

**The Role of Neutrophil Extracellular Traps in
Lung Epithelial Cell Death**

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IV. List of abbreviations

2-D gel electrophoresis	= Two-dimensional gel electrophoresis
ALI	= Acute lung injury
APC	= Activated protein C
ARDS	= Acute respiratory distress syndrome
BAL	= Broncho-alveolar lavage
BCA	= Bicinchoninic acid
BSA	= Bovine serum albumin
CGD	= Chronic granulomatous disease
COPD	= Chronic obstructive pulmonary disease
CXCR	= Chemokine-CXC-motif Receptor
DAPI	= 4', 6-diamidino-2-phenylindole
DIGE	= Differential in gel electrophoresis
DMEM	= Dulbecco's modified Eagle medium
DNA	= Deoxyribonucleic acid
DNase	= Deoxyribonuclease
DTT	= Dithiothreitol
Eap	= Extracellular adherence protein
EDTA	= Ethylenediaminetetraacetic acid
ESS	= Equilibration stock solution
ET	= Extracellular traps
FBS	= Fetal bovine serum
FITC	= Fluorescein isothiocyanate
FPR	= Formyl peptide receptors
GAS	= Group A <i>Streptococcus</i>
GPCR	= G protein-coupled receptor
HBP	= Heparin binding protein
HBSS	= Hank's buffered salt solution
HEPES	= Hydroxyethyl piperazineethanesulfonic acid
HNP	= Human neutrophil peptides
HOCl	= Hypochlorous acid
HRP	= Horseradish peroxidase

List of abbreviations

HSA	= Human serum albumin
HUVEC	= Human umbilical vein endothelial cells
IgG	= Immunoglobulin G
IL	= Interleukin
kDa	= kiloDalton
LDH	= Lactate dehydrogenase
LPS	= Lipopolysaccharide
MALDI	= Matrix-assisted laser desorption/ionization
MMP	= Matrix metalloproteinase
MNase	= Micrococcal nuclease
MPO	= Myeloperoxidase
MS	= Mass Spectrometry
NEI	= Neutrophil elastase inhibitor
NET	= Neutrophil extracellular trap
NF-kappa B	= Nuclear factor-kappa B
NO	= Nitric oxide
PAGE	= Polyacrylamide gel electrophoresis
PAMPs	= Pathogen associated molecular patterns
PBS	= Phosphate buffered saline
PFA	= Paraformaldehyde
PMA	= Phorbol myristate acetate
PMN	= Polymorphonuclear neutrophils
PPACK	= Phenylalanyl prolyl arginine chloromethyl ketone
PRRs	= Pattern recognition receptors
PSA	= Polysialic acid
ROS	= Reactive oxygen species
SDS	= Sodium dodecyl sulfate
SLE	= Systemic lupus erythematosus
SVV	= Small-vessel vasculitis
TEMED	= N,N,N',N'-Tetramethylethylenediamine
TLR	= Toll-like receptor
TNF	= Tumor necrosis factor
TOF	= Time of flight

V. Summary

Neutrophils play an important role in innate immunity by defending the host against invaded microorganisms. Antimicrobial activity of neutrophils is mediated by phagocytosis, release of reactive oxygen species and lytic enzymes as well as formation of neutrophil extracellular traps (NET). These structures are composed of DNA, histones and certain granular proteins such as neutrophil elastase as well as some cytoplasmic proteins. NET formation in different organisms, its bactericidal activity, and its presence in several pathophysiological conditions have been documented; however, little is known about the direct effect of NET on the host cells and its potential role in the pathogenesis of acute lung injury (ALI). This study aims to decipher the influence of NET on host cells, particularly alveolar epithelial cells as major cells responsible for gas exchange in the lung.

Herein, NET formation was documented in the lung tissue and the bronchoalveolar lavage fluid in a mouse model of ALI. Upon direct interaction with epithelial and endothelial cells, NET induced cytotoxic effects in a dose-dependent manner, and digestion of DNA in NET did not change NET-mediated cytotoxicity, indicating that NET associated proteins are responsible for this effect. NET associated proteins were also identified by mass spectrometry. Following treatment of epithelial cells with NET, different proteins with roles in cell death processes were upregulated. Pre-incubation of NET with antibodies against histones significantly reduced NET-mediated cytotoxicity, suggesting that the cell-destructive activity of NET was at least in great part mediated by histones. Activated protein C (APC) did decrease the histones-induced cytotoxicity in a purified system, but did not change NET-induced cytotoxicity, indicating that histones in NET are protected against APC degradation. Inhibition of neutrophil elastase, another abundant component of NET, did not decrease NET-mediated cytotoxicity, although elastase activity increased after DNA digestion. Inhibition of myeloperoxidase, another granular component of NET, decreased NET-mediated cytotoxicity. Moreover, in epithelial cells, NET induced a biphasic change in cellular permeability; at earlier stages decrease and at the later stages increase was observed.

These data reveal the important role of protein components in NET, particularly histones and partly myeloperoxidase, which may lead to host cell cytotoxicity and may be one of the inducers of organ destruction associated with inflammatory diseases including ALI. Thus, NET in a spatio-temporal manner can influence the cell functions.

VI. Zusammenfassung

Neutrophile erfüllen eine wichtige Rolle bei der angeborenen Immunabwehr, indem sie in den Körper eindringende Mikroorganismen bekämpfen. Ihre antimikrobielle Aktivität wird durch Phagozytose, die Ausschüttung reaktiver Sauerstoffspezies ("reactive oxygen species", ROS) und lytische Enzyme vermittelt. Eine alternative Bekämpfung der Pathogene kann über die Ausbildung von sogenannten "Neutrophil Extracellular Traps" (NET) erfolgen, einer speziellen Art des Zelltods, bei dem die Neutrophilen DNA, Histone, verschiedene granuläre Proteine (wie z.B. Neutrophile Elastase), aber auch zytoplasmatische Komponenten netzartig ausschleudern. Obwohl die Ausbildung von NET in verschiedenen Organismen und pathophysiologischen Bedingungen und ihre bakterizide Wirkung dokumentiert wurde, ist wenig bekannt über die Wirkung von NET auf körpereigene Zellen und im speziellen bei der Pathogenese des akuten Lungenschadens (ALI). In dieser Arbeit wurde die Auswirkung der NET-Bildung auf Endothel- und Epithelzellen studiert, vor allem im Bezug auf funktionelle Veränderungen von alveolären Epithelzellen, die mit für den Gasaustausch in der Lunge verantwortlich sind.

In einem Mausmodell des ALI konnte die Bildung von NET in Lungengewebe und Lavage der erkrankten Tiere gezeigt werden. Direkte Interaktion von NET mit endothelialen und epithelialen Zellen führte nicht nur zu Änderungen in der Permeabilität des Zellverbandes, sondern auch zu einer erhöhten Synthese pro-apoptotischer Proteine und wirkte sich in konzentrationsabhängiger Weise zytotoxisch auf die Zellen aus.

Da der DNase-Verdau von NET keinen Einfluss auf deren zytotoxischen Effekt hatte, können NET-assoziierte Proteine dafür verantwortlich gemacht werden. Hier waren es vor allem Histone, die auch in isolierter Form zytotoxisch wirkten, was durch verschiedene Antikörper gegen Histone verhindert werden konnte. Da aktiviertes Protein C (APC) Histone degradiert, wurde die Histon-induzierte Zytotoxizität *in vitro* reduziert, nicht aber die NET-induzierte Zytotoxizität. Dies legt den Schluss nahe, dass Histone in NET gegen den Abbau durch APC geschützt sind. Die Inhibierung von Neutrophiler Elastase, einem weiteren Proteinbestandteil von NET, hatte keine Wirkung auf deren Zytotoxizität, allerdings konnte eine stärkere Elastase-Aktivität nach DNase-Behandlung von NET festgestellt werden. Die Inhibierung von Myeloperoxidase, einer weiteren Komponente der NET verringerte allerdings die NET-induzierte Zytotoxizität. Die Proteomanalyse von mit NET inkubierten Epithelzellen zeigte einen deutlichen Anstieg an Apoptose-relevanten Proteinen.

Diese Arbeit zeigt, dass bestimmte Proteine in den NET und hier vor allem die Histone, aber auch Myeloperoxidase stark zytotoxisch auf Endothel- und Epithelzellen wirken. Damit können NET einer der Auslöser für die Zerstörung von Geweben und Organen bei entzündlichen Erkrankungen, wie beispielsweise des akuten Lungenversagens (ALI), sein.

1 Introduction

1.1 Immunity

The ability to respond to foreign substances including microbes or macromolecules such as proteins or polysaccharides is called “immunity”. Defending against infectious microbes is the physiologic function of the immune system which includes “innate” and “adaptive” immunity. Early reaction of the immune system is mediated by innate immunity which consists of various immune cells, mediators secreted by these cells and the complement system. These cells and mediators are present even before infection and therefore respond within minutes and hours after exposure to a noxious stimulus. The main components of the innate immunity are (a) physical and chemical barriers, for instance skin or mucosal epithelia and the substances produced by the epithelia; (b) phagocytic cells (neutrophils and macrophages) and natural killer cells; (c) the complement system and (d) proteins called cytokines which regulate the activities of the innate immunity cells. Innate immunity differs from adaptive immunity so that adaptive immunity is activated only after exposure to infectious agents and gains specific immunity against distinct stimulus. In adaptive immunity “lymphocytes” and their products have the main role. Adaptive immunity includes “humoral” and “cell-mediated” immunity. Humoral immunity is mediated by blood molecules called “antibodies” which are produced by B-lymphocytes. Antibodies recognize specific microbial antigens and eradicate the target microbes by different functional mechanisms. T-lymphocytes are involved in cell-mediated immunity. Antibodies cannot reach intracellular microbes such as viruses and several bacteria and therefore cell-mediated immunity involves in defence against these types of infection by destroying the infected cells. In contrast to innate immunity, adaptive immunity has the ability to “remember” and create much stronger responses after facing a specific microbe and therefore it is considered more specific (Abbas and Lichtmann, 2003).

Adaptive immunity uses several innate immunity mechanisms for obliteration of the microbes mostly by increasing the antimicrobial activities of the innate immunity system. For instance, some antibodies increase phagocytosis and other antibodies induce release of inflammatory mediators from the leukocytes. Recent studies show that cells and mediators in innate immunity can also regulate adaptive immunity and therefore innate immunity should not be considered as an ineffective and non-specific part of the immune system. In fact,

innate immunity can regulate and induce adaptive immunity in such a way which is more efficient for obliteration of infection (Kumar and Sharma, 2010).

1.2 Neutrophils

Polymorphonuclear neutrophils (neutrophils or PMN) are crucial cells of the innate immune system that form the first line of defense against bacteria and fungi (Borregaard and Cowland, 1997; Nathan, 2006). Neutrophils are a subgroup of granulocytes and the largest subpopulation of white blood cells. They differentiate in the bone marrow from hematopoietic stem cells to mature neutrophils. The differentiation starts from myeloblasts through promyelocytes to neutrophilic myelocytes and metamyelocytes. Neutrophils have a trilobulated nucleus and contain abundant granules. Primary or azurophilic granules are formed in the promyelocyte stage, whereas the secondary or specific granules are formed in the myelocytic stage. Later, during the metamyelocyte stage tertiary granules are generated and secretory vesicles are produced by endocytosis when neutrophils are circulating in the blood stream (Borregaard *et al.*, 1987). Approximately 300 different proteins are found in the neutrophils granules (Lominadze *et al.*, 2005). Granules which are produced later in differentiation of neutrophils are released earlier during the recruitment of neutrophils from the bloodstream to the site of infection. Secretory vesicles are released immediately after establishment of contact between neutrophils and endothelium. Tertiary granules are released during the transmigration of neutrophils through endothelium and secondary and primary granules are released at the site of inflammation (Faurischou and Borregaard, 2003).

1.2.1 Neutrophil granules

1.2.1.1 Granules components

Secretory vesicles, which originate from endocytosis, contain a collection of membrane-associated receptors and will be integrated into the plasma membrane after the release of vesicles (Sengelov *et al.*, 1993). Some of these receptors include formyl peptide receptors (fpr), β -2 integrins, CD14 and CD16 (Fc receptor) and CD10. Integration of these receptors on the PMN leads to interaction of neutrophils with the endothelium, dendritic cells and monocytes. Albumin and heparin binding protein (HBP or azurocidin or CAP37) are stored in

the matrix of secretory vesicles. HBP has an important role in increasing vascular permeability mediated by neutrophils (Tapper *et al.*, 2002; Gautam *et al.*, 2001).

Tertiary or gelatinase granules are secreted more easily than secondary granules and contain matrix degrading enzymes (matrix metalloproteinase-9 and lysozyme) and membrane receptors (fpr and CD11b) required for PMN extravasation (Mollinedo *et al.*, 1997).

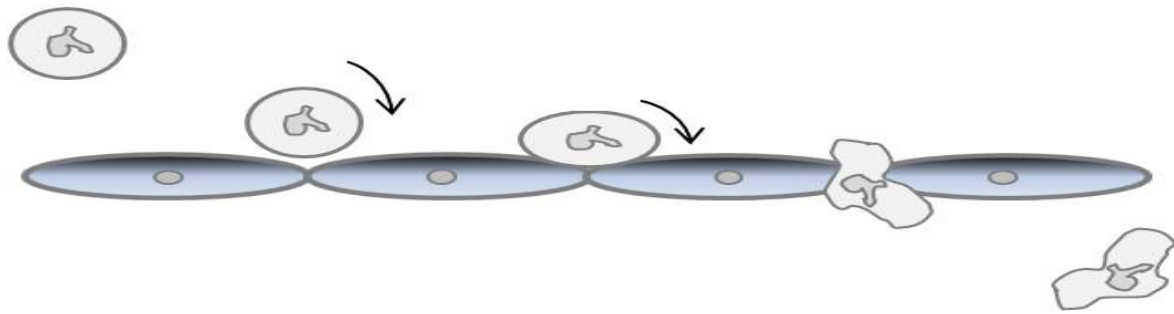
Secondary or specific granules are bigger than tertiary granules and contain antimicrobial proteins (lactoferrin, lysozyme and hCAP18, the proform of LL-37) which are used for antimicrobial activity in phagolysosome or exterior of the cells. The membrane proteins of the secondary granules include laminin receptor, vitronectin receptor, fpr and TNF receptor (Soehnlein *et al.*, 2009).

Primary or azurophilic granules contain several antimicrobial proteins while their membranes almost lack receptors. These granules contain myeloperoxidase (MPO), HBP, human neutrophil peptides 1-3 (HNP 1-3 or α -defensins) and different serine proteases such as elastase, cathepsin G and proteinase 3 (Rice *et al.*, 1987; Sinha *et al.*, 1987; Salvesen *et al.*, 1993; Campanelli *et al.*, 1990).

1.2.1.2 Neutrophil granules and neutrophil-endothelial interaction

PMN granules are safely stored within the PMN until they get activated by inflammatory stimuli, such as *N*-formyl-methionine containing peptides released by bacteria, complement products such as C5a and chemokines such as interleukin (IL)-8. Upon initiation of inflammation, PMN start to interact with the endothelium, resulting in the release of secretory vesicles. These constitute a pool of membrane-bound receptors that are integrated into the PMN plasma membrane (Fig. 1.1). A PMN rolling along the endothelium adheres to it and then both pattern recognition and chemokine receptors distinguish signals from the environment. Secretory vesicles are released at the luminal side when PMN establish an interaction with the endothelium, while exocytosis of tertiary granules is initiated upon PMN transendothelial migration. At this point, discharge of the metalloproteinases MMP-8 and MMP-9 most likely allows the PMN to cut its way through to the basement membrane. During the subsequent migration, PMN primary and secondary granules undergo partial exocytosis (Lacy and Eitzen, 2008; De Yang *et al.*, 2000; Soehnlein *et al.*, 2009).

1. Free flow 2. Rolling (Crawling) 3. Adhesion 4. Transmigration 5. Migration



	Secretory vesicles	Tertiary granules	Secondary and primary granules
Membrane	FPR, β 2- integrins, CD16, CD14, proteinase-3	FPR, β -2 integrins, TNF-R	Laminin-R, FPR, TNF-R, CD63, CD66
Matrix	Azurocidin, albumin	MMP-9, lysozyme, arginase	Collagenase, LL-37, HNP1-3, azurocidin, cathepsin G, elastase, MPO, proteinase-3

Figure 1.1: Granule subsets are released at different stages of PMN extravasation. PMN migrate from the vessel through a series of cell–cell and cell– matrix interactions. Granule subsets are released at different steps of PMN extravasation. Release of secretory vesicles following PMN adhesion leads to the deposition of PMN on the endothelial cell surface and integration of receptors (CD14, CD16, β 2-integrins) into the PMN cell surface. Transmigrating PMN release tertiary granules, which contain proteases to help penetration of the basement membrane. The migrated PMN deposit proteases (e.g. cathepsin G, elastase) and antimicrobial polypeptides (e.g. LL-37, azurocidin, HNPs) in the extravascular tissue, leading to interaction with nearby cells. Abbreviations: FPR, formyl peptide receptor; HNP, human neutrophil peptide; MMP-9, matrix metalloproteinase 9; MPO, myeloperoxidase; TNF-R, tumor necrosis factor receptor (Soehnlein *et al.*, 2009 with modifications).

This first recruitment and extravasation of PMN into the inflamed tissue is further replaced by mononuclear leukocytes (monocytes and lymphocytes) and macrophages. PMN granules have an essential role in recruitment of monocytes to the inflamed tissue by directly inducing monocyte adhesion to the endothelial cells, increasing the expression of adhesion molecules on the endothelial cell surface and elevating the expression of chemokines which regulate the recruitment of leukocytes (Rebuck and Crowley, 1955; Soehnlein *et al.*, 2009).

1.3 Mechanism of neutrophil bacterial killing

1.3.1 Phagocytosis: an intracellular killing mechanism

PMN have a variety of pattern recognition receptors (PRRs) such as Toll-like receptor (TLR) which recognize wide molecular patterns called PAMPs (pathogen associated molecular patterns) on infectious agents. Binding of infectious agents via TLRs results in phagocytosis and release of inflammatory cytokines (IL-1, TNF-alpha and IL-6) by PMN. Moreover, bacteria with IgG antibody on their surface have the Fc region exposed and this part of the Ig molecule can bind to the receptor on phagocytes. Binding to the Fc receptor requires prior interaction of the antibody with an antigen. Binding of IgG-coated bacteria to Fc receptors results in enhanced phagocytosis (Hickey and Kubes, 2009). Besides PRRs and Fc receptors, phagocytic cells have a receptor for the third component of the complement system, C3b. Binding of C3b-coated bacteria to this receptor also results in enhanced phagocytosis and stimulation of the respiratory burst (Fig. 1.2).

At the site of infection, neutrophils engulf microbes. Phagosomes, the vesicles containing the pathogen, fuse with primary and secondary neutrophil granules. The antimicrobial contents of these neutrophil granules are released into the lumen of the phagosome, which is then called phagolysosome. In the phagolysosome the pathogens are killed after exposure to reactive oxygen species (ROS), enzymes, and antimicrobial peptides.

In the phagolysosome, antimicrobial peptides and proteins such as defensins, bactericidal/permeability-increasing protein and the enzyme lysozyme, mainly function by disrupting the anionic bacterial surfaces. Proteases, such as neutrophil elastase and cathepsin G, degrade bacterial proteins, including virulence factors. Moreover, the NADPH oxidase complex assembles at the phagosomal membrane and produces O₂ radical, which is converted to hydrogen peroxide by the enzyme superoxide dismutase. Hydrogen peroxide is then converted to hyperchlorous acid (HOCl) by MPO. HOCl seems to play a role in bacterial killing. The combination of antimicrobials and ROS kill phagocytosed bacteria (Hampton *et al.*, 1998; Chapman *et al.*, 2002; Mayer-Scholl *et al.*, 2004; Hickey and Kubes, 2009).

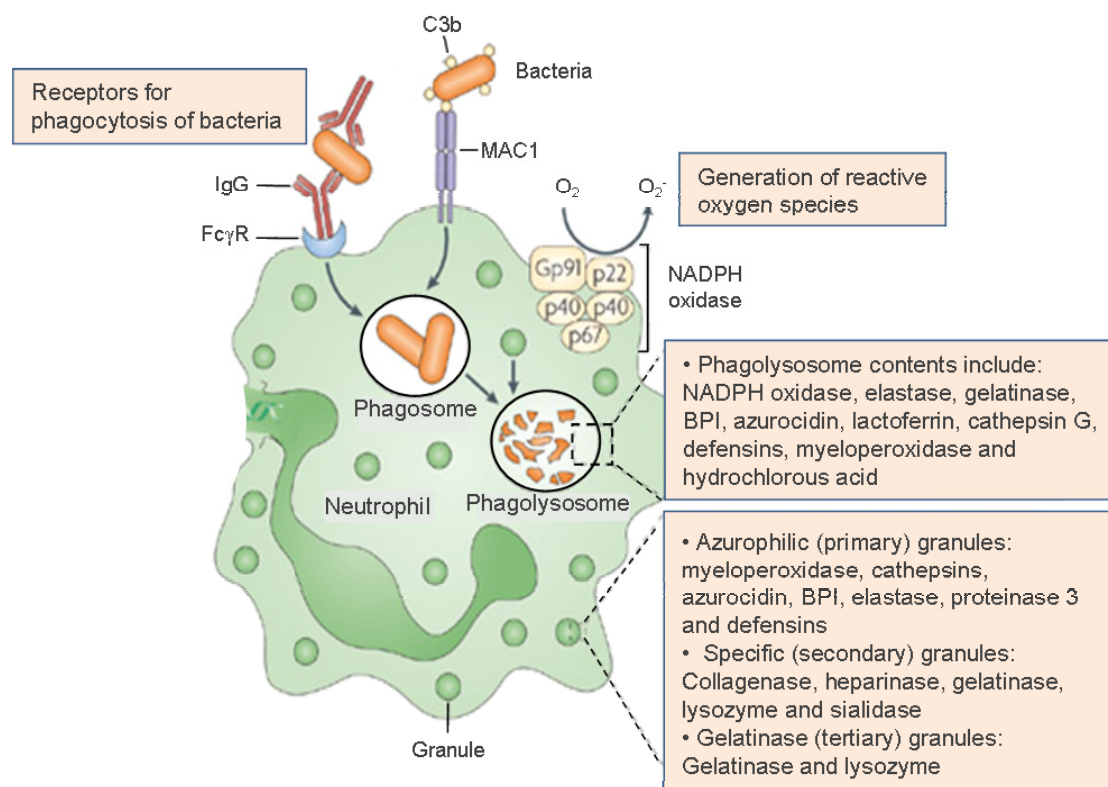


Figure 1.2: Neutrophil phagocytosis of bacteria. Neutrophils express various receptors that assist bacterial phagocytosis, including macrophage receptor 1 (Mac1; also known as $\alpha_M\beta_2$ integrin and CR3), which binds complement component 3b (C3b) on the surface of opsonized bacteria. Neutrophils also express Fc receptors for IgG (Fc γ R), which enable binding of IgG-coated particles. These molecules aid the uptake of bacteria into membrane-bound compartments, known as phagosomes. Phagosomes subsequently fuse with lysosomes and neutrophil granules, which contain a wide range of proteolytic and antibacterial enzymes and peptides, thereby forming phagolysosomes. The azurophil (primary), specific (secondary) and gelatinase (tertiary) granules contain characteristic proteases, antimicrobial proteins and peptides, and enzymes. NADPH oxidase complex assembles at the phagosomal membrane and produces O $_2^-$, which is rapidly converted to hydrogen peroxide and together with various enzymes such as myeloperoxidase, result in the production of further potent oxidants that destroy the bacteria. BPI: bactericidal/permeability-increasing protein (Hickey and Kubes, 2009 with modifications).

1.3.2 Neutrophil extracellular traps (NET): an extracellular killing mechanism

Recently, another antimicrobial mechanism of neutrophils was described. Activated neutrophils eradicate microorganisms in the extracellular space by a process which is called neutrophil extracellular traps (NET) formation (Brinkmann *et al.*, 2004). NET formation is an active process; upon activation of neutrophils e.g. by IL-8, the protein kinase C activator phorbol myristate acetate (PMA), lipopolysaccharide (LPS), gram positive and negative bacteria, fungi or activated platelets the chromatin de-condenses and hetero- and euchromatin are mixed. Later, the nuclear envelope and the granule membranes disintegrate and NET

components which are mainly chromatin and granular proteins are combined. Finally, the cell membrane is ruptured and NET are expelled from the cell (Fig. 1.3).

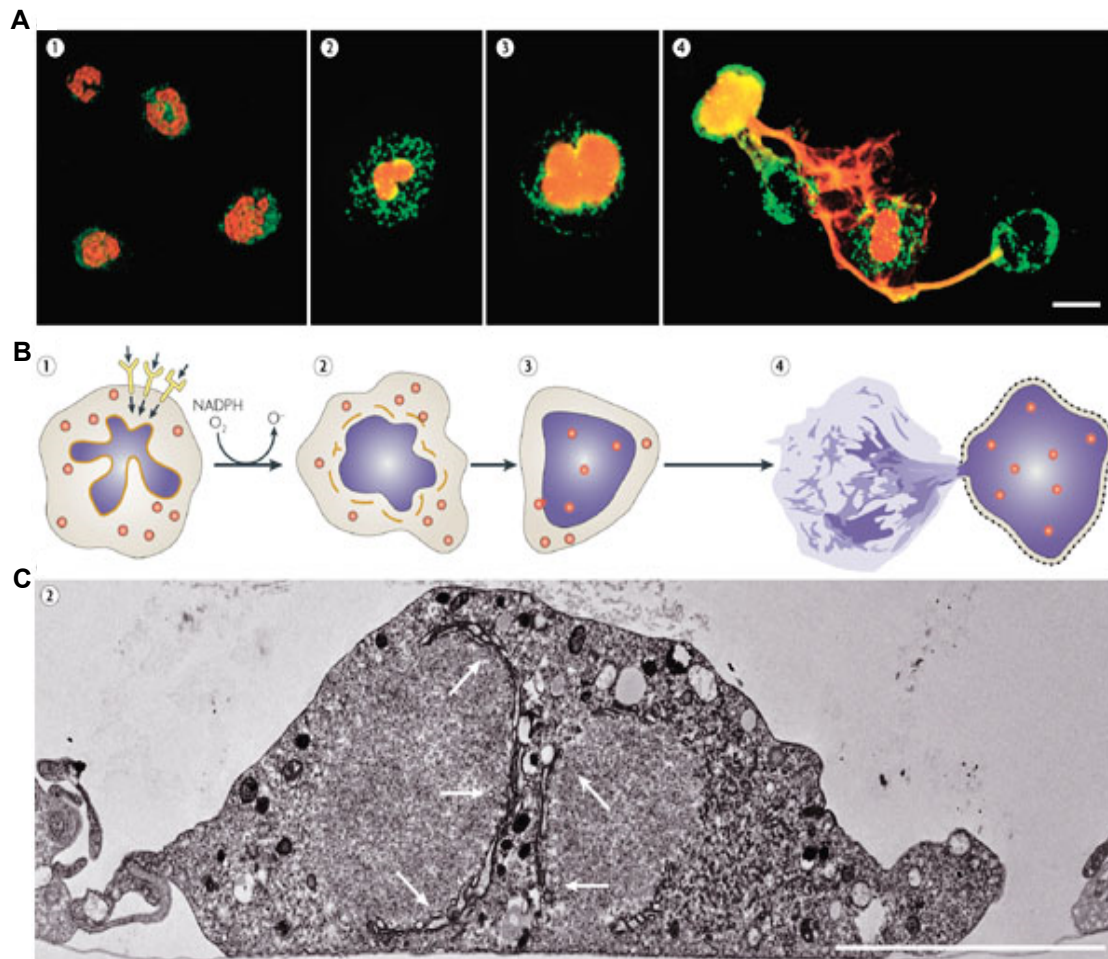


Figure 1.3: Neutrophil extracellular traps (NET) formation. (A) Fluorescent micrographs showing the steps of NET formation. DNA is shown in red and granules are in green. (B) Activation of neutrophil leads to the formation of reactive oxygen species (step 1). The nuclear membranes start to disintegrate into vesicles and the integrity of the granules is lost (step 2). Nuclei then lose their lobules and mix with the contents of the granules (step 3). During the final stage, the cells round up, contract and finally expel NET (step 4). (C) A transmission electron micrograph of a neutrophil at step 2 in the process. The arrows show the disintegration of the nuclear membrane, which allows the karyoplasm to mix with the cytoplasm (Brinkmann and Zychlinsky, 2007).

NET formation is a “beneficial suicide” of neutrophils; these structures can trap microorganisms, prevent their invasion and by providing a high local concentration of antimicrobial peptides, they facilitate the antimicrobial activity of neutrophils (Brinkmann and Zychlinsky, 2007; Fuchs *et al.*, 2007; Clark *et al.*, 2007).

Chromatin is considered the backbone of these structures, as NET can be degraded by DNases but not by proteases. Interestingly, several Gram-positive pathogens secrete DNases or have DNases bound to their cell wall, and these bacteria are more pathogenic. Strains of

group A *Streptococcus* (GAS) that did not secrete DNases were significantly less pathogenic than the isogenic wild-type strains which secrete DNase, and degradation of NET by secreted DNase increases the virulence of DNase-positive strains. DNase-expressing strains of GAS and *S. pneumoniae* can escape killing by NET and are thus more pathogenic than strains without DNases (Sumby *et al.*, 2005; Buchanan *et al.*, 2006). Moreover, the expression of a positively charged capsule in *S. pneumoniae* reduces binding to the NET, and could therefore be one mechanism that bacteria use to avoid getting trapped in NET (Wartha *et al.*, 2007).

It seems reasonable that NET formation is a common mechanism of the innate immune system in vertebrates, as it has been observed in humans (Brinkmann *et al.*, 2004; Gupta *et al.*, 2005; Marcos *et al.*, 2010), mice (Sumby *et al.*, 2005; Buchanan *et al.*, 2006; Wartha *et al.*, 2007), rabbits (Brinkmann *et al.*, 2004), horses (Alghamdi and Foster, 2005), cows (Lippolis *et al.*, 2006) and fish (Palić *et al.*, 2007). Interestingly, NET-like structures were also found in the hemolymph of insects and it was shown that they have a role both in innate immunity and hemolymph coagulation of the insects (Altincicek *et al.*, 2007). Recently, it has also been demonstrated that the neutrophil serine proteases and extracellular nucleosomes enhance coagulation in liver microvessels and reduce bacterial invasion into tissue (Massberg *et al.*, 2010). Therefore, NET and extracellular nucleic acids probably promote host survival by improving defense mechanisms against pathogens at sites of tissue damage/infection in both insects and mammals. Furthermore, extracellular structures similar to the extracellular traps described for neutrophils have been shown for mast cells and eosinophils which are called extracellular traps (ET) (Yousefi *et al.*, 2007; von Köckritz-Blickwede *et al.*, 2008). Activated eosinophils can release mitochondrial DNA in a reactive oxygen species-dependent manner, but independent of eosinophil death. In the extracellular space, the mitochondrial DNA and the granule proteins form extracellular structures able to bind and kill bacteria both *in vitro* and under inflammatory conditions *in vivo*.

NETs contain not only nuclear and granular proteins but also cytoplasmic proteins including cytoskeletal and peroxisomal proteins and glycolytic enzymes. Urban and co-workers identified and quantified the composition of NET by proteome approach which is summarized in table 1.1.

Table 1.1 Identified NET proteins

Cellular localization	Protein name
Granules	Leukocyte elastase
	Lactotransferrin
	Azurocidin
	Cathepsin G
	Myeloperoxidase
	Leukocyte proteinase 3
	Lysozyme C
	Neutrophil defensin 1 and 3
Nucleus	Histone H2A
	Histone H2B: a) Histone H2B b) Histone H2B like
	Histone H3
	Histone H4
	Myeloid cell nuclear differentiation antigen
Cytoplasm	S100 calcium binding protein A8
	S100 calcium binding protein A9
	S100 calcium binding protein A12
Glycolytic enzymes	Alpha-enolase
	Transketolase
Cytoskeleton	Actin (β and/or γ)
	Myosin-9
	Alpha-actinin (1 and/or -4)
	Plastin-2
	Cytokeratin-10
Peroxisomes	Catalase

Proteins that localize to NETs. Proteins are organized by their localization in unstimulated neutrophils (Urban *et al.* 2009 with modifications).

1.3.2.1 Mechanism of neutrophil extracellular trap formation

NET can be generated after three hours stimulation with potent inducer of NET, PMA. Papayannopoulos and Zychlinsky have been proposed that neutrophils use three major strategies to fight and clear microbes: phagocytosis, degranulation, and NET formation and these strategies operate over different timescales (Fig. 1.4) (Papayannopoulos and Zychlinsky, 2009). However, Clark and colleagues have found that NET are made only minutes after activation by stimulated platelets under flow (Clark *et al.*, 2007). Recently, a novel mechanism of rapid NET formation in response to *Staphylococcus aureus* has been described which happens very rapidly (5-60 min) and is oxidant-independent (Pilszczek *et al.*, 2010).

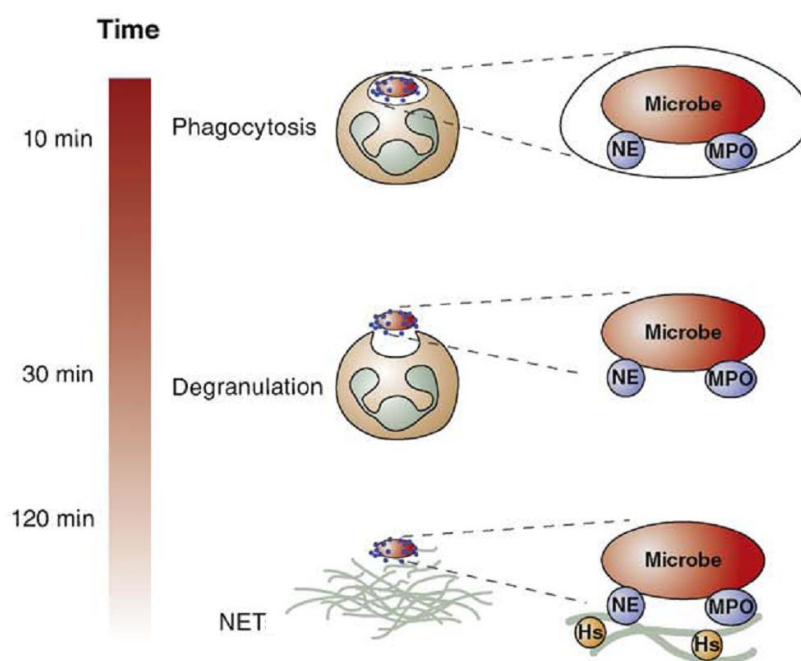


Figure 1.4: The antimicrobial strategies of neutrophils. Neutrophils use three main strategies to fight and clear microbes: phagocytosis, degranulation, and NET formation. These strategies function over different timescales. Red: microbe, Blue: granular proteins in the phagolysosome, extracellular space, or tethered within the NET, Gray: decondensed DNA of the NET. Abbreviations: Hs, histones; MPO, myeloperoxidase; NE, neutrophil elastase (Papayannopoulos and Zychlinsky, 2009 with modifications).

The process of NET formation is different from both apoptosis and necrosis. In necrosis, the nuclei of necrotic cells lose the segregation between eu- and heterochromatin inside an intact nuclear envelope. Apoptotic nuclei show condensed chromatin and are separated into several membrane-bound apoptotic bodies. By contrast, during NET formation, activated neutrophils

gradually dissolve their nuclear membranes and the granules, thus allowing mixing of NET components in the cytoplasm. In addition, the cell death that leads to NET formation is not accompanied by DNA fragmentation and does not require apoptosis-specific caspases. Moreover, NET formation is not the result of a direct disruption of the cell membrane; the situation which occurs when cells are attacked by bacterial toxins. It was shown that during the process of NET formation, neutrophils are alive before the NET are expelled from the cells (Fuchs *et al.*, 2007).

It was demonstrated that NET formation is dependent on the generation of ROS by NADPH oxidase and NADPH oxidase inhibitor, diphenylene iodonium, can block the formation of NET. Moreover, neutrophils isolated from patients suffering from chronic granulomatous disease (CGD) fail to make NET when stimulated with either bacteria or PMA, that is, when stimulated upstream of NADPH oxidase. CGD patients carry mutations in one of the subunits of NADPH oxidase and suffer from catastrophic infections. These neutrophils do make NET when they are stimulated downstream of NADPH oxidase with hydrogen peroxide (Heyworth *et al.*, 2003; Fuchs *et al.*, 2007). However, neutrophils from neonate infants are not able to form NET even if they are stimulated downstream of NADPH oxidase (Yost *et al.*, 2009). Remijnsen and co-workers have recently shown that NET formation needs both autophagy and superoxide generation, and inhibition of either autophagy or NADPH oxidase activity prevents NET formation and leads to neutrophil cell death by apoptosis (Remijnsen *et al.*, 2010). Furthermore, Li and co-workers showed that histone hypercitrullination catalyzed by peptidylarginine deiminase 4 is essential for chromatin decondensation during NET formation (Li *et al.*, 2010). Recently, Papayannopoulos and co-workers have shown that neutrophil elastase and MPO are necessary for regulation of NET formation. They showed that first elastase escapes from azurophilic granules and translocates to the nucleus, degrades specific histones and promote chromatin decondensation. Later, MPO, independent of its enzymatic activity, synergizes with elastase in chromatin decondensation (Papayannopoulos *et al.*, 2010). Moreover, using a new chemical genetic analysis Hakkim and co-workers have shown that the Raf-MEK-ERK pathway is involved in NET formation by activation of NADPH oxidase and upregulation of antiapoptotic proteins (Hakkim *et al.*, 2011). Therefore, NET formation seems to originate from the activation of different signaling pathways and cellular processes.

1.4 The role of neutrophils in inflammation

Inflammation is a multifaceted set of interactions between the cells and soluble factors that can occur in any tissue in response to infectious, traumatic, post-ischaemic, toxic or autoimmune injury. This procedure usually leads to healing and recovery from infection; however, if destruction and repair are not accurately phased, inflammation can lead to constant tissue damage by cells such as leukocytes and lymphocytes (Nathan, 2002).

Neutrophils as one of the body's major cellular components for the destruction of microorganisms also damages tissue and cells of the host. Neutrophil-mediated tissue damage at the infected sites is one of the major sources that launch inflammation, which in turn induces immunity. In fact, a normal immune response results from the ongoing detection of signals that report injury and signals that report infection. Neutrophils make essential contributions to the recruitment, activation and programming of dendritic cells and macrophages. In turn, the adaptive immune system controls the rate of neutrophil production in the bone marrow (Nathan, 2006).

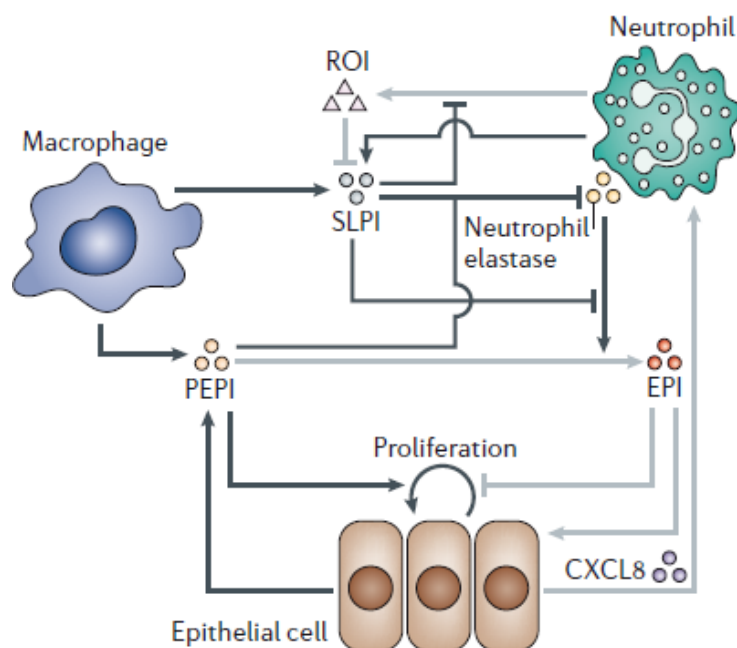


Figure 1.5: The role of neutrophils in wound healing. The picture demonstrates one pathway out of many that are functioning in wounds. Neutrophil- and macrophage-derived secretory leukocyte protease inhibitor (SLPI) blocks neutrophil elastase. SLPI alone, and in synergistic combination with macrophage- and epithelial-cell-derived proepithelin (PEPI), blocks cytokine-induced release of proteolytic enzymes and reactive oxygen intermediates (ROIs) by neutrophils. These actions decrease the neutrophil-dependent proteolytic conversion of PEPI to epithelins (EPIs), declining the ability of EPI to promote epithelial-cell production of CXC-chemokine ligand 8 (CXCL8; also known as IL-8), an important neutrophil chemoattractant. Intact PEPI promotes epithelial-cell proliferation, accelerating closure of the wound. Black arrows indicate processes involved in tissue repair and regeneration. Gray arrows indicate processes involved in host defence (Nathan, 2006).

Neutrophils also have significant roles in wound healing (Fig. 1.5). The main role of neutrophils to wound healing is microbial sterilization. Wounds tend to heal weakly in individuals with inadequate neutrophils. Neutrophils contribute in wound healing by production of signals that decrease the rate of accumulation of more neutrophils, suppressing their own activation and by initiation of a macrophage-based program that switches the state of damaged epithelium from pro-inflammatory and nonreplicative, to anti-inflammatory and replicative (Nathan, 2006).

Two of the most important mechanisms of damage by neutrophils are the respiratory burst and the degranulation response (Fig. 1.6). ROS and granular proteins can demolish microorganisms or their products; however, long exposure or high concentrations of these mediators can be detrimental for the host and lead to tissue injury (Nathan, 2006; Singer and Clark, 1999).

Excessive inflammation has a main role in different diseases such as rheumatoid arthritis, osteoarthritis, chronic obstructive pulmonary disease (COPD) and multiple sclerosis. The usual goal of neutrophil-targeted pharmacology is not to increase inflammation but is to suppress it. Unfortunately, common approaches to neutrophil-based anti-inflammatory therapy have their own disadvantages since suppressing neutrophil migration or activities impair host defence against infection (Nathan, 2006).

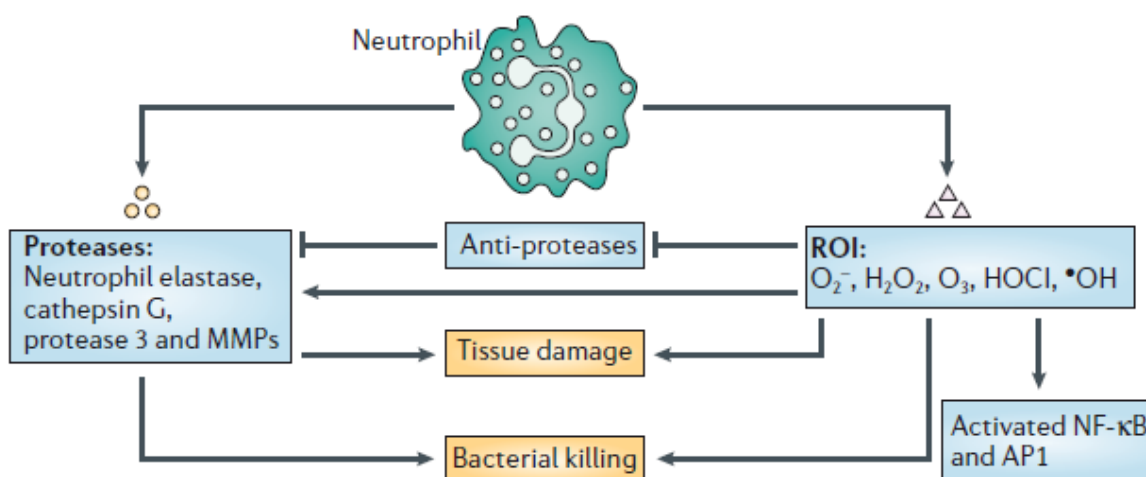


Figure 1.6: Anti-microbial activity with tissue destruction cross-talk in neutrophils. Two of the most important mechanisms of destruction by neutrophils are the respiratory burst and the degranulation response. Both can lead to bacterial killing and tissue damage. AP1, activator protein 1; ROI, reactive oxygen intermediates; H₂O₂, hydrogen peroxide; HOCl, hypochlorous acid; MMP, matrix metalloproteinase; O₂⁻, superoxide; O₃, ozone; •OH, hydroxyl radical; NF-kappaB; nuclear factor-kappaB (Nathan, 2006).

1.4.1 NET and inflammation

NET have also been seen *in vivo* in experimental model of dysentery and human appendicitis samples (Brinkmann *et al.*, 2004). During severe sepsis, platelets via TLR-4 bind to sequestered neutrophils, leading to NET formation within minutes which trap the bacteria present in the systemic circulation but this also causes endothelial cell and hepatic damage (Clark *et al.*, 2007).

Neutrophils isolated from patients suffering from CGD are not able to form an effective NET (Fuchs *et al.*, 2007). *In vitro*, Neutrophils of CGD patients are not capable to kill *Aspergillus nuidilans* but gene therapy in these patients restored NET formation and resulted in recovery from Aspergillosis (Bianchi *et al.*, 2009).

Moreover, NET formation occurs in autoinflammatory disorders in the absence of microbial infection. In small-vessel vasculitis (SVV), which is a chronic autoinflammatory disease and linked to antineutrophil cytoplasm autoantibodies, NET deposit in inflamed kidneys and triggers vasculitis and promotes the autoimmune response against neutrophil components in individuals with SVV (Kessenbrock *et al.*, 2009). Furthermore, patients with systemic lupus erythematosus (SLE) have autoantibodies against NET proteins as well as against dsDNA. A subset of SLE patients' sera degraded NET poorly. These patients have either DNase1 inhibitors or anti-NET antibodies which prevented DNase1 access to NET and it was shown that impairment in NET degradation is associated with lupus nephritis (Hakkim *et al.*, 2010).

ET, which are released by eosinophils, contain mitochondrial DNA as well as eosinophil granular proteins. Theses extracellular structures are able to bind and kill bacteria and were also found in colon samples of patients with Crohns disease (Yousefi *et al.*, 2008).

Fuchs and co-workers have recently shown that NET can induce thrombosis by stimulating platelets *in vitro*, and NET components were abundant in thrombi induced in baboons subjected to deep vein thrombosis, an example of inflammation-enhanced thrombosis (Fuchs *et al.*, 2010). All of these examples show a potential proinflammatory role of NET.

1.5 Inflammatory lung diseases

Inflammatory lung diseases refer to a group of diseases associated with different types and degrees of inflammatory cell accumulation in the lungs (Table 1.2). Diseases typically included in this group are acute lung injury/acute respiratory distress syndrome (ALI/ARDS), idiopathic pulmonary fibrosis/usual interstitial pneumonia (IPF/UIP), collagen vascular disease-associated interstitial pneumonia, nonspecific interstitial pneumonia, cryptogenic organizing pneumonia/bronchiolitis obliterans organizing pneumonia, sarcoidosis, hypersensitivity pneumonitis/extrinsic allergic alveolitis, and eosinophilic pneumonia. In a broad sense, COPD, bronchial asthma, and infectious diseases can also be included in this list (Fujishima, 2011).

Table 1.2 Clinical course and inflammatory cells in inflammatory lung diseases

Diseases	Clinical course	Major inflammatory cells observed
Acute lung injury / Acute respiratory distress syndrome (ALI/ARDS)	Acute	Neutrophils
Idiopathic pulmonary fibrosis / Usual interstitial pneumonia (IPF/UIP)	Chronic / acute exacerbation	Alveolar macrophages, neutrophils?
Hypersensitivity pneumonitis (HP)	Acute / chronic	CD8 lymphocytes, (CD4 lymphocytes)
Sarcoidosis	Chronic	CD4 lymphocytes
Eosinophilic pneumonia (EP)	Acute / chronic	Eosinophils
Chronic obstructive pulmonary disease (COPD)	Chronic / acute exacerbation	CD8 lymphocytes/ neutrophils
Asthma	Chronic / acute attack	Eosinophils, basophils, Th2 lymphocytes

Different inflammatory lung diseases, the clinical course and the major inflammatory cells which are involved in each disease (Fujishima, 2011).

1.5.1 Neutrophils and ALI

ALI and its more severe form ARDS represent a form of lung dysfunction characterized by impairment in the functions of the alveolar-capillary barrier, hypoxemia, non cardiogenic pulmonary edema, low lung compliance and widespread capillary leakage. They result from complex responses of the lung to a multitude of direct and indirect insults, any stimulus of local or systemic inflammation, principally sepsis (Kollef and Schuster, 1995; Bernard, 1994; Wheeler and Bernard, 2007). Alveolar epithelial cell function and barrier integrity are crucial to preserve normal gas exchange, and injury or loss of epithelial cells leads to progression of ALI/ARDS (Ware and Matthay, 2000; Manicone, 2009). Although its pathogenesis is largely uncertain, neutrophil-dominant acute inflammation and resultant tissue destruction are the main pathological features. Since ALI/ARDS carries high mortality rates between 40 and 60% (Rubenfeld *et al.*, 2005), understanding of the mechanisms in the development of ALI/ARDS is essential for developing novel therapeutic options to treat these patients (Liu, 2005; Liu and Slutsky, 1997).

Acute inflammation features neutrophil accumulation and plasma exudates outside of the blood vessels. Neutrophil recruitment is directed by the lung cells. Adhesion molecules induced on the lung cells and chemokines and colony-stimulating factors from lung cells stimulate neutrophil production from the bone marrow and chemotaxis (Christopher and Link, 2007; Mizgerd, 2002; Mizgerd, 2008). Moreover, intratracheal administration of bacterial toxin such as pneumococcal virulence factor pneumolysin into the lungs of mice draws the alveolar accumulation of neutrophils (Maus *et al.*, 2007). When neutrophils reach the site of infection, they generate proinflammatory signals, including TNF- α , IL-1 and chemokines which recruit and activate dendritic cells, monocytes, B-lymphocyte and T-lymphocytes. Therefore, adaptive and innate immune responses against microbes in the lungs are shaped by signals derived from neutrophils (Mizgerd, 2008; Tateda *et al.*, 2001; Liang *et al.*, 2006).

Moreover, after recruitment to the inflammatory site, neutrophils apply a variety of functions including production of ROS, phagocytosis, and degranulation of lytic enzymes that enable clearance of the invading pathogen. However, excessive inflammation and increased activation of neutrophils leads to injury and organ damage (Burns *et al.*, 2003; Nathan, 2006). Neutrophil accumulation has been observed in lung tissue (Bachofen and Weibel, 1977; Bachofen and Weibel, 1982) and broncho-alveolar lavage (BAL) fluids of ARDS patients

(Pittet *et al.*, 1997). Therefore, the recruitment and activation of neutrophils are considered crucial to the pathogenesis of ALI. Activated neutrophils contribute to lung injury by releasing proteolytic enzymes, ROS and other proinflammatory mediators (Lee *et al.*, 2005; Abraham, 2003) and lung epithelial cell death represents a potentially important mechanism contributing to the loss of this cell type in the development of ALI (Perl *et al.*, 2008; Bachofen and Weibel, 1982). It has been shown that in different models of ALI depletion of neutrophils leads to decrease injury associated with ALI (Lomas-Neira *et al.*, 2006; Inoue *et al.*, 1995; Clark *et al.*, 2003; Looney *et al.*, 2006; Abraham *et al.*, 2000).

2 Aim of the study

Since high amounts of NET seem to be associated with pathophysiological conditions, it was hypothesized that neutrophil derived NET, in excessive amounts, may contribute to epithelial and endothelial cell death and injury in the pulmonary system.

Thus the aims were:

- To identify the protein components of NET by mass spectrometry; identification of NET components will give a better understanding of the function of NET.
- To investigate NET formation in lung tissue and BAL fluid in the established animal model of LPS-induced acute lung injury; recognition of the presence of NET in the lung injury model may explain the deleterious role of NET.
- To analyze, qualify and quantify NET in mice and human neutrophils; evaluation of NET is a key factor to compare the effect of different stimuli on NET formation.
- To investigate the effect of extracellular adherence protein of *Staphylococcus aureus* in NET formation; the interaction between neutrophils and *S. aureus* in respect of NET formation has been described. However, the effect of extracellular adherence protein of *S. aureus* on NET formation has not been reported.
- To study the direct effect of NET on endothelial and lung epithelial cytotoxicity and cell death; to this point, the direct effect of NET on host cells has not been documented.
- To fractionate NET in order to analyze the cytotoxic components of NET; fractionation of NET, based on the molecular weight, will make the analysis of NET easier.
- To examine the effect of separate components of NET on epithelial and endothelial cytotoxicity; analyzing the effect of NET components, particularly histones as major components of NET as well as elastase and myeloperoxidase, will reflect which factors in NET are responsible for the cytotoxicity.
- To analyze the proteomic profile of epithelial cells after treatment with NET; proteome analysis of host cells after incubation with NET will clarify how they react to NET.
- To study the effect of NET on epithelial permeability; change in cellular permeability after treatment with NET will elucidate one of the host cell responses to NET.
- To investigate the presence of NET in cystic fibrosis patients; presence of NET in cystic fibrosis sputum implies the injurious role of NET in these patients and their complications.

3 Material and methods

3.1 Materials

3.1.1 Reagents

2.5-dihydroxybenzoic acid (Sigma-Aldrich, Germany)

2D-Quant kit (GE Healthcare, Germany)

A549-human lung adenocarcinoma cell line (ATCC, USA)

Acetone (Roth, Germany)

Acrylamide-Bisacrylamide (Roth, Germany)

Activated protein C (APC) (Xigris, Eli Lilly)

Alexa Fluor 488 donkey anti-rabbit IgG (Invitrogen, Germany)

Alexa Fluor 546 Monoclonal Antibody Labeling Kit (Invitrogen, Germany)

Alexa Fluor 555 donkey anti-mouse IgG (Invitrogen, Germany)

Amicon ultra-4 centrifugal filter units (Millipore, Germany)

Ammonium persulphate (Roth, Germany)

Anti-caspase-3 antibody (Cell Signaling Technology, Germany)

Anti-CD46 (Santa Cruz, Germany)

Anti-CD66b (Antibody-online, Germany)

Anti-DNA Histone H1 (Millipore, Germany)

Anti-Ly-6G MicroBead (Miltenyi Biotec, Germany)

Anti-neutrophil elastase (M-18); goat polyclonal (Santa Cruz, Germany)

Anti-neutrophil elastase; rabbit polyclonal (Calbiochem, Germany)

Anti- β -actin antibody, clone AC-15 (Sigma Aldrich, Germany)

Apoptotic/Necrotic/Healthy cells detection kit (PromoKine, Germany)

Bovine serum albumin (Sigma-Aldrich, Germany)

Bromophenol blue (Roth, Germany)

Calcium chloride (Roth, Germany)

CHAPS (GE Healthcare, Germany)

Collagen I (BD Biosciences, Germany)

Coomassie Brilliant Blue R-250 (Serva electrophoresis, Germany)
Cy2, 3 and 5 (GE Healthcare, Germany)
Cytotoxicity detection kit (Roche Applied Science, Germany)
DAPI (Vectashield mounting medium with DAPI, USA)
Dimethylsulfoxide (Roth, Germany)
DL-Dithiothreitol (DTT) (Sigma-Aldrich, Germany)
DNase I, RNase free (Fermentas, Germany)
Dulbecco's modified Eagle medium (DMEM) (GIBCO-Invitrogen, Germany)
ECL plus western blotting detection system (GE healthcare, Germany)
Ethanol (Roth, Germany)
Ethylenediaminetetraacetic acid (EDTA) (Roth, Germany)
Fetal bovine serum (GIBCO-Invitrogen, Germany)
FITC rat anti-mouse Ly-6G and LY-6C (BD Pharmingen, Germany)
FITC rat IgG2b (BD Pharmingen, Germany)
Flamingo fluorescent gel stain (Bio-Rad, Germany)
Green multi-caspase staining kit (Promokine, Germany)
HBSS (GIBCO-Invitrogen, Germany)
HEPES (PPA, Germany)
Histone H2A antibody (Cell signaling, Germany)
Histone H2B antibody (Millipore, Germany)
Histone H3 (citrulline 2 + 8 + 17) antibody [CitH3] (Abcam, Germany)
Histone H3 antibody (Millipore, Germany)
Histone H4 antibody (Cell signalling, Germany)
Histone type IIA from calf (Sigma-Aldrich, Germany)
Histopaque-1077 (Sigma-Aldrich, Germany)
Histopaque-1119 (Sigma-Aldrich, Germany)
HRP-conjugated anti-goat (Dako Cytomation, Denmark)
Human neutrophil elastase (Serva electrophoresis, Germany)
Human neutrophils (Primary, isolated)

Human Serum Albumin (Behring, Germany)

Human umbilical vein endothelial cells (HUVEC) (Primary, isolated)

Hybond-C polyvinylidene difluoride (PDVF) membrane (GE Healthcare, Germany)

IPG-strips (Bio-Rad, Germany)

LoBind tubes (Eppendorf, Germany)

LPS; L4391 (Sigma-Aldrich, Germany)

Methylendiphosphonic acid (Fluka, Germany)

Micro-BCA protein assay reagent kit (Pierce, Germany)

Micrococcal nuclease from Staphylococcus aureus (MNase) (Sigma-Aldrich, Germany)

Mouse IgG isotype antibody; M4509 (Sigma -Aldrich, Germany)

N-(Methoxysuccinyl)-Ala-Ala-Pro-Val 4-nitroanilide (Sigma-Aldrich, Germany)

N-(Methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone (Sigma-Aldrich, Germany)

N,N,N',N'-Tetramethylethylenediamine (TEMED) (Roth, Germany)

Native Gel Starter Kit (Serva electrophoresis, Germany)

Nitrate silver (Sigma-Aldrich, Germany)

Non-fat dry milk powder (Roth, Germany)

Paraformaldehyde (Roth, Germany)

Penicillin/streptomycin (GIBCO-Invitrogen, Germany)

Pharmalyte 5-9 (Serva electrophoresis, Germany)

Phenol red-free RPMI 1640 (GIBCO-Invitrogen, Germany)

Phenylalanyl prolyl arginine chloromethyl ketone (PPACK) (Calbiochem, Germany)

Phorbol myristate acetate (PMA) (Sigma-Aldrich, Germany)

Picogreen dsDNA kit (Molecular Probes, Invitrogen, Germany)

Protease Inhibitor cocktail complete (Roche, Germany)

Rabbit IgG isotype antibody (Dianova, Germany)

Rat anti-mouse CD16/CD32; Mouse BD Fc Block (BD Pharmingen, Germany)

Sodium dodecyl sulfate (SDS) (Roth, Germany)

Spermine-Nitric oxide complex hydrate (Sigma-Aldrich, Germany)

Staurosporine (Sigma-Aldrich, Germany)

Tris (hydroxymethyl) aminomethane (TRIS) base (Roth, Germany)

Trisodium citrate dehydrate (Roth, Germany)

Triton X-100 (Sigma-Aldrich, Germany)

Trypan blue (Sigma-Aldrich, Germany)

TrypLE Express (GIBCO-Invitrogen, Germany)

Trypsin; sequencing grade (Promega, Germany)

Urea (Sigma Aldrich, Germany)

Vectashield mounting medium with DAPI (Vector Laboratories, USA)

Vector M.O.M. Immunodetection Kit (Vector Laboratories, USA)

VersaDoc system (BioRAD, Germany)

3.1.2 Equipment

96-well black flat bottom polystyrene not treated microplate (Corning Life Science, Germany)

96-well white flat bottom polystyrene not treated microplate (Thermo Scientific, Germany)

CASY Cell Counter System (Schaerfe Systems, Germany)

Cell culture incubator (Heraeus, Germany)

Disposable pipettes (2 ml, 5 ml, 10ml, 25 ml, 50 ml) (Greiner Bio-One, Germany)

ELx 808 absorbance ultra microplate reader (BIO-TEK Instruments, Germany)

Eppendorf tubes (0.5 ml, 1.5 ml, 2.0 ml) (Eppendorf, Germany)

ExQuest spot cutter (Bio-Rad, Germany)

Facscalibur flowcytometer (BD Biosciences, Germany)

Falcon tubes (Greiner Bio-One, Germany)

Fluorescence and light microscope (Leica Microsystems, Germany)

FLx 800 fluorescence microplate reader (BIO-TEK Instruments, Germany)

Gel blotting paper (GE Healthcare, Germany)

Hyperfilm ECL (GE healthcare, Germany)

Labofuge 400 R (Heraeus, Germany)

Lab-Tek chamber slides (Thermo Scientific, Germany)

Micro centrifuge 22 R (Hettich, Germany)

Petri dishes (Greiner Bio-One, Germany)

Pipetboy (Integra bioscience, Germany)

Pipettes tips: 2; 20; 200; 1000 μ l (Eppendorf, Germany)

Protean II XI cell (Bio-Rad, Germany)

Spectrophotometer; Specord 10 (Zeiss, Germany)

Tissue culture dishes (Greiner Bio-One, Germany)

Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Germany)

VersaDoc system (Bio-Rad, Germany)

Vortex machine (VWR, Germany)

Water bath (Mettler, Germany)

3.2 Methods

3.2.1 Animal treatment

C57BL/6J mice were purchased from Charles River (Sulzfeld, Germany). To generate acute lung injury, mice (n=3 per group) were treated with a 50 μ l PBS suspension of 10 μ g LPS administrated intratracheally via a cannula. Control mice were treated with 50 μ l PBS. Following LPS treatment, mice were sacrificed after 3, 6, 12, 24 and 48 h and BAL fluid was collected for neutrophil counting or isolation. The lungs were perfused and shock frozen for 24 h at room temperature. All experiments were performed in accordance with the guidelines of the Ethics Committee of the University of Giessen, School of Medicine, and approved by local and national authorities.

3.2.2 Isolation of neutrophils from BAL and immunofluorescence of isolated neutrophils

Mice treated with LPS for 24 h were sacrificed, BAL was collected and mouse neutrophils were isolated by anti-Ly-6G MicroBead kit according to the manufacturer's protocol. Isolated neutrophils were seeded on coverslips and treated with 25 nM PMA for 1.5 h at 37°C. Cells were fixed with 2% PFA, blocked (3% BSA in PBS) and incubated with primary antibodies anti-DNA/histone H1 and anti-CD46, which were detected with secondary antibodies

coupled to Alexa Fluor 555 donkey anti-mouse IgG and Alexa Fluor 488 donkey anti-rabbit, respectively. Controls were done with isotype-matched controls or by omitting the primary antibodies. Vectashield mounting medium with DAPI was used for nucleus detection. The images were taken by fluorescence microscopy using Metamorph imaging software version series 7.0.

3.2.3 BAL collection, MNase digestion and neutrophil elastase activity

C57BL/6 mice were treated intratracheally with LPS as mentioned above and BAL was collected after 3, 6, 12, 24 and 48 h following treatment. Control mice were treated with PBS. BAL was centrifuged 1000 g for 5 min. The supernatants (each 500 μ l) were collected and each pellet was resuspended with 500 μ l PBS. The lysates were digested with 500 mU/ml micrococcal nuclease (MNase) from *Staphylococcus aureus* (Fuchs *et al.*, 2007). Both digested lysate (to detect NET-derived elastase) and supernatant (to detect free elastase) were incubated with peptide substrate N-(methoxysuccinyl)-Ala-Ala-Pro-Val 4-nitroanilide for 15 min and the optical density was measured at 405 nm (ELx 808 absorbance ultra microplate reader). BAL collection was performed with the help of Dagmar Hensel (Department of Internal Medicine II, Giessen).

3.2.4 Isolation of neutrophils from mice bone marrow

Mice were sacrificed, the skin was separated from the legs and the muscles were removed from the bones. The thigh and the shank were cut and put in a Petri dish containing wash buffer (HBSS + 25 mM Hepes + 10% FCS). The ends of the bones were cut and bones were washed with a (insulin) syringe filled with wash buffer. The bone-marrow suspension was filtered with cell-filter (70 μ m pore-size) and centrifuged at 300 g for 10 min. The supernatant was removed and the pellet was resuspended in 1 ml HBSS + Hepes. The suspension was put carefully on a gradient containing 4 ml Histopaque-1119 under 4 ml Histopaque-1077 (in a 15 ml tube). After centrifugation at 700 g for 30 min without break, the upper phase was removed and the second phase (around 4 ml) was put in a 50 ml tube with 20 ml wash buffer. The suspension was centrifuged again 300 g for 10 min. Following centrifugation, 12.5 ml supernatant was removed and 12.5 ml wash buffer was added to the tube, resuspended and centrifuged again at 300 g for 10 min. Then, the whole wash buffer was removed; the cells were washed again with 20 ml wash buffer and centrifuged at 300 g for 10 min. Thereafter, the wash buffer was removed and the cells were resuspended in RPMI 1640 medium without phenol red. The cells were counted and viability was assessed by trypan blue dye. Purity was checked by flow cytometry using FITC rat anti-mouse Ly-6G and

Ly-6C. FITC Rat IgG2b and purified rat anti-mouse CD16/CD32 were used as isotype control and blocking antibody, respectively. The protocol for mouse neutrophil isolation was kindly provided by Dr. Friedemann Kiefer's group (Max-Planck-Institute for Molecular Biomedicine, Muenster).

3.2.5 Isolation of neutrophils from human blood

Human neutrophils were isolated from healthy donors using density gradient separation according to the protocol (Costa *et al.*, 2006). Briefly, a double gradient was formed by layering an equal volume of histopaque-1077 over histopaque-1119. Venous blood was collected in EDTA tubes and carefully layered onto the upper histopaque-1077. The blood was centrifuged at 700 g for 30 min. The granulocytes are found at the 1077/1119 interphase. The cells were collected, washed with PBS and erythrocytes were lysed by incubation in cold ddH₂O for 1 min. Purity was assessed 96% by labeling the cells with neutrophil-specific marker CD66b, and viability was measured 99% by trypan blue dye exclusion.

3.2.6 Treatment of neutrophils with extracellular adherence protein

Human neutrophils were incubated with different concentrations of extracellular adherence protein (Eap) from *Staphylococcus aureus* strain Newman (crude protein gift from Prof. Mathias Herrmann, Saarland University; purified by Uwe Schubert, Biochemistry Department, Medical School, Giessen) in the absence or presence of PMA, and cell morphology and NET formation were evaluated. To quantify NET formation in the presence of Eap, the ratio of anti-DNA/histone H1 antibody area per total cell numbers was evaluated by Metamorph imaging software. Minimum 1200 cells were counted for each condition. Besides PMA, neutrophils were also incubated with other NET-inducing stimuli such as LPS or NO donor (Spermine–Nitric oxide complex hydrate, gift from Dr. Mike Althaus, Institute of Animal Physiology, Giessen) and NET formation was evaluated in the absence or presence of Eap.

3.2.7 Mouse lung immunofluorescence

Frozen 5 µm lung tissue sections from mice treated intratracheally with 10 µg LPS or PBS were equilibrated to room temperature and fixed in acetone for 10 min. Vector M.O.M. Immunodetection kit was used with some modifications in the protocol. Briefly, after fixation the sections were washed with PBS and blocked with working solution of M.O.M mouse Ig blocking reagent for 1 h. The sections were incubated with primary antibodies anti-CD46 or anti-neutrophil elastase (M-18) followed by a secondary antibody coupled to Alexa Fluor 488

and anti-DNA/histone 1 coupled with Alexa Fluor 546. Negative control slides were obtained by either omitting the primary antibodies or by using the isotype-matched controls. DAPI was used for nucleus detection.

3.2.8 Epithelial and endothelial cell treatment with histones

A549-human lung adenocarcinoma cell lines were obtained from American Type Culture Collection (ATCC). Human umbilical vein endothelial cells (HUVEC) were isolated as described (Jaffe *et al.*, 1973). Human A549 cells and HUVEC were treated with 10 to 200 µg/ml of histones type IIA from calf. The supernatants were collected for cytotoxicity assay or analysis of the release of lactate dehydrogenase (LDH) enzyme from the cells. Cell morphology was analyzed under the light microscope (Leica Microsystems) and the cell numbers were counted by CASY Cell Counter System.

3.2.9 NET production, isolation and NET-DNA quantification

Human neutrophils were resuspended in phenol red-free RPMI 1640, and 1.8×10^6 cells per well were seeded in 6-well plate. One well left untreated and the others were stimulated with 50 nM PMA for 4 h. Thereafter, the media from all wells were carefully removed. To remove the remaining components which are not bound tightly to the NET, 1 ml RPMI was added per well and carefully removed again. To collect NET, 2 ml RPMI was added per well and NET (the smear on the wells) was collected in 15 ml tubes by vigorous agitation. After centrifugation at 20 g for 5 min, NET were collected in the supernatant and subjected to different treatments, including partial digestion by 500 U/ml MNase for 10 min at 37°C or complete digestion by 10 U/ml DNase I (Fermentas, Germany) for 20 min at 37°C or kept undigested. An aliquot from each tube was used for quantification of DNA in NET by picogreen dsDNA kit according to the manufacturer's instructions. The protocol for NET isolation was kindly provided by Dr. Volker Brinkmann (Max-Planck-Institute for Infection Biology, Berlin).

3.2.10 NET Protein quantifications

Protein quantification in NET was performed by micro-BCA protein assay reagent kit. For two-dimensional gel electrophoresis (2-D gel electrophoresis) analysis, 2-D Quant kit was used for protein quantification. Both methods resulted in the same values of protein concentration.

3.2.11 Treatment of lung epithelial cells with NET

A549 cells were seeded in 24-well plate to become confluent, washed once with PBS, and NET in different concentrations was added to each well, whereby 800 µl or 2400 µl from isolated NET equal to 3.4 (NET) and 10.1 (3 x NET) µg/ml DNA-NET, respectively, were added to different wells. Some wells were treated with 3.3 µg/ml staurosporine as a cell death inducer and some left untreated as controls. The total volume of each well was kept constant by adding RPMI medium. Cells were analyzed after 4 h or 16 h with different measurements (such as qualification of LDH and multicaspase activity).

3.2.12 Cytotoxicity assay

A549 cells were treated with 1% Triton X-100 (maximum LDH release or “high control”), with NET or kept untreated (spontaneous LDH release or “low control”). LDH release in the supernatant was assessed by cytotoxicity detection kit according to the manufacturer's instructions. Cytotoxicity percentage was calculated as follows:

$$\text{Cytotoxicity (\%)} = \frac{\text{exp.value} - \text{low control}}{\text{high control} - \text{low control}} \times 100$$

“Exp.value” refers to the average absorbance of the experimental cells (cells treated with histones or NET).

3.2.13 Detection of activated caspases in living cells

Green multi-caspase staining kit was used to detect activated caspases in living cells according to the manufacturer's protocol. In brief, A549 cells were left untreated or treated with NET, staurosporine or caspase inhibitor Z-VAD-FMK as an additional negative control. After incubation, the supernatants were collected in different tubes, and cells were trypsinized and added to the collected supernatants. The tubes were centrifuged at 900 g for 5 min and the supernatants discarded. Then 300 µl from the induced and control cells were aliquoted into reaction tubes. FITC-VAD-FMK (1 µl) was added into each tube and incubated for 30 min at 37°C with 5% CO₂. The cells were centrifuged at 900 g for 5 min and the supernatant was removed. The cells were resuspended in 0.5 ml of wash buffer and cell suspensions (100 µl from each sample) were transferred to wells of a black microtiter 96-well plate. The fluorescence intensity was measured at excitation and emission wavelengths of 485 nm and 535 nm, respectively (FLx 800 fluorescence microplate reader).

3.2.14 Apoptotic/necrotic detection by fluorescence microscopy

NET- and staurosporine-treated or untreated A549 cells were stained with FITC-annexin V, ethidium homodimer III and Hoechst 33342 according to the manufacturer's instructions. In brief, after culturing the epithelial cells on coverslip, cells were treated with NET or staurosporine for 16 h, then washed with binding buffer and incubated with the staining solution (5 μ l of FITC-annexin V, 5 μ l of ethidium homodimer III and 5 μ l of Hoechst 33342 into 100 μ l of binding buffer) for 15 min at 37°C. After washing the slides with binding buffer, cell staining was evaluated with the fluorescence microscope (Leica Microsystems) using separate filters for FITC, rhodamine and DAPI.

3.2.15 Histones and NET treatment with activated protein C (APC)

Histone type IIA (100 or 200 μ g/ml) from calf thymus was incubated with 6 μ g/ml (100 nM) APC for 1 h at 37°C (Xu *et al.*, 2009). In addition, NET (approximately 10 μ g/ml protein content), digested or non-digested, was incubated with 100 nM APC for different time intervals. Moreover, NET was incubated with APC at different mass ratios of APC: NET (1:5, 1:2, and 1:1) for 1 h at 37°C. APC alone or APC plus 6 μ M APC inhibitor (PPACK) were used as controls. LDH release by A549 cells was measured after treatment with histones, APC, NET, APC plus histones and APC plus NET.

3.2.16 Western Blot analysis

Confluent HUVEC or A549 cells in 24-well plate were stimulated with different concentrations of NET for 4 h or left untreated. The cells were harvested and lysed with 70 μ l per well ice-cold modified RIPA buffer (150 mM NaCl, 1mM EDTA, 1% sodium deoxycholate, 50 mM Tris, pH 7.4, 0.1% SDS) that was supplemented with protease inhibitor cocktail tablets on ice. Cellular lysates were centrifuged at 17000 g for 15 min at 4°C to remove insoluble material. For Western blotting, 20 μ l from lysates were resolved on a 15% SDS-PAGE gel and blotted onto a polyvinylidene fluoride membrane. A polyclonal rabbit anti-caspase-3 antibody was used to detect the endogenous level of full length caspase-3 (35 kDa) and the large fragment of caspase-3 resulting from cleavage (17 kDa). The membrane was incubated for 5 min with ECL detection reagent to detect caspase antibody. Then, the membrane was stripped with stripping buffer (0.1 M glycine, pH 3), washed, blocked and reprobed with an anti- β -actin antibody for visualizing loading control.

3.2.17 NET fractionation and native-gel electrophoresis

DNase digested NET (4 ml, containing 10 µg/ml protein content) were centrifuged at 2500 g, and the supernatant were subjected to 30 and 50 kDa Amicon ultra-4 centrifugal filter unit in order to fractionate NET proteins based on the size. The final volumes of both concentrate (on top of the filter) and eluent were reached to 4 ml with RPMI medium and added to A549 cells or subjected to SDS-PAGE or blue native gel (Serva). For SDS-PAGE, 3.5 µg protein per well were loaded and stained with Flamingo. For native gel electrophoresis, 6.8 µg protein were loaded, run for 2 h at 200 V (constant) and stained with silver nitrate. Bands were cut and proteins were identified with matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

3.2.18 Two-dimensional gel electrophoresis

2-D gel electrophoresis was performed for untreated neutrophils, PMA-treated neutrophils, NET samples or NET-treated epithelial cells. For proteomic analysis of epithelial cells after NET treatment, confluent A549 cells in 24-well plates were treated with 800 µl non-digested or DNase digested NET (10 µg/ml protein) for 1, 3, 6 or 8 h, and untreated cells were used as controls. After incubation, the supernatants of the wells were discarded, wells were washed once with PBS, and the cells were collected and treated with lysis buffer for 2-D gel electrophoresis. Protein extraction was performed by ultrasonication in 100 µl 6 M urea, 2 M thiourea, 4% CHAPS, 1% Dithiothreitol (DTT) and 2% Pharmalyte 3-10, and protein quantification was performed with 2-D Quant kit. IPG-strips (pH 3-10 NL for epithelial proteins, pH 3-10 and pH 7-11 NL for NET-proteins) were rehydrated at 20°C with the protein extract. On each strip, 400 µg protein were applied and isoelectric focusing was performed with 32.05 kVh. After focusing, the IPG-strips were equilibrated for 10 min in 2 ml equilibration stock solution (ESS; 6 M urea, 0.1 mM EDTA, 0.01 % bromphenol blue, 50 mM Tris-HCl pH 6.8, 30 % glycerol) for 15 min in 2 ml ESS I (10 ml ESS containing 200 mg SDS, 100 mg DTT) followed by 15 min in ESS II (10 ml ESS containing 200 mg SDS, 480 mg iodacetamide). Protein separation in the second dimension was performed by electrophoresis on 12.5% SDS polyacrylamide gels according to Laemmli (Laemmli, 1970). Electrophoresis was carried out in a Hoefer 600 system with the following program: 15 min at 15 mA/gel and 5 h at 110 mA at 25°C. Gels were stained with coomassie brilliant blue R-250 or silver nitrate and scanned with a GS-800 densitometer (BioRAD).

3.2.19 Differential in gel electrophoresis (DIGE) derivatization

For DIGE derivatization using the minimal labeling kit, 100 µg of NET-proteins before or after treatment with APC were derivatized with Cy3 and Cy5, respectively, according to the instructions of the manufacturer (GE Healthcare). From both samples, 50 µg proteins were mixed and labeled with Cy2 as an internal standard. All three samples were mixed and subjected to 2-D gel electrophoresis, and proteins were separated according to their pI by isoelectric focusing on IPG strips (13 cm, pI 7-11 NL) with 32.05 kVh. For the second dimension, 12.5% polyacrylamide gels were used. Gels were scanned with a VersaDoc system from BioRAD, and gel images were analyzed with PdQuest software.

3.2.20 Tryptic in-gel digestion of proteins

Spots of interest were excised with the ExQuest spot cutter (BioRAD) and proteins were digested with trypsin on a liquid handling roboter system (MicroStarlet, HamiltonRobotics).

3.2.21 MALDI-TOF-MS analysis

MALDI-TOF-MS was performed on an Ultraflex TOF/TOF mass spectrometer equipped with a nitrogen laser and a LIFT-MS/MS facility. The instrument was operated in the positive-ion reflectron mode using 2,5-dihydroxybenzoic acid and methylenediphosphonic acid as matrix. Sum spectra consisting of 200–400 single spectra were acquired. For data processing and instrument control the Compass 1.1 software package consisting of FlexControl 2.4, FlexAnalysis 3.0 and BioTools 3.0 was used. All 2-D gel electrophoreses, gel digestions and mass spectrometry were performed by Dr. Guenther Lochnit (Biochemistry Institute, Medical School, Giessen).

3.2.22 Database search

Proteins were identified by MASCOT peptide mass fingerprint search (<http://www.matrixscience.com>) using the human IPI database. For the search a mass tolerance of 75 ppm was allowed and carbamidomethylation of cysteine as global modification and oxidation of methionin as variable modification were used.

3.2.23 Cystic fibrosis sputum staining

Sputum from 6 cystic fibrosis patients (inhaled with 5.85% hypertonic sodium chloride to collect sputum) was treated with 10% DTT for 20 min at 37°C and seeded on the coverslip. Moreover, DTT-treated sputum was centrifuged at the highest speed and the supernatant was seeded on the coverslip. The samples were fixed with 2% PFA, blocked (3% BSA in PBS) and incubated with primary antibodies anti-DNA/histone H1 and anti-neutrophil elastase

which were detected with secondary antibodies coupled to Alexa Fluor 555 donkey anti-mouse IgG and Alexa Fluor 488 donkey anti-rabbit, respectively. Cystic fibrosis sputum collection and the protocol for sputum treatment were kindly provided by Dr. Lutz Naehrlich (Pediatric Clinic, UKMG, Medical School, Giessen).

3.2.24 Macromolecule permeability of epithelial cells

The permeability of trypan blue-labeled albumin across human lung epithelial cells (A549) was studied in a two-compartment system separated by a filter membrane as described previously (Null *et al.*, 1999; Pfeil *et al.*, 2009). Cells were grown in DMEM full medium until they became confluent, and at the day of experiment they were grown in RPMI phenol red-free medium for controls or they were incubated with 2.5 ml (approximately 30 µg/ml protein content) of undigested or DNase-digested NET. There was no hydrostatic pressure gradient between both compartments. The "luminal" compartment containing the monolayer had a volume of 2.5 ml, and the "abluminal" of 6.5 ml. The fluid in the "abluminal" compartment was constantly stirred. Trypan blue-labeled albumin (60 µM) was added to the luminal compartment. The appearance of the labeled albumin in the abluminal compartment was monitored by pumping the liquid through a spectrophotometer (Specord 10). The concentration of labeled albumin in the luminal compartment was determined every min of incubation.

The albumin flux [F, expressed as mol/(sec x cm²)] across the monolayer with the surface (S) was determined from the increase of albumin concentration (d[A]₂) during the time interval (dt) in the abluminal compartment (volume V):

$$F = (d[A]_2 / dt \times V) / S.$$

The permeability coefficient (P, expressed as cm/sec) of the combined system of monolayer and filter support was calculated from F according to Fick's law of diffusion as follows

$$P = F / ([A]_1 - [A]_2)$$

where [A]₁ and [A]₂ denote tracer concentrations in the luminal and abluminal compartments, respectively. The driving force ([A]₁ - [A]₂) remained almost unchanged in the course of the described experiments, therefore the relative changes in F correspond to similar changes in the permeability coefficient. Here, the permeability (F) is shown in percentage in comparison to the control cells, while the permeability of the control cells was considered 100%.

Permeability experiments and the analysis were performed together with Dr. Muhammad Aslam (Physiology Department, Medical School, Giessen).

3.2.25 Inhibition of NET cytotoxicity

To test the influence of NET on cytotoxicity, 800 μ l NET (approximately 10 μ g protein content) was pre-incubated with 1:100 of antibodies against H2A, H3, CitH3 (citrulline 2 + 8 + 17) or H4, or with 8 μ g of antibodies against DNA/histone H1, H2B, mouse IgG isotype or rabbit IgG isotype for 1 h at room temperature before incubation of NET with epithelial cells. In addition, 800 μ l NET was pre-incubated with 37 ng/ml myeloperoxidase inhibitor dihydrolipoic acid (gift from Dr. Oliver Soehnlein, Ludwig-Maximilians-University Munich), 2 μ l (0.25 mM) neutrophil elastase inhibitor N-(methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone or 50 μ g/ml polysialic acid (PSA) (gift from Dr. Sebastian Galuska, Biochemistry Institute, Medical School, Giessen) for 1 h at room temperature before incubation of NET with epithelial cells.

3.2.26 Statistical analysis

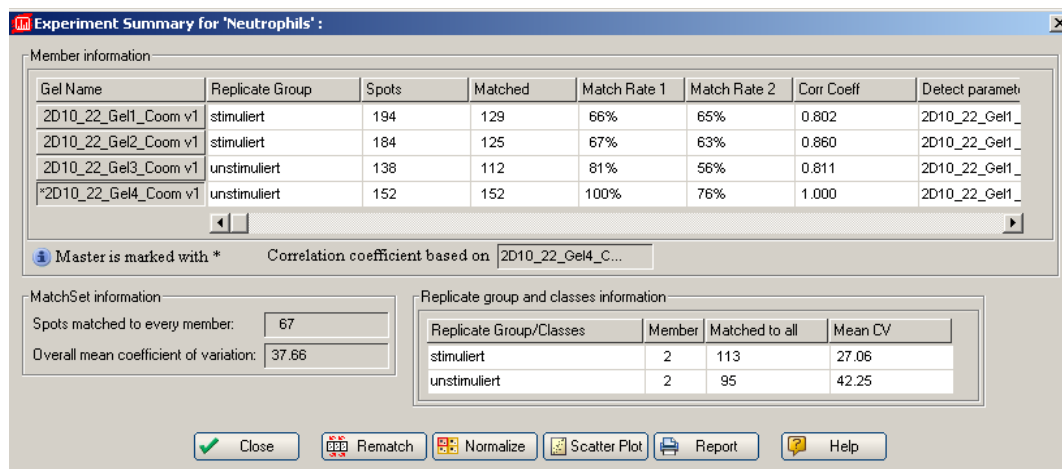
Data were analyzed by GraphPad Prism 5.02 software using one-way analysis of variance (ANOVA) with Tukey post-tests for multiple comparisons or by student's t-test for single measurements. Each experiment was performed at least 3 times on independent occasions, unless otherwise stated. Differences were considered statistically significant at $P < 0.05$ (except for proteomics analysis; $P < 0.02$) and data were expressed as mean \pm SD ($n \geq 3$).

4 Results

4.1 PMA-stimulated neutrophils show upregulation of proteins in comparison to the unstimulated ones.

Stimulation of neutrophils to form NET was done mainly by PMA. In order to compare PMA-stimulated neutrophils with the unstimulated ones, 2-D gel electrophoresis was performed for both conditions (Fig. 4.1A and B). From 28 randomly selected spots, almost all spots (27 spots) showed upregulation in stimulated cells (Fig. 4.2A and B).

A



B

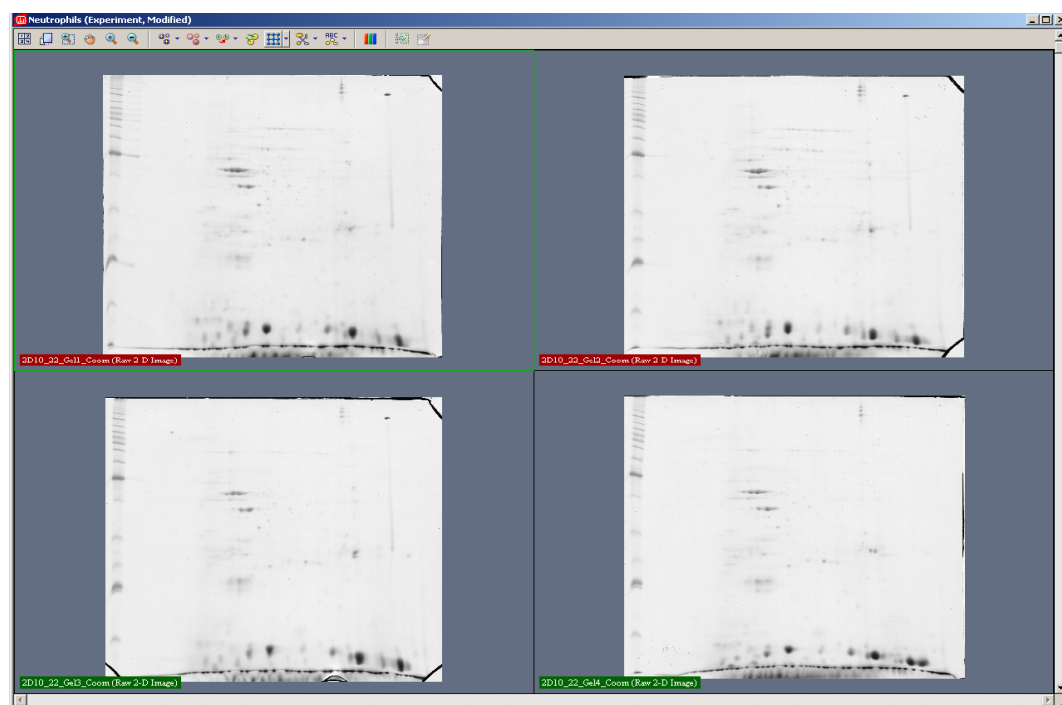


Figure 4.1: Two-dimensional gel electrophoresis for unstimulated and PMA-stimulated neutrophils. (A) and (B) Comparison of 2-D gel electrophoresis (pI 3-10) of unstimulated and PMA-stimulated neutrophils (two gels per each group). Stimulated and unstimulated are shown by red and green, respectively.

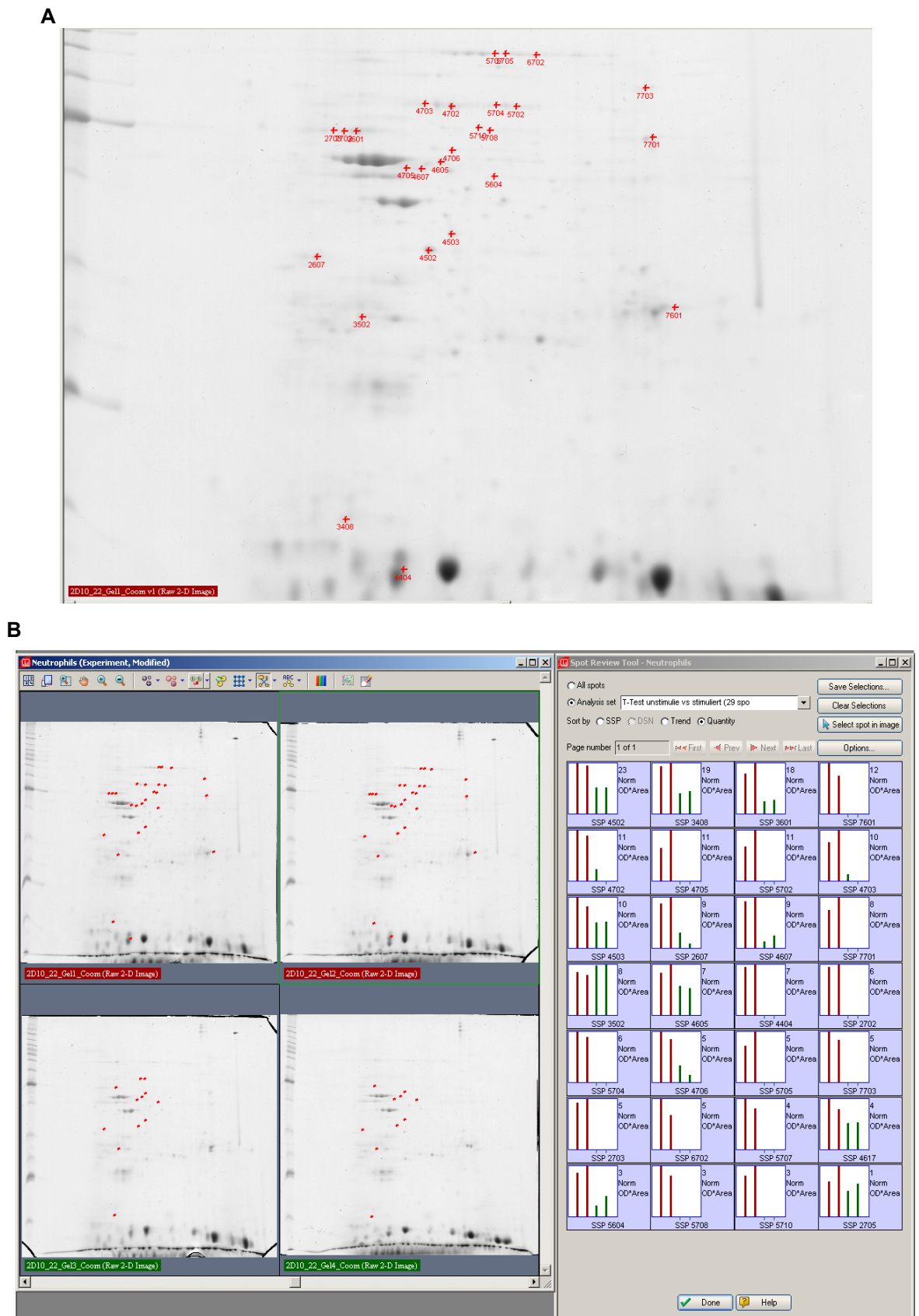


Figure 4.2: Upregulated proteins in PMA-stimulated neutrophils in comparison to the unstimulated ones. (A) Selected spots in the stimulated gel were shown. (B) Stimulated spots showed upregulation (red) in comparison to the unstimulated matched spots (green). Differences were considered statistically significant at $P < 0.02$.

4.2 Identification of NET proteins by 2-D gel electrophoresis and MALDI-TOF-MS

2-D gel electrophoresis was performed for NET-proteins at different pI ranges (3-10 and 7-11). From 32 randomly chosen spots in NET, we identified 13 different kinds of proteins (Table 4.1) from which 9 proteins have already been identified by another group (Urban *et al.*, 2009). Four different proteins were identified for the first time (indicated by *).

Table 4.1 Identified NET proteins by 2-D gel electrophoresis and MALDI-TOF-MS and their functions

NET-proteins	* Newly identified NET-proteins	Functions (source: uniprot)
Annexin A1	*	Membrane fusion, exocytosis, calcium/phospholipid-binding protein
Lactoferrin		Cellular iron ion homeostasis, iron ion transport, antimicrobial activity
Profilin-1	*	Actin cytoskeleton organization, platelet activation
Alpha-enolase		Glycolysis, Plasminogen activation, Transcription regulation
Protein S100-A8 and A9		Calcium-binding protein, chemotaxis, antimicrobial activity
Glyceraldehyde 3-phosphate dehydrogenase	*	Glycolysis, nitrosylase activities, transcription, RNA transport, DNA replication, apoptosis
Actin, cytoplasmic 1 and 2		Adherens junction organization, cytoskeleton, cell motility
Leukocyte elastase inhibitor (Serpin B1)	*	Regulation of proteolysis
Plastin-2		Actin-binding protein, T cell activation
Myeloperoxidase		Microbicidal activity
Proteinase 3		Serine protease which degrades extracellular matrix

4.3 NET formation in lung tissue and BAL fluid of LPS-induced lung injury model

Under physiological conditions and in the absence of infectious or inflammatory stimuli, there is less than one million PMN in the lung tissue and no PMN in the BAL fluid (Reutershan *et al.*, 2005). To recruit PMN to the lung in considerable number, LPS was instilled intratracheally into the mice to induce LPS-mediated acute lung injury. NET formation was observed in mouse lung tissue after LPS treatment as compared to PBS controls. NET formation was demonstrated by the appearance of extracellular chromatin and disintegration of cell membranes (Fig. 4.3A) as well as co-localization of extracellular chromatin with neutrophil elastase (Fig. 4.3B). In general, decondensated chromatin in NET showed weak signal for DAPI staining, while it was detected strongly by DNA/histone antibody. Presence of short fragments of DNA–protein complexes in the airways (Douda *et al.*, 2011) may explain the non-frequent NET detected structures in the tissue.

Moreover, after 3 to 48 h of stimulation with LPS, BAL fluid was collected and the numbers of PMN were counted. Application of LPS provoked maximal recruitment of neutrophils after 24 h (Fig. 4.4A). Treatment of NET with DNase such as MNase leads to dissociation of NET-DNA from NET-proteins. Treatment of BAL pellet with MNase (NET-related elastase, filled bars) showed significant increase in neutrophil elastase activity in comparison to the activity in the BAL supernatant (free elastase, open bars) after 12 h LPS stimulation which indicates NET formation (Fig. 4.4B). Together, these data show NET formation in the BAL and lung tissue in LPS-induced lung injury model. To characterize NET formation in BAL, mouse neutrophils in BAL after 24 h LPS stimulation were stained for chromatin and cell membrane marker CD46. Since non-LPS stimulated neutrophils, as negative controls, could not be isolated from BAL, stimulation with PMA was used as positive control. Therefore, some of these neutrophils were further stimulated with PMA, a potent inducer of NET formation, for 1.5 h *in vitro*. While LPS, which induces neutrophil recruitment, provoked NET formation to a certain extent, it was more pronounced in cells stimulated with PMA (Fig. 4.4C).

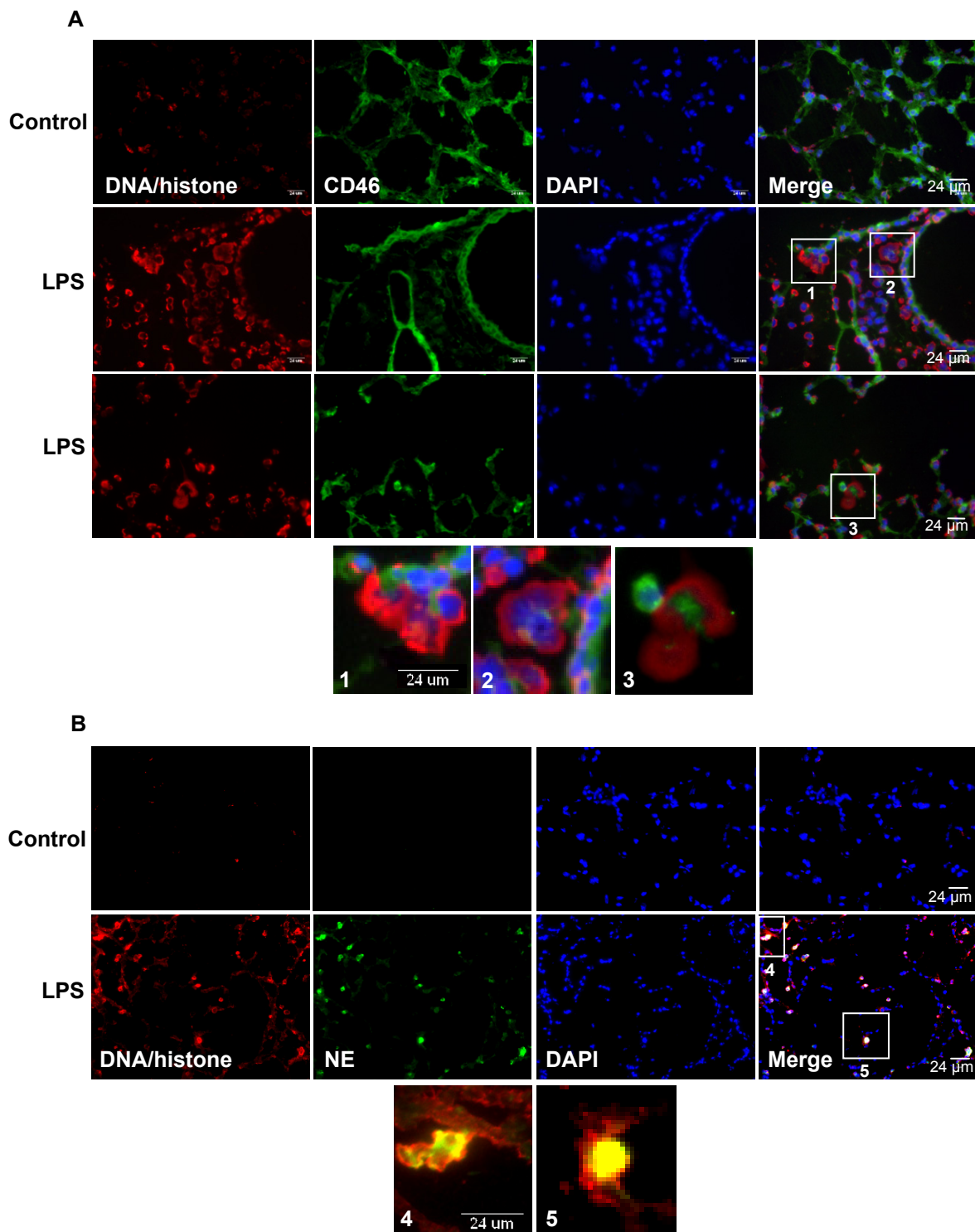


Figure 4.3: NET formation in mice lung tissue after LPS treatment. (A) Immunofluorescence staining of lung sections from mice after 24 h intratracheal LPS administration was performed, as compared to the control section, for DNA/histone (red), CD46 (green) as a cell membrane marker and DAPI (blue). The insets (1, 2 and 3) showed NET formation in LPS-treated lungs in higher magnification views. Note that in NET regions, DAPI had been blurred as opposed to sharp signal detected for DNA/histones which identified the decondensed chromatin. (B) Immunofluorescence staining of sections from control or LPS-treated mice were performed for DNA/histone (red), neutrophil elastase (green) and DAPI (blue). The higher magnification views of the insets (4 and 5) showed co-localization of neutrophil elastase (green) and DNA/histone (red) in NET structures.

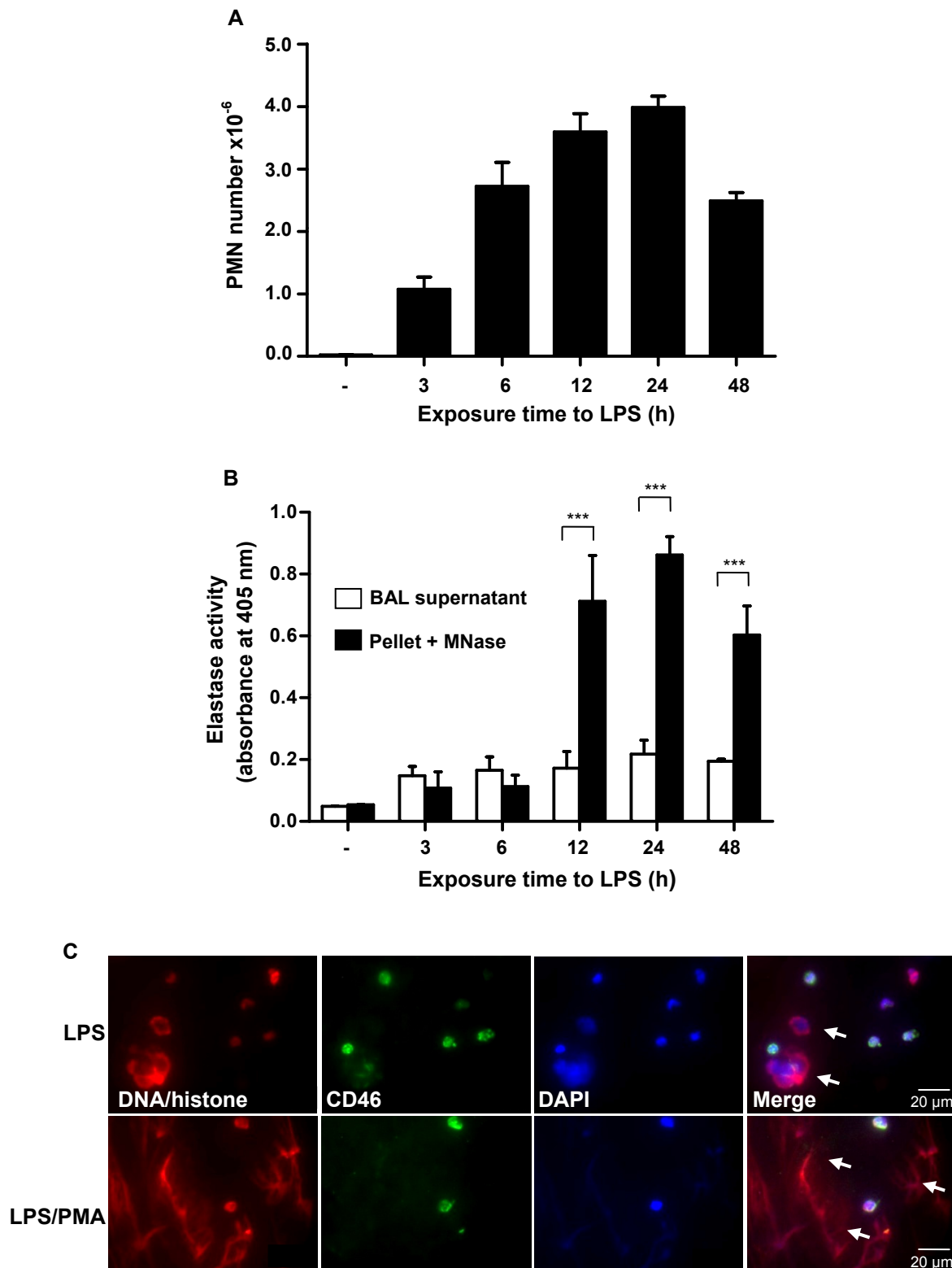


Figure 4.4: NET formation in mice BAL fluid after LPS treatment. (A) Following intratracheal LPS administration, PMN in the BAL fluid of mice were counted at different time intervals. (B) At the same time intervals, neutrophil elastase activity was measured in the supernatant of BAL fluid (free elastase, open bars) as well as in the MNase-digested pellet of BAL fluid (NET-related elastase, filled bars). Differences were considered statistically significant at *** $P < 0.001$. (C) PMN were isolated from BAL fluid of mice stimulated intratracheally with LPS for 24 h, and immunofluorescence staining of isolated cells was performed for DNA/histone (red), CD46 (green) and DAPI (blue) (upper row). Isolated PMN were further stimulated with PMA for 1.5 h (lower row); arrows indicate NET formation.

4.4 NET qualification and quantification

Different methods were used for NET quantification in mice and human including immunocytochemistry, picogreen dye, sytox orange and FACS. Immunocytochemistry with DAPI and DNA/histone was the best method for qualification and quantification since the structure of the nucleus, which is very important for NET detection, can also be evaluated; decondensation of the nucleus indicates NET formation (Fig. 4.5A). Picogreen dye was also a very sensitive method to quantify NET-DNA concentration. Sytox orange was a fast and quite easy method for quantification; however, the sensitivity was not as high as other methods (Fig. 4.5B). For the first time FACS analysis for NET quantification in mice neutrophils was used by measuring the signal for labeled-DNA/histone antibody and the amount of degranulation: The more NET formation, the more signal for labeled-DNA/histone and the more degranulation (Fig. 4.5C). For practical reasons, the following experiments were performed with human neutrophils which exhibited NET formation in a similar way.

4.5 Eap inhibits NET formation in a dose-dependent manner

The interaction between neutrophils and bacteria including *Staphylococcus aureus* in respect to NET formation has been described (Pilszczek *et al.*, 2010). However, there is no report concerning the role of extracellular adherence protein of *S. aureus*, Eap, in NET formation. Eap is a secreted protein from *S. aureus* which has a very wide range of binding interactions to host extracellular matrix components (Harraghy *et al.*, 2003; Chavakis *et al.*, 2005). Eap has an anti-inflammatory function by disrupting integrin-dependent neutrophil–endothelial interactions, and *S. aureus* may use this property to escape the host immune system (Chavakis *et al.*, 2002). Interestingly, incubation of human neutrophils by Eap from strain Newman did not induce NET formation but promoted neutrophil aggregation (Fig. 4.6A). DNA/histone antibody has more affinity for decondensated chromatin such as chromatin in NET structure. Surprisingly, Eap inhibited PMA-induced NET formation in a dose-dependent manner, as it was shown by the reduction in DNA/histone antibody areas (Fig. 4.6B and C). Eap had the same effect when neutrophils were treated with different NET-inducing stimuli such as LPS (Fig. 4.6D) or NO donor (Fig. 4.6E). Co-incubation of Eap with NO donor also led to aggregation of neutrophils. Pre-incubation of cells with Eap for 30 min had similar effects when cells were pre-incubated with PMA, LPS or NO donor.

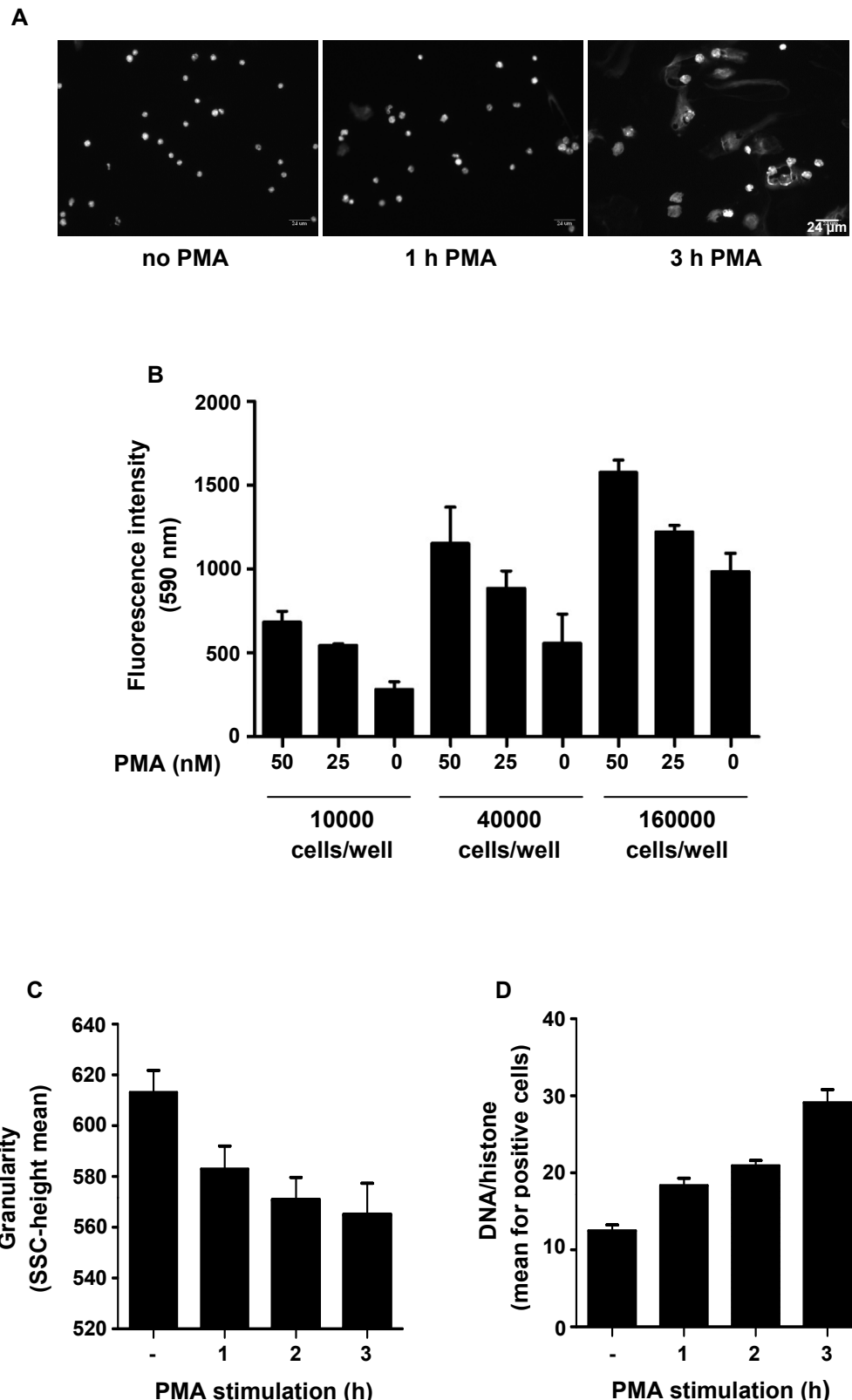


Figure 4.5: Different NET quantification methods. (A) Nucleus structural changes in PMN after 1 h or 3 h stimulation with 25 nM PMA were shown by staining the cells with DAPI. Note that decondensation of the nucleus indicates NET formation. (B) NET Quantification was performed for different PMN numbers and PMA concentrations using sytox orange. (C) and (D) FACS analysis was used to quantify NET formation. Decrease in side scatter (SSC) intensity and increase in signal for the labeled-DNA/histone after 1 h to 3 h stimulation with 25 nM PMA showed NET formation.

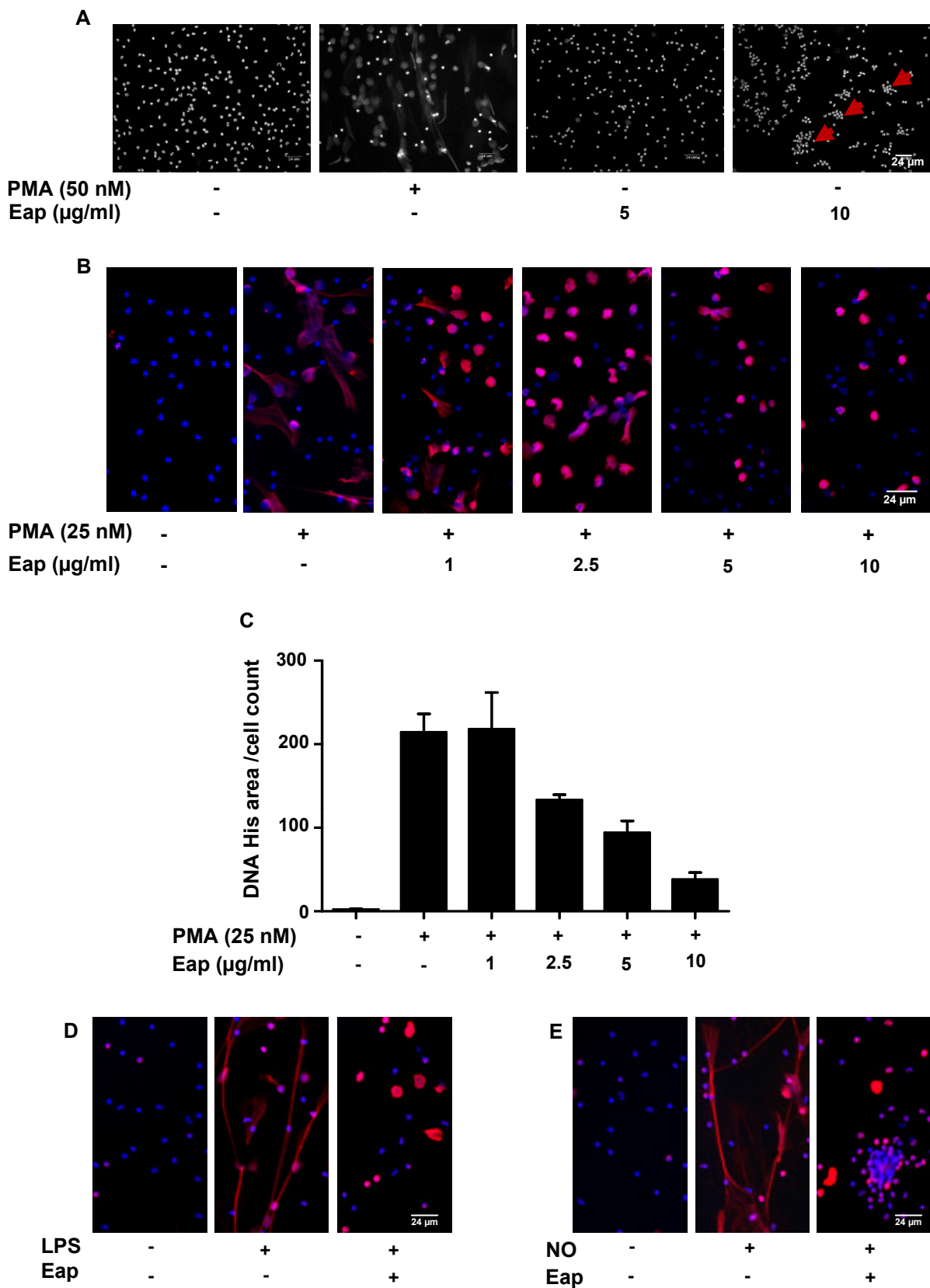


Figure 4.6: Eap induces neutrophil aggregation and inhibits NET formation. (A) Neutrophils were incubated with Eap or PMA and nucleus structures (DAPI: gray) were shown. Note that Eap alone did not induce NET formation but triggered neutrophil aggregation (arrows). (B) and (C) Eap inhibited PMA-induced NET formation in a dose-dependent manner; DAPI (blue) and DNA/histone (red). (D) Eap (10 μg/ml) inhibited LPS (10 μg/ml)-induced NET formation. (E) Eap (10 μg/ml) inhibited NO (100 nM)-induced NET formation.

4.6 NET induce cell death in epithelial cells in a dose-dependent manner

A549 cells were incubated with MNase-digested NET or staurosporine for 4 h or 16 h. While untreated cells grew normally and became confluent, cells treated with NET or staurosporine did not reach confluency (Fig. 4.7A). Multicaspase activity in A549 cells increased significantly after exposure to staurosporine or NET in a dose-dependent manner (Fig. 4.7B). Moreover, NET exposure increased the fractions of annexin V and ethidium homodimer (a membrane-impermeable fluorescent dye which binds to DNA in dead cells) positive cells (Fig. 4.7C). NET also increased caspase-3 cleavage in both epithelial and endothelial cells (Fig. 4.7D). Together, these data indicate that endothelial and lung epithelial cell death is induced by NET in a dose-dependent manner.

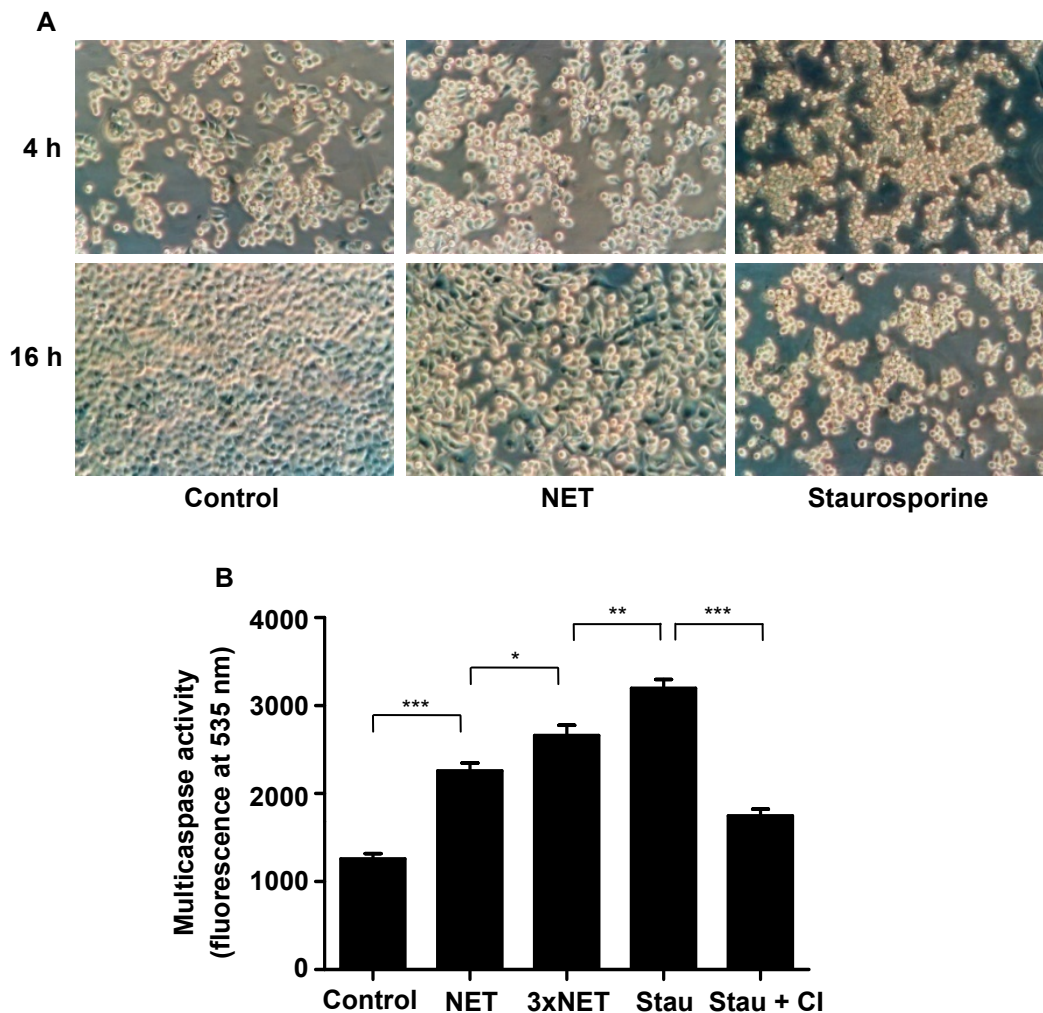


Figure 4.7: NET induce cell death in epithelial cells in a dose-dependent manner. (A) The morphology of A549 cells was evaluated after 4 or 16 h treatment with medium (control), NET or staurosporine; magnification: 20x. (B) Multicaspase activity of A549 cells was measured after 16 h treatment with NET, staurosporine (Stau) or staurosporine together with caspase inhibitor (CI). DNA-NET concentration in NET and 3 x NET were 3.4 and 10.1 $\mu\text{g/ml}$, respectively. Differences were considered statistically significant at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

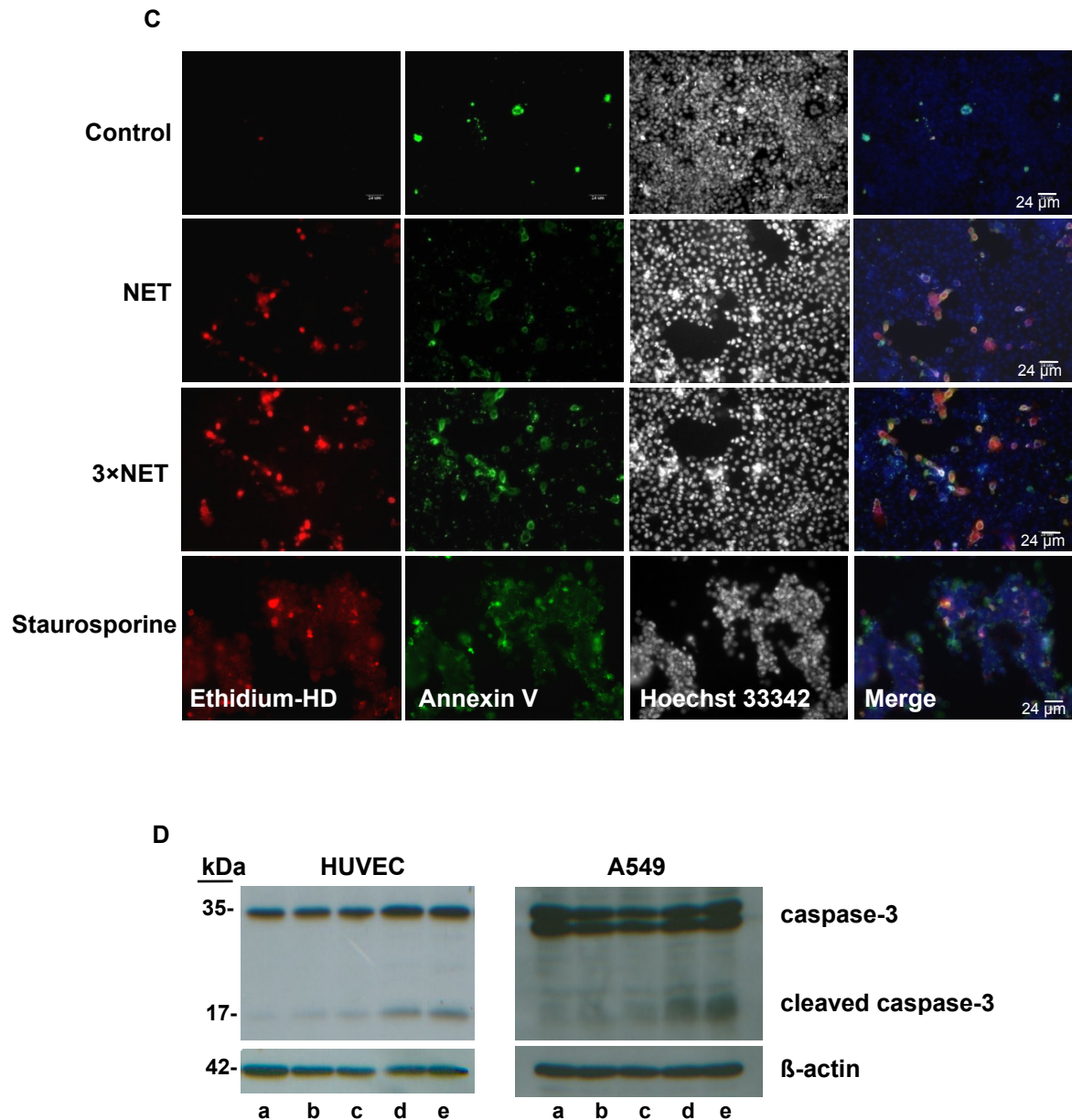


Figure 4.7: NET induce cell death in epithelial cells in a dose-dependent manner. (C) Immunofluorescence staining of A549 cells was performed after 16 h treatment with NET or staurosporine for Hoechst filter (gray), Hoechst 33342 (blue), annexin V (green) and ethidium homodimer III (red). (D) Immunoblot for caspase-3 cleavage of A549 and HUVEC cells was carried out after 4 h treatment with NET or staurosporine. a: untreated cells, b: cells treated with NET without digestion, c: cells treated with NET after MNase digestion, d: cells treated with 3 times higher amount of NET from c, e: cells treated with staurosporine.

4.7 NET induce cytotoxicity in lung epithelial and endothelial cells independent of NET digestion

In order to investigate in detail the role of DNA digestion in NET cytotoxicity, three forms of NET were used: undigested, completely digested by DNase or partially digested by MNase.

Cytotoxicity in A549 cells after incubation with NET was determined by LDH release, where cytotoxicity from untreated cells and cells treated with Triton X-100 were considered 0% and 100%, respectively. Cells treated with NET showed substantially increased cytotoxicity in comparison to untreated cells. Moreover, DNase or MNase treatment of NET did not change their cytotoxic activity. Incubation of cells with DNase, MNase or DNA alone did not provoke any appreciable cytotoxicity. These data indicate that NET induce cytotoxicity in epithelial cells independent of the type of DNA digestion (Fig. 4.8 and Fig. 4.7D).

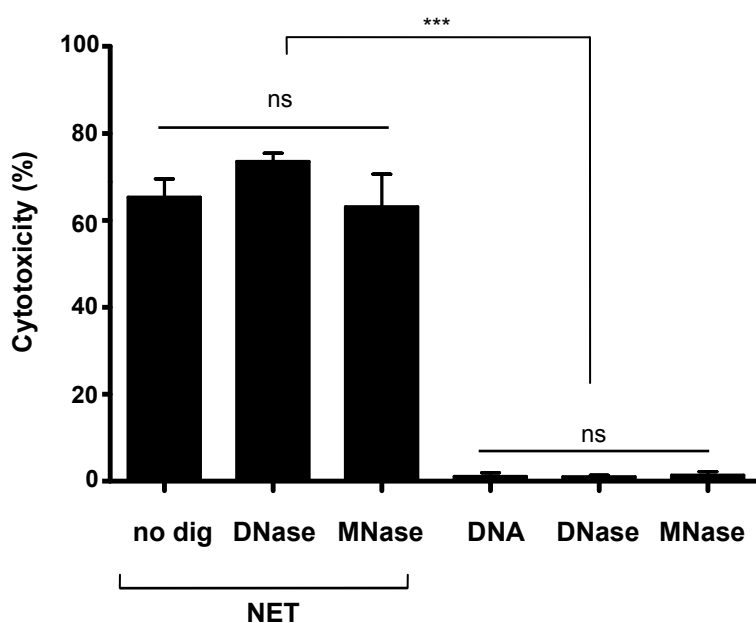


Figure 4.8: NET induce cytotoxicity in epithelial cells independent of digestion. LDH release from A549 cells was measured after 16 h treatment with undigested NET (no dig), completely or partially digested forms of NET by DNase and MNase, respectively. The same concentration of DNA alone as DNA-NET (3.4 $\mu\text{g/ml}$) as well as DNase or MNase alone were used as controls. Differences were considered statistically significant at *** $P < 0.001$.

4.8 Proteomic analysis in NET-treated epithelial cells

To compare the proteomic profile of epithelial cells before and after NET treatment, epithelial cells were incubated from 1 to 8 h with NET or kept untreated. Subsequent 2-D gel electrophoresis showed upregulation of different spots in NET-treated epithelial cells in comparison to the untreated ones. Identification of proteins by MALDI-TOF-MS revealed a wide range of proteins with different functions and cellular localizations (Table 4.2) and interestingly, several of these proteins have been reported to play a role in cell death (www.uniprot.org).

Table 4.2: Upregulated proteins in epithelial cells after treatment with NET

Identified proteins (*role in cell death; source: uniprot)	Cellular component
Elongation factor 1-beta	Cytosol
Heterogeneous nuclear ribonucleoprotein C-like 1	Nucleus
Isoform C2 of Heterogeneous nuclear ribonucleoproteins C1/C2	Nucleus Spliceosome
Isoform 2 of Heterogeneous nuclear ribonucleoprotein A1	Cytoplasm Nucleus Spliceosome
Heterogeneous nuclear ribonucleoprotein K	Cytoplasm Nucleus Spliceosome
Proliferating cell nuclear antigen	Nucleus
Isoform 2 and 1 of Tropomyosin alpha-3 and 4 chain	Cytoplasm Cytoskeleton
Aldo-keto reductase family 1 member C3	Cytoplasm
Annexin A1	Cell membrane Cell projection Cilium Cytoplasm Membrane Nucleus
Annexin A2	Basement membrane Extracellular matrix Secreted
Glyceraldehyde-3-phosphate dehydrogenase*	Cytoplasm Membrane Nucleus
Protein disulfide-isomerase	Cell membrane Endoplasmic reticulum lumen

Results

Prohibitin*	Membrane Mitochondrion Mitochondrion inner membrane
Ubiquitin carboxyl-terminal hydrolase isozyme L1	Cytoplasm
F-actin-capping protein subunit alpha-1	Cytoplasm Cytoskeleton
Aldo-keto reductase family 1 member B10	Cytoplasm
Transaldolase	Cytoplasm
Glutathione S-transferase omega-1	Cytoplasm
Protein DJ-1 (Oncogene DJ1, Parkinson disease protein 7) *	Cytoplasm Nucleus Mitochondrion
Retinal dehydrogenase 1	Cytoplasm
Isoform 1 of Triosephosphate isomerase	Cytosol
Nitrilase homolog 2	Cytoplasm
Poly (rC)-binding protein 1	Cytoplasm Nucleus
Stress-induced-phosphoprotein 1	Cytoplasm Nucleus
Peptidyl-prolyl cis-trans isomerase A	Cytoplasm
Superoxide dismutase [Mn], mitochondrial*	Mitochondrion
Vitamin K epoxide reductase complex subunit 1-like protein 1	Membrane
Isoform 1 of Mitochondrial import receptor subunit TOM40 homolog	Membrane Mitochondrion Mitochondrion outer membrane
Phosphoglycerate kinase 1	Cytoplasm
Isoform 1 of Phosphoserine aminotransferase	Cytoplasm

Profilin-1	Cytoplasm Cytoskeleton
60 kDa Heat shock protein, mitochondrial*	Mitochondrion
Heat shock protein beta-1*	Cytoplasm Nucleus Cytoskeleton
Proteasome subunit beta type-2*	Cytoplasm Nucleus Proteasome
Proteasome subunit beta type-3 *	Cytoplasm Nucleus Proteasome
Proteasome subunit beta type-4*	Cytoplasm Nucleus Proteasome
Isoform 1 of Proteasome activator complex subunit 3 *	Cytoplasm Nucleus Proteasome
Proteasome 26S non-ATPase subunit 13 isoform 2 *	Proteasome

4.9 NET fractionation and native gel electrophoresis

In order to investigate which proteins in NET are cytotoxic, NET proteins were fractionated based on the size, and NET fractions are treated with epithelial cells. In all cases, only the high molecular weight fractions (> 50 kDa when NET proteins are fractionated by 50 kDa filter) had cytotoxic properties (Fig. 4.9A). In fact, insufficient amounts of proteins from the smaller fractions (< 50 kDa) were obtained. Moreover, boiled NET did not show cytotoxicity activity, which is an indication that proteins in NET are responsible for the cytotoxicity. However, SDS-gel electrophoresis of the fractions > 50 kDa showed bands lower than this size which maybe the indication of protein complexes in NET (Fig. 4.9B and Table 4.3). Similar results had been also seen when NET fractionated by 30 kDa filter. Therefore, native-gel electrophoresis, which is a non-denaturing condition, was performed for NET proteins to

investigate possible protein complexes in NET. From randomly selected seven bands, we could identify protein complex only in one band (band 6, Fig. 4.10).

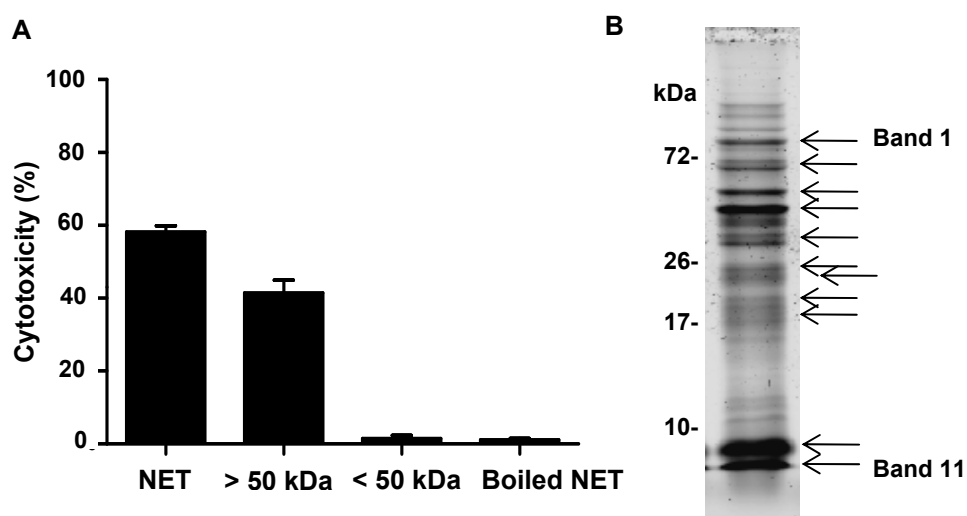


Table 4.3: Identified NET proteins; fraction > 50 kDa

Identified proteins	Band number
Transketolase	1
Glucose-6-phosphate isomerase	2
Alpha-enolase	3
Actin, cytoplasmic 1	4
Glyceraldehyde 3-phosphate dehydrogenase	5
Annexin A1	7
Actin-related protein 2/3 complex subunit 2	7
Actin, cytoplasmic 1	7
highly similar to Actin, cytoplasmic 1	8
Protein kinase C inhibitor protein 1	8
Protein S100-A9	10
Profilin-1	10
Protein S100-A8	11

Figure 4.9: Presence of the protein complexes in NET. (A) Total NET proteins were fractionated by 50 kDa filter, and NET or NET fractions were treated with epithelial cells for 16 h to check the cytotoxicity. Only total NET proteins and NET fractions > 50 kDa showed cytotoxic affect on epithelial cells. Total boiled NET did not induce cytotoxicity, indicating proteins in NET induce cytotoxicity. (B) SDS-gel electrophoresis (15%) was carried out for NET fractions > 50 kDa; note that bands < 50 kDa were appeared. **Table 4.3** shows the identified NET proteins (fraction > 50 kDa) from Fig. 4.9B by MALDI-TOF-MS. Bands 6 and 9: unidentified proteins.

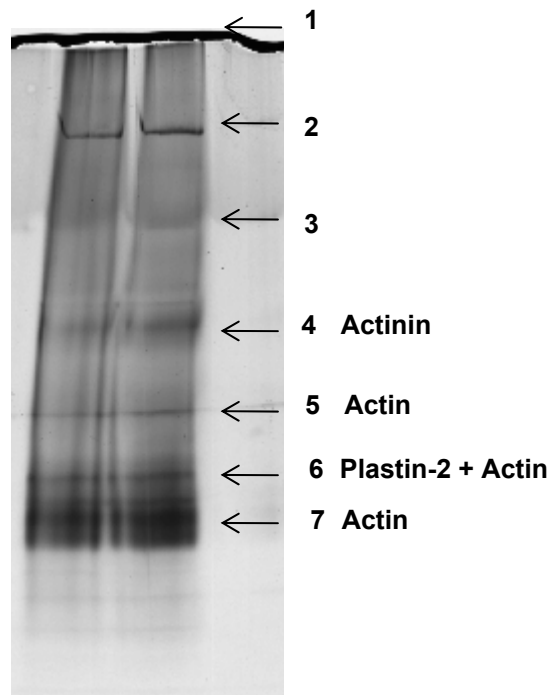


Figure 4.10: Native-gel electrophoresis for NET proteins. Blue native-gel electrophoresis (4-16%) was performed for NET proteins. Subsequent MALDI-TOF-MS for the specified bands showed protein complex only in band 6. Two lanes are NET proteins. Bands 1, 2 and 3: unidentified proteins.

4.10 Histones induce epithelial and endothelial cell death

To elucidate the relevance of histones (as major protein components of NET structure) for NET-mediated cytotoxicity, the influence of pure histones on both epithelial and endothelial cell death was investigated. Incubation of epithelial and endothelial cells for 16 h with high concentration of purified histones (histone type-IIA, which include all types of histones) induced cell death (Fig. 4.11A). Histones triggered cell death in epithelial cell in a dose-dependent manner (Fig. 4.11B and C). Likewise, histones induced cytotoxicity in endothelial cells in a dose-dependent manner and similar to epithelial cells, NET provoked cytotoxicity in endothelial cells whether DNA is digested in NET or not (Fig. 4.11D).

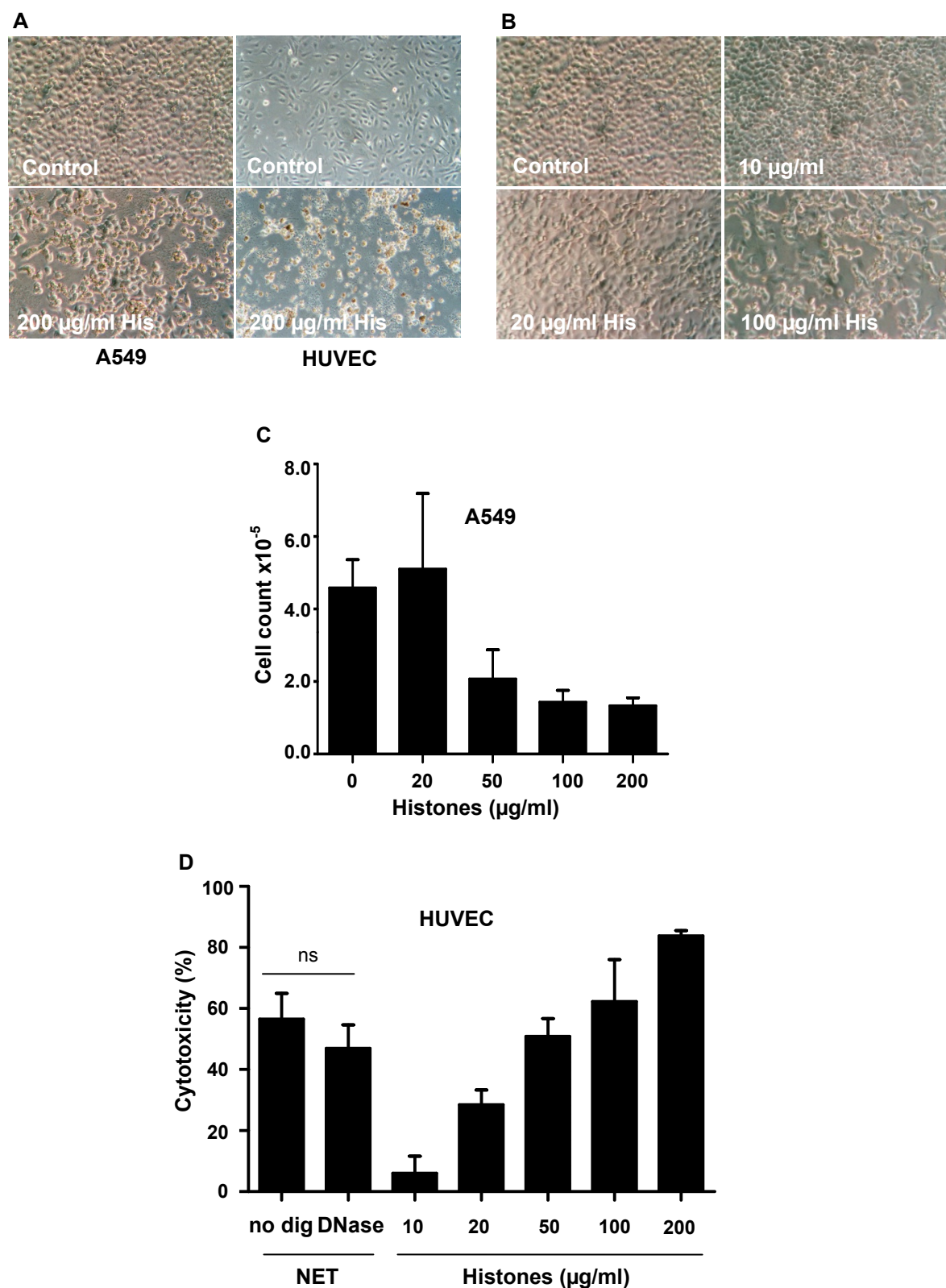


Figure 4.11: Histones induce epithelial and endothelial cell death. (A) HUVEC and A549 cells were treated with 200 $\mu\text{g/ml}$ calf histones for 16 h or left untreated (control), and cell morphology was evaluated; magnification: 20x. (B) A549 cells were treated with different concentrations of calf histones for 16 h and (C) the corresponding cell numbers were counted. (D) HUVEC were treated with different concentrations of calf histones, undigested NET (no dig) or completely digested with DNase (3.4 $\mu\text{g/ml}$ DNA-NET) for 16 h, and the cytotoxicity was measured.

4.11 Histone antibodies reduce NET-induced cytotoxicity

To investigate whether histones are the cytotoxic part of the NET, different histone antibodies were pre-incubated with NET before incubation of NET with epithelial cells. Pre-incubation of NET with histone antibodies against DNA/H1, H2A, H2B and H4 but not H3 and citrullinated H3 significantly decreased NET-mediated cytotoxicity (Fig. 4.12). These data suggest that the cytotoxicity of NET is at least partly mediated by histones.

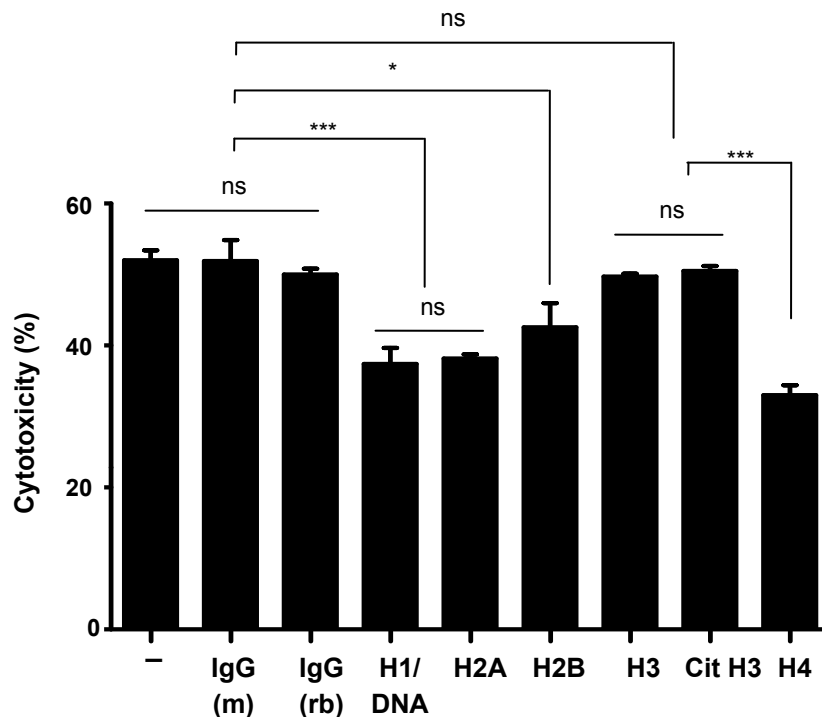


Figure 4.12: Histone antibodies decrease NET-induced cytotoxicity of epithelial cells. NET alone or NET pre-incubated with histone antibodies was treated with epithelial cells for 16 h, and cytotoxicity was evaluated. Antibodies against DNA/histone H1, H2A, H2B and H4 reduced NET-induced cytotoxicity in epithelial cells, while antibodies against histone H3, citrullinated H3 (cit H3) and isotype-matched control antibodies (m: mouse, rb: rabbit) had no effect on NET-induced cytotoxicity. * $P < 0.05$ and *** $P < 0.001$.

4.12 Activated protein C (APC) decreases epithelial cytotoxicity induced by histones but not by NET

Recently, it was described that APC can decrease histone-mediated cytotoxicity in endothelial cell (Xu *et al.*, 2009). Likewise, the cytotoxic activity of purified histones on A549 cells was substantially decreased by APC (Fig. 4.13A). In contrast, incubation of NET with APC even in high concentrations did not reduce NET-mediated cytotoxicity (Fig. 4.13B). Moreover, incubation of DNase or undigested forms of NET with APC up to 80 min could not decrease NET-mediated cytotoxicity (Fig. 4.13C and D).

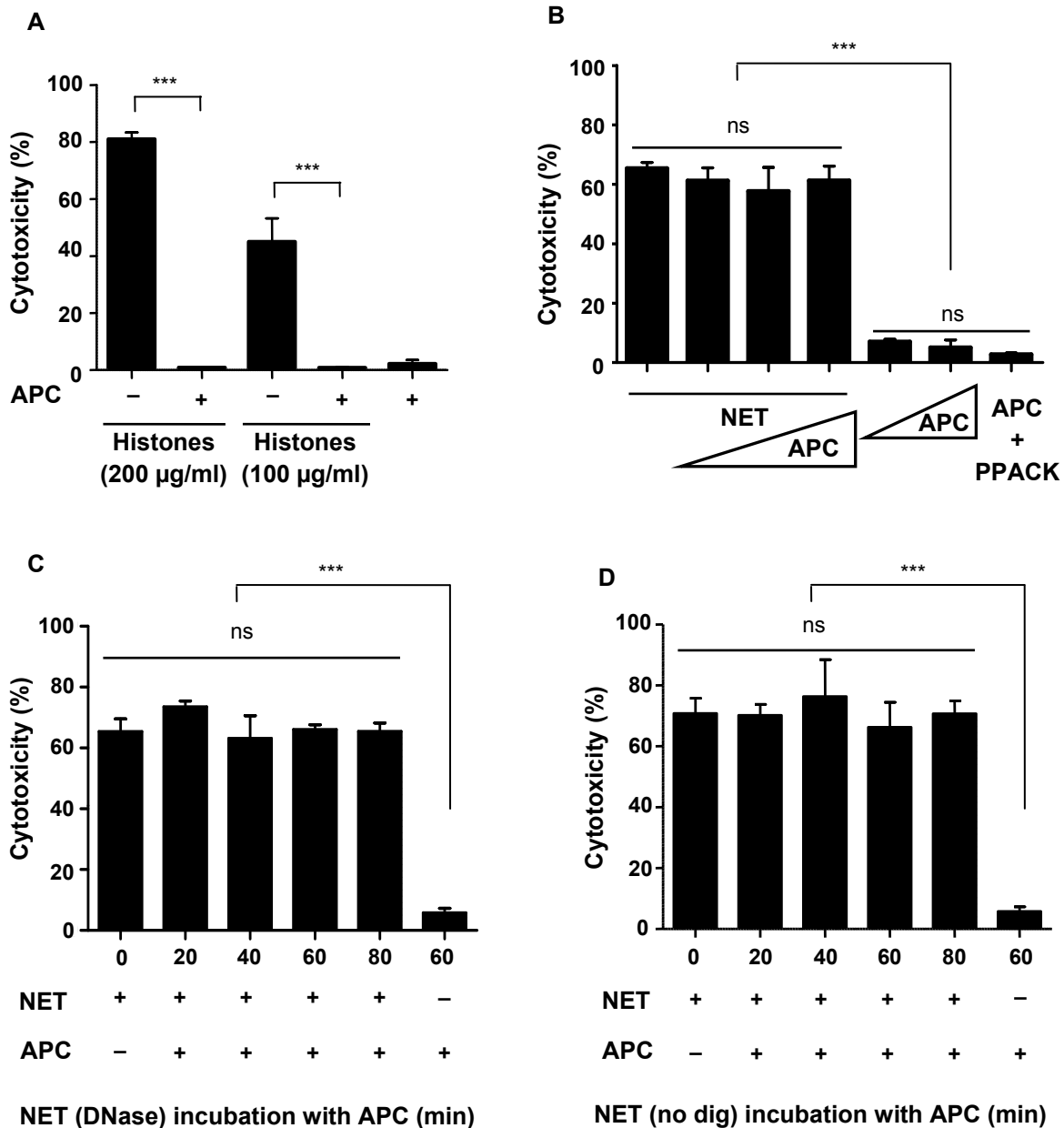


Figure 4.13: APC decreases epithelial cytotoxicity induced by histones but not by NET. (A) Calf histones (200 and 100 µg/ml) were pre-incubated without or with 100 nM human APC for 1 h at 37°C and then incubated with A549 cells for 16 h to measure LDH release. (B) NET were pre-incubated with APC (protein mass ratio APC: NET proteins, 1:5, 1:2 and 1:1) for 1 h at 37°C and then NET treated with A549 cells for 16 h to measure LDH release. APC alone and APC with APC inhibitor (PPACK) were also incubated with A549 cells as controls. (C) DNase digested or undigested forms of NET were pre-incubated with 100 nM APC for 20 min to 80 min before incubation with A549 cells for 16 h to measure LDH release. *** $P < 0.001$.

4.13 APC degrades histones but not NET-proteins

Similar to what Xu and colleagues showed (Xu *et al.*, 2009), APC can degrade histones (Fig. 4.14A). However, NET treated with APC did not show any degradation of NET proteins (Fig. 4.14B, C). Likewise, 2-D fluorescence difference gel electrophoresis (2-D DIGE), which

enables accurate analysis of differences in protein abundance between samples, did not show any difference between NET treated without or with APC (Fig. 4.14D).

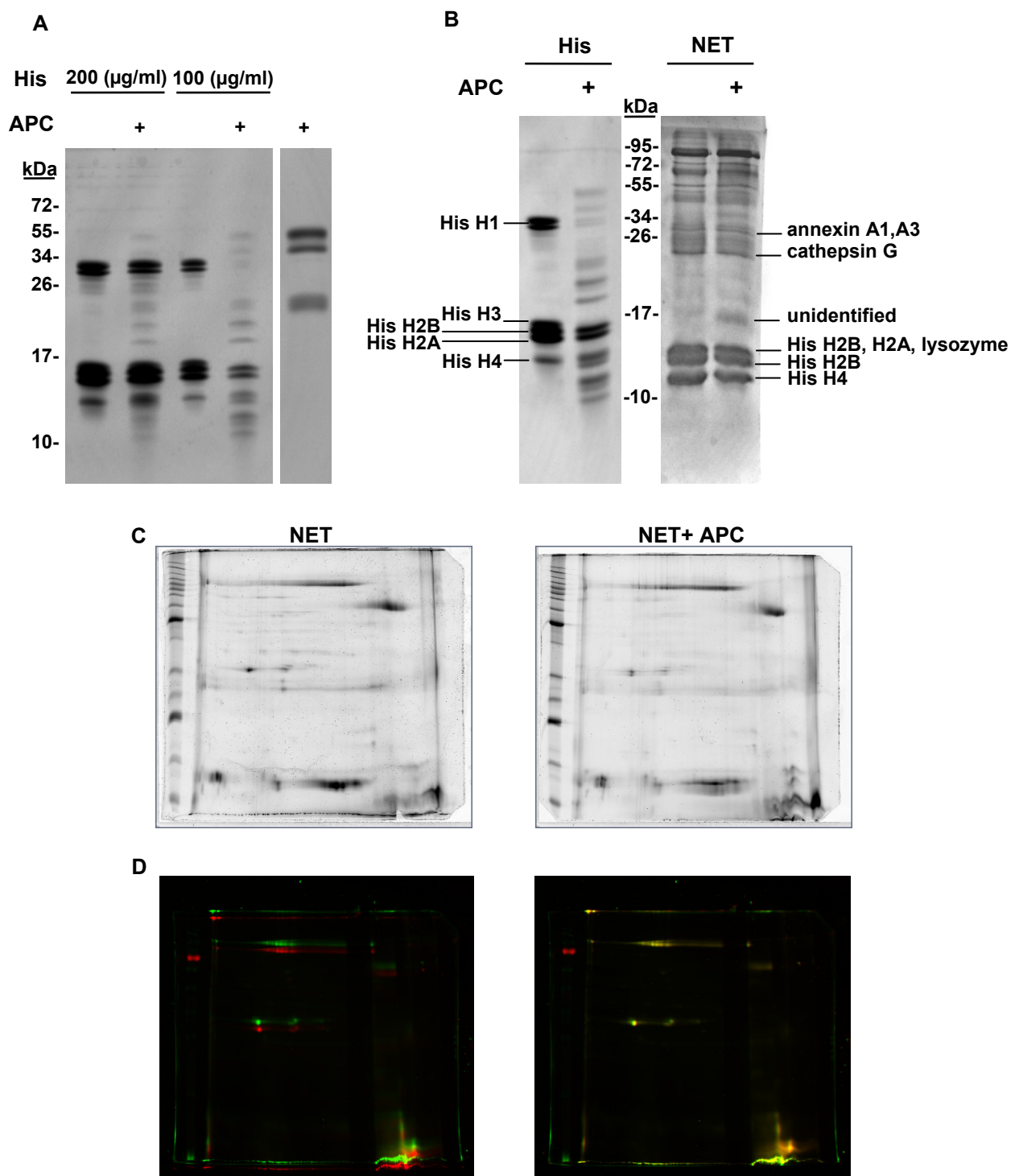


Figure 4.14: APC degrades isolated histones but not NET-associated proteins. (A) SDS-gel electrophoresis (15%) of histones and (B) NET proteins without or with incubation with 100 nM APC were carried out, and bands were identified with MALDI-TOF-MS. In (A) 10 times higher amount of APC was loaded as a control. (C) Two-dimensional (2-D) gel electrophoresis (pI 7-11) of NET (left) and NET treated with APC (right) were performed. (D) NET (Cy3, green) and APC-treated NET (Cy5, red) labeled protein was separated by 2-D gel electrophoresis. Gels from both samples were overlaid with a minor space in the vertical orientation (left gel) or exactly superimposed (right gel). No difference in the gel profiles was noted.

4.14 Polysialic acid (PSA) decreases histones and NET-induced cytotoxicity

PSA is a large and highly negatively charged glycan that plays essential roles in nervous system function and development. Mishra and co-workers have recently shown that histone H1 is a binding partner of PSA, and this interaction has a key role for nervous system development and regeneration (Mirsha *et al.*, 2010). Based on these findings, the potential role of PSA on histone- and NET-induced cytotoxicity was also investigated. PSA was able to reduce both histone- and NET-mediated cytotoxicity, even more than histone antibody (histone H4 antibody) did; PSA alone did not induce cell death (Fig. 4.15).

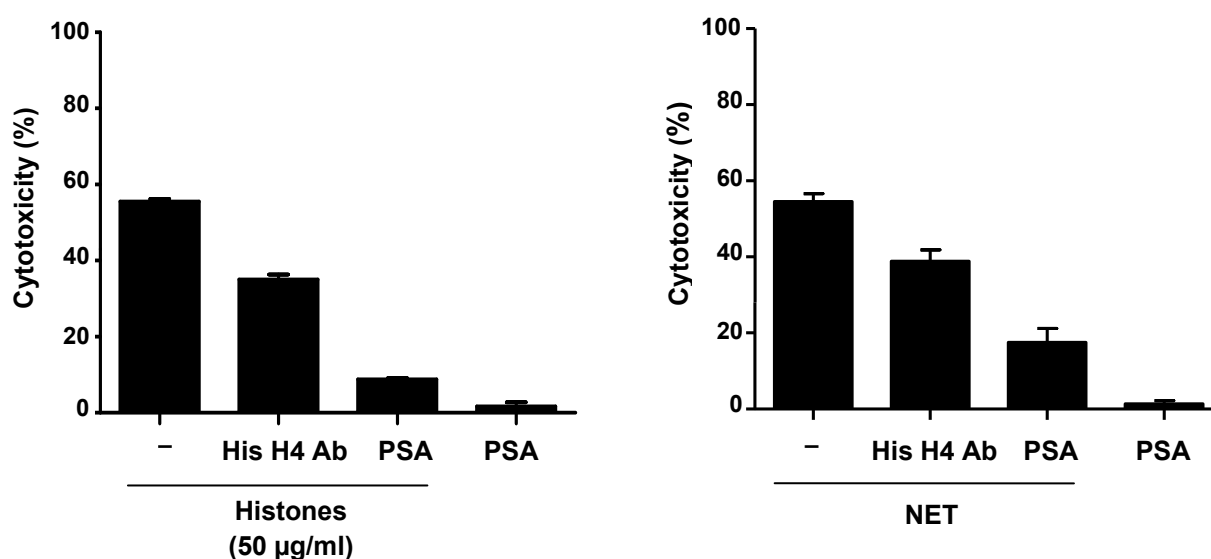


Figure 4.15: PSA reduces histones- and NET-mediated epithelial cytotoxicity. Histones or NET pre-incubated with PSA and histone H4 antibody for 1 h and then incubated with epithelial cells for 16 h to measure cytotoxicity. PSA alone had no cytotoxic effect.

4.15 Inhibition of neutrophil elastase does not inhibit NET-induced cytotoxicity

Another abundant component of NET is neutrophil elastase, which has important roles in clearance of invading pathogens (Urban *et al.*, 2009). However, it also mediates neutrophil-induced tissue damage and degrades extracellular matrix efficiently (Perl *et al.*, 2008). Neutrophil elastase in NET showed increase in activity after digestion of DNA either by DNase or MNase (Fig. 4.4B and Fig. 4.16A). Neutrophil elastase inhibitor significantly inhibited elastase activity in NET (Fig. 4.16A), indicating that neutrophil elastase is active in NET and its activity can be abolished by elastase inhibitor. However, elastase inhibitor could not reduce NET-mediated cytotoxicity in either digested or undigested NET (Fig. 4.16B).

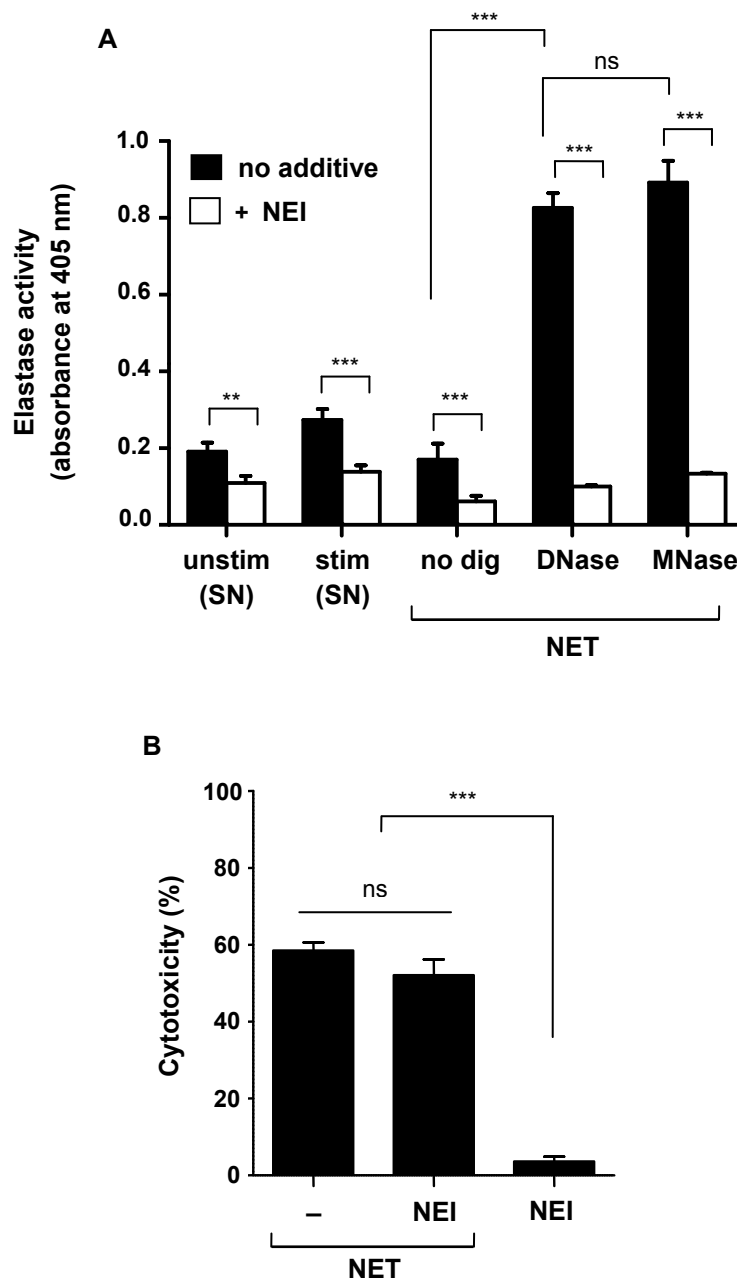


Figure 4.16: Inhibition of neutrophil elastase does not inhibit NET-induced cytotoxicity. (A) Neutrophils were stimulated with 50 nM PMA for 4 h or kept unstimulated. The supernatants of the stimulated (Stim) and unstimulated (Unstim) cells were collected for elastase activity assay. Moreover, NET were isolated from stimulated cells and digested with DNase or MNase or kept undigested. The undigested and digested forms were centrifuged and the supernatant were collected for elastase activity assay. Neutrophil elastase activities of the samples were measured in the absence (filled bars) or presence (open bars) of neutrophil elastase inhibitor (NEI). Elastase concentration for 800 μ l supernatant of SN (Ustim), SN (Stim), NET (no dig) and NET (MNase or DNase) were 0.07 U/ml, 0.15 U/ml, 0.07 U/ml and 0.33 U/ml, respectively. (B) Cytotoxicity of A549 cells was measured after 16 h treatment with NET (DNase-digested) in the absence or presence of NEI. Similar results were seen for MNase- or non-digested NET. Differences were considered statistically significant at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

4.16 Inhibition of myeloperoxidase reduces NET-induced cytotoxicity

One of the other granular components of NET is MPO (Brinkmann *et al.*, 2004; Urban *et al.*, 2009). MPO has an important role in defence against bacteria, viruses and fungi by conversion of hydrogen peroxide to HOCl. However, MPO activity can also induce damage to adjacent tissue and, thus, contribute to the pathogenesis of different inflammatory diseases including pulmonary injury (Klebanoff, 1999; Van Der Vliet *et al.*, 2000). Pre-incubation of MPO inhibitor with digested or non-digested NET led to reduction of NET-induced cytotoxicity in epithelial cells (Fig. 4.17). These data suggest that the cytotoxicity of NET is partly mediated by MPO.

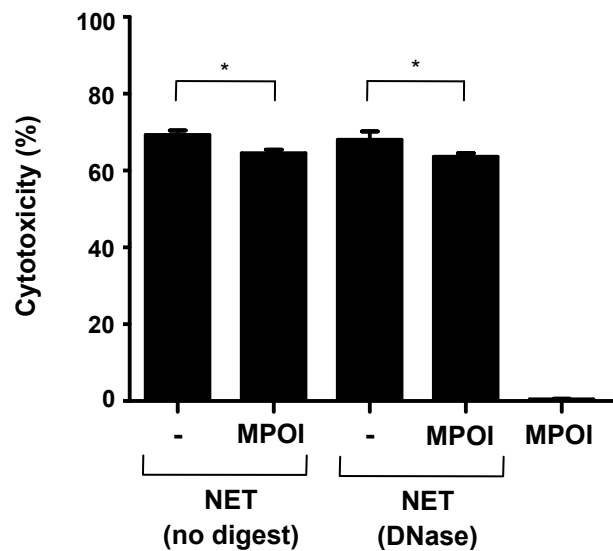


Figure 4.17: Myeloperoxidase inhibition decreases NET-induced cytotoxicity of epithelial cells. Non-digested or DNase-digested NET were pre-incubated without or with myeloperoxidase inhibitor (MPOI), followed by incubation of NET with epithelial cells (A549) for 16 h. Pre-incubation of NET with MPOI reduced NET-mediated cytotoxicity. MPOI alone (37 ng/ml) was not toxic for the epithelial cells. Differences were considered statistically significant at * $P < 0.05$.

4.17 NET mediate a biphasic characteristic in epithelial cell permeability

To scrutinize the effect of NET on epithelial cells, permeability of epithelial cells after incubation with NET was measured up to 10 h. Exposure with NET decreased permeability of epithelial cells in comparison to the untreated cells in the early phase (up to 6 h), whereas an increase in permeability was observed at the later stage (Fig. 4.18). NET digestion by DNase did not influence its impact on permeability.

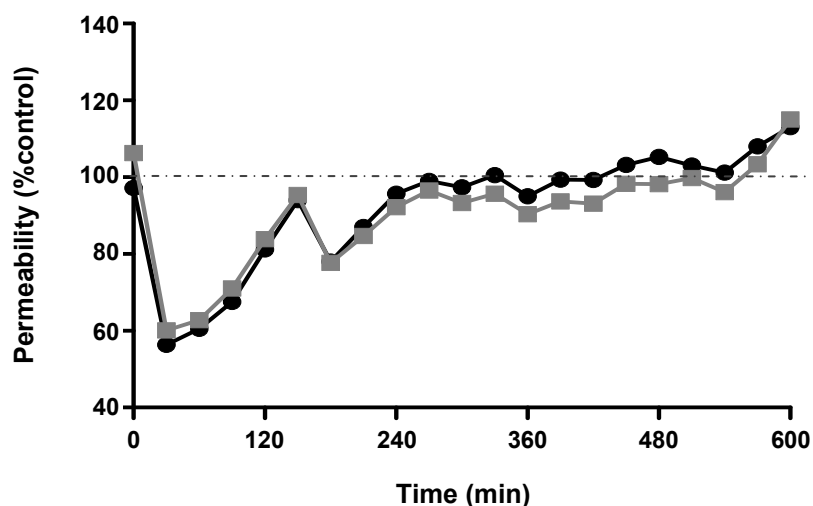


Figure 4.18: NET mediate a biphasic characteristic in the permeability of epithelial cells. Epithelial cells were incubated with NET up to 10 h, and cell permeability was evaluated by measuring the flux of trypan blue-labeled albumin across the cells. Permeability was shown in percentage, where permeability in the cells treated only with medium (control) was considered 100%. Decrease in the mean permeability up to approximately 6 h and increase at the later time points were noted. Undigested NET: black circle, DNase-digested NET: gray square.

4.18 Similarity between supernatant of cystic fibrosis sputum and isolated NET

Damage in the airways of several lung diseases such as cystic fibrosis is associated with lung epithelial cell death, and cystic fibrosis patients have accumulation of extracellular DNA in the airways (Hamutcu *et al.*, 2000). In order to investigate the relevance of NET on epithelial cell death in cystic fibrosis, sputum from these patients were evaluated for NET formation. Patient sputum (without any stimulation) demonstrated NET formation as illustrated by colocalization of DNA/histone and elastase (Fig. 4.19A) very similar to the appearance of NET from isolated neutrophils of healthy human blood that were stimulated with a NET-inducing agent such as PMA (Fig. 4.19B). Moreover, sputum supernatant and isolated NET showed

very similar structure (Fig. 4.19C and D) which further verifies the presence of NET in cystic fibrosis sputum.

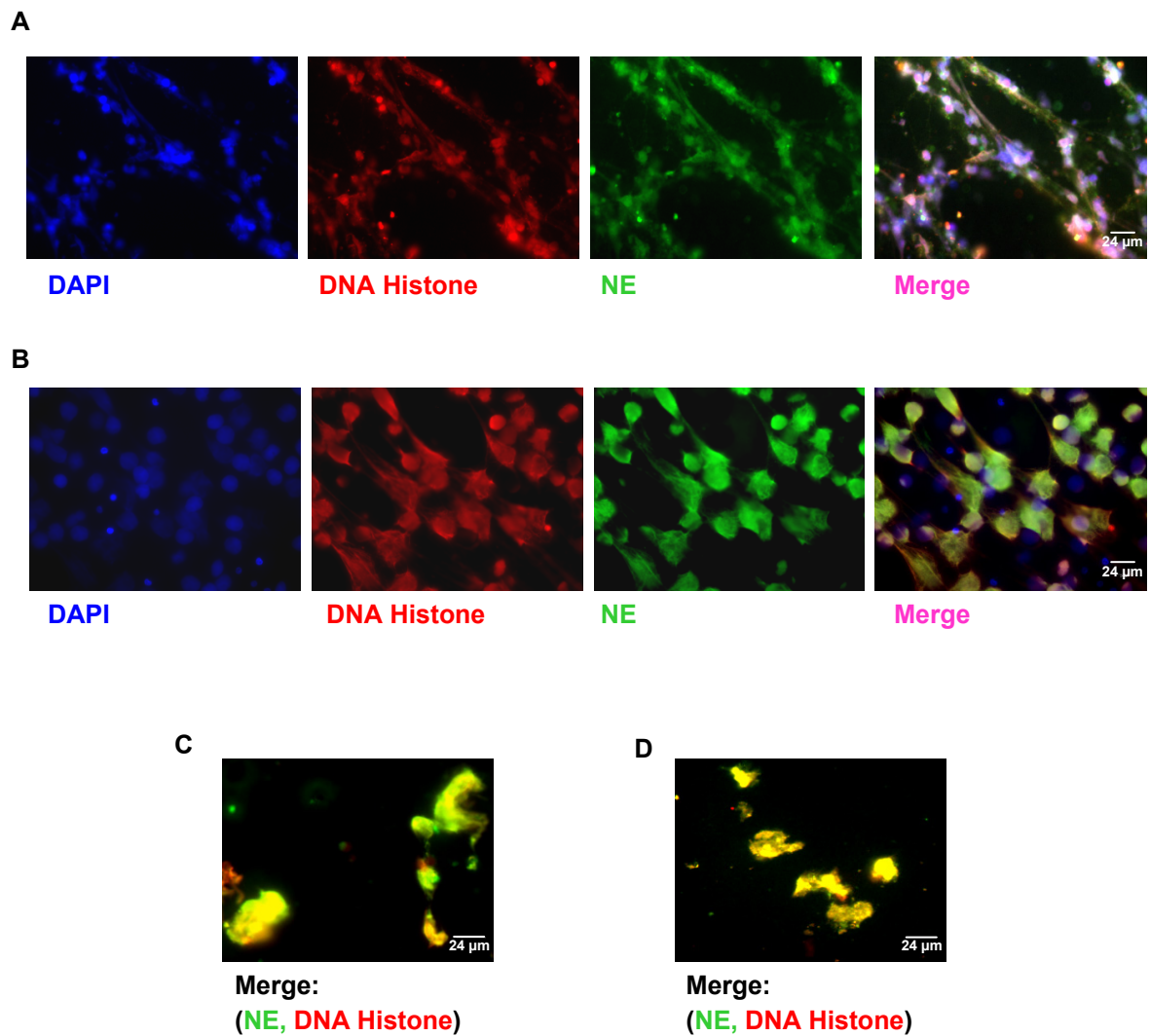


Figure 4.19: Similarity between supernatant of cystic fibrosis sputum and isolated NET. (A) Cystic fibrosis sputum and (B) PMA-treated isolated neutrophils from healthy human blood were stained for nucleus (DAPI), DNA/histone (red) and neutrophil elastase (NE, green). (C) Co-localization of neutrophil elastase and DNA/histone in isolated NET and (D) in the supernatant of cystic fibrosis sputum.

5 Discussion

This study focused on the role of NET in host cell functions, particularly on alveolar epithelial cells as major cells responsible for gas exchange in the lung as well as on endothelial cells. NET formation in different organisms, its bactericidal activity, and its presence in several pathophysiological conditions have been documented; however, little is known about the direct effect of NET on host cells and its potential role in inducing ALI. The following section will reveal the results of the current thesis and will discuss them in the context with the existing literature.

5.1 Protein identification of NET

Before investigating the effect of NET on host cells, the proteome profiles of PMA-stimulated neutrophils and NET proteins were studied. PMA, as a potent inducer of NET, was used mostly to induce neutrophils for NET formation. PMA-stimulated neutrophils showed upregulation of several proteins in comparison to the unstimulated cells. Identification of proteins in NET by mass spectrometry confirmed the presence of different kinds of proteins which have been identified by another group (Urban *et al.*, 2009) as well as several new proteins (Table 1.1 and 4.1). Besides granular proteins such as MPO, lactoferrin and protein S100-A8 and A9, which have antimicrobial activities, the presence of different cytoskeletal proteins were further confirmed. Presence of actin and actin binding proteins such as profilin-1 or platin-2 may reflect the role of these proteins for structural organization of NET. These proteins may also play important roles in the activity of other cells such as platelets or T lymphocytes.

Glycolytic proteins such as alpha-enolase or glyceraldehyde 3-phosphate dehydrogenase were also found in NET. It is appealing to recognize the function of these proteins in NET; whether they have a role in glycolysis of the NET-adjacent cells or whether they influence by their other activities such as regulation of DNA replication or transcription.

In addition, some NET-associated proteins, for instance elastase or proteinase-3, with antimicrobial functions are capable of degrading the extracellular matrix; which in turn make NET more susceptible to induce tissue injury. Interestingly, serpin B1 as an inhibitor of proteolysis was also identified in NET which may reveal that even in NET the activity of proteases may be regulated in order to prevent the deleterious role of proteases on host tissue.

One of the other NET-associated proteins, which was identified for the first time in this study, is Annexin A1. Annexin A1 is a calcium/phospholipid-binding protein which has a role in membrane aggregation as well as in inhibition of phospholipase A2, and is considered as an anti-inflammatory protein by blocking neutrophil trafficking (Perretti and D'Acquisto, 2009). The presence of this protein in NET implies that NET components not only have antimicrobial activities, but they can also regulate inflammation.

It should be noted that in the present study, due to the practical reasons, not all the NET-associated proteins were selected for the identification by mass spectrometry. Moreover, from the randomly selected protein spots, which were separated by 2-D gel electrophoresis, not all could be identified. Investigation of the other NET-associated proteins may uncover the effect NET on host cells more precisely. It should also be considered that different procedures for NET collection or various techniques for protein identification, such as gel-free mass spectrometry, may give rise to different results. So far, a standard procedure for NET collection, which truly confirms that the isolated NET contain all the materials and structures in the *in vivo*-formed NET, has not been established. Therefore, it is essential to mention the exact procedure of NET collection in studies dealing with NET.

5.2 NET formation in lung tissue and BAL fluid in LPS-induced lung injury model

Neutrophil products such as ROS, neutrophil elastase, cathepsin-G and other granular proteins can damage host cells and tissues and may lead to organ injury if not well-regulated (Mizgerd, 2008), and neutrophil-mediated tissue damage at the infected sites is one of the major sources that triggers inflammation (Nathan, 2006). Likewise, activated neutrophils contribute to lung injury by releasing proteolytic enzymes, ROS and other proinflammatory mediators (Lee *et al.*, 2005; Abraham, 2003).

To investigate the role of NET in ALI, an LPS-induced lung injury model was applied and both lung tissue and BAL fluid were examined for NET formation and presence. NET formation was observed for neutrophils in BAL fluid and lung tissue after LPS treatment. Moreover, activity of neutrophil elastase in BAL fluid was significantly increased after digestion of BAL with nuclease in comparison to the secreted elastase. Neutrophil elastase is either secreted from granules (free elastase) by activated neutrophils (Mizgerd, 2008; Nathan, 2006) or it can be expelled together with NET (NET-related elastase) in association with the chromatin (Brinkmann, 2004). Visualization of NET in lung tissue and BAL fluid in LPS-treated mice besides the increase of elastase activity in NET structure after DNA digestion

confirmed NET formation in LPS-induced lung injury model. Therefore, besides the key role of neutrophils and their secreted proteins, the presence of NET and their associated components may serve as additional causal factors which contribute to the pathogenesis of ALI and their complications.

It should be noted that NET formation is not the only activity of neutrophils. Neutrophils can damage lung cells by secretion of their components such as granular proteins which may further lead to the manifestations of ALI or other lung diseases dealing with epithelial cell death. To assure that epithelial cell death is caused only by NET components, the secreted materials of neutrophils, which are not in NET structure, should be eliminated. Designing such an *in vivo* model is not possible; therefore, for further experiments the *in vitro* model was performed by incubation of NET collected materials with the epithelial cells. However, whether the *in vitro*-formed NET have the same components and functions of the *in vivo*-formed ones has not been clarified.

One of the other challenging issues concerning NET detection in the *in vivo* model is the structure of NET in tissues. Immunocytochemistry and NET quantification in the isolated neutrophils are quite easy due to the distance between the cells. However, in the tissues such as lung when there is not enough space between the cells detection of NET is tricky. Besides, presence of short fragments of DNA–protein complexes in the airways as opposed to the long stringlike structures seen in *ex vivo* cultures (Doua *et al.*, 2011), as it has also seen in this study, make the detection of NET in tissue much more difficult.

5.3 The inhibitory role of Eap for NET formation

NET formation can be induced by incubation of neutrophils with gram positive and negative bacteria as well as fungi and other inflammatory stimuli. It is been also shown that *S. aureus* can induce NET formation (Pilszczek *et al.*, 2010). However, there is no report about the effect of bacterial products such as Eap on NET formation. Eap is a secreted protein from *S. aureus* which has a very wide range of binding interactions to host extracellular matrix components (Harraghy *et al.*, 2003; Chavakis *et al.*, 2005). Eap has an anti-inflammatory function by preventing integrin-dependent neutrophil–endothelial interactions, and *S. aureus* uses this property to escape the host immune system (Chavakis *et al.*, 2002). Interestingly, Eap suppressed NET formation in a dose-dependent manner, no matter whether the NET inducer was PMA, LPS or NO donor. This characteristic of Eap may be used to regulate NET formation.

Wang and co-workers have shown that Eap suppresses psoriasis, an inflammatory skin disease, by inhibiting T-cell recruitment (Wang *et al.*, 2009). Recently, it has been shown that mast cells and neutrophils are the main cell types in human skin that contain IL-17, which is a central cytokine to psoriasis pathogenesis. Interestingly, mast cells and neutrophils release IL-17 in the process of forming extracellular traps (Lin *et al.*, 2011). Inhibition of NET formation by Eap, as it was shown in the present study, may explain one of the mechanisms that lead to suppression of psoriasis after Eap administration.

5.4 The role of NET on host cell cytotoxicity

It has been reported that excessive NET formation or impairment of NET removal is linked to chronic inflammatory diseases such as preeclampsia, small-vessel vasculitis and systemic lupus erythematosus (Gupta *et al.*, 2005; Kessenbrock *et al.*, 2009; Hakkim *et al.*, 2010; Gupta *et al.*, 2010). Since alveolar epithelial cells are crucial to maintain normal gas exchange, and lung epithelial cell death represents a potentially important mechanism contributing to the loss of this cell type in the development of ALI (Perl *et al.*, 2008; Bachofen and Weibel, 1982), the direct effect of isolated NET on lung epithelial cells was further investigated.

Isolated NET induced cytotoxicity in epithelial cells in a dose-dependent manner. Interestingly, DNA alone or DNA-digested NET either completely digested or partially digested NET did not change NET-mediated cytotoxicity. Moreover, boiled or denatured NET could not induce cytotoxicity which is further an indication that proteins (and not DNA) in NET are involved in NET-mediated cytotoxicity of endothelial and lung epithelial cells.

NET-associated proteins include nuclear and granular proteins as well as cytoplasmic proteins such as cytoskeletal and peroxisomal proteins and glycolytic enzymes (Table 1.1 and 4.1). Most of these NET-associated proteins have high antimicrobial activity. Selective antimicrobial peptides differentiate between eukaryotic cells and bacterial cells based on variations in the composition of the cell membrane. Since the majority of antimicrobial peptides are positively charged at physiological pH, the popular theory is that selectivity results from electrostatic attraction of the cationic peptide to the anionic bacterial membranes (Zasloff, 2002; Henzler Wildman *et al.*, 2003; Wieprecht *et al.*, 2000; Wiesner and Vilcinskas, 2010). However, electrostatic interactions cannot be the only reason for selectivity, and other aspects of membrane composition and additional properties of these

peptides must also be important in determining selectivity (Dathe and Wieprecht, 1999; Henzler Wildman *et al.*, 2003).

Moreover, specific structures of NET-proteins may be responsible for host cell cytotoxicity. There could be unique post-translational modifications of proteins in NET which make them cytotoxic. Protein-protein interactions and formation of protein complexes in NET, as shown also in the present study by NET fractionation and native-gel electrophoresis, may create cytotoxic proteins which can be sensed by death receptors on the host cells.

Treatment of epithelial cells with NET induced upregulation of different proteins from nuclear to membrane proteins with different kinds of functions such as regulation of cell cycle, cellular component movement, oxidation-reduction process, cell surface receptor-linked signaling pathways, glycolysis, lipid metabolic process, regulation of proliferation, ubiquitin conjugation and proteasome degradation pathway and interestingly several of these proteins are upregulated during cell death and apoptosis (Table 4.2) which further confirm our data about NET-mediated cytotoxicity.

5.5 The role of histones in NET-mediated cytotoxicity

Bearing in mind that histones are one of the most abundant components of NET with bactericidal and leishmanicidal activity (Brinkmann *et al.*, 2004; Guimarães-Costa *et al.*, 2009), interference with histones such as histone degradation should decrease NET cytotoxicity. Histones can be degraded with different proteases such as cytotoxic T lymphocyte protease granzyme A (Zhang *et al.*, 2001), activated nuclear proteasome (Ulrich *et al.*, 1999) or APC. APC is a serine protease which plays an important role as natural anti-coagulant and also has been reported to degrade histones and thereby reduce cell death in sepsis (Xu *et al.*, 2009). While our data showed that APC significantly decreased histone-mediated epithelial cytotoxicity in a purified system, incubation of NET (either nuclease-digested or not) with APC could not reduce NET cytotoxicity, although much higher doses of APC were used for treatment with NET as in a purified system. Also, DIGE for NET and NET incubated with APC, which enables the accurate analysis of differences in protein abundance, did not show any difference in the gel profiles. This could be due to the formation of histone complexes with DNA or other proteins, to specific modifications of histones in NET structure (Urban *et al.*, 2009; Wang *et al.*, 2009), which may protect them against degradation by APC, or to the fact that histones are degraded during NET formation (Papayannopoulos *et al.*, 2010).

As reported before, antibodies against histones can reduce histone-mediated cytotoxicity during sepsis (Xu *et al.*, 2009). Likewise, pre-treatment of NET with different antibodies against histones could decrease NET-mediated cytotoxicity; however, it was not effective for all kinds of histones. Specific modification(s), unique structures of histones, histone degradation during NET formation or the protein complexes in NET could be responsible for such differences. Xu and colleagues mentioned that antibodies against histones had better effect in comparison with APC to reduce histone-mediated cytotoxicity, at least because of the bleeding complications which might occur by using APC (Xu *et al.*, 2009). Although they induced sepsis by purified histones, further recruitment of neutrophils and NET formation cannot be ignored. NET may lead to further cell death and this might be one of the reasons that explain why antibodies against histones were more effective than APC for treatment of sepsis.

The antimicrobial activity of histones has been discovered more than half a century ago. Final concentrations of less than 1 µg/ml histone kill susceptible microbes without detectable morphological alteration or lysis. However, among the microorganisms some are highly susceptible to histone, some are less and some are resistant to histones. The bactericidal effect of histone is antagonized by small amounts of certain basic substances (protamine, spermine), or by various acid polysaccharides (heparin, nucleic acids, bacterial lipopolysaccharides) (Hirsch, 1958). The present study also showed that pre-incubation of histones or NET with PSA showed decrease in histone- and NET-mediated cytotoxicity, respectively. PSA is a highly negatively charged glycan that binds to histone H1 and this interaction has a key role for nervous system development and regeneration. Histone H1 promotes axon outgrowth of cerebellar neurons in a PSA-dependent manner (Mirsha *et al.*, 2010). The present data may indicate that histones interact specifically with certain molecules on the host cell membrane and induction of cytotoxicity by histones is not unspecific. However, PSA, as a negatively charged molecule, may also neutralize the positive charge of antimicrobial peptides in NET, and lead to reduction in cytotoxicity. Further studies on the role of PSA in histone- and NET-mediated cytotoxicity are under investigation.

Moreover, histones can influence cell permeability (Zhdan-Pushkina *et al.*, 1975), and prolonged exposure of the epithelium to histone proteins causes irreversible loss of transepithelial resistance (Kleine *et al.*, 1995). The present data showed that NET induced a biphasic characteristic in epithelial cell permeability; a decrease in permeability at earlier time points and an increase at the later stage of NET incubation was observed. NET proteins

including histones may have a major role in this process. These proteins may be sensed by various response signals in bystander cells depending on the time and amount of NET exposure.

5.6 The role of non-histone components of NET in NET-mediated cytotoxicity

Besides histones, another abundant component of NET is neutrophil elastase (Urban *et al.*, 2009), which has an important role in clearing invading pathogens. Moreover, elastase is able to mediate neutrophil-induced tissue damage and can degrade extracellular matrix efficiently (Perl *et al.*, 2008). Neutrophil elastase activity in NET increased after digestion of DNA. These data support the presence of NET in BAL fluid after LPS induction, as they also showed increase in elastase activity after DNA digestion. However, inhibition of neutrophil elastase in DNase-digested or non-digested NET did not reduce NET-mediated cytotoxicity. Presence of more cytotoxic components of NET (such as histones) can explain this effect so that inhibition of neutrophil elastase is not sufficient to suppress NET-mediated cytotoxicity.

MPO, another granular protein with anti-microbial activity, was also investigated for NET-mediated cytotoxicity in the present study. MPO has an important role in defence against bacteria by conversion of hydrogen peroxide to HOCl. Nevertheless, MPO activity can also provoke damage to adjacent tissue and, therefore contribute to the pathogenesis of several inflammatory diseases including pulmonary injury (Klebanoff, 1999; Van Der Vliet *et al.*, 2000). It has been reported that MPO can provoke caspase-3 activation and apoptosis in HL-60 human leukemia cells (Myzak and Carr, 2002). Moreover, MPO can induce DNA strand breakage in lung epithelial cells (Haegens *et al.*, 2008). Pre-incubation of NET with MPO inhibitor decreased NET-mediated cytotoxicity of epithelial cells, indicating that besides histones MPO is also responsible for the cell damaging capacity of NET.

More than twenty different proteins have been identified in NET (Table 1.1 and 4.1), and investigation of all of these proteins on NET-mediated cytotoxicity is beyond the scope of the present study. It should be considered that not always the quantity but also the quality of NET components may have an important role in inducing cytotoxicity. The proteins (identified or non-identified) which encompass only a small quantity of NET components maybe even more toxic for the host cells in comparison to the other proteins. The diversity of NET proteins and their incomplete identification make the recognition and analysis of the cytotoxic parts of NET more complicated.

5.7 The pathophysiological role of NET

NET has been seen in different pathophysiological situations including appendicitis (Brinkmann *et al.*, 2004), severe sepsis (Clark *et al.*, 2007), autoinflammatory disorders such as small-vessel vasculitis (Kessenbrock *et al.*, 2009), systemic lupus erythematosus (Hakim *et al.*, 2010), Crohns disease (Yousefi *et al.*, 2008) and in vein thrombosis (Fuchs *et al.*, 2010). All of these examples show a potential proinflammatory role of NET.

Many lung diseases, including ALI, fibrosis or cystic fibrosis are associated with the lung epithelial cell death. Progressive infection and inflammation in the lower airways results in destruction of the small and medium airways in the lung in cystic fibrosis patients, and in these patients, extracellular DNA accumulates in the airway due to the chronic bacterial infection (Hamutcu *et al.*, 2002). In the present study, the structural similarity between isolated NET and the supernatant of cystic fibrosis sputum has been observed. Moreover, immunocytochemistry of patients sputum showed structures similar to NET; chromatin fibers decorated with granular proteins. Although the use of aerosol recombinant human DNase therapy is linked with an improvement in airflow obstruction and a decrease in the number of infectious respiratory exacerbations in some cystic fibrosis patients, this is not true for all cases (Fuchs *et al.*, 1994). It was seen that even the digestion of DNA in NET could not abolish NET cytotoxicity, and maybe this is one of the reasons that DNase therapy is not useful in all cystic fibrosis patients. Presence of NET in high amount in these patients may be one of the reasons which lead to pulmonary obstruction. Since cystic fibrosis sputum contains various cells and compounds, and handling the muocus sputum usually needs further treatments such as DTT-treatment, incubation of cystic fibrosis sputum with epithelial cells was not practical.

In this study, the direct effect of NET on endothelial and lung epithelial cells has been examined. However, the *in vivo* situation is far more complex. Phagocytosing cells remove dead cells and their debris (maybe NET too). Furthermore, the ciliary motion in the lung prevents staying of dead cells for a long time in one place. It has to be further investigated whether one of the reasons which promote lung destruction in patients with ALI or cystic fibrosis is impairment in the clearing mechanisms of NET.

It is also worthwhile to consider the variation of responses to NET in different individuals. Some individuals may produce the same amount of NET as others; however, they may have defect in resolution and removing the NET components. In contrast, others may have normal

resolution systems but they produce more NET. All of these aspects should be considered before designing any therapeutic agent for patients who suffer from complications of NET formation. It is also crucial to know where exactly in the tissues NET will form, and how a potential drug can reach NET.

5.8 Conclusion and further directions

Taken together, the results of the present study demonstrate that NET induce cytotoxicity in endothelial and lung epithelial cells in a dose-dependent manner and the type of DNA digestion in NET (either digested or not) does not influence NET-mediated cytotoxicity.

In addition to the results represented here, other components of NET may be responsible for NET-mediated cytotoxicity. Investigation of the protein components in NET, their unique structures and the possible receptors which sense NET will be useful to design therapeutic agents for the people who suffer from impairment of NET and its complications. Proteins such as Eap, which inhibits NET formation, histone antibodies or negatively charged glycan such as PSA, which decrease NET-mediated cytotoxicity, are some potential candidates that can be considered to decrease the deleterious effects of NET.

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7 Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

**Der Lebenslauf wurde aus der elektronischen
Version der Arbeit entfernt.**

**The curriculum vitae was removed from the
electronic version of the paper.**

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