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On Generation and Function of Neutrophil Extracellular Traps in Abdominal Sepsis

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Avin Hawez

LUND UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Sepsis is a clinical condition that develops when the immune system over-response to an infection. Sepsis can lead to organ failure as well as subsequent death. Sepsis-induced host response involves neutrophil over activation which can lead to self tissue damage. Neutrophils are the first line of defence, play a critical role in controlling the infection. Upon activation, neutrophils release neutrophil extracellular traps (NETs) as part of the host defence. NETs can be harmful in a certain conditions and involved in the pathogenesis of inflammatory diseases. The underlying mechanisms of NET formation in abdominal sepsis is still scarce. Thus, to understand the signaling mechanism of NET formation and it's role in abdominal sepsis, we investigated the role of c-Abl kinase in paper I. We detected NETs by electron microscopy in the lungs of septic mice. We found that inhibition of c-Abl kinase decreased CXC chemokines, neutrophil recruitment, and tissue injury in the lung. As well as it reduced plasma levels of DNA-histone complex in septic mice. In paper II, we show that miR-155 positively regulates neutrophil expression of peptidylarginine deiminase 4 (PAD4) which catalyzes histone hypercitrullination as part of NET formation. Bone marrow neutrophils were transfected with a mimic miR-155 which increased PAD4 expression and NET formation, while neutrophils transfected with antagomiR-155 decreased PAD4 expression and NET formation. In paper III, we studied the role of miR-155 in regulating NET formation in a mouse model of abdominal sepsis. In miR-155 deficient cecal ligation and puncture mice, we observed a reduction of CXC chemokines and neutrophil recruitment. In paper IV, by use of intravital microscopy, we observed that NET-microparticle aggregates participate in neutrophils-endothelium interactions. We found that neutrophil recruitment is mediated by HMGB-1 expressed on MPs via TLR2 and TLR4 signalling. Taken together our findings in the current thesis identified mechanisms of NET formation and tissue damage in abdominal sepsis. Thus, targeting these specific mechanisms of NET generation could be a useful strategy to ameliorate lung damage in abdominal sepsis.

Key words: abdominal sepsis, inflamation, neutrophil, neutrophil extracellular traps, mRNA, adhesion.

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On Generation and Function of Neutrophil Extracellular Traps in Abdominal Sepsis

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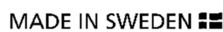

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بسم الله الرحمن الرحيم

يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ ﴿١١﴾

Allah will exalt those of you who believe and those who are given knowledge, to high degrees; and Allah is aware of what you do.

To my beloved family

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I. **Hawez A,** Ding Z, Rahman M, Taha D, Madhi R, and Thorlacius H. c-Abl kinase regulates neutrophil extracellular trap formation and lung injury in abdominal sepsis. Submitted to *J. Cell. Physiol*. 2020

II. **Hawez A,** Al-Haidari A, Madhi R, Rahman M, and Thorlacius H. MiR-155 Regulates PAD4-Dependent Formation of Neutrophil Extracellular Traps. *Front Immunol*. 2019; 10:2462. *

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Abbreviations

Ago2	Argonaute 2
ALI	Acute lung injury
APC	Allophycocyanin
AREs	AU-rich elements
BALF	Bronchioalveolar lavage fluid
CHX	Cycloheximide
CLP	Cecal ligation and puncture
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HMGB1	High-mobility group protein 1
H&M	Hematoxylin and eosin
LFA-1	Lymphocyte function antigen 1
Mac-1	Membrane activated antigen 1
MIP-2	Macrophage inflammatory protein 2
MNL	Monomorphonuclear leukocyte
MPO	Myeloperoxidase
MPs	Microparticles
mRNA	Messenger RNA
NETs	Neutrophil extracellular traps
PAD4	Peptidyl arginine deiminase 4
PBS	Phosphate buffered saline Phycoerythrin

PAMP	Pathogen associated molecular pattern
PE	Phycoerythrin
PMA	Phorbol 12- myristate 13-acetate
PMNL	Polymorphonuclear leukocyte
PSGL-1	P-selectin glycoprotein ligand-1
QPT-	Quantitative Reverse transcription polymerase chain reaction
RAGE	Receptor for advanced glycation end products
RIP	RNA immunoprecipitation
RNA	Ribonucleic acid
SIRS	Systemic inflammatory response syndrome
ROS	Reactive oxygen species
RPMI 1640	Rowsell Park Memorial Institute medium 1640
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TSB	Target site blocker
UTR	Untranslated region

Introduction

Sepsis is a complex disorder that develops from dysregulated host response to an infection. Sepsis is associated with acute organ dysfunction and high mortality (1). Despite the advances in intensive care management, sepsis is still associated with high mortality (2). The population-based incidence of sepsis is estimated to be 176–380 cases per 100,000 inhabitants each year with a mortality of up to 35% worldwide, resulting in approximately 20,000 deaths per day and is responsible for over 250,000 death in the United States annually (3, 4). Multiple organ failure is the main reason for mortality in sepsis. Host defence response against the pathogens induces a complex network of molecular cascades leading to tissue damages and organ failure including lung, liver, and kidney (5-7). The lung is the most sensitive organ that gets affected heavily during systemic inflammation (8, 9). Neutrophil activation responsible for the development of multiple organ failure during sepsis through the release of cytokine and other inflammatory mediators leading to neutrophil infiltration and tissue damage, later organ dysfunction (10). Neutrophils are the most abundant white cells in blood and constitute the first line of host defence against microbial infections and in inflammation (11). Neutrophils are generated from stem cells in the bone marrow and mature through several stages including myeloblast, promyelocyte, myelocyte, metamyelocyte, and finally, neutrophil (12). Neutrophil plays an important role in the innate immune system including chemotaxis, anti-microbial function, phagocytosis, degranulation (13, 14). It is the first leukocyte migrate from the blood to the site of infection or inflammation via a process called chemotaxis, then phagocyte pathogens or release NETs to kill the microbes (15). These cells also can negatively cause pathological outcomes in a wide range of diseases. In the site of infection, neutrophil engulfs and kill bacteria or form neutrophil extracellular traps (NETs) to trap and kill pathogens (16). Moreover, the excessive formation of NETs causes tissue damage in both infectious and non-infectious diseases (17). Neutrophil extracellular traps (NETs) release upon neutrophil activation to the extracellular space, which consists of decondensed chromatin including DNA, histones, neutrophil elastase, and myeloperoxidase (18). It has been shown that NET formation causes tissue damage in various inflammatory diseases (19). Thus, in this thesis, we focus on the mechanism of NETs formation and NETs-mediated tissue damage in abdominal sepsis. It is also known that neutrophil dies after completing its mission (20).

Background

Sepsis

Sepsis is an uncontrolled systemic inflammatory response to infection. Sepsis leads to the release of inflammatory mediators and recruitment of excessive inflammatory cells to the site of infection which causes tissue damage and organ failure (21-23). Sepsis is still a major cause of death despite significant research and the burden due to sepsis is several billion dollars in all over the world. Thus, sepsis is a major public health issue (24). SIRS is a hallmark in patient with sepsis which defined by several clinical variables including temperature >38°C or <35°C, heart rate >90 beats/min, respiratory rate >20 breaths/min or PCO2 < 32 mmHg, and WBC > 12000 cells/mm^3 or <4000 cells/mm^3 (25-27). In 2016, the diagnosis of sepsis changed, which depends on new criteria that focus more on organ dysfunction (21). The Sequential Organ Failure Assessment score (SOFA) is now used for sepsis identification and organ dysfunction (28). Based on the qSOFA score there is no severe sepsis, the categories changed to infection, sepsis and septic shock. Patients with sepsis should have an infection and at least two of the qSOFA criteria (29, 30). The SOFA score is (0-3) and consists of three criteria including respiratory rate, change in mental status, and systolic blood pressure. Organ dysfunction determines by the level of these criteria. Sepsis includes both pro-inflammatory and anti-inflammatory responses which are responsible for the early death of sepsis (31). Receptors on the innate immune cell surface such as toll-like receptors (TLRs) recognize bacteria and stimulate innate immune cells to produce IL6, interleukin-1β, and tumour necrosis factor (TNF). Systemic inflammatory response produces by these pro-inflammatory cytokines. The pro-inflammatory response was thought to drive early mortality in the first several days of sepsis, while the anti-inflammatory response was thought to induce organ failure, immune suppression, and then mortality (32). The balance between pro-inflammatory and anti-inflammatory mediators restricts the inflammation response to the local site of infections.

Pathogenesis of sepsis

Sepsis develops when the infection causes a local inflammatory reaction. Bacterial infections are common causes of sepsis (33, 34). However, viruses and fungi can also cause sepsis (35, 36). The septic process initiates when the pathogen enters the host and pathogen-pathogen associated molecule (PAMPs) interact with pattern recognition receptors (PRRs) (37, 38). Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) activate immune cells (39, 40) and present on pathogens, including lipopolysaccharide (LPS) in gram-negative bacteria, peptidoglycans, and lipoteichoic in Gram-positive bacteria and double-strand RNA in viruses (41). Gram-negative bacteria produce endotoxin, which is also known as lipopolysaccharide, is the major component of the cell membrane of gram-negative bacteria, mediated the activation of innate immune cells such as neutrophils, monocytes, lymphocyte, and macrophage. LPS binds to the CD14/TLR4/MD2 receptor and MyD88, leading to an oxidative reaction of nuclear factor-kB (NF-kB) (42). PAMPs and DAMPs are recognized by cells of the innate immune system via pattern-recognition receptors (PRRs) (41, 43, 44). Binding between PARs and PAMPs leads to activation of the immune system and then releases several pro-inflammatory mediators including IL6, TNF-α, IL1, high mobility group box-1 (HMGB-1) and, platelet-activating factor (PAF) (45, 46). TNF-α triggers the expression of pro-inflammatory cytokines (47), also causes tissue damage (48). Some of the isolated gram-positive bacteria in sepsis are *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeruginosa* (49). Exotoxins of streptococci and enterotoxin of staphylococcus induce TNF-α production and caused toxic shock in staphylococcus and streptococcus infections (50). While in fungal infections like LPS, TLR4 binds to the fungal proteins resulting in the production of pro-inflammatory mediators (51). Understanding the pathogenesis of sepsis will be an important first step to improve the survival rate of septic patients.

Neutrophil in sepsis-induced lung injury

While neutrophils consider the first line of defense in innate immunity, neutrophil activation causes tissue damage as well as organ failure in severe sepsis (52, 53). Bacterial infections of the lungs and abdomen are the most common causes of sepsis and often results in acute lung injury (ALI) (54), which is a critical factor for the mortality of septic patients (55). ALI is characterized by neutrophil infiltration,

acute lung inflammation with tissue injury, increased endothelial permeability, pulmonary edema, and respiratory failure. One study reported that migration of neutrophil into the extravascular space and tissue occurs at least two sequential events (56). The first event is the neutrophil activation followed by neutrophil recruitment. ALI initiates when chemoattractant signals such as TNF-α, IL-8, IL-1 recruit neutrophil to the site of infection (57, 58). Recruited neutrophil caused damage tissue by release of reactive oxygen species (59). There are two forms of ALI, primary acute lung injury which is caused by direct injury to the lung (e.g., pneumonia) and, secondary acute lung injury which is caused by an indirect insult (e.g., pancreatitis) (60, 61).

Neutrophil recruitment

Neutrophils are the first responding immune cells that recruited to the site of infection in response to chemotactic mediators (62), including IL-8, CXCL1, and CXCL2 which are consider as key mediators of neutrophil mobilization (63). Neutrophils play an important role in the pathogenesis of sepsis, as well as are essential for host defence against bacteria (64). The process of chemotaxis controlled by multiple intracellular pathways (65). Leukocyte accumulation is a multistep process (rolling-adhesion-emigration) (66), mediated by specific adhesion molecule expressed on endothelial cells and leukocytes (67), which can be grouped into following category: 1) Selectins (P-, E-, and L-selectin), 2) Integrins (β2-family CD11/CD18), 3)Adhesion molecule (ICAM-1, VCAM-1, and junctional adhesion molecule). Leukocyte rolling is supported by the selectin family, which interact with their glycoprotein counter-ligands (68). P-selectin glycoprotein ligand-1 (PSGL-1) is one of the selectin counter-receptor, which binds to P-selectin and E-selectin (69, 70). Leukocytes express β2-integrin, which mediates the adhesion of leukocyte to the endothelial cells. Initially, circulating neutrophil form L-selectin and β2 integrin-mediated interaction on the luminal face of the wall of post-capillary venule (71). This is induced by cytokine e.g. TNF-α which causes up-regulation of P-selectin, E-selectin, and integrin ligands including ICAM1. Several pro-inflammatory mediators like TNF-α and histamine have been shown to regulate rolling by controlling the regulation of P-selectin (72). Up-regulation of P-selectin and E-selectin on the surface of endothelial cells enables binding with their ligands expressed on neutrophils, such as P-selectin glycoprotein ligand 1 (PSGL1), which allow the tethering of circulating neutrophils to the post-capillary veins (73). Due to this binding they can move along the endothelium, a process called rolling (74). The interaction of lymphocyte function-associated antigen

1 (LFA-1) and membrane activate complex 1 (MAC-1) with intracellular adhesion molecule 1 (ICAM-1) and ICAM-2 expressed on the vessel walls contribute to the firm adhesion between rolling neutrophils and the endothelial cells (12, 75, 76). The β2- integrin expressed on the leukocyte surface, it is low under normal condition while up-regulating after activation (77). The interaction between β2-integrins and ICAMs has been reported in several studies (78, 79), which mediate firm leukocyte adhesion in the inflammatory response. Transmigration is the last phase of the neutrophil recruitment, where neutrophils pass through the junctions of the endothelium (80, 81).

Neutrophil extracellular traps in sepsis

Neutrophil extracellular traps were discovered more than a decade ago (16). Zychlinsky and his colleagues found that neutrophil uses an enzyme called neutrophil elastase to eliminate bacteria. By a deeper study of the mechanism, they observed that activated neutrophil kill's bacteria by the release of NETs. Today, it is widely accepted that NETs have both protective and pathological impacts in many inflammatory diseases including sepsis (16, 82). Neutrophil attack invading pathogens after recruitment to the site of infection by releasing antimicrobial peptide and ROS production followed by phagocytosis (83). In addition, activated neutrophil can expel nuclear DNA to form a web-like structure containing granular and cytoplasmic proteins as well as histones, which are referred to as neutrophil extracellular traps (16, 82). It has been reported that NETs formation causes tissue injury in models of inflammation and infections (19, 84). Moreover, NETs provoke several pro-inflammatory effects in the blood (17), pancreas (85), liver (86) as well as lung (87). However, MPO plays an important role in neutrophil antimicrobial response and it has been reported that neutrophil from deficient MPO failed to form NETs in the inflammatory disease (88, 89). In vitro study revealed critical steps of NET formation (16, 90). Peptidylarginine deiminase enzyme mediates citrullination by converting arginine to citrulline in certain proteins, such as histones (91-93). Peptidyl arginine deiminase 4 (PAD4) is a human protein that is encoded by PAD4 gene. PAD enzyme mediates citrullination which converts arginine to citrulline in certain proteins, including histones. (91). There are five known PAD (PAD1-PAD4 and PAD6) (94-96). The enzyme peptidyl arginine deaminase (PAD4) which participates in the NET formation and play a key regulator during this process by converting arginyl residues in the histone of chromatin to citrulline. (97). PAD4 is highly expressed in neutrophils. Mice lacking PAD4 are unable to form neutrophil extracellular traps (NETs) in response to activation (98).

c-Abl kinase in inflammation

c-Abelson (c-Abl) kinase is a nonreceptor tyrosine-protein kinase ubiquitously expressed in mammalian cells and was initially identified as a potent driver of myeloid cell transformation (99). Studies have shown that c-Abl kinase plays a key role in neutrophil accumulation by regulating β2-integrin-mediated neutrophil migration (100). Moreover, inhibition of c-Abl kinase activity has been reported to reduce endotoxin-induced neutrophilia and tissue damage in the lung (101). Subsequent data demonstrated that c-Abl kinase had a role in inflammatory disease, including immunoglobulin-mediated glomerular damage, nephrotoxicity, and endotoxin-induced vascular leakage (102). It is interesting to note that c-Abl kinase has been suggested to regulate ROS generation in neutrophils (103), and ROS has been shown to be involved in the expulsion of NETs from neutrophils (104). c-Abl kinase also regulates critical components of inflammation, such as endothelial cell integrity and neutrophil adhesion (105). c-Abl kinase plays an important role in neutrophils function and in different models of inflammation and the role of c-Abl kinase in NET formation and tissue damage in inflammatory disease.

MicroRNAs and miR-155

MicroRNA (miRNAs) are small 22-25 noncoding nucleotide RNAs that play a significant role in post-transcriptional gene regulation through complementary base pairing to the 3´ UTR of target mRNA (106). It is known to regulate cytoplasmic control of mRNA translation and degradation. miRNAs are produced from transcription, then miRNA precursors are processed to mature miRNA in two steps involving the RNase III family enzymes Drosha and Dicer. First processing the miRNA by Drosha in the nucleus to create precursor miRNA, which is exported to the cytoplasm by exportin-5, then undergo the final processing by Dicer complex followed by integrated into the RISC (RNA-induced silencing complex). This process is mediated by argonaute-2 protein (Ago-2) is found in RISC complexes. Several studies have shown that miRNA play important role in pathogenesis including systemic inflammatory and sepsis (107, 108). Several studies have been reported that miR-155 plays a critical role in the various physiological and pathological processes such as inflammation, cancer, haematopoietic lineage differentiation, and cardiovascular disease. Moreover, miR-155 plays an important function as a protective miRNA that locally down-regulated expression of certain MMPs, thus controls tissue damage during inflammation. The functional role of

miRNA in the immune system is mostly focused on neutrophil regulation (109). Recently, one study reported the role of miRNA in NETs formation (110), while another study showed negative regulation of neutrophil migration toward chemotactic stimuli (111).

Cellular microparticles

Microparticles (MPs) are small sphere-shaped intact vesicles, originating from the cell membrane of activated cells such as platelets, endothelial cells, leukocytes, and erythrocytes with a size less than 1 µm (112). MPs consist of the same composition of the mother cells. For example, both activated neutrophils and neutrophil-derived MPs express a high level of Mac-1 (113). Many studies have shown that the numbers of MPs increase during inflammation (114-119), which are evidence for the potential role of MPs in disease pathogenesis. In response to external stimuli, neutrophil-derived MPs generate pro-inflammatory compounds, such as reactive oxygen species (120). Some previous studies reported the interaction between MPs and extracellular DNA (121) and form complex with NETs via histone-phosphatidylserine interaction (113). It is known that NETs cause tissue damage in both infectious and non-infectious diseases (17, 85). The numbers of MPs shedding from granulocyte and platelet are reported to be elevated in sepsis patients (114).

Treatment of sepsis

Sepsis is a major cause of mortality in the intensive care unit (ICU) (122). Early diagnostic and rapid therapy improve the outcome and reduce the death rate in sepsis (123), which is the primary way of treatment in hospitals. There is no specific treatment for sepsis, since the level of sepsis varies including differences in source and different degrees of inflammation, using the same treatment will not exert a beneficial effect, therefore sepsis management focusing more on the diagnosis. International guidelines for the management of sepsis and the Centres of Medicare and Medicaid Services (CMS) recommend the earlier identification of sepsis for better and quick treatment of sepsis (124, 125). The recognition of sepsis requires clinicians to measure some parameters including, the level of serum lactate, amount of fluids in the body, the blood culture. Many therapies showed interesting results, this includes antibiotics, fluid, and surgical interventions, haemodialysis of the kidney, mechanical ventilation in the lung and, a low dose of corticosteroid (126, 127). Despite many therapies, the mortality rate in the ICU remains high. Therefore, there is an urgent need of septic research both in experiment and in clinical setups.

Aims

Paper I. To examine the role of c-Abelson (c-Abl) kinase in NET formation and inflammation in abdominal sepsis**.**

Paper II. To investigate the role of miR-155 in NET formation in vitro**.**

Paper III. To study whether miR-155 has a role in NET formation in a mouse model of sepsis.

Paper IV. To examine the role of NET-MP aggregates in leukocyte recruitment in vivo and to determine the adhesive mechanisms mediating leukocyte-endothelium interaction and recruitment in response to NET-MP aggregates.

Material and methods

Contents

- Animals
- Experimental sepsis model
- Systemic leukocyte count
- Lung edema and BALF
- Myeloperoxidase activity
- Enzyme-linked immunosorbent assay (ELISA)
- Isolation of bone marrow and blood neutrophils
- NETs detection in vivo
- NETs formation in vitro and NET quantification
- ROS formation in neutrophil
- Flow cytometry
- Western blot
- Confocal microscopy
- Transmission electron microscope and scanning electron microscope
- Cremaster muscle preparation
- Lung IVM
- Assessment of cytotoxicity
- Viability assay
- Histology
- Bacterial culture
- Quantitative real-time polymerase chain reaction (qRT-PCR)
- MicroRNAs transfection
- Bioinformatics analysis of binding sites
- RNA immunoprecipitation RIP assay

Animals

All experiments were performed using male C57BL/6 or miR-155 gene-deficient mice (20 to 25g body weight). The Regional Ethical Committee for Animal Experimentation at Lund University, Sweden has approved the study protocol (Permit number: M136-14 and 5.8.18-08769/2019., Animal were housed on an animal facility where standardized condition were maintained throughout the 12-12 h light-dark cycle. All animal placed in cage and provided standard laboratory diet and purified water ad libitum.

Experimental sepsis model

Abdominal sepsis was induced by puncture of cecum. Briefly, animals were anesthetized with ketamine and xylazine. The lower part of abdomen was shaved, and midline incision was made. The feces were pushed from ascending colon to the lower part of the cecum, ligated 75% by 5-0 silk suture, and then soaked with PBS. After ligature two puncture was made with a 21-gauge needle. The cecum was then relocated to the peritoneal cavity and the abdominal incision was sutured. Sham mice underwent the identical laparotomy without puncture and ligation. Mice then returned to their cages and immediately injected with pain killer subcutaneously, food and water were provided sufficiently. Animals were re-anesthetized for sample collection at 6 and 24 h after CLP induction. Blood was collected from inferior vena cava for later flow cytometric analysis and plasma was acquired by centrifugation and frozen at -20°C for CXCL1, CXCL2, and IL-6. The lower part of the left lung was ligated and excised for edema measurement. In total 3 ml of BAL fluid was collected from lungs using syringe and catheter and PMNLs were counted using Burker chamber. Next, the lung was perfused with PBS to remove the blood from the blood vessels, one lobe of right lung was fixed in formaldehyde for histology and the other lobe was quickly frozen in liquid nitrogen and stored at -80ºC for later assays.

Systemic Leukocyte counts

Blood sample were collected from tail vein. Total leukocyte count (monomorphonuclear (MNL) and polymorphonuclear (PMNL) were performed in Burker chamber using Turk´s solution (0.2 mg gentian violet in 1 ml glacial acetic acid, 6.25% vol/vol) in a 1:20 dilution.

Lung Edema and Broncheoalveolar

To quantify the lung edema in CLP mice, left lung was collected, rinsed in PBS and snap-dried by paper then weighted and recorded as wet weight. The tissue allowed to dry by incubation in 60 for 72 h and re-weighted (dry weight). Lung edema was calculated for each sample by wet/dry weight. BALF was collected by inserting catheter in the trachea and 1 ml of PBS containing 5 mM EDTA was injected 3 times. The sample were centrifuged, then the number of PMNL cells were counted in a Burker chamber.

Myeloperoxidase activity

MPO is an enzyme abundantly present in the azurophilic granules of the neutrophils. For quantitative measurement of MPO activity, lung tissue harvested and homogenised in 1 ml of 0.5% hexadecyltrimethylammonium bromide, then the homogenised tissue samples were kept in the freezer for overnight. Next day, frozen samples are thawed and sonicated. The supernatant were used to determine MPO activity my using TMB substrate. The absorbance of the redox reaction by H2O2 was determined suing a colorimeter in 450 nm wavelength light, with reference filter 540 nm at 25°C. Values were presented as MPO unit/g tissue.

Enzyme-linked immunosorbent assay (ELISA)

CXCL1, CXCL2, and IL6 level were detected in the supernatant of lung tissue samples. Briefly, samples were homogenised then centrifuged to collect the supernatant. Cytokine levels in the supernatant were detected using commercial ELISA kits (R&D Systems, Abingdon, UK) according to the manufacturer instructions. For detection of cytokine in blood samples, blood was collected from inferior vena cava (1:10 acid citrate dextrose) and centrifuged at 14,000 RPM for 10 min 4℃ and stored at -20℃ until use. Plasma levels of CXCL1, CXCL2, and IL6 were quantified by using a commercially available ELISA kit (R&D System). Recombinant CXCL1, CXCL2, and IL6 were diluted in a specific diluent provided by the ELISA kit and used to make standard curves.

Isolation of bone marrow and blood neutrophil

Bone marrow neutrophils were isolated from male C57BL/6 or mir-155 gene-deficient mice were euthanized by careful dislocation of the epiphysis. Muscles were removed from legs, femurs and tibia were flushed with a 25-gauge needle with a 2 ml syringe containing Roswell Park Memorial Institute medium 1640 (RPMI 1640, Invitrogen, Stockholm, Sweden) containing 10% fetal bovine serum (FBS, Invitrogen) and 2 mM EDTA (Sigma-Aldrich, Stockholm, Sweden). Blood were obtained from normal mice (1:10 acid citrate dextrose) from vena cava. Blood samples were added to RPMI 1640. Red blood cells were lysed with 5 ml ice-cold 0.2% NaCl for 45 s followed by addition of 5 ml 1.6% NaCl. Neutrophils were isolated from other blood cells using Ficoll-Paque gradient (GE Healthcare, Uppsala, Sweden) and centrifugation. The layer containing neutrophils was collected and washed with RPMI 1640 and finally isolated neutrophils were suspended at 5×10^6 cells/ml.

NET detection in vivo

DNA-histone complexes were detected in plasma. A sandwich ELISA based on monoclonal antibodies directed against histones and DNA was used to detect the complexes according to the manufacturer's instructions (Cell Death Detection ELISA plus; Roche Diagnostics).

NET formation in vitro and NET quantification

Bone marrow neutrophils were freshly isolated from bone marrow by density gradient centrifugation using a Ficoll-Paque gradient (GH Healthcare). Neutrophils were incubated with PMA, MIP2, and β-glucan for NET formation. NETs adhered on the bottom of the wells were collected by pipetting and then isolated by gradient centrifugation. After centrifugation, supernatant were discarded and pelleted NETs were collected and resuspended in 300 ml PBS for detection of DNA-histone complex using sandwich ELISA as described above. By use of flow cytometry, we quantified the expression of MPO and citrullinated histone H3 on neutrophil.

Ros formation in neutrophils

For ROS generation, bone marrow isolated neutrophils were incubated with dihydrorhodamine 123 (Sigma) for 15 min at 37°C and then stimulated with 50 µg/ml β-glucan for 20 min at 37°C, after that ROS generation were detected by flow cytometry.

Flow cytometry

In this thesis, I have used flow cytometry for the detection of the neutrophils in blood, ROS generation and neutrophil surface NETs markers. Samples were fixed with 2% formaldehyde for 10 min then washed twice with PBS containing 2% FBS, followed by incubation with primary antibodies against protein of interest in PBS containing 5% donkey serum. Cells were washed twice and incubated with secondary antibody specifically against primary antibodies. Flow cytometry analysis was performed on a CytoFLEX flow cytometer (Becton Dickinson, Mountain View, CA, USA) and histograms were made using cell-Quest Pro software (BD Biosciences).

Western blot analysis

This technique is used for the detection and quantification of a certain protein after various treatments. Isolated neutrophil from blood or bone marrow were homogenized in ice-cold RIPA buffer (RIPA lysis and extraction buffer, Thermo Fisher, USA) containing protease inhibitors (Halt Protease Inhibitor Cocktail; Pierce Biotechnology, Rockford, IL), cells were sonicated and stored at -20°C. Protein concentration were determined before heating up to near boiling for a few minutes. Proteins (20 µg) were loaded per lane then transferred onto polyvinylidene fluoride membrane (Novex, San Deigo, CA, USA). Before blotting, total protein gel images were taken. The membrane is then blocked to avoid non-specific binding before adding the primary antibody against the protein of interest and allowed to be incubated for 24 h. Secondary horseradish peroxidase (HPR) linked antibody specifically against the primary antibody is allowed to incubate for 2 h. Each target protein band was normalized against the total protein load of sample lane. Bio-Rad ChimiDocTM MP imaging system was used to detect the bands and LabTM software version 5.2.1 was used for analysing.

Confocal microscopy

In this thesis, I have used confocal microscopy to detect NETs in vitro. Neutrophils seeded over coverslip in 24 a 24 well plate, PMA or MIP-2, neutrophils washed and fixed with 4% paraformaldehyde for 10 min at room temperature, for intracellular staining cells were permeabilized with 0.1% Triton X-100, and washed, then blocked with 1% BSA for 45 min, followed by incubation with specific primary antibodies FITC-conjugated anti-mouse MPO antibody and rabbit anti-mouse citrullinated anti-H3 antibody for 2 h. Coverslips were collected and rinsed with distal water to remove residual salts and then incubated with rat anti-rabbit secondary antibody and the nucleus were counterstained with DAPI or Hoechst 33342 (Thermo Fisher Scientific). Confocal microscopy was performed using LSM 800 confocal (Carl Zeiss, Jena, Germany). Images were acquired by using ×63 oil immersion objective (numeric aperture = 1.25). The pinhole was set to ~ 1 airy unit and the scanning frame was set to 1024×1024 pixels. ZEN2012 software was used for final image preparation.

Transmission electron microscope and scanning electron microscope

Lung tissue samples were first deparaffinized, or bone marrow neutrophils were stimulated with PMA. After that both samples were fixed in 2.5% glutaraldehyde 0.15 mol/L sodium cacodylate, pH7.4 (cacodylate buffer), for 30 min at room temperature. Specimens were washed with cacodylate buffer. Samples were then dehydrated an ascending ethanol series from 50% (vol/vol) to absolute ethanol (10 min/step). Then, specimens were dried to critical point in carbon dioxide, with absolute ethanol as intermediate solvent, mounted on aluminium holders, and finally sputtered with 20 nm palladium/gold. Jeol/FEI XL 30 FGE scanning electron microscope was used to take images at the Core Facility for Integrated Microscopy at Panum Institute (University of Copenhagen, Denmark). The location of the individual target molecule was analysed at high resolution using ultrathin sectioning and transmission immunoelectron microscopy. Specimens were then embedded in Epon 812 and sectioned into a 50-nm-thick ultrathin section with a diamond Knife using an ultramicrotome. For immunohistochemistry, sections were incubated overnight at 4°C with primary antibody against elastase (ab68672, 10 µg/ml, Abcam, Cambridge, UK) and citrullinated histone H3 (ab5103, 10 µg/ml, Abcam, Cambridge, UK) in paper 1 and 3, or against Mac-1, citrullinated histone H3 and HMGB1 (Abcam, Cambridge, UK) in paper 4. Controls without primary antibody

were also included. The grids then were incubated with gold-conjugated species-specific secondary antibodies (Electron Microscopy Sciences, Fort Washington, MD). Finally, the sections were post-fixed in 2% glutaraldehyde and post stained with 2% uranyl acetate and lead citrate. Joel/FEI CM100 transmission electron microscope operated at 80-kV accelerating voltage was used to observe the specimens at the Core facility for Microscopy at Panum Institute.

Cremaster muscle preparation

Cremaster muscle of mice was prepared for intravital microscopy to study the cremaster microvasculature. The surgical procedure was performed on a transparent pedestal by a midline incision of the skin and foscia, and incised tissue were retracted to expose the cremaster muscle, then separated from the epididymis and the testis was pushed back to the side of the preparation. The preparation was performed on a transparent pedestal allowing transillumination and microscopic observations of the cremaster muscle microcirculation after a 10-min equilibration time. Intrascrotal injection of NETs (0.5-4.5 µg) and TNF-α (0.5 µg, R & D Systems Europe, Ltd., Abingdon, Oxon, U.K.) diluted in 0.15 mL PBS was performed at 3 h before microscopic observation. To delineate the role of the selectins and integrin in leukocyte-endothelium interactions, monoclonal antibodies directed against P-selectin (2 mg/kg, clone RB40.34, rat IgG, BD Biosciences, San Diego, CA, U.S.A), PSGL-1 (2 mg/kg, clone 2PH1, rat IgG, BD Biosciences), Mac-1 (4 mg/kg, clone M1/7, rat IgG, BD Biosciences Pharmingen) and LFA-1 (4 mg/kg, clone M17/4, rat IgG, BD Biosciences) as well as a control antibody (clone R3-34, rat IgG1, BD Biosciences) were given IV immediately before intrascrotal administration of NETs. In separate experiments, monoclonal antibodies against HMGB1 (0.4 mg/kg, clone DPH 1.1, mouse IgG, HMGBiotech, Milano, Italy), TLR2 (1 mg/kg, clone T 2.5, mouse IgG, Biolegend, London, U.K) and TLR4 (2 mg/kg, clone MTS510, rat IgG, Biolegend) were administered IV immediately before intrascrotal administration of NETs. All animals were randomized for treatment.

Lung IVM

Leukocyte-endothelium interactions and leukocytes flux was observed in cremaster microcirculation using an Olympus microscope (BX50WI, Olympus Optical Co. GmbH. Hamburg, Germany) equipped with water immersion lenses (40/NA 0.75 and 63/NA 0.90). The microscopic image was recorded in a computer using a

charge-coupled device video camera (FK6990 Cohu, Pieper GmbH, Berlin, and Germany) for subsequent off-line analysis. Analysis of leukocyte flux and leukocyte-endothelium interactions (rolling and adhesion) were made in venules (inner diameter between 20 and 40 µm) with stable resting blood flow. Rolling leukocyte flux was determined at indicated time points by counting the number of rolling leukocyte per 20 s passing a reference point in the microvessel and expressed as cells per min. leukocyte rolling velocity was determined by calculating the velocity of 10 leukocytes rolling along the endothelial cell lining and given as µm s-1. Leukocyte adhesion in venules (stationary for 20 s) was counted 100 µm long vascular segments and expressed as the number of adhered cells per mm2. Leukocyte emigration was quantified by counting by the number of extravascular leukocytes within an extravascular area of 100 × 70 µm immediately adjacent to the venules. And expressed as the number of extravascular cells per mm^2. Diameters were measured in micrometers perpendicularly to the vessel path. Microvascular hemodynamic were determined after injection of 0.1 ml 5% FITC- dextran (MW 150 000, Sigma-Aldrich, Stockholm, Sweden) for contrast enhancement by intravascular staining of plasma and cremaster muscle was visualized by a 100-W mercy lamp and filter set for blue (450-490 nm excitation and >520 nm emission wavelength) and green (530-560 nm excitation; >580 nm emission) blight epi-illumination. Computer-assisted image analysis using the line shift method was used to measure flow velocity. Venular wall shear rate was calculated using the Newtonian definition: wall shear rate = 8 (red blood cell velocity/ venular diameter) (House et al., 1987). All quantitative analysis of micro hemodynamic parameters in the cremaster microcirculation was performed by using the computer-assisted image analysis system Caplmage (Zeintl, Heidelberg, Germany).

Assessment of cytotoxicity

Lactate dehydrogenase LDH is an enzyme found in all living cells. High level of Lactate dehydrogenase (LDH) indicate tissue damage, Therefore LDH was used as an indicator for cytotoxicity. To examine the cytotoxicity of translation inhibitor we used different concentration. Cells were pre-incubated with 1, 10, 200, and 500 µg/ml cycloheximide and puromycin for 30 min. After that cells were stimulated with PMA for 3 h (same as other experiments) at 37°C. After the experiment supernatants were collected and activity of LDH in the medium was determined by using LDH cytotoxicity Clorometric Assay Kit II according to the manufacturer’s instructions. Percentage of toxicity was calculated using the following formula:

(Test sample-Low control)/ (High control-Low control). Absorbance in the samples without any drug was considered as a low control and absorbance from lysed samples was considered as a high control.

Viability assay

Cell viability was performed on neutrophil and assessed by using trypan blue dye exclusion assay. Transfected neutrophils were collected by low-speed centrifugation. Viability assay was performed by adding equal volume of samples and trypan blue dye. The percentage of viable cells were estimated by Burker chamber.

Histology

Lung samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated. After dehydration samples were embedded in paraffin and then cut into six µm sections and stained with hematoxylin and eosin. Lung injury was quantified in a blinded manner by using a modified scoring system, including alveolar collapse, thickness of alveolar septae, alveolar fibrin deposition, and neutrophil infiltration graded on a zero (absent) to four (extensive) scale. In each tissue sample, 5 random areas were scored, and the mean value was calculated. The histology score is the sum of all parameters.

Bacterial cultures

To evaluate the bacterial clearance, 24 hours after CLP Inferior vena cava blood was taken. Sample were diluted using a serial logarithmic manner. Blood samples were cultured on trypticase soy agar II with 5% sheep blood (Becton Dickinson GmbH, Heidelberg, Germany). Plates were incubated at 37 °C for 24 hrs in aerobic condition and colonies counted. The systemic bacterial load was presented as the number of CFU ($\times 10^5$) per ml of blood.

Quantitative real-time polymerase chain reaction (qRT-PCR)

For gene expression studies, we have used qRT-PCR. The total RNA of neutrophil from different experimental settings was isolated and followed by concentration and purity determination using the NanoDrop spectrophotometer at 260 nm absorbance. Reverse transcription was conducted using a (0.1-2.5 µg) of total RNA. QRT- PCR was conducted using SYBER Green dye. The mRNA reference sequence was used to design primers using web-based primer design tools of the national centre of biotechnology information. The primers sequences used in this study are listed in (Table 2). Relative expression to control house-keeping gene U6 and GAPDH were determined using $2^{-\Delta\Delta CT}$ methods.

MicroRNA transfection

Isolated neutrophils (5×10^6 cells) were seeded in a six-well plate in 2 ml of DMEM containing 10% FBS and 20 ng/ml recombinant mouse G-CSF. Cells were transfected after 3h with mmu-miR-155-5p mimic (50 nM), Ctrl-Mimic, antagomiR-155-5p (50 nM) or ctrl-antagomiR (50 nM) for 24 h in Opti-MEM reduced serum media according to manufacturer's instructions.Twenty-four hours post-transfection , cells were washed and lysed for RNA extraction or harvested for further experiments. Transfection efficiency was confirmed by quantitative real-time polymerase chain reaction (qRT-PCR).

Bioinformatics analysis of binding sites

Target prediction algorithm was done using RNAhybrid web-based bioinformatics. The binding site for miR-155-5p at the 3′-UTR of PAD4 mRNA was identified for experimental validation (http: bibiserv.techfak.uni-bielefeld.de/rnahybrid). However, based on evidence of AU-rich elements (AREs) it is known that miR-155-5p plays a critical role in the regulation of certain important proteins by binding at ARE sites of mRNA, specially AUUA and AUUUA motifs and therefore our analysis was limited to these sites. We confirmed the functional role of this binding site by designing a target site blocker (TSB, 22 nucleotides sequence) which binds selectively to a sequence overlapping with the miR-155-5p ARE sites in the 3′-UTR of PAD4 mRNA. The blocker was a fully phosphorothiolated Locked Nucleic Acids (LNA) in the DNA sequences to increase their affinity and selectivity for the target. The TSB_PAD4_miR-155-5p; 5′-TTAATTTTATTAAATATATAT-3′ and TSB

negative Control _PAD4_miR-155-5p; 5′-TAACACGTCTATACGCCCA-3′ were co-transfected with the miR-155-5p mimic in different concentration (12.5-50 nM) as described above. PAD mRNA levels were quantified using qRT-PCR. We validate the specific interaction between miR-155 and pad4 mRNA by using immunoprecipitation (RIP) assay.

RNA immunoprecipitation RIP assay

For experimental validation of miR-155p binding to PAD4 mRNA, RIP assay were performed to immune-precipitate Ago protein complex that contains functionally related miRNAs: mRNA complex using EZ-Magna RIP kit following the manufacture's protocol. Cells were scraped off 24 h after transfection with mmu-miR-155-5p Mimic, Ctrl-Mimic, antagomiR-155-5p or ctrl-antagomiR. Cells were then lysed in complete RIP lysis buffer containing protease inhibitor cocktail, after which 100 µl of whole cell extract was incubated with RIP buffer containing magnetic beads conjugated with an anti-Ago2 antibody or ctrl-IgG antibody and rotated for 3 h at 4°C. After several washes, samples were incubated with proteinase K with shaking to digest proteins at 55°C. RNA was then isolated, and concentrations were measured using a NanoDrop spectrophotometer. The co-immunoprecipitated (co-IP) RNA, including microRNA: mRNA complexes, were analyzed by qRT-PCR to measure the potential association between miR-155 and PAD4 mRNA.

Statistics

Results were expressed as mean values ± standard errors of means (SEM). Statistical evaluation for multiple comparism were performed using Kruskal-Wallis one-way analysis). For comparison of two groups Mann-Whitney rank-sum test was used. *P* values below 0.05 was considered statistically significant and n represent the number of animals or experiments in each group. Graphpad prism 8 software was used for data analysis.

Table 1. Histology scoring system

Alveolar space: Alveolar space were scored using medium power field 40X

Score	Definition
0	normal alveolar microarchitecture
1	occasional reduction of alveolar space
2	progressive reduction of alveolar space
3	diffuse of alveolar space
4	extensive destruction of tissue architecture

Thickness of the alveolar septa: The thickness of the alveolar septa were scored in oil emersion high power field

Score	Definition
0	thin alveolar septa
1	occasional thickening of alveolar septa
2	progressive thickening of alveolar septa
3	diffuse thickening of alveolar septa
4	massive thickening of alveolar septa

Fibrin deposition: The fibrin deposition within the alveolar space were scored in oil emersion high power field (HPF)

Score	Definition
0	absent of fibrin deposition within the alveolar space
1	occasional fibrin deposition within the alveolar space
2	progressive fibrin deposition within the alveolar space
3	diffuse fibrin deposition within the alveolar space
4	massive fibrin deposition within the alveolar space

PMN infiltration: Infiltrated PMN were counted in intestinal and intra alveolar space in high power field 100X (HPF)

Score	Definition
0	0-10 PMN cells
1	11-20 PMN cells
2	21-30 PMN cells
3	31-50 PMN cells
4	More than 50 PMN cells

Table 2. Primers sequence used for mRNAs chemokine receptors gene expression

Gene	Primers
PAD4	Forward 5′-TGTGACCCGAAAGCTCTA-3′ Reverse 5′-CTGCTGGAGTAACCGCTATT-3′
mmu-miR-155-5p	Forward 5′UUAAUGCUAAUUGUGAUAGGGGU-3′
ICAM-1	Forward 5′-AGCACCTCCCCACCTACTTT-3′, Reverse 5′-AGCTTGCACGACCCTTCTAA-3′
U6	Forward 5′-GCTTCGGCAGCACATATACTA-3′ Reverse 5′-CGAATTTGCGTGTCATCCTTG-3′
GAPDH	Forward 5′-CATGTTCGTCATGGGGTGAACCA-3′, Reverse 5′-AGTGATGGCATGGACTGTGGTCAT-3′.

Results and Discussion

Role of c-Alb kinase in regulating NET formation and inflammation in abdominal sepsis

C-Abelson (c-Alb) kinase is a protein tyrosine kinase that regulates the signalling process in various health and diseases (128, 129). Several studies have shown that c-Alb kinase is involved in controlling cell growth, survival, adhesion and migration (130-132). In addition, it has been shown that c-Alb kinase participates in vascular leakage in sepsis (102). However, the role of c-Alb kinase in NETs formation in abdominal sepsis remains elusive.

In the first paper, we demonstrated that c-Alb kinase plays a critical role in NETs formation in septic lung injury. Thus, targeting c-Alb kinase activity decreased systemic inflammation in sepsis. Suggesting that c-Alb kinase plays a critical role in the development of abdominal sepsis. We asked whether c-Alb kinase is activated in abdominal sepsis. We checked the expression of c-Alb phosphorylation in circulating neutrophils in CLP mice, we found that c-Alb kinase enhanced phosphorylation in circulating neutrophil, while treatment with GZD824, a specific c-Alb kinase inhibitor, markedly decreased c-Alb kinase activation in circulating neutrophils, 24 h after CLP induction, suggesting that GZD824 is an effective inhibitor of c-Alb kinase in vivo (Figure 1).

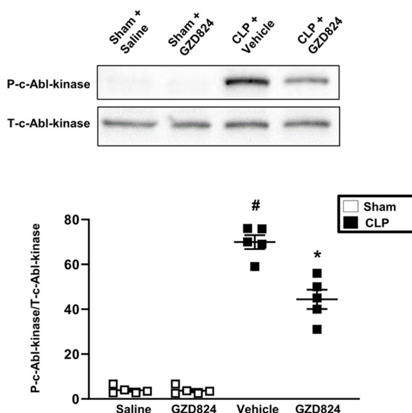

Figure 1. c-Abl kinase activity in neutrophils. Phosphorylation of c-Abl kinase in circulating neutrophils were examined by western blot as described in Materials and Methods. Animals were treated with GZD824 (5 mg/kg) or vehicle prior to CLP induction. Mice treated with saline (sham) or GZD824 alone without CLP. Samples were collected 24 hours after induction of CLP. Data are presented as mean values ± standard error of the mean (SEM) and n = 5. #P < 0.05 vs. Sham and *P < 0.05 vs. Vehicle + CLP.

Activated neutrophil expels neutrophil extracellular traps (NETs), composed of neutrophil-derived DNA forming extracellular web-like structures decorated with nuclear histones as well as granular and cytoplasmic proteins (16, 82). NETs is caused by uncontrolled inflammatory response during sepsis, which leads to the tissue damage (133, 134). However, mechanisms in regulating NETs expulsion in sepsis remain very elusive. Herein, we examined the role of c-Abl kinase in NETs formation and tissue damage in sepsis. We performed scanning electron microscopy and transmission immunoelectron microscopy to demonstrate the presence of NETs in the lung, we observed that CLP induced formation of extracellular fibrillary and web-like structure compatible with NETs (Figure 2A). In addition, transmission immunoelectron microscopy showed that neutrophil-derived granule protein elastase and citrullinated histone H3 co-localized with these extra fibrillary and web like structures (Figure 2B), while in the normal lung not observed. Interestingly, GZD824 greatly reduced generation of NETs in the septic lung (Figure 2 A, B). To further study the role of c-Abl kinase in NETs generation and whether directly regulates NET formation in neutrophil, we examined the level of DNA-histone complex in plasma and in neutrophils isolated from bone marrow which were stimulated with β-glucan, the results show that administration of GZD824 reduced NET formation in plasma and stimulated neutrophil (Figure 2 C,D). Upon activation neutrophil produce reactive oxygen species (135), which is involved in NETs formation (82, 136), it is interesting to know that c-Abl kinase has been implicated in the neutrophil formation of ROS (103). One study has shown that induction of ROS is pivotal in β-glucan-induced NET formation in neutrophil (137). In a separate in vitro study, flow cytometry used to detect ROS generation in neutrophils were incubated with dihyrorhodamine 123 for 15 min and then stimulated with 50 µg/ml β-glucan for 20 min 37°C. Co-incubation of neutrophil with GZD824 decreased β-glucan-provoked generation of ROS in neutrophils. Thus, c-Abl kinase-dependent generation of ROS might be involved in β-glucan induced formation of NETs in neutrophils.

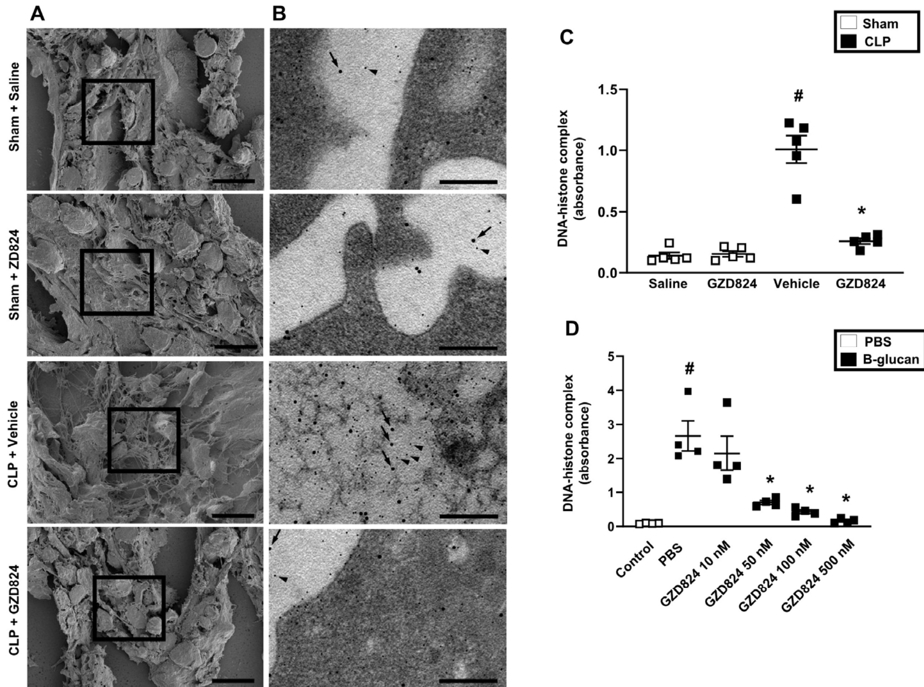

Figure 2. NET formation in sepsis. A) Scanning electron microscopy showing extracellular web-like structures in the lung from CLP mice. Scale bar = 5 µm. B) Transmission electron microscopy of the indicated area of interest from Figure 2A. Scale bar = 0.25 µm. All images are representative of five independent experiments. C) DNA-histone complex formation. Animals were treated with GZD824 (5 mg/kg) or vehicle prior to CLP induction. Mice treated with saline (sham) or GZD824 alone without CLP. Samples were collected 24 hours after induction of CLP. D) DNA-histone complexes in the supernatant determined by ELISA. NETs were generated from isolated neutrophils by β-glucan-stimulation, co-incubated with or without GZD824 (50 µg/ml). Non-stimulated neutrophils served as a control. Data are presented as mean values ± standard error of the mean (SEM) and n = 5. #P < 0.05 vs. Sham and *P < 0.05 vs. Vehicle + CLP.

Since neutrophil infiltration consider as a key factor of sepsis-induced lung injury (75) we tested whether c-Abl kinase affects neutrophils infiltration in the lung, the result shows that blocking c-Abl kinase decreases total PMNL in the BALF (Fig 3A) and CXCL1 CXCL2 lung (Figure 3B, C). Additionally, blocking c-Abl kinase decreased sepsis-induced edema in the lung (Figure 3D). In histological analysis, we observed that CLP induced pulmonary injury, characterized by destruction of tissue microarchitecture, edema of interstitial tissue, and neutrophil infiltration, which were minimizing by administration of GZD824 (Figure 3E-H). These finding

are in line with the observations that CLP-evoked lung damage was reduced in GZD824-treated animals.

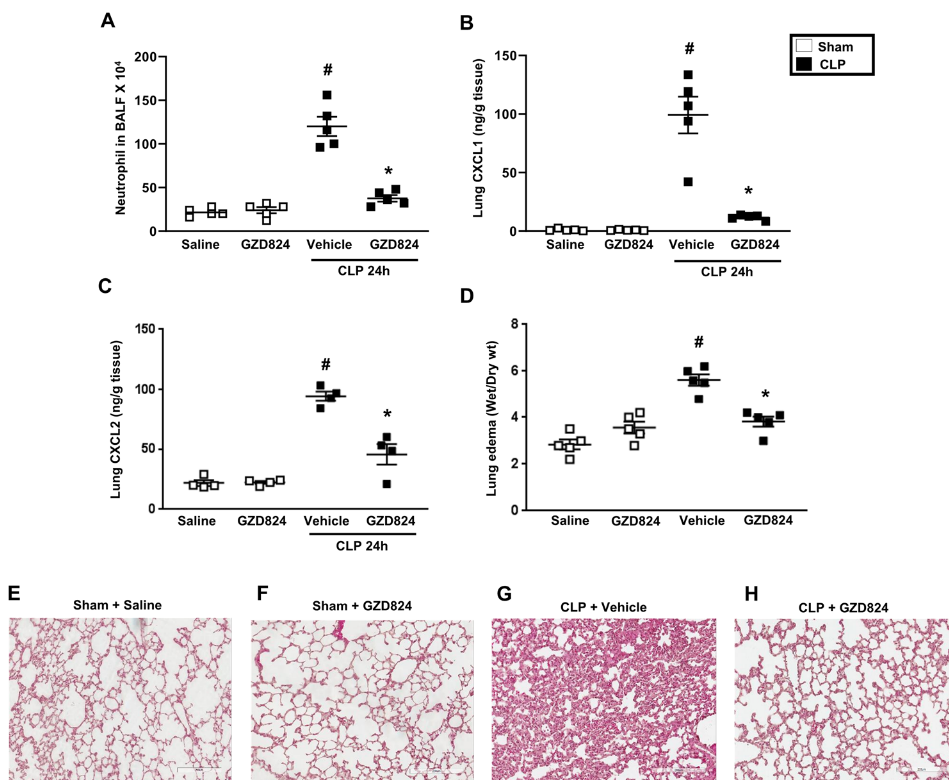

Figure 3. c-Abl kinase regulates CLP-induced infiltration of neutrophils in the lung. A) number of BALF neutrophils were determined 24 h after CLP induction. Pulmonary levels of B) CXCL1 and C) CXCL2. D) Level of edema. Representative haematoxylin & eosin sections of the lung. E) Animals were treated with saline or F) GZD824 alone. G) vehicle or H) treated mice. Data are presented as mean values ± standard error of the mean (SEM) and n = 5. #P < 0.05 vs. Sham and *P < 0.05 vs. Vehicle + CLP.

Another marker for systemic inflammation is IL-6 which is an important pro-inflammatory cytokine. Several studies reported that IL-6 is an important mediator which markedly increased during sepsis (138-145). Herein, we show that inhibition of c-Abl kinase activity reduced plasma level of IL-6 in septic mice (Figure 4). These results may support the concept that inhibition of c-Abl kinase reduce systemic inflammation in sepsis. Our finding indicates the role of c-Abl kinase in

NET generation in neutrophil and in septic lung injury. Targeting c-Abl kinase activity reduced pulmonary formation of CXC chemokines, neutrophil recruitment and tissue damage in lung. Finally, blocking c-Abl kinase activity decreased systemic inflammation. Thus, this study not only delineates a novel signalling mechanism regulating NET formation in sepsis but also suggests that blocking c-Abl kinase might be a useful strategy to ameliorate local and systemic inflammation in sepsis.

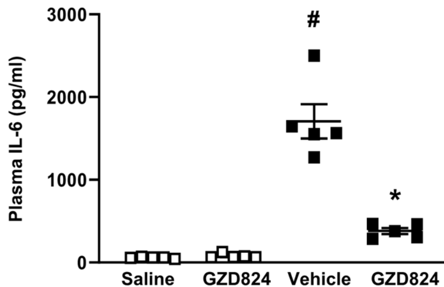

Figure 4. Plasma levels of IL-6. Animals were treated with GZD824 (5 mg/kg) or vehicle prior to CLP induction. Mice treated with PBS (sham) or GZD824 alone without CLP. Data are presented as mean values ± standard error of the mean (SEM) and n = 5. #P < 0.05 vs. Sham and *P < 0.05 vs. Vehicle + CLP.

Role of miR-155 in regulating NET formation and inflammation

MicroRNAs are a small non coding RNA 21-25 nucleotides, which are involved in immune regulation by regulate gene expression in transcriptional and posttranscriptional levels (146). miR-155 is a multifunctional microRNA, like other miRNAs regulates gene expression and has a critical role in various pathological processes such as inflammation, immunity and viral infection (147-151). Many studies have demonstrated that several miRNA like miR-223, miR-142, and miR-451 regulate neutrophil functions (152), and one study reported that miR-146 regulates NETs formation (110). miR-155 plays an important role in the immune response (153). It has been shown that expression of miR-55 inversely regulates neutrophil migration (111), however, the effect of miR-55 in NETs generation is unknown.

Based on these considerations we hypothesized that miR-55 might control NETs generation via regulating PAD4 function. In our experiment, three different methods were used to quantify NETs including DNA-histone complex assay, confocal

microscopy, and the surface expression of MPO and citrullinated histone H3 by flow cytometry. Herein, we showed for the first time that protein translation involved in NETosis. Pre-incubation of neutrophils with translational inhibitors cyclohexamide or puromycin 1 and 10 µg/ml for 30 min markedly decreased PMA-induced generation of NETs (Figure 5B), while pre-incubation with 10 µg/ml of translational inhibitors for 5 min had no effect (Figure 5B). In separate experiment, we found that translational inhibitors have the same effect in neutrophils exposed to MIP-2. Citrullinated histone H3 is a biomarker for NETs formation (154, 155). We examined the expression of MPO and citrullinated H3 on neutrophils using flow cytometry after stimulation with PMA for 24 hrs, we found that of MPO and citrullinated H3 is unregulated (Figure 5 C, D), and pre-incubation with 10 µ/ml translational inhibitors for 30 min reduced PMA and MIP-2 induced expression of MPO and citrullinated H3 on neutrophils.

Transfection process has no effect on cell viability after 24 h transfection. In addition to flow cytometry, confocal microscopy were used to detect MPO and histone H3, we observed that the MPO and citrullinated histone H3 increased in PMA stimulated neutrophil, while decreased in neutrophils stimulated with PMA pre-incubated with 10 µg/ml of cycloheximide or puromycin for 30 min. The finding of our study are inconsistent with three other studies which they showed that protein translation is not involved in the NETs generation (93, 156, 157), which could be because a difference in methodology. Use of Sytox Green and orange as DNA binding dyes to measure NETs are fraught with significant drawbacks. Sytox green is a cell permeable dye and generates non-specific false positive fluorescence signal with respect to NETs generation as described by manufacturer (157), using just 100 nM of Sytox Green which is a 10-50 time lower concentration than those used in other studies (156), could be rapid and dose-dependent leakage into living cells. One study reported that actinomycin D had no effect on NET formation (156), while Khan et al found the effect of actinomycin D on NETs formation (158), which means opposite conclusion about role of transcription in NET formation. Moreover, pre-incubation of neutrophil with cyclohexamide for 5 min. and stimulation with PMA for 15 hours (156), considering with our results 5 min is too short time for translation inhibitors and 15 hours stimulation is too long time for stimulation, which might increase non-specific fluorescence signal. In this context, it has been shown that cycloheximide reduce endotoxin-induced deamination of histone H3 (93), which is consider as an important component in the process of NETs release.

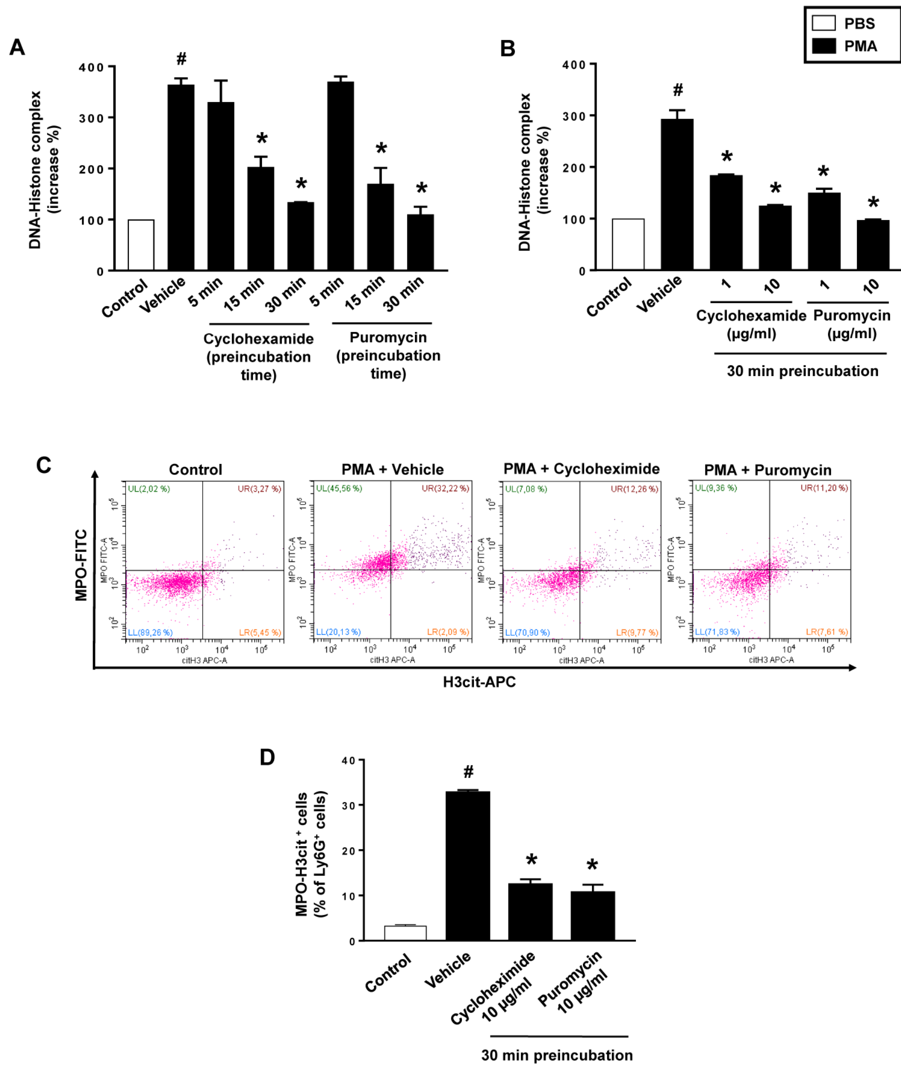

Figure 5. NET formation in neutrophils. (A) Neutrophils were preincubated with 10 µg/ml of cycloheximide or puromycin for 5–30 min and stimulated with PMA (500 nM) for 3 h at 37°C. (B) Neutrophils were preincubated with indicated concentrations of cycloheximide or puromycin for 30 min and stimulated with PMA (500 nM). DNA-histone complexes were quantified in supernatants by ELISA. (C) Levels of citrullinated Histone H3 and MPO in neutrophils (Ly6G+ cells) were determined by FACS. Neutrophils were preincubated with 10 µg/ml of cycloheximide or puromycin for 30 min and stimulated with PMA (500 nM). (D) Aggregate data of flow cytometry. Data represent mean ± SEM and n = 5. #P < 0.05 vs. control and *P < 0.05 vs. vehicle.

PAD4 is essential in regulating NET formation (159). Interestingly we found that the level of PAD4 and mRNA increased in PMA-stimulated neutrophil. Therefore, we asked whether miR-155 regulate PAD4 expression in neutrophils, it was found that a mimic miR-155 transfected neutrophil increased PAD4 mRNA, while antagomiR-155 transfected neutrophils decreased PAD4 mRNA. In addition, transfection with mimic miR-155 or antagomiR-155 increased and decreased the level of DNA-histone complex, respectively as expected (Figure 6A, B). Moreover, the level of PAD4 protein and histone H3 were quantified by western blot analysis, we observed that transfection with mimic miR-155 had no effect on PAD4 and citrullinated histone H3 protein level in PMA stimulated neutrophil, while transfection with antagomiR markedly decreased the protein level of PAD4 and citrullinated histone H3 in PMA stimulated neutrophil, suggesting that miR-155 an important regulator of PAD4 mRNA. In addition, recently it has been shown that miRNAs are involved in NETs generation, that the level of cell-free DNA and citrullinated histone 3 increased in the neutrophils of miR146 gene-deficient mice (110).

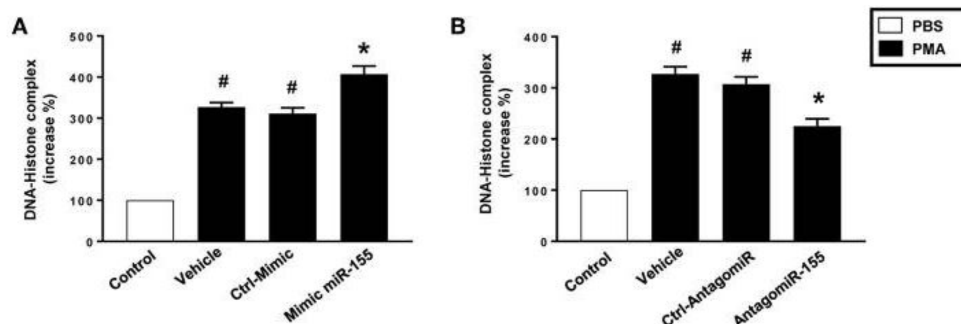

Figure 6. Levels of DNA histone complexes in neutrophils transfected with (A) Ctrl Mimic or mir-155 mimic and (B) Ctrl-antagomiR or antagomiR-155-5p. Data are expressed as mean ± SEM and n = 4–5 and represented as fold change. #P < 0.05 vs. control and *P < 0.05 vs. vehicle.

3′-UTR region of mRNA involved in positive regulation mRNA translation by miRs (147, 160, 161) RNA hybrid web-based bioinformatics target prediction algorithm (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid) were performed to know if PAD4 is a direct target of miR-155. Herein, we focused in AU-rich elements in the 3′-UTR of mRNA, which plays important role in regulation and translation of mRNA (162-164), we found AREs binding site complementary to the seeding region of miR-155. Therefore, we have designed specific blocker targeting this site

by transfecting neutrophil with multiple doses of TSB. Interestingly, we found that transfection with specific blocker reduced mimic miR-155 induced expression of PAD4, indicating that this specific ARE region of 3′-UTR of PAD4 mRNA is a functional miR-155 target. Thus, this study identifies a new target site regulating translational activation of PAD4 mRNA by miR-155. These results are in line with another study reporting the role of miR-155 on RhoA gene expression and migration of colon cancer cells (165). It should be noted that miRs might act on multiple gene targets. Thus, RIP assay was performed to examine the potential association between miR-155 and PAD4, we found that neutrophils were enriched with PAD4 mRNA and miR-55, in the Ago2-containing miRNAs relative to control IgG immunoprecipitates (Figure 7 A, B). Altogether, these novel findings show that miR-155 regulates NETs formation via positive regulation of PAD4. However, existing evidence showed that AREs in the 3′-UTR of PAD4 mRNA plays a central role in mediating miR-155 induced PAD4 gene expression. These results could be potential strategies to prevent NET-dependent tissue damage in acute inflammation.

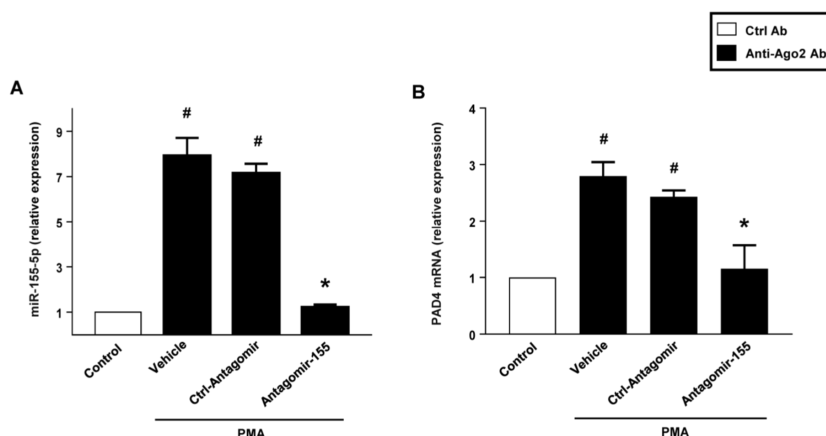

Figure 7. Neutrophils were transfected with ctrl-antagomiR, antagomiR-155-5p and then stimulated with or without PMA (500 nM). The amount of miR-155-5p and PAD4 mRNA were determined in input RNA by qRT-PCR in RIP assays. AntagomiR-155-5p decreased relative enrichment of (A) miR-155-5p and (B) PAD4 mRNA in Ago2 immunoprecipitates. Data are presented as fold change compared to anti-IgG ctrl. U6 and GAPDH were used as a house-keeping gene to normalize miR-155-5p and PAD4 expression, respectively. Relative expression was determined using $2^{-\Delta\Delta CT}$ method. Data are expressed as mean ± SEM and n = 5. #P < 0.05 vs. control Ab and *P < 0.05 vs. anti-Ago2 Ab-Ctrl-antagomiR treated cells.

Role of miR-155 in regulating NET formation and lung injury in abdominal sepsis

Sepsis is a systemic inflammatory response syndrome caused by an uncontrolled host response to the infection (166). Neutrophils play an essential role in immune system and consider as a first line of immune defence against pathogen (167). More recently, it was reported that neutrophil can expel a chromatin structure called neutrophil extracellular traps (168), which is associated with several pathological conditions, and also mediated tissue damage in abdominal sepsis (169). miRNA play essential role in biological process by regulating mRNA expression (170, 171). miR-155 is one of the most important miRNA which involved in several immunological disease (172-174) and inflammation (175, 176). Since we have shown in our previous study that miR-155 regulates NET formation in vitro. Thus, we presume that miR-155 may regulate systemic inflammation and pulmonary injury in abdominal sepsis via regulating NET formation. Therefore, we used miR-155 deficient mice to study the role of miR-155 in NETs generation. First, we

wanted to investigate the role of miR-155 in regulating NET formation in neutrophil, we examine DNA histone complex, citrullinated histone H3 and neutrophil derived granule protein MPO in MIP2 stimulated neutrophil which markedly decreased in miR-155 gene-deficient neutrophils. By now, we were encouraged to study the role of miR-155 in regulating NET in vivo. Abdominal sepsis was induced in wild-type C57BL/6 and miR-155 gene-deficient mice by cecal ligation and puncture. NETs have been visualized by scanning electron microscopy and transmission electron microscopy. We observed that CLP-induced generation of NETs in the lung significantly decreased in miR-155 gene-deficient compare to the wild-type mice. We confirmed this finding by measuring DNA-histone complex, which were decreased in mice lacking miR-155 (Figure 8).

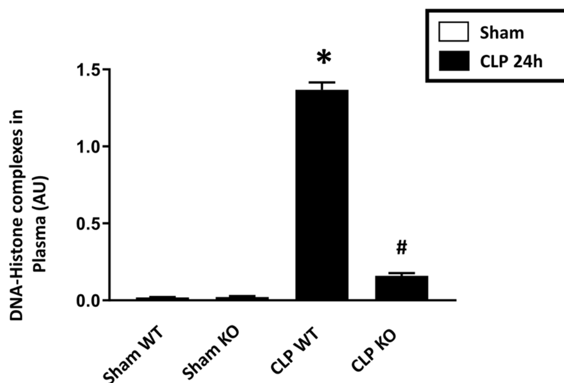

Figure 8. NET formation in sepsis. A) DNA-histone complexes in the plasma from wild-type and miR-155 gene-deficient mice. Samples were collected 24 hours after induction of CLP. Data are presented as mean ± SEM and $n = 5$. $^{\#}P < 0.05$ *vs*. WT Sham and $^{*}P < 0.05$ *vs.* WT CLP.

PAD4 is indispensable in NETs formation (177) and mediated chromatin decondensation, which converts histone arginine to citrulline (91, 92). PAD4 is more expressed in neutrophils (97), and it has been shown that PAD4 regulates NET formation (98). PAD4 and citrullinated histone 3 expression were analysed by western blot. We detected a significant decrease of PAD4 and citrullinated histone 3 in septic mice lacking miR-155 vs septic wild-type mice. This result is in line with our previous study (178), showing that miR-155 regulate NET formation .via PAD4 mRNA. Herein, we observed that PAD4 and citrullinated H3 decreased in animals lacking miR-155, suggesting that miR-155 regulates histone citrullination is abdominal sepsis.

Misdirected neutrophil results in tissue injury and organ failure (179). Thus, we asked whether miR-155 is involved in CLP-provoked pulmonary injury. Morphological analysis demonstrated that CLP-induced severe pulmonary damage in wild type mice, characterized by alteration of microarchitecture of the lung massive necrosis, capillary congestion, alveolar space collapse and excessive neutrophils infiltration (Figure 9C), while significantly decreased in miR-155 gene-deficient (Figure 9D). We also observed that CLP-induced lung edema decreased in miR-155 gene deficient mice, indicating that miR-155 mediates septic lung injury.

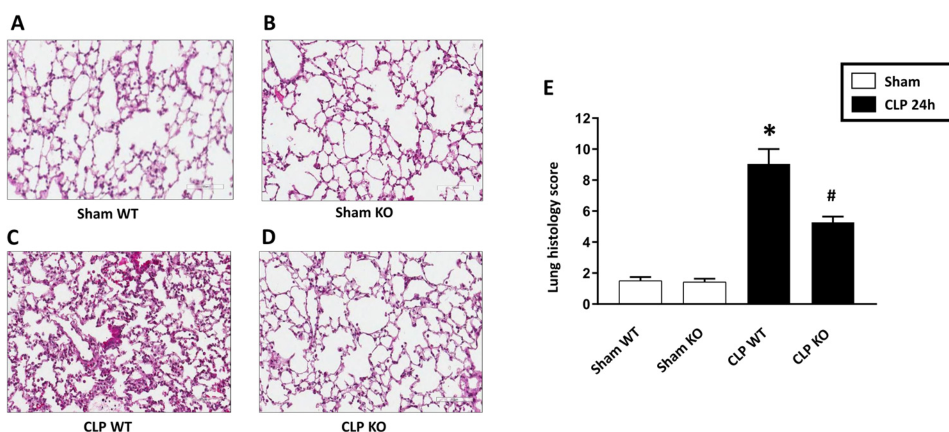

Figure 9. Lung injury. A-D) Representative haematoxylin & eosin sections of the lung from wild-type (WT) and miR-155 gene-deficient mice. Samples were collected 24 hours after induction of CLP. E) Aggregate data on lung injury score. Data are presented as mean ± SEM and $n = 5$. $^{\#}P < 0.05$ *vs.* WT Sham and $^{*}P < 0.05$ *vs.* WT CLP.

Neutrophil infiltration is another marker in the pathophysiology of septic lung injury (180, 181). Number of neutrophils in BALF were determined to investigate neutrophil accumulation in the lung. Number of BALF neutrophils were increased in CLP induced mice and decreased in miR-155 deficient mice, suggesting that miR-155 involved in pulmonary neutrophil in sepsis.

CXC chemokines including KC and MIP-2 chemokines secretion increased in the plasma and lung during sepsis (182), and they mediate neutrophil trafficking to the sites of inflammation (183-186). As expected, plasma and pulmonary level of KC and MIP-2 in wild-type septic mice was high (Figure 10A-D), and greatly reduced in septic mice lacking miR-155 (Figure 10A-D), indicating that miR-155 regulates CXC chemokine formation in the blood and lungs in sepsis, which helps to explain how miR-155 effects on neutrophil infiltration in septic lung damage.

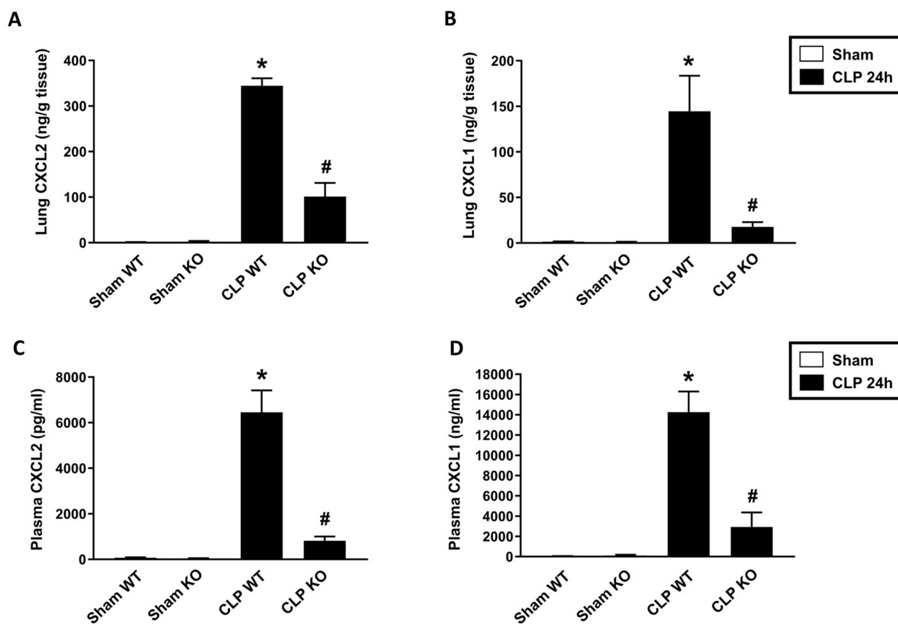

Figure 10. Chemokine formation in sepsis. Pulmonary A-B) and plasma C-D) levels of CXCL1 and CXCL2 from wild-type (WT) and miR-155 gene-deficient mice. Samples were collected 24 hours after induction of CLP. Data are presented as mean ± SEM and n = 5. #P < 0.05 vs. WT Sham and *P < 0.05 vs. WT CLP.

Mac-1 play important role in adhesion and accumulation of neutrophil in the lung (187, 188). The surface expression of Mac-1 on neutrophil changes upon activation from low avidity to the high avidity. Flow cytometry was used to measure the surface expression of Mac-1 on neutrophil. We found that Mac-1 expression increased in CLP animals, while markedly decreased in miR-155 deficient mice.

Indicating that miR-155 regulates neutrophil activation and Mac-1 expression in abdominal sepsis.

In summary, the current study shows the role of miR-155 in regulating pulmonary formation of NETs in abdominal sepsis. Lack of miR-155 decreased NETs generation and accumulation of neutrophils in septic lungs as well as decreased damage to the lung tissue. Thus, targeting miR-155 might be a useful target to reduce pulmonary damage in abdominal sepsis.

Role of NET-MP complexes in neutrophil recruitment

Herein, we examine the role of NETs-MPs aggregates in leukocyte recruitment in vivo. We studied leukocyte-endothelium interactions by use of intravital microscopy in the mouse cremaster microcirculation. The preparation of cremaster muscle was performed on a transparent pedestal allowing transillumination and microscopic observations of the cremaster muscle microcirculation after a 10-min equilibration time. In the present study, we found that the intrascrotal injection of NET-MP aggregates dose-dependently (0.5-4.5 µg) increase in firm leukocyte adhesion (Figure 11A and D). In contrast, NETs reduced leukocyte rolling velocity, while the number of rolling leukocytes did not increase. Additionally, we studied the number of extravascular leukocytes, we found that 1.5 and 4.5 µg NETs increased leukocyte migration. Thus, we selected 1.5 µg NETs for further studies. We next asked whether TNF-α has the same effect. Similar to NETs, it was found that TNF-α stimulation has no effect on venular leukocyte rolling (Figure 11B), while the leukocyte rolling velocity reduced (Figure 11C), which is mediated by up-regulation of endothelial adhesion molecules (189), as well as leukocyte adhesion and emigration.

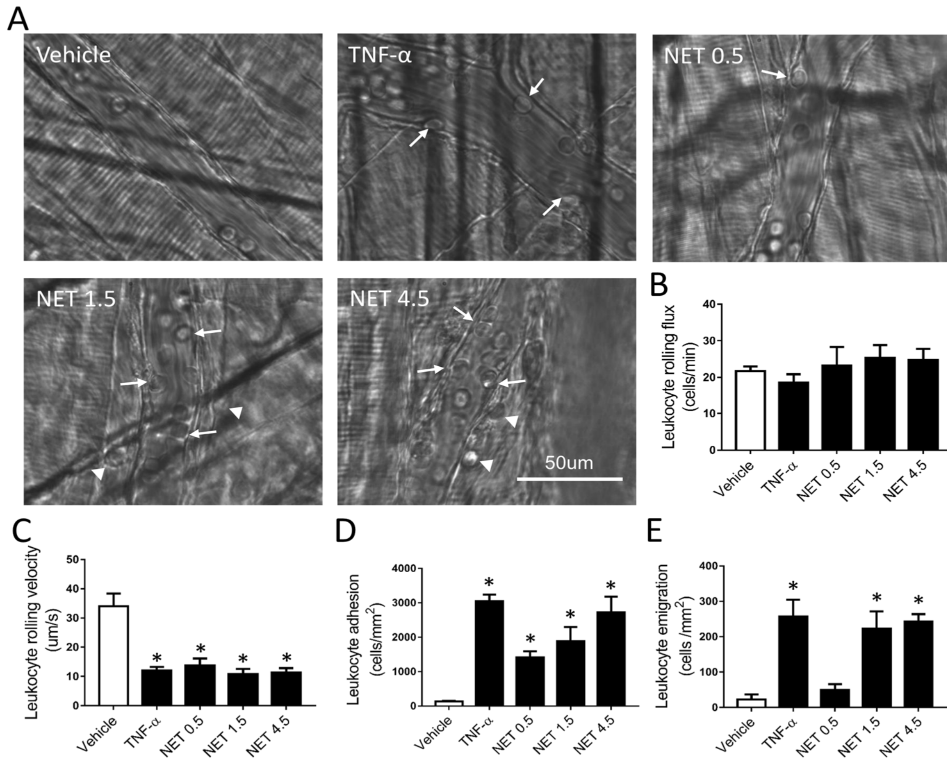

Figure 11. NET-induced leukocyte recruitment in vivo. A) Intravital images of the cremaster microvasculature in response to vehicle, TNF-α, NET 0.5, 1.5 and 4.5. Leukocyte B) rolling flux C) rolling velocity D) adhesion and E) emigration in mouse cremaster muscle 3 hours after intrascrotal challenge with vehicle, TNF-α, NET 0.5, 1.5 and 4.5. Data are mean ± SEM and n = 5. *P < 0.05 vs. Vehicle.

In this context, we wanted to know which leukocyte is responding to challenge with NETs. By using antibody against Ly-6G on neutrophil, we found that the numbers of neutrophil decreased by 99%. Interestingly, depletion of neutrophil abolished NET-induced leukocyte rolling, adhesion and migration, suggesting that neutrophils are the main leukocyte sub-type responding to NET challenge.

It has been shown in our experiment that pre-treatment with an antibody directed against P-selectin reduced NET-induced neutrophil rolling, firm adhesion as well as emigration. In addition, immunoneutralization of PSGL-1 decreased NET-evoked neutrophil rolling, adhesion and emigration. Next, we wanted to investigate the role of β2-integrins in NET-triggered neutrophil accumulation, we found that administration of the antibody against Mac-1 reduced the number of firmly adherent and emigrated neutrophils in NET exposed tissues. Moreover, we found that

blocking LFA-1 had no effect on rolling, while NET-evoked neutrophil adhesion and extravasation decreased, suggesting that both these molecules involved in extravasation of neutrophils in NET-provoked inflammation. Our findings are further supported by other studies showing that LFA-1 and Mac-1 mediates leukocyte adhesion while having no effect on leukocyte rolling (190-196). During neutrophil activation, MPs form and bind to NETs forming NET-MP complex, which play important role in thrombin generation in sepsis (113). For NETs generation without microparticles, we pre-treated neutrophil with caspase and calpain inhibitors which resulted in formation of NETs depleted of MPs (113). Electron microscopy were used to confirm that co-incubation of stimulated neutrophil with caspase and calpain inhibitors resulting of NETs formation without MPs. In addition, we observed that, NETs without MPs attenuated neutrophil adhesion and emigration, and this reduction was similar as in DNase treatment, indicating that MPs attached to NETs have important role in NET-induced neutrophil recruitment. Thus, considering our finding that NET-MP complex are a potent inducer of neutrophils recruitment and might have effect on several neutrophil-dependent diseases, such as sepsis as well as acute pancreatitis (17, 85). HMGB1 is a ubiquitous protein present in both nuclei and cytoplasm of various cells like neutrophils, macrophage, and monocytes (197, 198), which releases to the extracellular space upon cell injury and acts as pro-inflammatory cytokine. HMGB1 involved in sepsis and causes organ failure (199-202). Then we asked whether NET-MPs contain HMGB1. By use of transmission electron microscopy, we found that NET-MPs express HMGB1. Moreover, we found that immunoneutralization of HMGB1 reduced adhesion and emigration of NET-induced neutrophil in vivo. Thus, we indicate that HMGB1 play an essential role in pro-inflammatory effects on NET-MP aggregates. TLR2, TLR4 and RAGE consider the main receptors of HMGB1 (203). Herein, we showed that inhibition of TLR2 and TLR4 markedly decreased the number of adherent neutrophils in response to NET-MP aggregate challenge, which indicate that both TLR2 and TLR4 signalling mediated NET-triggered neutrophil recruitment. This result is supported by another experiment showed that both TLR2 and TLR4 are critical for HMGB1-induced accumulation of stem cells along microvascular endothelial cells in vivo (204). Taken together, our findings clearly showed that NET-induced neutrophil recruitment is dependent on neutrophil-derived MPs expressing HMGB1 and mediated via TLR2 and TLR4 signalling in vivo. Blocking MP binding to NET or inhibition of HMGB1-TLR2/TLR4 axis might provide useful targets to reduce NET-dependent tissue damage in acute inflammation.

General discussion and future perspectives

Abdominal sepsis is an inflammatory disease that is associated with high morbidity and mortality. Specific treatment is currently lacking in part due to an incomplete knowledge of the mechanisms. Because of the complexity of the disease process, the treatment of abdominal sepsis is limited to antibiotics and supportive care. Emergence of antibiotic resistance bacterial also put a challenge on existing sepsis management practices. There are some specific treatments for specific population of the septic patients such as recombinant activated protein C, low dose corticosteroid, intensive insulin therapy, however, each of this therapy fail to improve survival rate more than 10% (205). When bacteria leak into the bloodstream, a systemic inflammation is induced which among other things, leads to inflammation and multiple organ failure importantly lung failure. Neutrophils are a type of white blood cell that have been shown to play a central role in the development of septic lung damage. As part of defense mechanism of the body, neutrophils secrete oxygen radicals and enzymes that cause pathological inflammation in the organs. Recently, it has been shown that neutrophils can also release so-called neutrophil extracellular traps (NETs) and aggravate the overall inflammation (19). Several studies have shown that the numbers of MPs increase during inflammation (114, 119), suggesting that they might play important role in disease pathogenesis. The overall purpose of the current dissertation is to study how NETs are formed in abdominal sepsis and its significance in inflammation and lung damage.

To study the mechanisms of NETs formation and role of NETs-MPs in abdominal sepsis, we used a surgical technique called cecal ligation and Puncture (CLP). In this model, cecum is first ligated with a suture and then punctured twice to release intestinal contents into the abdominal cavity. Fecal bacteria stimulates local immune cells and release various inflammatory substances that in turn released into the circulation In addition, bacteria themselves can penetrate gut-blood barrier and initiate systemic inflammation (206). There are several experimental sepsis models such as endotoxemia and bacteremia models, colon ascendens stent peritonitis (CASP) model and CLP model, of which CLP is considered as gold standard because this model closely mimic the pathophysiology of intra-abdominal clinical sepsis in terms of early hyperdynamic phase and late hypodynamic phase of sepsis

(207). The major challenge in this model is the consistency or reproducibility. One paper described detail procedures to standardize the CLP method in which they described how length of ligated cecum, puncture size and fluid resuscitation could determine the grade and reproducibility of sepsis (208). Over the years, many different animal models of sepsis are used including non-human primates model. The translation of preclinical results from animal to human sepsis remains as a major challenge. There could be many different reasons, but one possible reason could be heterogeneity of septic populations in the emergency room of the clinic. Another possible reason could be rapid shifting of animal research to human trials without evaluating wide range of models. Mouse is one of the most widely used animal model for septic research. Mouse and human share 95% of protein coding genes (highly conserved), but non-coding genes and regulatory regions are less conserved. This notion underlines the importance of confirming our findings in other animal models, possibly to higher animals before translating laboratory results to clinical setup.

It has been established that neutrophil extracellular traps (NETs) involved in the body's defense against pathogen by trapping microorganism, but also the excessive formation of NETs linked to numerus disease and contribute to the pathology of systemic inflammation. Thus, we believe that understanding the mechanism and targeting NET formation would be interesting strategy for such pathological conditions. In this dissertation, we showed that how c-Abl kinase regulates NETs formation and lung injury in abdominal sepsis. Our results indicate that targeting c-Abl kinase could be a useful strategy to inflammation and tissue damage in sepsis. We further dug into the mechanisms of NETs formation and our results show microRNA-155 plays a central role in the formation of NETs that also appears to be dependent on de novo protein synthesis. Inhibition of c-Abl kinase activity and inhibition of PAD4 function by microRNA-155 could be effective therapeutic strategy for the treatment of septic lung injury. In addition, our work shows that NETs form aggregates with MPs and they are functional in their ability to facilitate neutrophil recruitment in acute inflammation. NET-MP-induced neutrophil accumulation appears to be a result of HMGB1-TLR2/TLR4 signaling and blocking of this axis could also be a relevant target for the treatment of patients with abdominal sepsis. Our studies show the regulation and function of NETs, as well as the role of NETs in the pathogenesis of sepsis. Taken together, all results in this study have shown that controlling NETs might be useful to counteract the infectious disease.

Many successful preclinical anti-inflammatory and anti-coagulant therapies failed to show any promising results in the randomized human trials (209, 210). Sepsis usually manifest itself into many different processes within very short term, thus making the interventions difficult, indicating that much more preclinical research is required. The role of c-abl kinase signaling and translation of PAD4 in NETs formation has pointed out that several signaling pathways are at interplay in the development of abdominal sepsis. The interplay among other immune cells, microvascular of the organs and the coagulation system is beyond the scope of this thesis. Nevertheless, it should be noted that certain adhesion molecules expressed on the endothelial cells play a critical role in neutrophils adhesion and transmigration to the site of infection. Platelets are another important immune cell, which is capable of activating neutrophils directly and indirectly. Accumulating data also suggest that platelets can recruit neutrophil to the site of infection and facilitate NETs formation in various inflammatory diseases. Thus, it could be speculated that other host cells could be involved in NETs generation and NETs mediated tissue injury in abdominal sepsis. It should be noted that extensive clinical studies are warranted to confirm these findings in humans.

In a placebo-controlled human clinical trials, use of aerosolized recombinant DNase enzyme (Pulmozyme) showed promising results for the treatment for cystic fibrosis. DNase treatment effectively reduced viscoelasticity of neutrophil released DNA with improving pulmonary function and well-being of patients in phase 3 trials (211). During the current coronavirus disease 2019 (COVID-19) pandemic, one study has shown that patients with COVID-19 have elevated levels of NETs marker such as myeloperoxidase-DNA and citrullinated histone H3 in the serum (212). While another study proved the presence of NETs in the lung specimens of deceased COVID-19 patients (213). Thus, considering the fact of NETs presence in the lung and complications similar to sepsis, the concept of DNase treatment could also be extended to extremely sick COVID-19 patients. It should be kept in mind that DNase treatment could reduce bacteria killing ability of host immune system. Hence, in future treating septic patients with a combination of DNase and antibiotics could be considered as a useful treatment strategy to reduce pulmonary NET contents and improve lung function. In conclusion, mechanisms revealed in this dissertation open up the possibility of future therapies in abdominal sepsis.

Conclusion

Paper I

c-Abl kinase plays a significant role in NET generation in neutrophils and in septic lung injury. Targeting c-Abl kinase might be an effective way to ameliorate lung damage in abdominal sepsis.

Paper II

MiR-155 plays a key role in regulating NET formation by positive regulation of neutrophil expression of PAD4 via a specific ARE element in the 3′-UTR region of PAD4 mRNA. Thus, targeting miR-155 could be useful strategy to control NET formation in inflammatory diseases.

Paper III

MiR-155 plays a key role in regulating pulmonary formation of NETs in abdominal sepsis, thus targeting miR-155 could be a useful target to reduce pulmonary damage in abdominal sepsis.

Paper IV

MPs attached to NETs play a key role in acute inflammatory disease. Blocking MP binding to the NET or inhibition of the HMGB1-TLR2/TLR4 axis might be an effective way to attenuate NET-dependent tissue damage.

Populärvetenskaplig sammanfattning på Svenska

Buksepsis är en inflammatorisk sjukdom som är associerad med associerad med hör morbiditet och mortalitet. Behandlingen av buksepsis är begränsad till antibiotika och stödjande behandling. Specifik behandling saknas idag delvis beroende på en ofullständig kunskap om de mekanismer som orsakar och är viktiga för hur det går för patienter med buksepsis. När bakterier läcker ut i blodbanan orsakas en systemisk inflammation som bland annat leder till en inflammation och organskada i flera organ av vilka lungorna utgör det viktigaste. Neutrofila granulocyter är en typ av vita blodkroppar som har visat sig spela en central betydelse för utvecklingen av septisk lungskada. Neutrofiler utsöndrar syreradikaler och enzymer som orsakar patologisk inflammation i lungan. På senare tid har det visat sig att neutrofiler också kan bilda och utsöndra så kallade neutrofila extracellulära fällor (neutrophil extracellular traps, NETs). Det övergripande syftet med den aktuella avhandlingen var att studera hur NETs bildas vid buksepsis och dess betydelse för inflammation och skada i lungorna.

Delarbete 1. Detta arbete var fokuserat på intracellulära signalvägar för regleringen av NET syntes. C-Abl kinase är ett enzym som finns i alla celler och det har visat sig att c-Abl kinas kan reglera bildningen av syreradikaler i neutrofiler. Syreradikaler är viktigast för bildningen av NETs och målsättningen blev därför att studera betydelsen av c-Abl kinas vid buksepsis med fokus på NET bildning. Först observerades att c-Abl kinas fosforylerades (aktiverades) och att GZD824 effektivt inhiberade forsforylering av c-Abl kinas i neutrofiler. Inhibition av c-Abl kinas aktivering minskade bildningen av NETs i blodet och i lungorna vid sepsis. Dessutom kunde det konstateras att GZD824 minskade ackumulering av neutrofiler i lungorna och att bildningen pro-inflammatoriska ämnen (kemokiner) minskade i lungorna. Behandling med GZD484 minskade också lungödemet vid septisk lungskada. Behandling med GZD484 minskade också den systemiska inflammationen mätt som ökning av IL-6 och kemokiner i plasma.

Delarbete 2. Nyare forskning har visat att vissa mikroRNA kan reglera uttrycket av många proteiner relevanta för inflammation och NET bildning. Tidigare har man ansett att NET bildning sker utan de novo syntes av proteiner och därmed skulle man kunna tolka situation så att mikroRNA inte är relevanta för NET bildning. Dock

har tidigare studier använt ganska trubbiga och oprecisa metoder att mäta NETs så vi ville omvärdera uppfattningen att NETs inte skulle vara beroende av de novo proteinsyntes. Det kunde konstateras att cyklohexamine och puromycin som hämmar RNA translation effektivt minskade bildningen av NET oavsett olika typer av stimuli vilket antyder att NET bildning de facto är beroende av proteinsyntes. Vidare studeras betydelsen av mikroRNA-155 som tidigare visat sig vara viktig för regleringen av inflammation. Det kunde visas att transfektion med mikroRNA-155 ökade och att transfektion med en mikroRNA-155 antagonist minskade NET bildningen vilket inte bara stärker betydelsen av de novo proteinsyntes och visar av specifika mikroRNA kan reglera NET bildningen. Det kunde också påvisas att mikroRNA-155 reglerade enzymet PAD4 som är ett hastighetsreglerande enzym i bildningen av NETs.

Delarbete 3. Detta arbete var en vidareutveckling av delarbete 2 där mikroRNA-155 betydelse för NET bildning och septisk lungskada studerades in vivo. Möss som saknade genen för mikroRNA-155 visade sig bilda mycket mindre NET i samband med buksepsis. Vidare sågs att möss som saknade genen för mikroRNA-155 uppvisade minskade halter av PAD4 och citrullinerat histone H 3 som är viktiga för bildningen av NETs och kan förklara det reducerade mängden NETs i septiska lungor från dessa möss. Dessutom observeras mindre mängd kemokiner i både blodet och lungorna samt mindre mängd neutrofiler och svullnad i lungorna i septiska möss utan genen för mikroRNA-155 vilket visar att mikroRNA-155 reglerar patologisk inflammation i lungorna vid buksepsis.

Delarbete 4. Vi har i tidigare studier funnit att NETs och mikropartiklar (MP, delar av celler som kan knoppas i samband med aktivering) kan bilda så kallade NET-MP aggregat. I detta arbete avsågs att studera om dessa NET-MP aggregat har pro-inflammatoriska effekter in vivo. Det kunder konstateras att NET-MP aggregat dos-beroende stimulerade neutrofil-endotelcells interaktioner in vivo. Behandling med caspase och calpain inhibitorer kunde minska bildning av NETs med MP. Sådana NETs utan MP var mycket mindre potenta stimuli för neutrofil rekrytering in vivo, vilket visar att dessa NET-MP aggregat har funktionell betydelse vid inflammatoriska reaktioner. Dessutom kartlades vilka adhesionsmolekyler som var viktiga på endotelceller och neutrofiler i samband med NET-MP-inducerad neutrofil ackumulation. Framförallt P-selectin, LFA-1 och Mac-1 visade sig mediera neutrofil-endotelcells interaktioner i mikrocirkulation efter stimulering med NET-MP aggregat. Dessutom observerades att en pro-inflammatorisk molekyl HMGB1

fanns på MP och att HMGB1 var viktig för stimuleringen av neutrofil rekrytering med NET-MP aggregat. Blockering av TLR2 och TLR4 minskade också NET-MP-inducerad neutrofil ackumulation vilket talar för att NET-beroende rekrytering av neutrofiler är medierad via en HMGB1-TLR2/TLR4 axel vid akut inflammation.

Sammantaget visar detta avhandlingsarbete att c-Abl kinas och mikroRNA-155 spelar en central roll för bildningen av NETs som dessutom verkar var beroende av de novo proteinsyntes. Inhibiiton av c-Abl kinas och mikroRNA-155 skulle kunna utgöra effektiva relevanta terapeutiska måltavlor för behandling av septisk lungskada. Dessutom visar detta arbete att NETs bildar aggregat med MP som är funktionella med avseende på stimulering av neutrofil rekrytering vid akut inflammation. NET-MP-inducerad neutrofil ackumulation verkar vara ett resultat av HMGB1-TLR2/TLR4 signalering och blockering av denna axel skulle också kunna vara en relevant måltavla för behandling av patienter med buksepsis.

Acknowledgment

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