBS + 0.2% Triton X-100 was carried out overnight at 4°C as previously described (Jerjomiceva et al., 2014). Samples were washed with PBS and subsequently incubated with an Alexa-Fluor-488-labelled goat-anti-mouse antibody (1:1000 Invitrogen) for 45 min at room temperature. After washing, slides were mounted in ProlongGold® antifade with DAPI (Invitrogen) and analyzed by confocal fluorescence.
microscopy using a Leica TCS SP5 confocal microscope with a HCX PL APO 40x 0.75-1.25 oil immersion objective. Settings were adjusted in accordance to control preparations using an isotype control antibody.

Due to donor-specific variations in spontaneous NET-release, each experiment was performed with neutrophils derived from a minimum of three independent healthy blood donors. For each preparation, a minimum of six randomly selected images were acquired per slide and used for quantification of NET-producing cells. Data are expressed as percentages of NET-forming cells in relation to the total number of cells visualized with DAPI to stain the nuclei.

2.4.4 Boosting HIF-1α degradation by adding external iron ions

Human blood derived neutrophils (isolation as described above), were seeded at a concentration of $5 \times 10^5$ cells/well in 250 µl medium containing iron citrate ($\text{Fe}^{3+}$) or iron chloride ($\text{Fe}^{2+}$) (both 250µM) in a 24-well plate. Control cells were cultured in `pure´ RPMI medium. Subsequently, cells were stimulated with DFO (300µM) or L-Mimosine (300µM) for 3h at 37°C and 5%CO$_2$. The subsequent fixation and NET visualization as well as quantification were performed as described above.

2.4.5 NET entrapment assay

Bacteria were grown in Brain-Heart Infusion (BHI) medium at 37°C under agitation. Fresh overnight cultures were diluted 1:100 in BHI and then grown to mid-logarithmic growth phase ($\text{OD}_{600} = 0.7$). The bacteria were washed and FITC (0.33mg/ml) labelled for 30min in the dark. Subsequently, neutrophils were infected for 90 min at 37°C and 5%CO$_2$. After incubation, non-entrapped bacteria were washed away and bacterial entrapment within the NETs was analyzed by measuring the fluorescence signal at 485/538 nm compared to total amount of bacteria.
2.4.6 Immunostaining of PAD4 and H2A-H2B-DNA complex for PAD4 quantification and NET visualization

The procedure is the same as described above but additional antibodies were used. Besides the PL2-6 mouse IgG2b, a polyclonal rabbit anti PAD4 antibody (Wang et al., 2004) was used as a primary antibody besides the respective isotype IgG rabbit (Jackson Immunoresearch) as control staining. As additional secondary antibody Alexa-Fluor-633 goat anti rabbit (Invitrogen) was used.

ImageJ software was used for quantification of the PAD4-expression. Thus, the fluorescent intensity caused by the excited secondary antibody Alexa-Fluor-633 which binds to the anti PAD4 antibody was compared to the intensity of the respective isotype control.

2.4.7 Blocking PAD4 activity by adding external chloramidine

Human blood derived neutrophils (isolation as described above) were treated with chloramidine at a final concentration of 200 µM per well shortly before stimulation with HIF-1α boosters and PMA as NET inducers. The stimulation itself as well as incubation and subsequent fixation were performed as described above.

2.4.8 PAD4 detection in Western blot analysis

Neutrophils have been isolated and stimulated as described above. The cells were lysed in standard lysis buffer with proteinase inhibitors and proteins were separated via 10% SDS PAGE. After electrophoresis, proteins were transferred on to a PVDF membrane for 100 min. at 240 mA and blocked in 0.1% TBST + 5% nonfat dry milk for 45 min. For PAD4 detection, the blot was incubated with the polyclonal rabbit anti-PAD4 antibody (Wang et al., 2004) over night at 4°C with agitation. Additionally a monoclonal mouse anti-β-Actin antibody (Santa Cruz) was used as a loading control. The respective secondary antibody (goat anti-rabbit HRP and goat anti-mouse HRP) was added for 45 min at RT with agitation. Proteins were detected using SuperSignal West Femto Chemiluminescent Substrate reagents (Pierce, Thermo Scientific). The software Image J was used for signal quantification. Therefore, the PAD4 signal was normalized against the β-Actin control signal.
2.4.9 Statistical analysis

For statistical analysis GraphPad Prism 5.0 (Graph Pad Software, La Jolla, CA) was used. Data derived from a minimum of 3 independent experiments were analyzed. For analysis of time-dependent effect of DFO on NET-formation, 2-way ANOVA, followed by a Sidak’s multiple comparison to control group (no matching) was used. For concentration-dependent effect of DFO on NET-formation, non-parametric Kruskal-Wallis test followed by a Dunn’s multiple comparison to control group (no matching) was applied. For all other data a student’s t test (paired/non-paired, one-tailed) was performed. For all tests p was defined as * p<0.05; ** p<0.005; *** p<0.001, **** p<0.0001.
2.5 Material and methods for chapter 3.5:

Novel role of the antimicrobial peptide LL-37 in the protection of neutrophil extracellular traps against degradation by bacterial nucleases

2.6 Additionally used methods

2.6.1 NET induction with Dipyridyl

Human blood derived neutrophils (isolation as described above) were either treated with Dipyridyl or its solvent ethanol at a final concentration of 1 mM. Cells stimulated with 25nM PMA served as a positive control and media only as negative control. The stimulation itself as well as incubation and subsequent fixation were performed as described above.

2.6.2 Bioinformatics

To perform bioinformatics the online promoter prediction server tool from the center for biological sequence analysis (CBS) as well as the software Vector NTI (Invitrogen) was used. In addition the National Center for Biotechnology Information (NCBI) and its features were utilized for determination of protein sequences and localization within the gene.
3 Results

3.1 A novel role for the transcription factor HIF-1α in the formation of mast cell extracellular traps

ACCELERATED PUBLICATION

Short title: Role of HIF-1α in mast cell extracellular traps

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¹ Contributed equally to the work.

Abstract:

MCs (mast cells) are critical components of the host innate immune defence against bacterial pathogens, providing a variety of intra- and extra-cellular antimicrobial functions. In the present study we show, for the first time, that the transcriptional regulator HIF-1α (hypoxia-inducible factor-1α) mediates the extracellular antimicrobial activity of human and murine MCs by increasing the formation of MCETs (MC extracellular traps).

The extent of Lena Völlger’s contribution to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: A
2. Performing of the experimental part of the study: B
3. Analysis of the experiments: B
4. Presentation and discussion of the study in article form: A
3.2 Characterization of the antimicrobial activities of HL-60 cells as an alternative model to study neutrophil functions

Technical note

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The extent of Lena Völlger’s contribution to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: A
2. Performing of the experimental part of the study: B
3. Analysis of the experiments: B
4. Presentation and discussion of the study in article form: A
Highlights

- Differentiated HL-60 cells show less NET-formation compared to primary neutrophils.
- HL-60 cells show less antimicrobial activity compared to primary neutrophils.
- HL-60 cells are of limited usage as a model to study neutrophil functions.

Keywords:
Neutrophil extracellular traps, Staphylococcus aureus, Myeloperoxidase

Abstract

The human leukemia cell line HL-60 is considered an alternative cell culture model to study neutrophil differentiation and migration. The aim of this study was to characterize the suitability of HL-60 cells differentiated to neutrophil-like cells (nHL-60) as substitute for blood-derived human neutrophils to investigate the interaction of neutrophils with bacteria. For this purpose, bacterial killing and the release of neutrophil extracellular traps (NETs) of nHL-60 cells were analyzed and compared to blood derived primary neutrophils using Staphylococcus aureus as a prototype bacterium. Overall, the antibacterial activities of nHL-60 cells were distinctly lower than in blood-derived neutrophils. Furthermore, NET formation was clearly impaired in nHL-60 cells. In conclusion, these cells are of limited usage as an alternative model to study antimicrobial functions of neutrophils.

Introduction

The usage of primary blood-derived neutrophils to study host-pathogen interactions in vitro constitutes a limitation to the experimental design. One restriction is the total number of cells available at one occasion. On the other hand, interindividual differences between donors hamper comparisons between experiments. In addition, the isolation of neutrophils from whole blood is laborious and requires specific equipment. A cell-line based model to substitute blood-derived neutrophils is therefore highly wanted. The human leukemia cell line HL-60 is considered an alternative cell
culture model to study neutrophil functions. In this case, DMSO and all trans-retinoic acid (ATRA) are widely used to differentiate HL-60 cells to neutrophil-like cells (Wang et al., 2009, Verstuyf et al., 1995). Although the differentiated neutrophil-like cells show many characteristics of primary neutrophils, the differentiation is somewhat incomplete and defective (An et al., 2005; Herwig et al., 1996; Nordenfelt et al., 2009).

Neutrophils possess different antimicrobial activities to fight against invading pathogens. The most prominent one is phagocytosis, where pathogens are internalized and killed intracellularly by non-oxidative and oxidative mechanisms (Nathan, 2006). Another strategy is degranulation, meaning to deplete the granular content, e.g. antimicrobial peptides, into the extracellular space. More recently, the formation of extracellular traps (ETs) by neutrophils (Brinkmann et al., 2004) and other leukocytes (reviewed by von Köckritz-Blickwede and Nizet, 2009) has been discovered as an additional mechanism to entrap and kill pathogens extracellularly. Key mediators to trigger neutrophil ETs (NETs) release are reactive oxygen species (ROS), generated by the membrane-bound NADPH oxidase enzyme complex.

The goal of this study was to characterize the antimicrobial activity of differently differentiated HL-60 cells against the pathogen Staphylococcus aureus in comparison to primary human blood-derived neutrophils, with special emphasis on the formation of NETs.

**Materials and methods**

**Bacterial strains and growth conditions**

For bacterial killing assays, *S. aureus* Newman was used; for NET induction assays, a nuclease-deficient derivative of *Staphylococcus aureus* USA 300 LAC (*S. aureus* AH1787) was used (Berends et al., 2010). The absence of bacterial nuclease activity ensured to capture total NET formation without interference with NET degradation. Bacteria were grown in brain heart infusion (BHI) medium at 37°C shaking. An overnight culture was diluted 1:100 into fresh medium and grown to mid-logarithmic phase (OD\textsubscript{600}=0.5). Bacteria were then harvested by centrifugation, suspended in PBS and adjusted to the desired concentration by optical density at 600 nm. Further dilutions were prepared in cell culture medium.
HL-60 cells

HL-60 cells were received from the “Deutsche Sammlung für Zellkulturen” (DSMZ, DSMZ-No.: ACC-3).

Cultivation and differentiation of HL-60 cells

The myeloid leukemia cell line HL-60 was propagated in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from PAA). To induce a neutrophil-like phenotype, cells were treated with either 1.25% DMSO for 3 days, 1.25% DMSO for 4 days or 1 µM retinoic acid for 4 days, reaching a maximum cell count of $1 \times 10^6$ cells/ml. For experiments, differentiated cells were collected by centrifugation for 10 minutes at 118 g, washed once with PBS and finally adjusted to a density of $2 \times 10^6$ cells/ml in RPMI 1640 supplemented with 2% nuclease-free FBS (heat-inactivated at 70°C). HL-60 cells cultured and differentiated according to this protocol will further be referred to as nHL-60.

Isolation of human blood-derived neutrophils

Human neutrophils were isolated from freshly taken venous blood from healthy donors by density gradient centrifugation using PolymorphPrep according to the manufacturer’s protocol (Axis-Shield). Neutrophils were adjusted to a density of $2 \times 10^6$ cells/ml in RPMI 1640 supplemented with 2% nuclease-free FBS.

Bacterial killing assay

To determine the bactericidal activity of nHL-60 cells or neutrophils, cells were co-incubated with bacteria at a multiplicity of infection (MOI) of 2 in a final volume of 500 µl in 48-well non-treated cell culture plates. All incubations were carried out at 37°C and 5% CO₂ in a humidified incubator. Prior to infection, cells were pre-stimulated for 20 minutes with phorbol 12-myristate 13-acetate (PMA; 25 nM), with or without addition of cytochalasin D (10 µg/ml) to block phagocytosis. Control cells received the vehicle (DMSO) in the same dilution. Bacteria were then added to the cells, the plates were centrifuged for 5 minutes at 472 g and incubated for 30 minutes. Cells were lysed by addition of 50 µl of 0.25% Triton X-100 in PBS and
serial dilutions were plated on Todd-Hewitt agar plates for viable count. All conditions were analyzed in duplicate. Results were expressed as surviving bacteria compared to bacterial growth under the same conditions in the absence of neutrophils.

**NET induction assay**

The capacity of nHL-60 cells and blood-derived neutrophils to form NETs was assessed after stimulation with PMA and *S. aureus* AH1787. Cells were seeded on 8-mm cover slips coated with poly-L-lysine, stimulated with 25 nM PMA and/or bacteria at a MOI of 2 as indicated and centrifuged for 5 minutes at 472 g. The plates were then incubated at 37°C and 5% CO₂ in a humidified incubator for 1, 2, 3 or 4 hours. Cells were fixed by addition of paraformaldehyde (PFA) in PBS to a final concentration of 4% PFA. For all conditions, preparations were performed in duplicate.

**NET visualization and quantification**

Fixed cells were washed three times with PBS and permeabilized and blocked with 2% BSA in 0.2% Triton X-100/PBS for 45 minutes at room temperature. Incubation with a mouse monoclonal anti-H2A-H2B-DNA complex (clone PL2-6 (Losman et al., 1992), 0.5 µg/ml in 2% BSA in 0.2% Triton X-100/PBS) was carried out overnight at 4°C, followed by washing (3 times with PBS) and subsequent incubation with an AlexaFluor488-labelled anti-mouse antibody for 45 minutes at room temperature. After washing, slides were mounted in ProlongGold antifade including DAPI and analyzed by confocal fluorescence microscope using a Leica DMI6000CS confocal microscope with a HCXPLAPO 40 × 0.75 - 1.25 oil objective. Preparations with an isotype control antibody were used for setting adjustment. For each preparation, three randomly selected images were acquired and used for quantification of NET producing cells. Data were expressed as percentages of NET-forming cells in relation to the total number of cells. The mean value derived from *n*=6 images for each condition per experiment was used for statistical analysis.
Myeloperoxidase (MPO) detection assay

The cells were treated in the same way as for the NET induction assay. The visualization and quantification differed only in the used antibodies. For the myeloperoxidase detection a polyclonal rabbit anti myeloperoxidase antibody (Dako firma) in 2% BSA in 0.2% Triton X-100/PBS was used. The secondary antibody was an Alexa-Fluor 488-labelled anti-rabbit antibody (1:1000; Invitrogen). All other steps were identical compared to the NET visualization and quantification.

Statistical analysis

The average values derived from independent experiments performed in duplicate were used for statistical analysis and are depicted as mean and standard error of the mean (SEM). Comparisons between stimulated and non-stimulated cells of equally differentiated cells were performed by paired one-tailed \( t \)-test as indicated. Comparisons between differently differentiated cells or different cell types were performed as unpaired one-tailed \( t \)-test. In both cases, differences with \( P<0.05 \) were considered statistically significant.

Results

nHL-60 cells exhibit antimicrobial activity

To investigate whether differentiated HL-60 (nHL-60) cells act antibacterial, we co-incubated \( S. \) aureus Newman with nHL-60 (Figure 3.2.1A). Prior to infection, cells were stimulated with PMA, a widely used neutrophil activator, or left untreated. After differentiation with DMSO for three days, bacterial growth was reduced compared to incubation in cell-free medium and the antibacterial activity of both DMSO-differentiated nHL-60 cells could be enhanced by PMA, although this effect did not reach significance. Cells differentiated with retinoic acid were not antimicrobial active, even if pre-stimulated with PMA.
In general, the inhibitory properties of nHL-60 cells were considerably lower than the bactericidal activity exhibited by blood-derived neutrophils, regardless of the differentiation method. In addition, the stimulatory effect of PMA was less efficient in HL-60 cells differentiated for three days with DMSO compared to neutrophils (reduction of bacterial growth to 86% by nHL-60 versus 45% by neutrophils compared to non-treated cells, $P=0.004$). In both other cases PMA-stimulation did not lead to bacterial killing.

**Decreased phagocytosis in nHL-60 cells**

Stimulation with PMA triggers both intracellular killing after phagocytosis and killing by extracellular traps (ETs) in neutrophils. We sought to further specify the antibacterial activity of nHL-60 cells. Therefore, phagocytosis was pharmacologically blocked by cytochalasin D, an inhibitor of actin polymerization (Ding et al., 2010).
Figure 3.2.1B: Bacterial killing by PMA-stimulated nHL-60 cells and blood-derived neutrophils was determined in the presence of the phagocytosis inhibitor cytochalasin D. Results from three independent experiments are depicted as mean and SEM (n=3). Comparison between non-treated cells (Co) and cells treated with cytochalasin D (CytD) was performed by unpaired one-tailed t-test, comparison between nHL-60 cells and blood-derived neutrophils was performed by unpaired two-tailed t-test; **P<0.01, ***P<0.001.

However, this treatment did not influence the antibacterial action in nHL-60 cells, indicating that phagocytosis is inefficient in these cells under the experimental conditions applied here (Figure 3.2.1B). In blood-derived neutrophils in contrast, bacterial killing was greatly reduced in the presence of cytochalasin D (Figure 3.2.1B).

**nHL-60 cells form NETs**

Since phagocytosis was not an effective antibacterial strategy in nHL-60 cells, we investigated the ability of nHL-60 cells to form NETs in response to chemical and biological stimuli. While PMA-induced NET formation in blood-derived neutrophils was evident after two hours and complete after four hours of incubation, only single nHL-60 cells released NETs even at the end of the experiment (Figure 3.2.2).
Figure 3.2.2: NET formation of PMA-stimulated primary neutrophils and DMSO-differentiated HL-60 cells. (A) NET formation by HL-60 cells differentiated with DMSO for three days and blood-derived neutrophils was assessed after stimulation with PMA for indicated periods of time. Results from 3-4 experiments are depicted as mean and SEM (n=3-4). Comparison between nHL-60 cells and neutrophils at each time point was performed by unpaired two-tailed t-test; #P<0.05, ####P<0.0001. Comparison to non-stimulated cells was performed by unpaired one-tailed t-test; **P<0.01, ***P<0.001. (B) Representative images from experiments shown in (A). nHL-60 or blood-derived neutrophils were stimulated with PMA for 4 hours, fixed and stained with an antibody directed against histone-DNA-complexes and a secondary AlexaFluor488-labelled anti-mouse antibody (green). The nuclei were stained with DAPI (blue). The scale bar is 100 µm.

By additional stimulation with the nuclease-deficient \textit{S. aureus} AH1787, NET release by nHL-60 cells was triggered to up to 28% (Figure 3.2.3), however, still far lower than NET formation by blood-derived neutrophils.

Differentiation with retinoic acid or longer differentiation with DMSO (four days) did not substantially increase the ability to form NETs (Figure 3.2.4). Actually, after differentiation with retinoic acid the amount of produced NETs was decreased (11.8% and 7.5% after stimulation with PMA only and additional \textit{S. aureus} infection, respectively).
Figure 3.2.3: NET formation of PMA-stimulated nHL-60 cells and blood-derived neutrophils infected with a nuclease-deficient S. aureus USA 300 LAC strain. (A) PMA-stimulated nHL-60 cells were infected with S. aureus AH1787 and compared to non-infected PMA-stimulated cells. Comparison was performed by unpaired one-tailed t-test; #P<0.05. (B) Representative images from experiments shown in (A). PMA-stimulated nHL-60 without (non-infected) or infected with S. aureus AH1787 for 4 hours were fixed and stained with an antibody directed against histone-DNA-complexes and a secondary AlexaFlour488-labelled anti-mouse antibody (green). The nuclei were stained with DAPI (blue). The scale bar is 100 µm.

Figure 3.2.4: NET formation by differently differentiated HL-60 cells. NET formation by differently differentiated HL-60 cells with and without PMA stimulation and S. aureus infection after 4 hours. The results of three independent experiments are shown. Comparisons between none-stimulated, PMA-stimulated and S. aureus-infected cells and between differently differentiated HL-60 cells were performed by unpaired, one-tailed t-test. No statistical differences were detected.
Discussion

In this study we aimed to examine whether differentiated HL-60 cells could provide a model for replacing primary blood-derived neutrophils for *in vitro* experiments to investigate antimicrobial functions. Compared to neutrophils, the overall antibacterial activity of nHL-60 cells against the model organism *S. aureus* was greatly reduced, even in the presence of the chemical stimulant PMA. Based on the results from this study, we conclude that all three major antibacterial mechanisms exhibited by neutrophils, i.e. degranulation, phagocytosis and NET formation, are derogated in nHL-60 cells.

It is an established fact that HL-60 cells do not harbor the entire arsenal of granules, making their differentiation somewhat incomplete. As a marker for cell differentiation MPO expression of the differentiated HL-60 cells was assessed in this study (Figure 3.2.S1). The data demonstrated similar amounts of nHL-60 cells compared to primary neutrophils, indicating a neutrophil-like phenotype. Nonetheless, the overall antimicrobial functions were decreased in nHL-60. Since HL-60 cells lack secondary granules and secretory vesicles, which contain the vast proportion of \( b_{558} \), the membrane-bound component of the NADPH oxidase enzyme complex, their ability to produce reactive oxygen species (ROS) might be impaired. Supportive for this hypothesis is a study conducted by Nordenfelt et al., 2009. Using *Streptococcus pyogenes* as a model organism, the authors conclude that HL-60 cells can replace neutrophils in models which do not rely on respiratory burst activity, pointing towards reduced capacity of HL-60 cells to generate ROS. The presence of MPO would not overcome this issue, as it acts downstream of NADPH. However, reports regarding the presence of NADPH oxidase and ROS generation in HL-60 cells are controversial (Vong et al., 2014). Additionally to \( b_{558} \) the cathelicidin hCAP18/LL-37 is stored in secondary granules. Hence, an absence of these granules implicates also the lack of LL-37. Furthermore, An et al. (2005) showed that peripheral blood cells from patients with acute myeloid leukemia do not produce this cathelicidin, even though gene-specific mRNA is detectable. In HL-60 cells, this deficiency could not be converted by differentiation using all-*trans* retinoic acid (An *et al.*, 2005). Primary granules, carrying most of the antimicrobial peptides including neutrophil defensins, on the other hand can be found in HL-60 cells (Herwig *et al.*, 1996). Nevertheless, the lacking of LL-37
and impaired ROS production might be explanations for the reduced killing of \textit{S. aureus} by nHL-60 cells in comparison to primary neutrophils.

Consistent with the results of other studies (Vong et al., 2014; Kawakami et al., 2014; McInturff et al., 2012), also in these experiments nHL-60 cells produced NETs, although to a much lower amount than primary neutrophils. A putative defect in the neutrophil NADPH oxidase enzyme complex appears also a feasible reason for the failure of nHL-60 cells to efficiently produce NETs. It is known that ROS production by NADPH oxidase is essential for an efficient NET production (Fuchs et al., 2007), therefore a lack of this enzyme would lead to a downgraded NET formation. In addition, neutrophil elastase, a component in the primary granules, has recently been identified being indispensable for NET formation (Papayannopoulos et al., 2010). Lysates of HL-60 cells failed to induce NET release from isolated nuclei, indirectly indicating that the primary granules of HL-60 cells lack this enzyme.

These findings together show that the development of neutrophil characteristics is insufficient in HL-60 cells: HL-60 cells after chemical differentiation with DMSO or ATRA do not exert similar antibacterial activities compared to blood-derived neutrophils. Thus we conclude that, HL-60 cells differentiated with DMSO or ATRA are of limited value to replace primary cells in \textit{in vitro} experiments to investigate host-pathogen interactions.

\textbf{References (style adjusted to respective Journal)}


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Supplementary data

Figure 3.2.S1: Percentage of myeloperoxidase-positive cells of differently differentiated HL-60 cells. (A) Percentage of myeloperoxidase-positive cells of HL-60 cells after 2 hours of incubation with PMA. Comparisons between the differently differentiated HL-60 cells were performed by unpaired, one-tailed t-test. (B) Representative fluorescence micrographs of data shown in (A). nHL-60 cells were stimulated with PMA for 2 hours, fixed and stained with an antibody directed against myeloperoxidase and a secondary AlexaFluor488-labelled anti-rabbit antibody (green). The nuclei were stained with DAPI (blue).
3.3 Influence of cell density on phorbol 12-myristate 13-acetate (PMA) mediated NET-formation

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A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: B
2. Performing of the experimental part of the study: C
3. Analysis of the experiments: C
4. Presentation and discussion of the study in article form: C
Abstract

In 2004, neutrophil extracellular traps have been described as fundamental immune defense of neutrophils against various microbes. Since that time publications are increasing that characterize the stimuli which can activate the cells to release NETs. Mostly, the phorbol ester phorbol-myristate acetate (PMA) is used as a positive control, since PMA has been shown to efficiently induce NET formation via increasing the oxidative burst in the cell. However, different protocols of neutrophil cultivation can lead to varying results in NET formation, comparing results from different laboratories. Thus the aim of the study was to analyze the effect of cell density on PMA stimulated NET formation. Using immunofluorescence microscopy, we showed that the more cells were seeded, the more NETs were detected (higher x-fold-increase) in PMA stimulated cells compared to unstimulated cells. Thus, the effect of cell density on PMA mediated NET formation may explain variable results that are found between different laboratories and should be considered in future NET experiments.

Introduction

Within the innate immune system, Neutrophils are a key player of the first line of defense against pathogens. They exert a variety of intra- and extracellular antimicrobial functions and additionally contribute in processes of tissue remodeling and tissue repair. Besides phagocytosis and degranulation, extracellular trap (ET) formation (reviewed by von Köckritz-Blickwede and Nizet, 2009) has been described as additional fundamental antimicrobial activity of neutrophils. ETs are released by different immune cells such as neutrophils (Brinkmann et al., 2004), macrophages/monocytes (Chow et al., 2010), mast cells (von Köckritz-Blickwede et al., 2008) and eosinophils (Yousefi et al., 2009). NETs are decondensed chromatin structures which contain antimicrobial components like histones and proteases as well as antimicrobial peptides and can enable the immobilization of microbes. NET formation (NETosis) can be activated by pathogens or microbial derived factors like lipopolysaccharide (LPS) [Brinkmann et al., 2004], pro-inflammatory cytokines (IL-8) [Keshari et al., 2013] or different substances like phorbol 12-myristate 13-acetate (PMA) [Brinkmann et al., 2004, Fuchs et al., 2007]. PMA is frequently used to induce PET
formation by activating protein kinase C (PKC) [Tahara et al., 2009]. PKC, in turn, triggers the NADPH oxidase complex [Dekker et al., 2000] that produces superoxide anions which serve as a starting product for ROS that constitute the oxidative burst. NADPH oxidase activation by PMA therefore leads to the formation of ROS, which is also initiating PET formation. Since PMA has been proven as efficient NET-inducing agent [Fuchs et al., 2007], it is mostly used as positive control when quantifying NETs in response to a specific stimulus [Brinkmann et al., 2004, Fuchs et al., 2007, Urban et al., 2009, Menegazzi et al., 2012]. Within the past ten years, research in the field of neutrophil extracellular traps (NETs) increased widely. Nevertheless, when comparing different literature on NET studies, it attracts attention that authors use different protocols, which result in different results between laboratories. Especially, no standardized protocol that mentions a specific cell density of neutrophils is available. Therefore, the aim of this study was to analyze the effect of cell density on PMA stimulated NET formation.

Results and Discussion

Since neutrophils are naturally non-adherent cells, it needs to be ensured that the cells adhere to the cover slip, which is later microscopically analyzed. We used Poly-L-lysine, a nonspecific attachment factor. The polypeptide of the essential amino acid L-lysine promotes cell adhesion by enhancing electrostatic interaction between positively-charged surface ions of the solid cell culture surface and negatively charged ions on the cell membrane [Sitterley, 2008]. Human blood-derived neutrophils were isolated by density gradient centrifugation and seeded at the indicated cell density onto Poly-L-Lysine coated glass coverslips. The cells were then either treated with RPMI medium (Ctr.) or RPMI + a final concentration of 25nM PMA for 1, 2, 3 and 4h at 37°C, 5%CO₂.

Then, we analyzed if the number of cells that attached to the surface does not differ between PMA stimulated and unstimulated cells. We therefore counted the total number of cells per slide.
Figure 3.3.1a-d: Number of analyzed cells for each sample (Ctr., PMA) and time point at the respective cell density. Human blood-derived neutrophils were isolated by density gradient centrifugation and seeded at the indicated cell density onto Poly-L-Lysine coated glass coverslips. The cells were then either treated with RPMI medium (Ctr.) or RPMI + a final concentration of 25nM PMA for 1, 2, 3 and 4h at 37°C, 5%CO₂. Formation of NETs was visualized microscopically, using an antibody against H2AH2B-DNA complexes in combination with DAPI to stain the nuclei. NET releasing cells were counted using the Image J software. The number of counted cells within the two samples is consistent for the cell densities $2 \times 10^4$ (a), $5 \times 10^4$ (b) and $1 \times 10^5$ (c). At a cell density of $5 \times 10^5$, slightly more cells were counted in the PMA treated sample in comparison to the unstimulated control (d).

Figure 3.3.1 a-c show that there is no significant difference between the number of cells counted for the control sample in comparison to the PMA stimulated sample at cell densities of $2 \times 10^4$, $5 \times 10^4$ and $1 \times 10^5$ cells per well at all tested time points (1-4h). At a cell density of $5 \times 10^5$ cells per well, a slight but not significant increase in the amount of cells counted in the PMA sample in comparison to the control sample can be observed for the time points 1h-3h but not for the 4h time point (Figure 3.3.1 d). Thus, a significant impact of the adhesion to the glass slides in the presence or
absence of PMA can be excluded. Figure 3.3.1e-h show representative immunofluorescence images of the data represented in Figure 3.3.1c) and d).

![Fluorescent images](image)

Figure 3.3.1e-h: Representative fluorescent images of the data displayed in Figure 3.3.1c and d. The green numbers represent cells that were counted as NET negative; the red numbers show cells that were counted as NET positive. The quantification was done using the software ImageJ. Due to the previous immunostaining the NETs were displayed in green, nuclei and nuclear DNA in blue.

Then, the percentage of cells releasing NET-structures was quantified between 1 and 4 hours in the presence or absence of PMA. Figure 3.3.2 a-d) shows the percent of cells releasing NET-structures and x-fold NET induction of PMA in comparison to unstimulated cells over time (1-4h) and for each tested cell density. Within the first two hours, the detected NET-induction does not increase more than 2-fold in the PMA stimulated cells in comparison to the control cells for all tested cell densities (Figure 3.3.2 a-d).
Figure 3.3.2a-d: Percent of cells releasing NET-structures and x-fold NET-induction of PMA in comparison to unstimulated cells over time and for each tested cell density. Within the first two hours, the detected NET-induction does not increase more than 2-fold in the PMA stimulated cells in comparison to the control cells for all tested cell densities (a-d). Higher NET-formation was detected for 3 and 4 hour stimulation. The maximum NET-release of 8.9 fold was detected after 4 hours of incubation at a cell density of 5x10^5 cells (d).

Higher NET-formation was detected for 3 and 4 hours of stimulation. The maximum NET-release of 8.9 fold was detected after 4 hours of incubation at a cell density of 5x10^5 cells (Figure 3.3.2d). The number of cells releasing NETs after stimulation with 25nM PMA generally increases over time. However, the number of NET-positive cells within the unstimulated control decreases with the number of cells seeded per well (Figure 3.3.3a).

Importantly, the data presented here show that the more cells were seeded, the more NETs were detected (higher x-fold-increase) in PMA stimulated cells in comparison to unstimulated cells: A 3.6 fold increase in cells releasing NETs was detected after
3h of PMA stimulation using $2 \times 10^4$ cells per well (Figure 3.3.2.a), whereas a 9.4 fold induction was detected when $5 \times 10^5$ cells per well were seeded (Figure 3.3.2d).

![Graph](image)

Figure 3.3.3a/b: NET-release after 4h of incubation at 37°C, 5%CO$_2$ for different cell densities. (a) Unstimulated neutrophils show higher NET-formation at lower cell densities. The number above the columns represents the mean number of cells counted in a total of six images per cell density. (b) The x-fold increase in the amount of PMA treated cells releasing NETs in comparison to unstimulated cells increases with the cell density. Differences were analyzed by using a one-tailed Student's t-test. The significance is indicated as **$p$ < 0.005 and ***$p$ < 0.001.

It may be hypothesized that during NET formation, neutrophils release factors that stimulate surrounding neutrophils to additionally form NETs. Those factors might be any proteins that have been found to be associated with NETs e.g. elastase, NADPH oxidase or MPO. Since it is also known that those three factors may contribute to NET formation [Munafo et al., 2009] it might be hypothesized that the closer the proximity of the individual neutrophils, the stronger might be the impact of those factors to subsequently also release NETs from neighboring neutrophils.

Finally, the effect of cell density on PMA mediated NET-formation may explain variable results that are found between different laboratories or authors and should be considered in future NET experiments. Current work in our lab is focusing on the question, if and how activated neutrophils can stimulate neighboring cells to release NETs.
Material and Methods

Isolation of human blood derived neutrophils

Human primary blood-derived neutrophils were isolated from freshly heparinized blood of healthy donors as previously described [von Köckritz-Blickwede et al., 2010]. For the performed in vitro NET assays, the neutrophils were seeded on poly-L-lysine-coated (0.01%, Sigma) cover slides in 24-well plates at a concentration of $2 \times 10^4$, $5 \times 10^4$, $1 \times 10^5$ or $5 \times 10^5$, cells/well (250µl/well). RPMI 1640 without phenol red (PAA) was used for cultivation of the cells at 37°C and 5% CO₂. The cells were either stimulated with 25 nM PMA (InvivoGen Corp., San Diego, CA, US) or RPMI medium alone for 1, 2, 3 and 4h. After incubation, the cells were fixed by adding PFA (#15710 Electron Microscopy Science) at a final concentration of 4% for 15 min at room temperature. Subsequently the samples have been immune stained as described below.

Visualization of NETs

Fixed cells were washed three times with PBS, blocked and permeabilized with 2% BSA in 0.2% Triton X-100/PBS for 45 min at room temperature. Incubation with a 1:5000 dilution of a mouse monoclonal anti-Histone1-DNA complex (Millipore MAB3864, 2.2 mg/ml in 2% BSA in 0.2% Triton X-100/PBS) was carried out overnight at 4°C. Samples were washed with PBS and subsequently incubated with an Alexa-Fluor-488-labelled goat-anti-mouse antibody (1:1000; Invitrogen) for 45 min at room temperature. After washing, slides were mounted in ProlongGold® antifade with DAPI (Invitrogen) and analyzed by confocal fluorescence microscope using a Leica TCS SP5 confocal microscope with a HCX PL APO 40x 0.75-1.25 oil immersion objective. Settings were adjusted with control preparations using an isotype control antibody. For each preparation, three randomly selected images were acquired and used for quantification of NETs. The mean value derived from $n = 6$ images for each condition per experiment was used for statistical analysis.
**NET quantification**

To quantify the percentage of cells releasing NETs, the software ImageJ was used. The quantification took place by counting the cells which released NET structures and are associated with a NET within the image (Figure 1e-h). The green numbers represent cells that were counted as NET negative; the red numbers show cells that were counted as NET positive. Due to the previous immunostaining, the NETs were displayed in green, nuclei and nuclear DNA in blue.

**Statistical analysis**

Data were analyzed using Excel 2010 (Microsoft) and GraphPad Prism 5.0 (GraphPad Software). Due to donor-specific variations in spontaneous NET-release, each experiment was performed with neutrophils derived from a minimum of three independent healthy blood donors. For each preparation, a minimum of six randomly selected images were acquired per slide and used for quantification of NET-producing cells. Data are expressed as percentages of NET-forming cells in relation to the total number of cells visualized with DAPI to stain the nuclei. Differences were analyzed by using a one-tailed Student’s t-test. The significance is indicated as *p<0.05; **p<0.005 and ***p<0.001.

**References (style adjusted to respective Journal)**


3.4 Iron chelating agents Desferrioxamine and L-Mimosine stimulate formation of neutrophil extracellular traps (NETs)

Running title: Desferrioxamine induces NETs

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3. Analysis of the experiments: C
4. Presentation and discussion of the study in article form: C
Abstract

Neutrophil extracellular trap (NET) formation is a significant innate immune defense mechanism against microbial infection that complements other neutrophil functions including phagocytosis and degranulation of antimicrobial peptides. NETs are decondensed chromatin structures in which antimicrobial components (histones, antimicrobial peptides and proteases) are deployed and mediate immobilization of microbes. Here we describe an important effect of iron chelation on the phenotype of NET formation. Iron chelating agents desferrioxamine (DFO) and L-Mimosine significantly induced the production of NETs by freshly isolated human neutrophils as visualized and quantified by immunohistochemistry against histone-DNA complexes. Further analysis revealed that NET induction by iron chelation required NADPH-dependent production of reactive oxygen species as well as protease and peptidylarginine deiminase 4 (PAD4) activities, three key mechanistic pathways previously linked to NET formation. Our results demonstrate that iron-chelation contributes to the formation of NETs and suggest a target for pharmacological manipulation of NET activity.

Key words
extracellular traps; innate immunity; neutrophils

Introduction

Neutrophils are part of the first line of defense against pathogens within the innate immune system. These specialized leukocytes support a variety of intra- and extracellular antimicrobial functions and collaborate in processes of tissue remodeling and tissue repair. Three principal modes of neutrophil antimicrobial function are known: phagocytosis, degranulation and extracellular trap (ET) formation (reviewed by von Köckritz-Blickwede and Nizet, 2009). ETs are released by different immune cells such as neutrophils (Brinkmann et al., 2004), macrophages/monocytes (Chow et al., 2010), mast cells (von Köckritz-Blickwede et al., 2008) and eosinophils (Yousefi et al., 2009) after stimulation with host cytokines or microbial-derived factors. Neutrophil ETs (NETs) consist of a backbone of DNA fibers, closely associated with antimicrobial peptides, histones and proteases which exert antimicrobial activity.
These structures have been shown to entrap and immobilize several bacterial and fungal pathogens (Brinkmann et al., 2004; von Köckritz-Blickwede and Nizet, 2009; Beiter et al., 2006; Urban et al., 2006; Mc Donald et al., 2012) thereby restrict their spread within the infected host.

The cellular processes that lead to the formation of NETs are not fully elucidated. Different inflammatory mediators like interleukin-8 (IL-8) (Brinkmann et al., 2004), interferon (IFN) α/γ (Martinelli et al., 2004), phorbol myristate acetate (PMA) (Brinkmann et al., 2004) or hydrogen peroxide (H₂O₂) (Fuchs et al., 2007) are strong NET stimulants, and direct exposure to bacteria or fungi (Fuchs et al., 2007; Buchanan et al., 2006; Ramos-Kichik et al. 2008; Grinberg et al., 2008; von Köckritz-Blickwede et al., 2010; von Köckritz-Blickwede and Nizet, 2009) or bacterial-derived factors such as lipopolysaccharide (LPS) (Brinkmann et al., 2004) can induce NET formation. These signals can lead to NADPH oxidase activation and formation of reactive oxygen species (ROS), which has been shown to be essential for NET formation in response to some stimuli e.g. bacteria (Fuchs et al., 2007), and contributory to NET formation with others e.g. enrofloxacin (Jerjomiceva et al., 2014). Ultimately, the nuclear membrane of the activated neutrophil dissolves and chromatin is decondensed. The disrupted nuclear membrane allows nuclear components to mix with the cytoplasmic granular proteins of the cell (Fuchs et al., 2007). Finally those mixed nuclear and cytoplasmic components are released into the extracellular space as ETs. In 2009 Wang et al. showed that histone hypercitrullination by peptidylarginine-deiminase 4 (PAD4) regulates the unfolding of chromatin during the formation of NETs (Wang et al., 2009). Furthermore, neutrophil elastase has been shown to proteolytically modify histones and contribute to NET formation (Papayanopouloos et al., 2010).

Dysregulation of NET formation is thought to have serious consequences: As reviewed by Saffarzadeh and Preissner (Saffarzadeh and Preissner, 2013), too much NET formation can lead for example to the development of autoimmune diseases or thrombosis, and too little can lead to ineffective defenses against infection. Understanding the mechanisms that regulate NET formation might lead to potential drug targets for treating infections or autoimmune diseases. Here we show that iron-chelating agents desferrioxamine (DFO) and L-Mimosine lead to the formation of NETs in human blood-derived neutrophils.
Results

Iron chelating agents L-Mimosine and DFO mediate formation of NETs

To examine the effect of iron chelation on NET formation, human blood-derived neutrophils were treated with the iron-chelating agents DFO (300 µM) or L-Mimosine (300 µM) for 3 h at 37°C and 5% CO₂.

Figure 3.4.1A: Increased NET formation was detected for neutrophils stimulated for 3h with 300µM each, DFO and L-Mimosine. All graphs represent the mean ± SEM of 18 images derived from 3 independent experiments. ** p<0.005; *** p<0.001.

As shown in Figure 3.4.1A, both iron chelators significantly induced NET formation. Representative immunofluorescent micrographs of DFO-mediated NET induction compared to control are shown in Figure 3.4.1B, with NET structures visible as histone-DNA extrusions of the nuclei of the cells.
Figure 3.4.1B: Representative fluorescent micrographs of human neutrophils incubated in media only (Ctr) or media containing 300µM DFO representing the results of the column bar graph in Figure 3.4.1A.

Figure 3.4.1C and D corroborate that NET formation was related to the iron chelation activity of the drugs, as addition of excess iron decreased the induction phenotype: both ferrous and ferric forms of iron (Fe^{2+} or Fe^{3+}) supplementation had the same effect in this assay.

Figure 3.4.1C/D: Addition of divalent (c) or trivalent (d) iron ions abolishes the NET-induction effect. All graphs represent the mean ± SEM of 18 images derived from 3 independent experiments. ** p<0.005; *** p<0.001.
To confirm that NETs induced by iron chelation are functional, we demonstrated that extracellular entrapment of methicillin-resistant *Staphylococcus aureus* (MRSA, USA300 strain) was increased after neutrophil treatment with DFO (Figure 3.4.2).

Figure 3.4.2A/B: DFO induces entrapment of *Staphylococcus aureus* in NETs released by human neutrophils. (A) Human blood-derived neutrophils were isolated by density gradient centrifugation, seeded, stimulated with media only or media containing 300µM DFO for 2h, infected with FITC-labeled *S. aureus* (MOI=10) for 90 min and washed to remove unbound bacteria. The FITC signal was measured and compared to total amount of bacteria. Data are shown as mean ± SEM of 4 independent experiments. * p<0.05. (B) Representative fluorescent micrograph of FITC-labeled *S. aureus* (green) entrapped in DAPI stained DFO-induced human NETs (blue).

DFO-mediated NET-induction was both time and concentration dependent (Figure 3.4.3A and B), and a similar effect was documented in bovine derived neutrophils, indicating that the DFO-mediated NET-induction is not restricted to human cells (Figure 3.4.3C).
Figure 3.4.3A/B: DFO induced NET formation is time- and concentration dependent. Human blood derived neutrophils were isolated by density gradient centrifugation, stimulated and the formation of NETs was visualized using the PL2-6 antibody against H2AH2B-DNA complexes in combination with DAPI to stain the nuclei. (A) Human neutrophils were stimulated with 300µM DFO for 1, 2, 3 and 4h and subsequently fixed in 4% PFA. NET formation was determined in comparison to the unstimulated control. A significant increase in the amount of cells that form NETs was observed over time. The numbers on top of the bars represent the fold increase of NET-release from cells treated with DFO compared to the unstimulated control. (B) Different DFO concentrations (150µM, 300µM, 600µM, 900µM) were tested on their ability to induce NETs in human neutrophils after an incubation period of 3h. The graphs represent the mean ± SEM of the 24 (A), 30 (B) images derived from 4 (A), 5 (B), independent experiments. * p<0.05; *** p<0.001, **** p<0.0001.

Figure 3.4.3C: DFO induced NET formation is not limited to human neutrophils. Bovine neutrophils were isolated by density gradient centrifugation, stimulated and the formation of NETs was visualized. NET formation of bovine neutrophils after stimulation with either media only or media containing 300µM DFO for 3 and 5h. The graph represents the mean ± SEM of 12 images derived from 2 independent experiments. *** p<0.001.
ROS and proteases contribute to DFO-mediated NET formation

Since NADPH-oxidase-dependent formation of ROS has been shown to contribute to NET formation (Fuchs et al., 2007; Akong-Moore et al., 2012), diphenylene iodonium (DPI) was used to inhibit NADPH oxidases to test the role of NADPH oxidases in DFO-induced NET formation in human neutrophils. As shown in Figure 3.4.4A, we found that DPI significantly blocks the formation of NETs, indicating that a NADPH-oxidase-dependent process of NET formation is induced by DFO.

Figure 3.4.4A: NADPH oxidase contributes to DFO-mediated NET formation. Cells were incubated for 3h in media only or media containing 300µM DFO in the presence and absence of DPI (10µg/ml). Graph represents the mean ± SEM of a minimum of 18 images derived from 3 independent experiments. * p<0.05.

Recently, the proteolytic activity of neutrophil elastase was shown to modify histones and contribute to NET formation (Papayannopoulos et al., 2010). The trypsin inhibitor and anti-fibrinolytic drug, aprotinin, has further been shown to block the activity of serine proteases such as neutrophil elastase (Smith et al., 2010). As shown in Figure 3.4.4B, treatment of neutrophils with aprotinin also significantly blocked the DFO-mediated NET formation, indicating that the activity of serine proteases (e.g. elastase) is also involved in the induction of this phenotype.
Results

Figure 3.4.4B: Elastase contributes to DFO-mediated NET formation. Cells were incubated for 3h in media only or media containing 300µM DFO in the presence and absence of aprotinin (40µg/ml). Graph represents the mean ± SEM of a minimum of 18 images derived from 3 independent experiments. *** p<0.001.

PAD4 is partially involved in DFO-induced NET formation

Hypercitrullination of histones by PAD4 was shown to be a key early step in chromatin unpacking during NET formation induced by TNFα (Wang et al., 2009). We quantified PAD4 expression in DFO-treated human blood derived neutrophils in comparison to untreated neutrophils using immunofluorescence microscopy (Figure 3.4.5A). An increased level of PAD4 was present in cells treated with the iron chelator DFO. Figure 3.4.5B and C show representative immunofluorescence-micrographs of neutrophils stained with a DNA-histone-complex antibody (green) in combination with a PAD4 antibody (red) to visualize PAD4 in NET structures as well as DAPI (blue) to visualize the nucleus.
Figure 3.4.5A: DFO increases PAD4 protein level. PAD4 protein level was quantified in human blood derived neutrophils treated with 300µM DFO in comparison to untreated neutrophils using immunofluorescence microscopy. The fluorescence intensity of the PAD4 signal was measured using Image J. Statistical analysis was performed using 24 images of four independent experiments. * p<0.05.

Figure 3.4.5B/C: (B) and (C) show representative immunofluorescent micrographs of neutrophils stained with an antibody to visualize PAD4 (red) within the NET structures (green). DAPI was used to counterstain the nuclei in blue. A higher level of PAD4 was detected in cells that release NETs and that were treated with the iron chelator DFO (C) compared to the untreated control (B).

Furthermore, we used semi quantitative Western Blot analysis, which only showed a trend towards higher PAD4-protein content in DFO-treated cells (p = 0.2; Figure 3.4.5D).
Next, PAD4 activity was blocked by addition of the known inhibitor chloramidine (Cl-amidine) (Luo et al., 2006; Wang et al., 2009). In the presence of Cl-amidine, a significant reduction in DFO-induced NETs was observed, though this level still exceeded that in untreated control cells (Figure 3.4.5E). These data suggest that PAD4-mediated histone modifications partially contribute to the observed DFO mediated NET formation.
Discussion

The data presented in this paper show that the tested iron chelators DFO and L-Mimosine significantly induce NET formation in human blood-derived neutrophils. Our data are consistent with a previous publication showing that the iron chelator Mimosine can boost the antimicrobial effect of neutrophils against *S. aureus* infections (Zinkernagel et al., 2008), while providing a new mechanistic insight implicating NET formation and subsequent entrapment of the pathogen. Other studies have already shown that NETs can play a protective role against *S. aureus* infections based on their ability to entrap and immobilize the bacteria (Berends et al., 2010; Chow et al., 2010).

Some important key mechanisms that have been identified to be involved in NET formation are the NADPH-dependent formation of ROS and the elastase-mediated histone degradation (Fuchs et al., 2007; Papayannopoulos et al., 2010). When blocking the respective enzyme biochemically during our assay, we could significantly diminish the DFO-mediated NET formation. These data indicate that both enzymes are involved in the process.

The DFO-mediated NET formation effect can also be abolished by an iron excess mediated by supplementation with Fe$^{2+}$ or Fe$^{3+}$. If abnormalities in NET formation are detectable in patients suffering from chronic iron deficiency anemia or iron overload (e.g. hemochromatosis) still remains to be determined. Improper regulation of NET formation may contribute to sepsis, systemic inflammatory response syndrome, small vessel vasculitis or vascular injury associated with systemic lupus erythematosus (Clark et al., 2007; Kessenbrock et al., 2009; Villanueva et al., 2011; Garcia-Romo et al., 2011).

Iron is an essential factor involved in the general stress response of a cell by regulating activity of key enzymes such as prolyl hydroxylases (PHD). Iron- and oxygen-dependent PHDs are the key factors responsible for the degradation of the α subunit of the hypoxia inducible factor HIF-1 during normoxia (Liu and Semenza, 2007). During hypoxia or following an acute inflammatory stimulus, PHD-mediated degradation of HIF-α is reduced (Frede et al., 2007) leading to altered gene regulation in the cell (Schäfer et al., 2013; Frede et al., 2007). An Fe(II) ion is located within the catalytic site of PHDs and is coordinated by one aspartate residue and two histidine
residues (Semenza, 2004). Proline and asparagine residues of HIF-1α are hydroxylated by PHDs, which enables HIF-1α to bind to the von Hippel–Lindau tumor suppressor protein (vHL), a protein with ubiquitin ligase activity (Figure 3.4.S1)).

As a consequence HIF-1α is no longer able to bind to coactivators such as CREB binding protein (CBP) and p300 and in the end gets ubiquitinated and thereby labelled for 26S proteasomal degradation (Huang et al. 1998; Kallio et al., 1999; Salceda et al., 1997). Under hypoxic conditions or iron-limited conditions, HIF-1α is accumulating due to interruption of the degradation pathway by inhibition of the PHD-mediated hydroxylation. The heterodimeric transcription factor is formed and is able to interact with its coactivators which leads to binding of HIF-1α to specific binding sites, so called hypoxia responsive elements (HREs) (Nizet and Johnson, 2009; Zinkernagel et al., 2007; Peyssonnaux et al, 2005; Zaremba and Malech, 2005). HIF-1α binding regulates the transcription of target genes that encode eryth-
ropoietin, glucose transporters, glycolytic enzymes, antimicrobial factors and the angiogenic factor VEGF (Zinkernagel et al., 2007; Zarember and Malech, 2005).

By virtue of their iron chelation, DFO and L-Mimosine are known HIF-1α agonists (Wang and Semenza, 1993; Jiang et al., 1997; Pugh et al., 1997; Semenza, 2004; Zinkernagel et al., 2008; Fine et al., 2012; Zhang et al., 2013, Chung et al., 2012; Yu et al., 2012). HIF-1α regulates elastases and NADPH-oxidase expression on transcriptional level (Peyssonnaux et al., 2005) and HRE-binding sites (Semenza et al., 1996) are found in the promoter region of PAD4 (Figure 3.4.S2).

![Figure 3.4.S2: Schematic figure of the PAD4 gene and several putative HIF-1 binding sites, consisting of the well-known motifs RCGTG and CACAG (Semenza et al., 1996), located upstream of its promotor. CBS, access: 15.08.2011, 09:48 MEZ](image)

Thus, HIF-1α mediated activation of those enzymes might represent a key trigger for the NET formation induced by DFO and L-Mimosine. In line with this hypothesis, we were also able to show that the HIF-1α protein stabilizing agents cobalt chloride or dimethyloxalylglycine (DMOG) (Maxwell and Salnikow, 2004; Kumar et al., 2014; Li et al., 2014; Wang et al., 2014) showed modest but statistically significant increase in NET-formation (Figure 3.4.S3).
Figure 3.4.S3: Additional HIF-1 agonists CoCl$_2$ and DMOG slightly induce NET formation. Human blood derived neutrophils were isolated by density gradient centrifugation and treated with media alone as a control, DFO (300µM), CoCl$_2$ (300µM), or DMOG (500µM) and its respective control for 3h. Afterwards, NET formation was determined using immunofluorescence microscopy. This graph represents the mean ± SEM of 18 images derived from 3 independent experiments. * p<0.05; *** p<0.001.

Recently, we have observed that the HIF-1α-agonist AKB4924 facilitated the formation of mast cell extracellular traps (MCETs) (Branitzki-Heinemann et al., 2012) in murine and human mast cells. Others recently reported that HIF-1α contributes to rapamycin induced NET formation in human leukemic HL-60 cells (McInturff et al., 2012). Thus, it may be hypothesized that stabilization of HIF-1α might facilitate formation of ETs in myeloid cells in hypoxic or iron-deficient tissue as it occurs during infection (Zinkernagel et al., 2007; Melican et al., 2008; Colgan and Taylor, 2010; Karhausen et al., 2004; Pacifico et al., 2014). Future studies in our lab will focus on the role of HIF and hypoxia in formation of ETs among several myeloid cell types.

In conclusion, our study shows, that iron chelating agents DFO and L-Mimosine boost the formation of NETs in human primary blood-derived neutrophils, an effect that can be abolished by iron supplementation. Since DFO and L-Mimosine are described as well-known HIF-1α-agonists, our data support the hypothesis of other recent publications with mast cells and a human leukemic cell line (HL-60 cells) that stabilization of HIF-1α might facilitate formation of NETs. Iron chelating prolyl hydrox-
ylase inhibitors are in advanced clinical development for anemia therapy, and might be explored in a novel context of NET induction to support innate immune clearance of problematic pathogens.

Materials and Methods

Bacterial strains

S. aureus strain LAC (pulsed-field type USA300), a community-acquired CA-MRSA strain (Voyich et al., 2005), was used for entrapment studies as previously described (Berends et al., 2010).

Microscopy to visualize the effect of DFO and L-Mimosine on NET formation

Human neutrophils were isolated from fresh heparinized blood by density centrifugation at 500 x g using Polymorphpreptm (Axis-Shield PoC). Neutrophils have been seeded on cover slides covered with 0.01% Poly-L-lysine (# P4707, Sigma). 5×10^5 cells in 250 µl RPMI 1640 (# E15-848, phenol red free, PAA) were seeded per well in a 24-well-plate. The cells were either stimulated with 25 nM PMA (Cat. tlr1-pma InvivoGen Corp., San Diego, CA, US), 300 µM DFO (D9533, Sigma) or 300 µM L-Mimosine (M0235, Sigma) for 3h at 37°C with 5% CO₂. After incubation, the cells were fixed by adding PFA (#15710 Electron Microscopy Science) to each well at a final concentration of 4% for 15 min at room temperature and kept at 4°C until subsequent immunostaining.

Immunostaining of H2A-H2B-DNA complex for NET visualization

Fixed cells were washed three times with PBS, blocked and permeabilized with 2% BSA PBS + 0.2% Triton X-100 for 45 min at room temperature. Incubation with a mouse monoclonal anti-H2A-H2B-DNA complex (clone PL2-6, 0.5 µg/ml) in 2% BSA PBS + 0.2% Triton X-100 was carried out overnight at 4°C as previously described (Jerjomiceva et al., 2014). Samples were washed with PBS and subsequently incubated with an Alexa-Fluor-488-labelled goat-anti-mouse antibody for 45 min at room temperature. After washing, slides were mounted in ProlongGold® antifade with DAPI (Invitrogen) and analyzed by confocal fluorescence microscopy using a
Leica TCS SP5 confocal microscope with a HCX PL APO 40x 0.75-1.25 oil immersion objective. Settings were adjusted in accordance to control preparations using an isotype control antibody.

Due to donor-specific variations in spontaneous NET-release, each experiment was performed with neutrophils derived from a minimum of three independent healthy blood donors. For each preparation, a minimum of six randomly selected images were acquired per slide and used for quantification of NET-producing cells. Data are expressed as percentages of NET-forming cells in relation to the total number of cells visualized with DAPI to stain the nuclei.

Boosting HIF-1α degradation by adding external iron ions

Human blood derived neutrophils (isolation as described above), were seeded at a concentration of $5 \times 10^5$ cells/well in 250 µl medium containing iron citrate ($Fe^{3+}$) or iron chloride ($Fe^{2+}$) (both 250µM) in a 24-well plate. Control cells were cultured in ‘pure’ RPMI medium. Subsequently, cells were stimulated with DFO (300µM) or L-Mimosine (300µM) for 3h at 37°C and 5%CO$_2$. The subsequent fixation and NET visualization as well as quantification were performed as described above.

NET entrapment assay

Bacteria were grown in Brain-Heart Infusion (BHI) medium at 37°C under agitation. Fresh overnight cultures were diluted 1:100 in BHI and then grown to mid-logarithmic growth phase ($OD_{600}$ = 0.7). The bacteria were washed and FITC (0.33mg/ml) labelled for 30min in the dark. Subsequently, neutrophils were infected for 90 min at 37°C and 5%CO$_2$. After incubation, non-entrapped bacteria were washed away and bacterial entrapment within the NETs was analyzed by measuring the fluorescence signal at 485/538 nm compared to total amount of bacteria.

Immunostaining of PAD4 and H2A-H2B-DNA complex for PAD4 quantification and NET visualization

The procedure is the same as described above but additional antibodies were used. Besides the PL2-6 mouse IgG2b, a polyclonal rabbit anti PAD4 antibody
(Wang et al., 2004) was used as a primary antibody besides the respective isotype IgG rabbit (Jackson Immunoresearch) as control staining. As additional secondary antibody Alexa Fluor 633 goat anti rabbit (Invitrogen) was used.

ImageJ software was used for quantification of the PAD4-expression. Thus, the fluorescent intensity caused by the excited secondary antibody Alexa Fluor 633 which binds to the anti PAD4 antibody was compared to the intensity of the respective isotype control.

**Blocking PAD4 activity by treatment of neutrophils with Cl-amidine**

Human blood derived neutrophils (isolation as described above) were treated with Cl-amidine in a final concentration of 200 µM per well at the same time when stimulating the cells with the iron chelators or PMA as NET inducers. The stimulation itself as well as incubation and subsequent fixation were performed as described above.

**PAD4 detection in Western blot analysis**

Neutrophils have been isolated and stimulated as described above. The cells were lysed in standard lysis buffer with proteinase inhibitors and proteins were separated via 10% SDS PAGE. After electrophoresis, proteins were transferred on to a PVDF membrane for 100 min. at 240mA and blocked in 0.1% TBST + 5 % nonfat dry milk for 45 min. For PAD4 detection, the blot was incubated with the polyclonal rabbit anti-PAD4 antibody (Wang et al., 2004) over night at 4°C with agitation. Additionally a monoclonal mouse anti-β-Actin antibody (Santa Cruz) was used as a loading control. The respective secondary antibody (goat anti-rabbit HRP and goat anti-mouse HRP) was added for 45 min at RT with agitation. Proteins were detected using SuperSignal West Femto Chemiluminescent Substrate reagents (Pierce, Thermo Scientific). The software Image J was used for signal quantification. Therefore, the PAD4 signal was normalized against the β-Actin control signal.

**Statistical analysis**

For statistical analysis GraphPad Prism 5.0 (Graph Pad Software, La Jolla, CA) was used. Data derived from a minimum of 3 independent experiments were analyzed.
For analysis of time-dependent effect of DFO on NET-formation, 2-way ANOVA, followed by a Sidak’s multiple comparison to control group (no matching) was used. For concentration-dependent effect of DFO on NET-formation, non-parametric Kruskal-Wallis test followed by a Dunn’s multiple comparison to control group (no matching) was applied. For all other data a student’s t test (paired/non-paired, one-tailed) was performed. For all tests p was defined as * p<0.05; ** p<0.005; *** p<0.001, **** p<0.0001.

Acknowledgments

This work was supported by DFG grant KO 3552/4-1.

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3.5 Novel role of the antimicrobial peptide LL-37 in the protection of neutrophil extracellular traps against degradation by bacterial nucleases

Running head: Role of LL-37 in neutrophil extracellular traps

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Abstract:

Neutrophil extracellular traps (NETs) have been described as a fundamental innate immune defense mechanism. They consist of a nuclear DNA backbone associated with different antimicrobial peptides (AMPs) which are able to engulf and kill pathogens. The AMP LL-37, a member of the cathelicidin family, is highly present in NETs. However, the function of LL-37 within NETs is still unknown because it loses its antimicrobial activity when bound to DNA in the NETs. Using immunofluorescence microscopy, we demonstrate that NETs treated with LL-37 are distinctly more resistant to *S. aureus* nuclease degradation than nontreated NETs. Biochemical assays utilizing a random LL-37-fragment library indicated that the blocking effect of LL-37 on nuclease activity is based on the cationic character of the AMP, which facilitates the binding to neutrophil DNA, thus protecting it from degradation by the nuclease. In good correlation to these data, the cationic AMPs human beta defensin-3 and human neutrophil peptide-1 showed similar protection of neutrophil-derived DNA against nuclease degradation. In conclusion, this study demonstrates a novel role of AMPs in host immune defence: beside its direct antimicrobial activity against various pathogens, cationic AMPs can stabilize neutrophil-derived DNA or NETs against bacterial nuclease degradation.

The extent of Lena Völlger’s contribution to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: A
2. Performing of the experimental part of the study: B
3. Analysis of the experiments: B
4. Presentation and discussion of the study in article form: B
4 Discussion

Since the discovery of PETs for the first time in neutrophils by Brinkmann et al., in 2004, knowledge about the cellular processes that lead to the formation of PETs is emerging. The regulatory processes involved in PET formation on the other hand, are still unknown. There are hints, that HIF-1α might be involved in PET formation, since it was shown that boosting HIF-1α resulted in an increased extracellular capacity of human phagocytes to kill certain bacteria [Peyssonnaux et al., 2005; Zinkernagel et al., 2008]. However, it is not known so far, whether HIF-1α is involved in PET formation in neutrophils or mast cells.

Therefore, the overall aim of this study is to evaluate the role of HIF-1α in the formation of PETs. Hence, neutrophils and mast cells were chosen as representative PET forming cells and the specific aims were defined as follows:

Aim 1: Evaluation of the role of HIF-1α in PET formation by mast cells
Aim 2: Improvement of neutrophil culture conditions to characterize NET formation
Aim 3: Evaluation of role of HIF-1α in PET formation by neutrophils

The well-established cell culture conditions for permanent mast cell lines as well as murine primary bone-marrow-derived mast cells, allow to successfully induce and visualize PETs in vitro, but also provide possibilities to work with genetically modified mast cells. These characteristics are the basis to be able to evaluate the role of HIF-1α in PET formation by mast cells (Chapter 3.1).

The permanent mast cell line HMC-1 is known to constitutively express HIF-1α [Jeong et al., 2003]. Using immunofluorescence microscopy with HIF-1α-specific antibodies, we were also able to confirm this finding in the HMC-1 cell line and under our chosen experimental conditions. Pre-treating of HMC-1 cells or bone-marrow-derived mast cells (BMMCs) with echinomycin, which blocks hypoxia responsive element (HRE) binding sites of HIF-1 [Kong et al., 2005; Nickols et al., 2007; Vlaminck et al., 2007], completely abolished the antimicrobial activity against S. aureus Newman.

As a next step, BMMCs from mice lacking HIF-1α in the myeloid cell lineage were used to further elucidate the influence of HIF-1α in the antimicrobial activity. In good
correlation to the treatment of mast cells with echinomycin, HIF-1α-deficient BMMCs showed a significantly reduced antimicrobial effect against S. aureus compared to control BMMCs. Since it was shown that pharmacological augmentation of HIF-1α boosts the bactericidal activity of macrophages and neutrophils [Zinkernagel et al., 2008; Okumura et al., 2012], HMC-1 cells and BMMCs were treated with the new pharmacological agent AKB-4924. AKB-4924 [Okumura et al., 2012] inhibits prolyl hydroxylases, which are involved in the HIF-1α degradation pathway and thereby increase HIF-1α protein levels as assessed using immunofluorescence microscopy and Western Blot (Chapter 3.1, Figure 3.1.1A and Figure 3.1.S1). Further, the transcript expression of known HIF-1α target genes, like IL-6 and IL-8 [Kong et al., 2005] was significantly induced in a control experiment conducted with HMC-1 cells. HMC-1 cells treated with AKB-4924 additionally showed increased antimicrobial activity against S. aureus. This effect was abolished, if echinomycin was used to inhibit HIF-1α signaling. In good correlation to these data, echinomycin reduced the AKB-492-induced transcript expression of IL-6 and IL-8 in HMC-1 cells. Importantly, AKB-4924 also increased the antimicrobial activity of wild type BMMCs, but not of HIF-1α-deficient BMMCs. This finding further verifies the HIF-1α-specific effect.

To evaluate, if intra- or extracellular mechanisms are involved in the HIF-1α mediated boosting of mast cell antimicrobial activity, assays were repeated using the actin polymerization inhibitor cytochalasin D [Casella et al., 1981]. Due to its capacities to inhibit actin polymerization, it also blocks phagocytosis [Shi et al., 2013; Haberzettl et al., 2007]. Interestingly, inhibition of phagocytosis in the presence and absence of AKB-4924 did not alter the antimicrobial activity of HMC-1 cells. These data suggest that HIF-1α might primarily mediate extracellular bactericidal activity in HMC-1 cells. Treatment of HMC-1 cells with DPI, an NADPH oxidase inhibitor, led to a drastically decreased antimicrobial activity, no matter if AKB-4924 was present or not. These data go in line with a recent publication from von Köckritz-Blickwede et al., 2008. The authors conclude that ROS-dependent formation of mast cell extracellular traps (MCETs) is significantly contributing to the antimicrobial activity in mast cells. Therefore, AKB-4924 mediated MCET formation was analyzed in addition.

Importantly, treatment of HMC-1 cells and BMMCs with AKB-4924, to boost HIF-1α levels, resulted in a significant increase in the amount of NETs formed in comparison to unstimulated cells (Chapter 3.1, Figure 3.1.2C and Figure 3.1.2D/F). In good
correlation to the data obtained by von Köckritz-Blickwede et al., 2008, using a microscopic mammalian cytotoxicity assay that stains extracellular DNA and the nuclei of dead cells, it was also found that MCET-releasing cells are dead. Furthermore, visualization of bacterial viability entrapped in MCETs revealed, that entrapped bacteria are dead. Treatment of AKB-4924 stimulated HMC-1 cells with micrococal nuclease leads to MCET degradation and thereby to impaired antimicrobial activity. Moreover, there was no AKB-4924-induced MCET formation detectable in BMMCs derived from HIF-1α deficient mice.

In conclusion, the results obtained within chapter 3.1 suggest that the transcription factor HIF-1α is a key regulator of the extracellular antimicrobial activity and the formation of PETs in mast cells.

In contrast to mast cells, there is no standardized protocol for neutrophils, which allows the usage of a permanent neutrophil cell line to visualize NETs. Due to their short life span, primary neutrophils can only be cultured for 6-12h. Thus, the primary aim was to establish suitable cell culture conditions using a surrogate PMN cell line (differentiated HL-60 leukocytes) to mimic primary neutrophils. To gain the possibility to e.g. use genetically modified surrogate PMNs instead of primary blood derived PMNs that are limited in amount and availability and may show also donor specific variations, we aimed to examine whether differentiated HL-60 cells could provide an accurate model to replace primary blood derived neutrophils for in vitro experiments. Chapter 3.2 shows that the overall antibacterial activity of nHL-60 cells in comparison to primary neutrophils against the model organism S. aureus was distinctly reduced.

It is well known that the differentiation of HL-60 cells is incomplete [Herwig et al., 1996; An et al., 2005] and they further do not harbor the entire arsenal of granules and secretory vesicles that neutrophils do [Nordenfelt et al., 2009]. We therefore assessed myeloperoxidase (MPO) expression of differentiated HL-60 cells as a marker for cell differentiation (Figure 3.2.S1). However, the detected amount of MPO in our study was similar in neutrophils and nHL-60 cells, indicating a neutrophil-like phenotype of the nHL-60 cells. Importantly, the overall antimicrobial functions of nHL-60 cells were significantly decreased. A study by Nordenfelt et al., in 2009 used Streptococcus pyogenes as a model organism to study antimicrobial capacities of
HL-60 cells. The authors stated that HL-60 cells can replace neutrophils only in models that do not rely on respiratory burst activity. They concluded that HL-60 cells might have a reduced capacity to generate ROS and hypothesized that the lack of the flavocytochrome b\textsubscript{558}, the membrane-bound component of the NADPH oxidase enzyme complex, might affect an effective respiratory burst in HL-60 cells. The presence of NADPH oxidase and ROS generation in HL-60 cells is moreover controversially discussed within the literature [Vong 	extit{et al.}, 2014.]. The presence of MPO however, does not compensate this defect as it acts downstream of NADPH oxidase. Additionally, secondary granules not only harbor the flavocytochrome b\textsubscript{558} but also antimicrobial peptides like the cathelicidin hCAP18/LL-37. Thus, an absence of these granules might also implicate a lack of LL-37 in HL-60 cells. However, An 	extit{et al.}, showed that peripheral blood cells derived from patients with acute myeloid leukemia, express LL-37 gene-specific mRNA. But further experiments revealed that those cells still were not able to produce biological active LL-37. Differentiation of HL-60 with all-trans retinoic acid could not overcome the LL-37 deficiency [An 	extit{et al.}, 2005]. Nevertheless, HL-60 cells do harbor primary granules, which contain most of the antimicrobial peptides including neutrophil defensins [Herwig 	extit{et al.}, 1996] but the absence of LL-37 and impaired ROS generation might explain the reduced intracellular killing of \textit{S. aureus} by nHL-60 in comparison to primary neutrophils.

Consistent with the data obtained in other studies [Vong 	extit{et al.}, 2014; Kawakami 	extit{et al.}, 2014; McInturff 	extit{et al.}, 2012], we showed that nHL-60 cells produced NETs. But it is important to mention that nHL-60 cells release NETs to a much lower extent than human primary blood derived neutrophils. Since it is known that ROS production by NADPH oxidase is essential for efficient NET production [Fuchs 	extit{et al.}, 2007], a putative defect within the neutrophil NADPH oxidase enzyme complex might be a feasible reason to explain the failure of nHL-60 cells to efficiently produce NETs.

However, the obtained data in this study demonstrate that all three major antibacterial mechanisms exhibited by neutrophils, i.e. degranulation, phagocytosis and NET formation, are impaired in DMSO or all-trans retinoic acid (ATRA) stimulated HL-60 cells and the development of neutrophil characteristics in those cells is incomplete. Thus we conclude that, HL-60 cells differentiated with DMSO or ATRA are of limited value to replace primary blood derived cells in \textit{in vitro} experiments. Therefore, we decided to only use primary blood-derived neutrophils for our experiments.
When comparing different literature on NET studies, it attracts attention that authors use different protocols. Especially, no standardized protocol that mentions a specific cell density of neutrophils and the time period in which NET induction assays work best is available. Therefore, both components, cell density and time, were analyzed within this PhD thesis (Chapter 3.3).

Since neutrophils are naturally non-adherent cells, it needs to be ensured that the cells adhere to the cover slip, which is later microscopically analyzed. Therefore, Poly-L-lysine, a nonspecific attachment factor was used. The polypeptide of the essential amino acid L-lysine promotes cell adhesion by enhancing electrostatic interaction between positively-charged surface ions of the solid cell culture surface and negatively charged ions on the cell membrane [Sitterley, 2008]. The first but very important finding in regard to the amount of analyzed cells was, that the number of cells that was counted per view field does not differ between PMA stimulated and unstimulated cells. We therefore show that the counted number of NET negative cells is not influenced by the applied techniques to wash, stain and analyze the cells. Thus, a possible impact of the “stickiness” of NETs compared to non-netting cells can be excluded. Finally, the data presented in chapter 3.3 indicate that the more cells were seeded, the more NETs were detected (higher x-fold-increase) in PMA stimulated cells in comparison to unstimulated cells: A 3.6 fold increase in cells releasing NETs was detected after 3h of PMA stimulation using 2x10^6 cells per well, whereas a 9.4 fold induction was detected when 5x10^5 cells per well were seeded. It can be hypothesized that during NET formation, neutrophils release factors that stimulate surrounding neutrophils to additionally form NETs. Those factors might be e.g. elastase, NADPH oxidase or MPO, since it is known that they are able to induce NET formation and they are further also found within already formed NET structures [Munafo et al., 2009]. The closer the proximity of the individual neutrophils, the stronger might be the impact of those factors to subsequently also release NETs from neighboring neutrophils. However, MPO, elastase and NADPH-oxidase levels at the different tested cell densities have not been analyzed in this context. Nevertheless, based on these two technical studies, we decided to use primary blood derived neutrophils at a concentration of 2x10^6 cells /ml and a stimulation time of 3 hours for further studies.
To evaluate the role of iron chelating HIF-1α agonist in PET formation by neutrophils, NET formation was induced using DFO and L-Mimosine. Due to their iron chelation capacities, both agents are well known HIF-1α agonists [Wang and Semenza, 1993; Jiang et al., 1997; Pugh et al., 1997; Semenza, 2004; Zinkernagel et al., 2008; Fine et al., 2012; Zhang et al., 2013, Chung et al., 2012; Yu et al., 2012]. In this study, it is shown that HIF-1α boosting by the above mentioned iron chelators, leads to significantly induced NET formation in human blood derived neutrophils. Furthermore, DFO led to increased NET formation in primary neutrophils isolated from bovine blood, demonstrating that this effect is not specific to human neutrophils. This data is consistent with a previous publication, indicating that the iron chelator mimosine can boost the antimicrobial effect of neutrophils against S. aureus infections in mice [Zinkernagel et al., 2008], while providing a new mechanistic insight which implicates NET formation and subsequent entrapment of the pathogen. Furthermore, by utilization of externally added iron ions (Fe$^{2+}$, Fe$^{3+}$), it was found within this PhD study that the NET induction effect might be due to the iron chelator activity of those HIF-1α-inducers and not due to other characteristics that those chemicals might have. However, here it was not tested if other ions e.g. zinc (Zn$^{2+}$), calcium (Ca$^{2+}$) or magnesium (Mg$^{2+}$) are also able to prevent the DFO and L-Mimosine induced NET formation.

Interestingly, Hasan et al., 2013, recently showed that PMA induced NET formation was inhibited when the membrane permeable high affinity Zn$^{2+}$ selective chelator TPEN was used to sequester the zinc signal [Hasan et al., 2013]. Further, the authors also analyzed, if NET formation can be restored by providing a Zn$^{2+}$ excess. They found out that addition of equimolar concentrations (5µM) of inhibitor (TPEN) and Zn$^{2+}$ almost completely abolished the inhibiting effect of TPEN on NET formation. Therefore, they concluded that the ability to chelate metals is required for TPENs inhibitory influence on PMA induced NET formation [Hasan et al., 2013]. To further analyze the effect of different metal ions, they decided to additionally investigate the effect of excessive copper (Cu$^{2+}$), calcium (Ca$^{2+}$) and magnesium (Mg$^{2+}$) ions on TPEN inhibited NET formation. In case Zn$^{2+}$ and Cu$^{2+}$ were present at equimolar concentrations, PMA induced NET formation was rescued. Nevertheless, Ca$^{2+}$ and Mg$^{2+}$ showed no effect and could not restore NET formation in the presence of TPEN. Further, TPEN also inhibits H$_2$O$_2$ induced NET formation. Last but not least,
Hasan et al., also examined at what time point the zinc signal is required during the process of NET formation. Therefore, they administered TPEN 20min prior to, or 30 to 180min after the addition of PMA and found out that TPEN is able to block PMA induced NET formation until up to 30min after the PMA treatment. No significant inhibition of NET formation was detected at TPEN administration after PMA stimulation for 60min or longer. Thus, we can assume that different ion chelators can exhibit different effects on NET formation.

Moreover, in so far unpublished data, another iron chelating substance called Dipyridyl was analyzed within this study for its capacity to induce NET formation. However, these data are not published in one of the described results chapters, because Dipyridyl is discussed controversially in the literature. It is described as an iron chelator [Horky et al., 1998; Kallio et al., 1999; Demougeot et al., 2004; Thompson and Carabeo, 2011] as well as a PHD blocker [Martens et al., 2007]. But it is not mentioned, if it acts as a PHD blocker due to its ion chelating capacities or because it might block the binding site of the necessary co-factor or even directly bind to the active site of the PHD enzyme.

Figure 4.1: Dipyridyl does not induce NET formation in human neutrophils. Human blood derived neutrophils were treated with 1mM of Dipyridyl for 3h and NET formation was subsequently analyzed. Neutrophils treated with ethanol represent the amount of NETs induced by the solvent of Dipyridyl. To evaluate the amount of spontaneously formed NETs one portion of neutrophils was treated with RPMI medium only. PMA (25nM) treated neutrophils serve as a positive control.
In NET induction experiments, Dipyridyl, in contrast to DFO and L-Mimosine, does not induce NET formation in isolated human primary neutrophils (Figure 4.1), but is neither inhibiting NET release. Thus, there seems to be a certain ability that is shared by DFO and L-Mimosine which leads to the formation of NETs that is not present in Dipyridyl. The observed NET induction effect of DFO and L-Mimosine therefore does not seem to be a general iron chelator effect.

A possible reason for the observed differences might be variations within the membrane permeability of those substances. Dipyridyl is known in the literature to be membrane permeable in K562 cells, a human erythroleukemic cell line [Breuer et al., 1995], in the African green monkey kidney cell line BSC40 [Romeo et al., 2001], skin fibroblasts [Dong et al., 2008] and in human endothelial HEp2 cells [Thompson and Carabeo, 2011]. It is important to mention that DFO is considered as an iron chelator with reduced membrane permeability in hepatocytes [Porter et al., 1988], neuroblastoma cells [Richardson et al., 1994; Jayasena et al., 2007] and in K562 cells [Breuer et al., 1995]. Nevertheless, the membrane permeability of DFO, L-Mimosine and Dipyridyl in PMNs is not known so far and should be tested in future experiments.

Further research needs to be done to shed more light into the phenomenon of PETs e.g. perform experiments using DFO and L-Mimosine to induce NET formation in the presence and absence of different, not yet tested metal ions (Zn\(^{2+}\), Ca\(^{2+}\), Cu\(^{2+}\)) or perform NET induction experiments using other ion chelators to reveal a possible mechanism behind the DFO and L-Mimosine mediated NET formation.

In line with chapter 3.3, were we hypothesize that HIF-1α is involved in the DFO mediated NET formation, it was recently reported that mammalian target of rapamycin (mTOR), a highly conserved PI3K-like serine/threonine kinase, regulates the formation of NETs in human neutrophils through induction of HIF-1α protein expression [McInturff et al., 2012]. Interestingly, the recent study by McInturff et al., showed that the iron chelating HIF-1 agonist cobalt chloride (CoCl\(_2\)) induces NET formation [McInturff et al., 2012]. They further analyzed the mechanism behind the NET induction effect and whether HIF-1α might regulate NET formation in neutrophils. The authors state that HIF-1α is indeed regulating NET formation in neutrophils and surrogate PMNs (DMSO treated HL-60 cells) via mammalian target of rapamycin (mTOR), a posttranscriptional regulator of HIF-1α protein expression in human PMNs. Human neutrophils were treated with rapamycin, a specific inhibitor of mTOR
activity and NET formation was subsequently analyzed. Fewer NETs were detected if neutrophils were treated with rapamycin in comparison to a FK-506 treated control. FK-506 binds to the regulatory molecule FKBP12, but does not inhibit mTOR activity. However, within this present PhD study, I was not able to induce NET formation using DFO in HL-60 cells (Figure 4.2).

![Figure 4.2: DMSO differentiated HL-60 cells after a 3h treatment with 300µM DFO. In contrast to primary neutrophils, no NET formation was observed.](image)

Therefore, HL-60 cells were not used as surrogate neutrophils in this study. To obtain further evidence that HIF-1α is important for NET formation, McInturff et al., used short hairpin RNA (shRNA) to inhibit HIF-1α protein expression in HL-60 leukocytes that were used as surrogate PMNs. NET formation in genetically inhibited cells was dramatically decreased after 2h NET induction with LPS in comparison to cells treated with a scrambled version of the shRNA. Additionally, rapamycin treatment also decreased NET mediated killing of a pathogenic E.coli strain. Thus, mTOR and HIF-1α are shown to influence the formation of NETs and bacterial killing by human PMNs [McInturff et al., 2012]. However, the conducted experiments within this PhD study were not able to reproducibly demonstrate that iron chelating HIF-1 agonists indeed stabilize HIF-1α using Western Blot as well as immunofluorescence microscopy to determine a potential increase in HIF-1α protein level.
To conclude this chapter, this study shows, that the iron chelating HIF-1α agonists DFO and L-Mimosine boost the formation of NETs in human primary blood-derived neutrophils. Iron supplementation with an externally added excess of Fe$^{2+}$ and Fe$^{3+}$ can abolish this effect. In line with our hypothesis, we were also able to show that the HIF-1α-protein stabilizing agents cobalt chloride and dimethyloxalylglycine (DMOG) [Maxwell and Salnikow, 2004; Kumar et al., 2014; Li et al., 2014; Wang et al., 2014] led to modest but statistically significant increase in NET formation (Chapter 3.4, Figure 3.4.S3). This finding goes in line with our mast cell study (Chapter 3.1) and the rapamycin induced NET formation by McInturff et al., 2012, suggesting that HIF-1α is playing a role in the process of NET formation.

However, the final HIF-1α link still needs to be determined. In this study, I was not able to repeatedly and specifically detect HIF-1α using Western Blot experiments performed on DFO treated primary human neutrophils. Therefore, it is also not possible to determine HIF-1α deficiency in neutrophils, derived from mice that are deficient in their myeloid cell lineage. Consequently, experiments using HIF-1 deficient mice have not been performed yet.

![Figure 4.3: DFO treated, human blood derived neutrophils stained with a HIF-1α specific antibody (left, Novus, mouse IgG2b monoclonal anti HIF-1α, 4mg/ml, 1:50 diluted) or its isotype control (right, 20319 IgG2b (1,3mg/ml), in equal concentration to HIF-1α specific antibody), both in red and DAPI to visualize the nuclei (blue). The signal intensity of the isotype control antibody is heavily increased in comparison to that of the HIF-1α specific antibody.](image-url)
Also, no reliable results were obtained using immunofluorescence microscopy in human primary blood derived neutrophils. The signal intensity of the isotype control antibody was higher than the signal of the HIF-1α specific antibody (Figure 4.3).

In further experiments, the role of putative HIF-targets for NET formation was elucidated. The influence of ROS, neutrophil elastase (NE), PAD4 (Chapter 3.4) and LL-37 (Chapter 3.5) on NET formation was studied more in detail.

HIF-1α has been shown to regulate the expression of NADPH-oxidase and elastase on the transcriptional level [Peyssonaux et al., 2005], two key mechanisms involved in NET formation [Fuchs et al., 2007; Papayannopoulos et al., 2010]. Additionally, HRE-binding sites [Semenza et al., 1996] are found in the promoter region of PAD4 (Chapter 3.4, Figure 3.4.S2), another key enzyme involved in NET formation [Wang et al., 2009; Li et al., 2010; Leshner et al., 2012]. Thus, HIF-1α mediated activation of those enzymes might represent a key trigger for the NET formation induced by DFO and L-Mimosine.

In chapter 3.4 of this PhD thesis, it is shown that ROS play a role during DFO and L-Mimosine induced NET formation. Our data go in line with the literature [Fuchs et al., 2007, Keshari et al., 2013] that application of DPI, an NADPH oxidase inhibitor, leads to significantly reduced NET formation in response to PMA (Chapter 3.4, Figure 3.4.4A). We additionally confirmed that elastase mediated histone degradation also takes place during DFO induced NET formation. When blocking the respective enzyme biochemically using apronin during our assay, we significantly diminished the DFO mediated NET formation (Chapter 3.4, Figure 3.4.4B). These data indicate that both enzymes, NADPH oxidase as well as elastase are involved in the process of DFO induced NET formation.

Peptidylarginine deiminase 4 (PAD4) is, as already mentioned, another key biochemical component of PET formation. PAD4 deiminates histones and thereby facilitates chromatin decondensation, which is necessary for PET formation [Wang et al., 2004; Wang et al., 2009]. However, very little is known about the regulatory processes, especially on transcriptional level, behind this phenomenon. After performing bioinformatics to find potential promoter sequences and binding sites of already well known transcription factors within the PAD4 gene, HIF-1α turned out to be a potential
transcription factor which may be responsible for controlling NET formation mediated by PAD4. In addition it could be verified that PAD4 has an effect on HIF-1α dependent NET induction. Blocking PAD4 function, using chloramidine, results in less formed NET structures. Nevertheless, a significant NET induction compared to the control group was still observed when PAD4 activity was blocked with chloramidine. But the obtained results within this study also support the data of Li et al. in 2010, in which he worked with PAD4 knock-out mice and achieved corresponding results. Li et al., also showed that PAD4 is not the only factor that plays a role during NET induction [Li et al., 2010]. But it still remains to be determined what kinds of PAD4-independent mechanisms are involved in NET formation. However, within this study using immunofluorescence microscopy, higher PAD4 protein levels were detected in DFO treated samples compared to the control. Therefore, the described findings also support the hypothesis that due to the activation of the well-known transcription factor HIF-1α, PAD4 expression is enhanced, which contributes to the formation of NETs.

CRAMP, the mouse analogue to the antimicrobial cathelicidin LL-37 in humans, has been shown to be an additional HIF-target [Peyssonnaux et al., 2005; Berger et al., 2013]. The single mouse cathelicidin CRAMP is essential for the mammalian innate immunity towards group A streptococci (GAS) [Nizet et al., 2001]. Additionally, it is known that HIF-1α deficient mice lack mature CRAMP whereas vHL deficient mice showed increased levels of CRAMP [Peyssonnaux et al., 2005]. The authors further stated that this reduction is partly regulated via a reduction within CRAMP mRNA levels. The transcript levels of CRAMP in HIF-1α deficient mice were reduced by 80% whereas the transcript levels in vHL deficient mice were increased. In good correlation to these data, hypoxia, the natural stimulus of HIF-1α, also induced CRAMP mRNA levels in neutrophils derived from wild type mice [Peyssonnaux et al., 2005].

Therefore, we hypothesized that HIF-1α might also regulate the human cathelicidin LL-37 in neutrophils. However, a direct link between LL-37 and HIF-1 is not yet shown. LL-37 is constitutively expressed in neutrophils [Zanetti et al., 1995] and has been shown to also be associated with DNA fibers in NET structures [Urban et al., 2009; Chow et al., 2010]. Interestingly, it was shown to lose its bactericidal activity when bound to DNA [Weiner et al., 2003]. Thereby, its actual role within NET struc-
ture was unknown. Within chapter 3.5 the role of this putative HIF-1 target in NETs was elucidate. It was confirmed that LL-37 is present in NETs using immunofluorescence and electron microscopy. Further, it is known that LL-37 protects mammalian DNA as well as bacterial plasmid DNA against degradation by different serum nucleases [Molhoek et al., 2009; Lande et al., 2011]. Therefore, we analyzed the effects of LL-37 on the stability of neutrophil DNA and/or NETs in the presence of micrococcal nuclease. LL-37 treatment significantly protected NETs against the nuclease mediated degradation. However, addition of aprotinin, to block the proteinase 3-mediated activation of endogenous, neutrophil-derived LL-37 [Yamasaki et al., 2006] resulted in a significantly higher degree of NET degradation. Using an analytical protocol to detect double-stranded DNA (dsDNA), the PicoGreen assay, it was found that LL-37 significantly protected neutrophil as well as calf thymus derived DNA against degradation by \textit{S. aureus} nuclease. This may be due to the concentration dependent LL-37 mediated sequestration of the DNA. Similar results were obtained in case other DNA-intercalating dyes (Sytox green, ethidium bromide) were used. In addition, LL-37 was also able to protect the DNA against degradation mediated by nucleases from other bacterial species than \textit{S. aureus}. Purified EndA from \textit{S. pneumoniae} and nucleases from three different nuclease-producing GAS strains as well as the methicillin-resistant \textit{S. aureus} (MRSA) were not able to substantially degrade the DNA in the presence of LL-37. Moreover, LL-37 also enhances NET mediated entrapment and subsequent immobilization of bacteria. Using a fragment library of LL-37, it was aimed to determine possible biochemical features that lead to the LL-37 mediated protection of host DNA against bacterial nuclease degradation. Cationicity turned out to correlate with the ability to protect the DNA, since also other human cationic antimicrobial peptides like HNP-1 and hBD-3 significantly diminish bacterial nuclease mediated DNA degradation. This data shows, that different host derived cationic peptides can stabilize NETs against degradation by bacterial nucleases. Interestingly, \textit{in vivo} studies show that NET residues are still detectable in tissues of mice infected with nuclease producing bacteria [Berends et al., 2010]. Those residues might be stabilized by host derived antimicrobial peptides and thereby be prevented from degradation.

In summary, chapter 3.5 demonstrates a novel role of the antimicrobial peptide LL-37, in protecting neutrophil DNA and NETs against degradation by bacterial
nucleases. Thereby, the positive overall charge of LL-37 is responsible for LL-37 mediated stabilization of host DNA against bacterial nucleases. LL-37 also enhances NET mediated entrapment and subsequent immobilization of bacteria.

These data might facilitate the hypothesis that boosting of HIF-1α with iron chelating HIF-1 agonists like DFO might also lead to increased levels of LL-37, which then stabilize NETs and lead to increased entrapment of bacteria like *S. aureus* as shown in chapter 3.4.
5 Future outlook

Since the direct HIF-1α link is still missing, several techniques can be used to verify its role during NET formation. Are neutrophils derived from e.g. HIF-1α deficient mice impaired in NET formation and those derived from vHL deficient mice favorable to form NETs? The obtained results in this PhD study are in vitro data and should be deepened and verified within future studies using cells derived from HIF-1α knockout mice as well as vHL deficient mice, to confirm the specificity of HIF-1α in further in vitro experiments. Additionally, methods which allow conclusions on protein level like Western blot analysis should be performed and optimized.

To work with a natural HIF-1α stimulus, that allows to reliably induce the transcription factor, might increase the chance to develop and verify a technique to detect HIF-1α protein levels. Future studies in our lab will be therefore conducted under hypoxic conditions. In detail, we will focus on the role of HIF and hypoxia in the formation of ETs among several myeloid cell types. Therefore, PET formation under hypoxic conditions e.g. 1% oxygen will be compared to normoxic conditions. HIF-1α is expected to accumulate even stronger during hypoxic conditions, as during incubation with HIF agonists. An increased HIF-1α concentration might then also lead to successful Western blot experiments or RNA extractions.

Another future aim would be to further analyze the putative HIF-1α target genes e.g. PAD4/LL-37. Is HIF-1α indeed regulating PAD4 and/or LL-37 as we hypothesize within the discussion (Chapter 4)? Three different methods can be used to answer that question on DNA/RNA level. The first possible method would be si-RNA to silence HIF-1α or PAD4/LL-37 in respective cells and subsequently analyze NET formation. This will also help providing a direct HIF-1α link, showing, that HIF-1α is regulating those putative target genes. However, silencing experiments are only possible in cell lines like HMC-1 cells but not in primary cells. In case HIF-1α, PAD4 or LL-37 play a role, less NET formation would be expected. Very interesting would be, if it is possible to rescue the NET formation phenotype by providing a complemented vector containing the respective protein. Further, as a second method, real time PCR could be used to quantify the PAD4 and LL-37 mRNA level in wild type as well as silenced cells. Thereby, the amount of translatable mRNA and subsequent formation of PAD4/LL-37 protein can be determined. The possible induction effect of DFO and L-Mimosine via HIF-1α could be quantified and compared to e.g. a natural stimulus
like hypoxia or a bacterial infection. This would further verify the obtained results on DNA/RNA level. Furthermore, the possible PAD4 and LL-37 promotor binding of HIF-1α using chromatin immunoprecipitation (CHIP) assays can be analyzed. Additionally, other factors which may play a role in HIF-1α induced NET formation should be reviewed. Finally, a limit of this study is the lack of *in vivo* experiments.

The following questions need to be addressed in future experiments:

Is the effect of iron chelator mediated NET induction still detectable *in vivo* in e.g. HIF-1α knockout mice? Do iron chelator treated wild type mice (via food or water) show a favorable outcome during an infection? Does pretreatment influence a later occurring infection *in vivo*? The *in vivo* data would also provide a new level of significance to the already obtained *in vitro* data.

Altogether, this new knowledge will have significant implications for understanding the HIF-1α mediated immune response as a novel target against drug-resistant bacterial infections.
6 Final concluding remarks

In summary, we have observed that the HIF-1α agonist AKB-4924 facilitates the formation of mast cell extracellular traps (MCETs) [Branitzki-Heinemann et al., 2012] in murine and human mast cells. Furthermore, we have shown that the HIF-1α agonist DFO boosts NET formation in primary blood derived neutrophils derived from human and bovine blood. Well known HIF-1 targets were confirmed to be important in the process of DFO mediated NET formation and NET stabilization. Others recently reported that HIF-1α contributes to rapamycin-induced NET formation in human leukemic HL-60 cells [Mclnturff et al., 2012]. Thus, it may be hypothesized that stabilization of HIF-1α might facilitate the formation of ETs in myeloid cells in hypoxic or iron-deficient tissue, as it occurs during infection [Zinkernagel et al., 2007; Melican et al., 2008; Colgan and Taylor, 2010; Karhausen et al., 2004; Pacifico et al., 2014].

Already in 2008 Zinkernagel et al. could show that HIF-1α may represent a therapeutic target to improve the bactericidal activity of human phagocytes [Zinkernagel et al., 2008] but the underlying mechanism was still unclear. The authors found out that the phagocytic uptake of bacteria was not impaired by HIF-1α deficiency. The data of this thesis support the hypothesis that HIF-1α-boosting increases NET formation. Interestingly, it has been recently shown that statins are able to boost the formation of neutrophil and macrophage extracellular traps and that trap induction is linked to inhibition of the sterol pathway [Chow et al., 2010]. The infection progress is milder when NET formation was boosted with statins. Thus, the authors concluded that a drug therapy, which may alter antimicrobial cell function e.g. NET formation, may be of benefit for susceptible patients and may improve the host immune response against the pathogen. HIF-1α boosting might be a new possibility for therapeutic treatment by enhancing the local immune response against infections.
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8 Acknowledgement

First of all I would like to express my gratitude to my supervisor PD Dr. Maren von Köckritz-Blickwede for giving me the opportunity to complete my PhD under her supervision. Her professional competence and expertise as well as her enthusiasm for the topic resulted in a great project and I really enjoyed working with her. She provided me with excellent supervision and from the first day until the end of my PhD studies, Maren always supported and guided me. She always wanted to make sure that things go well. I will always be thankful for the opportunity to work in the Infection Biochemistry Research Group.

I would also like to thank Prof. Dr. Hassan Y. Naim for the opportunity to work in his lab. His constructive criticism and helpful advices facilitated a very fruitful collaboration between the two groups. I also learned to address research from a biochemical point of view and was lucky to be taught so many biochemical methods.

Thirdly, I would like to thank PD Dr. Christoph Baums and Prof. Dr. Ralph Goethe for their critical and constructive suggestions, for improvement of my work and the helpful and interesting discussions. They gave lots of helpful tips and comments on my work, also from a different perspective which was very important.

I really appreciate all the help and advice that PD Dr. med. Annelies S. Zinkernagel gave me during the last 3 years of my PhD study. Your guidance has improved this work substantially.

I thank all my collaboration partners, especially Victor Nizet and Kathryn Akong-Moore from UCSD for their excellent, productive and successful cooperation.

I would like to thank Oliver Goldmann for his expertise in real time PCR and for the chance to learn this method from him.

Further, thanks to all coauthors on the manuscripts for their successful cooperation.

I would also like to thank all my colleagues from the Department of Physiological Chemistry, for their daily support in lab and a great working atmosphere. Especially Ari, Natalja, Nat and Steffi, with whom I shared lots of scientific discussions. Working with all of you has made my time here a lot more enjoyable!

It is a pleasure to thank Rike for her excellent help in the lab.

I would also like to the thank the Deutscher Akademischer Austauschdienst (DAAD)
for funding my PhD studies, in particular my stay at University of California, San Diego, which has provided me not only with new insights into my research topic but also helped me develop as a researcher and as a general person.

I further want to thank the Deutsche Forschungsgemeinschaft (DFG) who financially supported this work by DFG grant KO 3552/4-1.

I am very grateful to all people, who provided me with blood samples. Without all the samples, my PhD thesis would not exist.

Last but not least, I would like to thank my family for their continuous support throughout all aspects of my life. Without them I would never have gotten this far. Thank you that you are always there for me.