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On neutrophil extracellular traps in leukocyte-dependent inflammation

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A microscopic image of a blood vessel, likely a capillary or small vein, showing a cross-section of the vessel wall and several neutrophils. The neutrophils are characterized by their multi-lobed nuclei and are seen adhering to the vessel wall. The background is a warm, reddish-orange color, suggesting a blood smear or a similar biological sample.

On neutrophil extracellular traps in leukocyte-dependent inflammation

FEIFEI DU

DEPARTMENT OF CLINICAL SCIENCES, MALMÖ | FACULTY OF MEDICINE | LUND UNIVERSITY



On neutrophil extracellular traps in leukocyte-dependent inflammation

Feifei Du



LUND
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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended via ZOOM on 21st of April 2022 at 09:00 am in room 9110014, Clinical Research Centre, Jan Waldenströmgata 35, Malmö

Faculty opponent

Professor Mihály Boros, MD, PhD

Institute of Surgical Research, University of Szeged, Hungary

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Author(s): Feifei Du	Sponsoring organization	
Title and subtitle: On neutrophil extracellular traps in leukocyte-dependent inflammation		
<p>Abstract</p> <p>Leukocyte recruitment to a site of an infection is a hallmarks of body's host response. However, inappropriate accumulation of leukocytes can cause tissue damage and organ failure. The mechanisms of leukocyte accumulation may vary depending on different stimuli and organs. Herein, we visualized the process and explored the underlying mechanisms of leukocyte recruitment in response to different stimuli or conditions by intravital microscopy. We examined the role of polyphosphates (PolyPs), neutrophil extracellular trap (NET)-associated proteins and endothelial Midline 1 (Mid1) in regulating leukocyte recruitment during inflammation. We observed that neutrophils constitute the main recruited cells in response to PolyP-induced inflammation, and that interactions between P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) play a dominant role in leukocyte rolling in microvessels. Moreover, both Mac-1 and LFA-1 contributed to leukocyte firm adhesion. In NET-induced inflammation, NET-associated microparticles (MPs) and S100A9 were found to play significant a role in regulating leukocyte accumulation. It was observed that NET-MP complexes trigger leukocyte recruitment via high mobility group box 1 (HMGB1) and toll-like receptor TLR2 and TLR4. S100A9 not only regulates NET-mediated leukocyte recruitment, but also regulates NET formation via reactive oxygen species (ROS) formation. Since leukocyte recruitment is the result of interactions between leukocytes and endothelial cells, we also evaluated a potential target on endothelial cells in septic lung. It was found that Mid1 is overexpressed in septic lung postcapillary venules and in TNF-α-stimulated endothelial cells in vitro. Silencing of Mid1 reduced ICAM-1 expression and leukocyte adhesion to the endothelial cells both in vitro and in vivo. In addition, we also found that protein phosphatase 2Ac (PP2Ac) is the target of Mid1 protein, indicating that the Mid1-PP2Ac axis regulate leukocyte accumulation via ICAM-1 expression. Taken together, these findings give us further understanding of leukocyte accumulation during inflammation and suggest that targeting these proteins would be an effective ways to ameliorate excessive leukocyte accumulation and tissue damage during local or systemic inflammation.</p>		
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On neutrophil extracellular traps in leukocyte-dependent inflammation

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Coverphoto: The image of venule and leukocytes was taken by Feifei Du, edited by Zuo Jing.

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To my parents

所谓致知在格物者，言欲致吾之知，在即物而穷其理也

《大学章句》

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- I. **Du F**, Wang Y, Ding Z, Laschke M, and Thorlacius H. Microvascular Mechanisms of Polyphosphate-Induced Neutrophil- Endothelial Cell Interactions in vivo. *Eur Surg Res.* 2019;60:53-62.
- II. Wang Y, **Du F**, Hawez A, Morgelin M, Thorlacius H. Neutrophil extracellular trap-microparticle complexes trigger neutrophil recruitment via high-mobility group protein 1 (HMGB1)-toll-like receptors (TLR2)/TLR4 signalling. *Br J Pharmacol.* 2019;176(17):3350-63.
- III. **Du F**, Ding Z, Rönnow C.-F, Rahman M, Schiopu A, Thorlacius H. S100A9 induces reactive oxygen species-dependent formation of neutrophil extracellular traps in abdominal sepsis. Submitted to *Sci Rep*.
- IV. **Du F**, Hawez A, Ding Z, Wang Y, Rönnow C.-F, Rahman M, Thorlacius H. E3 ubiquitin ligase Midline 1 regulates endothelial cell ICAM-1 expression and neutrophil adhesion in abdominal sepsis. Submitted to *Int J Mol Sci*.

Papers not included in the thesis

- I. Ding Z, **Du F**, Averitt V RG, Jakobsson G, Rönnow C.-F, Rahman M, Schiopu A, Thorlacius H. Targeting S100A9 Reduces Neutrophil Recruitment, Inflammation and Lung Damage in Abdominal Sepsis. *Int J Mol Sci* 2021;22:12923.

- II. Ding Z, **Du F**, Rönnow C.-F, Wang, Y, Rahman M, Thorlacius, H. Actin-related protein 2/3 complex regulates neutrophil extracellular trap expulsion and lung damage in abdominal sepsis. *American Journal of Physiology - Lung Cellular and Molecular Physiology*. 2022;(In press)

Abbreviations

PolyPs	Polyphosphates
NETs	Neutrophil extracellular traps
MPs	Microparticles
Mid1	Midline 1
IVM	Intravital microscopy
HMGB1	High mobility group box 1
PSGL1	P-selectin glycoprotein ligand-1
Mac-1	Membrane activated antigen-1
LFA-1	Lymphocyte function-associated antigen 1
ROS	Reactive oxygen species
TLR	Toll-like receptor
TNF	Tumor necrosis factor
ICAM-1	Intracellular adhesion molecule 1
PP2A	Protein phosphatase 2
RING	Really interesting new gene
LPS	Lipopolysaccharide
SIRS	Systemic inflammatory response syndrome
VLA-4	Very late antigen-4
rhAPC	Recombinant human activated protein C
CLP	Cecal ligation puncture
mTOR	Mammalian target of rapamycin

GM-CSF	Granulocyte-macrophage colony-stimulating factor
C5a	Complement factor 5a
TRAIL	TNF-related apoptosis-inducing ligand
TRIM	Tripartite motif family
PTP1B	Protein-tyrosine phosphatase 1B
STAT3	Signal transducer and activator of transcription 3
CTLs	Cytotoxic lymphocytes
IFN	Interferons
IRF3	Interferon regulatory factor 3
MPO	Myeloperoxidase
CXCR	C-X-C chemokine receptor
MAdCAM	Mucosal addressin cell adhesion molecule
iNOS	Nitric oxide synthase
VCAM-1	Vascular adhesion molecule 1
RAGE	Advanced glycation end-products

Introduction

Leukocyte recruitment and leukocyte-mediated tissue damage is a hallmark in many inflammatory diseases. The conventional leukocyte recruitment cascade is a multi-step process, which can be briefly divided into three consecutive phases, including leukocyte rolling, adhesion and extracellular migration [1]. Leukocyte rolling is mediated by selectins, such as, E-, P- L-selectin, which could work either independently or collaboratively according to different cell types, tissues or stimuli. Selectin-mediated rolling slow down the cells and enable adhesion to the blood vessel. Integrin-mediated leukocyte adhesion is a prerequisite for subsequent migration to the source of infection. Leukocytes finally cross blood vessels to the extracellular space which is known as migration, where leukocytes exert their antimicrobial role. However, increasing evidences have shown that excessive accumulation of leukocytes can cause tissue damage [2-4], thereby, ways to prevent abnormal accumulation of leukocytes is worth to study. Platelets are known to interact with leukocytes and regulate inflammation [5]. For instance, it has been shown that inflammatory mediators released by activated platelets could regulate leukocyte recruitment and tissue damage [6-8]. In mammalian cells, platelets are the main source of PolyPs, which have been reported as a pro-inflammatory molecule. However, the underlying mechanisms of PolyP-mediated inflammatory regulation is not well known. Neutrophils are considered as the first type of cells to recruit to the site of inflammation. Neutrophils eliminate pathogens by multiple mechanisms including phagocytosis, degranulation, cytokine release and newly discovered neutrophil extracellular traps (NETs)-mediated killing [9-11]. Interestingly, NETs are reported as a double-edged sword in the literatures [12, 13]. On the one hand, NETs could prevent microorganism spreading by exhibiting its classical bactericidal effect. On the other hand, excessive accumulation of NETs could also trigger unwanted inflammatory responses [14, 15]. The antimicrobial effect of NETs is mostly because of the NET associated components, such as histones, MPO etc. Microparticles and S100A9 are also found to be associated with NETs, however,

the role of NET associated microparticles and S100A9 in inflammation are less known.

Sepsis is an uncontrolled and dysregulated host response to infection and considered as a major challenge worldwide due to its high mortality [16, 17]. Leukocyte recruitment and neutrophil interactions with endothelium are considered to participate in the pathological process of multiple organ dysfunction in sepsis [18, 19]. Several studies have shown that leukocyte accumulation may be a rate-limiting step of lung injury in septic mice [20, 21], indicating that preventing excessive leukocytes migration could be an effective way to ameliorate lung injury in sepsis.

Mid1 belongs to the family of RING E3 ubiquitination ligase. It plays a central role in regulating embryonic development and mRNA translation [22]. Several studies have reported the pro-inflammatory role of Mid1 [23, 24] and regulation of cytotoxic T cell by Mid1 [25]. In one of our recent studies, we found that Mid1 expression is upregulated in septic lung post capillary venules [26], however, its functional role in sepsis remained unclear.

Thus, the role of platelet and neutrophil-derived mediators in leukocyte-endothelium interactions and tissue damage are need to be explored.

Background

Leukocyte recruitment

Leukocytes play vital roles in defending against the invading pathogens. The process of leukocyte leaving the intravascular compartment to the site of inflammation is called leukocyte recruitment, which is a hallmark feature in inflammation. However, other than its protective effects, leukocytes can also cause tissue damage when inappropriate activation resulting in excessive accumulation. Over the past few decades, the advances of *in vivo* imaging helped us to visualize the process of leukocyte recruitment in details. For example, by using intravital microscopy, researchers observed leukocyte recruitment in inflamed tissues, such as liver [2, 27], kidney [19], and lung [28] in sepsis-related inflammation. It reveals a dynamic process of leukocyte recruitment which consists multiple sequential steps. The initial interactions of leukocytes with endothelial cells lead leukocyte rolling along the vessel wall, followed by firmly leukocyte adhesion, and finally transmigration or extravagation to the extravascular areas (Figure. 1).

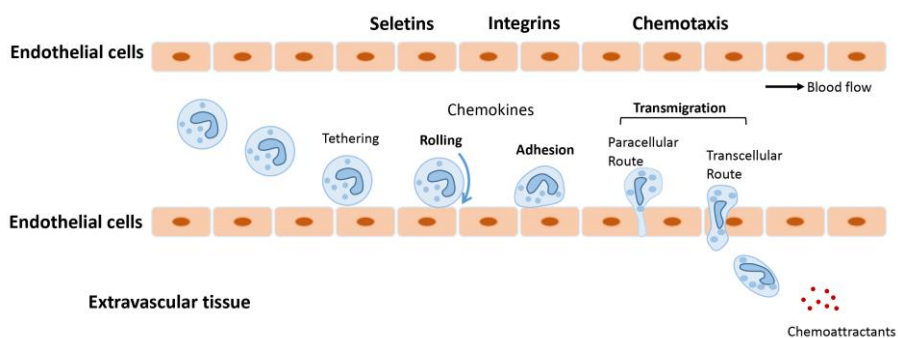


Figure 1. Dynamic paradigm of leukocyte recruitment. Modified from Ley et al. [29]. Leukocytes are first tethered and roll along the endothelium, which is mainly mediated by selectins. Chemokines activate the leukocytes and then leukocytes interact with endothelial cells which is mediated by integrins. Chemokines secreted by inflamed tissues and other immune cells attract leukocytes to the site of inflammation via paracellular or transcellular migration.

Leukocyte rolling

Leukocyte rolling is mediated by carbohydrate ligand binding to selectins on cell surface. Selectins are a transmembrane glycoprotein family of three members including E-selectin (CD62E), L-selectin (CD62L), and P-selectin (CD62P) [30, 31]. The process of selectins binding to their ligands is known as tethering and followed by leukocyte rolling along the endothelial cell surface [32]. Conventional, leukocyte tethering and rolling are regarded as the initial step of leukocyte recruitment and are prerequisite for subsequent firm adhesion, however, some studies suggest that selectin-mediated leukocyte rolling is not essential before adhesion. For example, Wong et al. did not observe leukocyte rolling in sinusoids [33]. Similarly, Vriese et al. did not observe any leukocyte rolling in glomerular capillaries before adhesion in a rat model of glomerulonephritis [34].

L-selectin is expressed on most leukocytes and mediates cell tethering and rolling. Researchers have found that L-selectin shed quickly from cell surface upon cell activation, this shedding regulates leukocyte rolling and promote their arrest in the inflamed endothelium together with other costimulatory effectors such as chemokines and shear stress [35, 36]. Inhibition of this shedding increases the leukocyte rolling flux and reduces the rolling velocity in vivo [37]. P-selectin is stored in α -granules in platelets and Weibel-Palade bodies of endothelial cells. It can be rapidly exposed on the cell surface and shed from cell membrane when platelets and endothelial cells get activated [38, 39]. One study found a clear increase in circulating neutrophil counts in P-selectin-deficient mice, however, these neutrophils were unable to exit from the blood stream and no leukocyte rolling was observed in the venules of mesentery, in the meantime, a delayed recruitment of neutrophil occurred in the induced inflammation, indicating a compromised role of leukocyte rolling in P-selectin deficient mice [40]. E-selectin get upregulated when endothelial cells are stimulated with inflammatory or immune mediators, such as TNF- α , IL-1 and lipopolysaccharide (LPS) [41, 42]. E-selectin is considered to mediate slow rolling of leukocytes, which could greatly prolong the transit time interacting with vessel wall and can be a rate-limit step for leukocyte delivering to the site of inflammation [43, 44]. L-selectin, P-selectin, and E-selectin all can interact with P-selectin glycoprotein ligand-1 (PSGL-1), they can work either separately or synergistically in mediating leukocyte rolling [45-47], the interaction of P-selectin with PSGL-1 triggers the activation of integrin, promoting leukocyte adhesion [48]. Inhibition of PSGL-1 with antibody significantly reduces the leukocyte rolling flux and increase the rolling velocity in the model of murine colitis [49]. Consistently, in PSGL-1-deficient mice, the ability of leukocyte infiltration in

experimental peritonitis is compromised, and leukocyte rolling is found to be decreased significantly [50].

Leukocyte adhesion

Leukocyte adhesion to endothelial cells is a prerequisite for neutrophils infiltration in the inflamed tissues. Exposure of rolling leukocyte to chemoattractants or other stimuli could induce integrin activation, which mediates leukocyte adhesion via interacting with immunoglobulin superfamily ligands on endothelial cells, such as intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1) (Figure 2). Integrins are a family of adhesion molecules expressing on extensive cell types and are cell surface glycoproteins consisting two subunits, a large α and a smaller β subunits [51, 52]. A various combination of 18 α subunits and 8 β subunits could form at least 24 integrins [53]. β 1-integrins are expressed almost on all vertebrate cells, whereas β 2-integrins are mainly expressed on leukocytes [54]. The most studied members are β 1-integrin very late antigen-4 (VLA-4), β 2-integrin lymphocyte function antigen-1 (LFA-1) and membrane-activated complex-1 (Mac-1). Blocking of these integrins with specific antibodies have shown to decrease the number of infiltrated leukocytes in different inflammatory models [55-57]. The avidity of integrin is the basis of adhesive interaction of leukocytes and regulated by both affinity and valency. Affinity is the binding force between integrin and its ligands which is determined by the changes of integrin conformation, while valency is the density or distribution of integrin receptors on cell surface that form adhesive bonds. When integrins are inactive, they have a bent conformation representing low-affinity state, which could later change to a conformation to expose the binding sites upon activation by inflammatory signals, such as chemokines. Meanwhile, the clustering of integrin driving by signals could be redistributed to the zone of contact, hence, increasing the affinity of binding sites and further enhancing the leukocyte recruitment [58, 59]. Due to the importance of integrins in leukocyte recruitment, they may be potential targets in many human diseases [60].

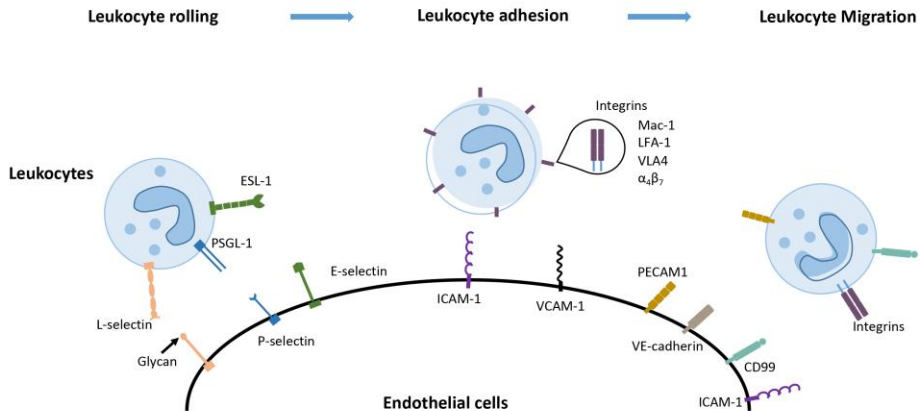


Figure 2. Interactions between leukocytes and endothelial cells. Selectins mainly conduct the leukocyte rolling. Then leukocyte get activated and integrins interact with immunoglobulin superfamily ligands on endothelial cells (ICAM-1, VCAM-1), which mediates leukocyte adhesion. In addition, integrins and other molecules, such as CD99, PECAM1 and VE-cadherin regulate the leukocyte migration.

Leukocyte migration

The final step of leukocyte recruitment is transmigration through vessel walls. Before crossing the endothelium, integrin-dependent leukocyte crawling is crucial for efficient transmigration [61, 62]. Literatures suggest that CXCL-1, LFA-1, Mac-1 involve in the neutrophil crawling, and blocking of LFA-1 and CXCL-1 with antibodies could reduce the neutrophil crawling [63, 64]. Mac-1-deficient neutrophils are disabled in crawling and would take longer time for extravasation [61].

Migration of leukocytes consists of two types of routes, transcellular route and paracellular route. Paracellular migration is a way that leukocytes extravasate from venules through endothelial cell junctions. This is the general consensus of transmigration for most leukocytes. However, increasing evidences have suggested that leukocytes could also migrate across the endothelium via transcellular route, a way to pass endothelial cells directly through the body of cells [29, 65, 66]. An in vitro study have found that majority of human neutrophils preferentially migrated at the tight junction of tricellular corners of HUVEC monolayers when treated with IL-1 [67], whereas Feng et al. reported that neutrophils migrated from skin venules in a manner of transcellular route in response to N-formyl-methionyl-leucyl-phenylalanine (FMLP) stimulation [68]. In Mac-1 deficient mice, more than half of the neutrophils transmigrated via transcellular pathway, in contrast, the wild-type

neutrophils were often found at the site of cell to cell junctions [61]. Those contradictory discoveries implicated different mechanisms of neutrophils migration in response to different stimulators.

Sepsis

Despite significant development in medical care and diagnosis during the past decades, sepsis is still one of leading causes of mortality and morbidity worldwide [16, 69]. It was estimated that nearly 50 million people are affected by sepsis each year, of which more than 50% are among children under 5 years old [70]. In 2017, the estimated burden of sepsis globally were 48.9 million cases, and 11.0 million were sepsis-related deaths, which accounted for 19.7% of total death that year [71]. In the same year, sepsis was considered as a global health priority by the World Health Assembly (WHA) and World Health Organization (WHO); and adopted a resolution to improve the prevention, diagnosis, and management of sepsis [72]. Sepsis was initially defined as the systemic inflammatory response syndrome (SIRS) triggered by infection, with the advances in understanding the progress of sepsis, the concept was defined as serious, life-threatening organ dysfunction caused by dysregulated host responses to infection in 2016 [72]. New definition gave rise to new criteria for stratifying and processing septic patients.

The progress of sepsis could be divided into two stages, initiating from hyper-inflammatory stage and then converted to immunosuppression stage as a result of compensatory down regulation of inflammatory responses, in some patients, this may finally cause septic shock and even death [73-75]. During sepsis, leukocytes get activated [76, 77] and released various inflammatory mediators, such as TNF- α , interleukins [78, 79], elastase [80] and reactive-oxygen productions [81]. In addition, sepsis induces the upregulation of integrins expression on the cell surface of leukocytes and show a high affinity binding to their ligands on endothelial cells of blood vessels [82-85]. Since the role of leukocytes in the pathophysiological process of sepsis is indispensable, targeting leukocyte function during sepsis could be beneficial to the patients.

Adhesion molecules in sepsis

Dysregulated neutrophil infiltration contributes to multiple organ dysfunction in sepsis. The expression of adhesion molecules was found elevated along with the

process of sepsis and some of them could be used as biomarkers for endothelial damage and sepsis [86, 87]. As the pathophysiology of sepsis progresses, soluble isoforms of adhesion molecules are shed from cell surfaces and considered to be potential biomarkers of this disease [88]. There are mainly five soluble adhesion molecules involving in, who have already mentioned above. Three selectin family members, E-selectin, P-selectin, and L-selectin mediate leukocyte rolling. Two immunoglobulin superfamily members, ICAM-1 interacting with Mac-1, LFA-1 and VCAM-1 binding with VLA-4, mediate firm adhesion and migration. The levels of those soluble adhesion molecules are all found elevated in septic patients [89]. Apart from these adhesion molecules, there are other factors that may regulate the neutrophil-endothelial cell interactions in sepsis. For example, an in vivo study revealed the role of iNOS-derived NO in inhibiting neutrophil rolling and adhesion to endothelial cells in cecal ligation puncture (CLP) model of sepsis [90]. Others, such as some anticoagulants were reported to remarkably increase the neutrophil rolling velocity in plasma of septic patients and reduce the number of adherent neutrophils to endothelial cells [91].

Polyphosphate-provoked inflammation

Inorganic polyphosphate (PolyP) is a chain of linear polymers of orthophosphate linked by phosphoanhydride bonds. It is widespread in almost all kinds of organisms and has been shown to be involved in various biological processes, such as coagulation, thrombosis and pro-inflammation [92-94]. The size of chain is the key factor for triggering different roles in pathophysiology. Long-chain PolyP consists of hundreds to thousands orthophosphate residues and is found mostly in bacteria, while it is also found in some mammalian cells and tissues [95]. It has been reported that long-chain PolyP is a potent initiator of clotting via activation of the contact system pathway compared to short-chain polymer [96]. Apart from that, it could also help *E. coli* to escape from host and accelerate mortality in *E. coli*-induced sepsis by affecting the function of macrophages [97]. Activated platelets could release short-chain PolyP that has the length of 60 - 100 orthophosphate units. It is found PolyP-65 could exacerbate LPS-induced inflammation response via promoting interactions between LPS and TLR4 receptors on macrophages and formation of LPS micelles [98]. Besides, PolyP could also work as a potent pro-inflammatory mediator by modulating endothelial cells activation and leukocyte recruitment. PolyP-45, 65 and 70 could increase vascular barrier disruption and

accelerate endothelial cells apoptosis. The expression of ICAM-1, VCAM-1 and E-selectin were found to be upregulated on PolyP-activated endothelial cells, promoting the adhesion of leukocytes to endothelial cells both in vivo and in vitro and subsequent leukocyte migration to peritoneal cavity in PolyP-treated mice [99, 100]. Several mechanisms may involve in the PolyP-induced inflammatory response, such as amplification of the effects of histone and HMGB-1, activation of NF- κ B, mTOR and contact pathway [101-103]. Although several previous studies have demonstrated an important role of PolyP in inflammatory diseases, microvascular mechanisms of polyphosphate-induced neutrophil-endothelial cell interactions are not investigated yet.

Neutrophil extracellular traps (NETs)

Neutrophils are well known as part of the first line of cells in host defense, exhibiting an important role in elimination of pathogens. Neutrophils exert immune responses by various mechanisms including, phagocytosis, cytokine release, degranulation, and NET formation [9, 104, 105]. Moreover, NET formation is described as a new way of neutrophils death, which is distinct from apoptosis and necrosis [106-109] (Table 1). NET consists of web-liked DNA structure and is decorated with various of antimicrobial components, such as histones, neutrophil elastase, myeloperoxidase (MPO) etc. In vitro experiments revealed that neutrophils die quickly in response to phorbol 12-myristate 13-acetate (PMA) stimulation and could undergo morphological change within short time of activation [106]. The process of NET formation is known as NETosis, based on the fate of the cells, NETosis is divided into suicidal NETosis and vital NETosis (Figure 3). Different stimulators could induce NET formation via different mechanisms. Suicidal NETosis is often induced by PMA stimulation and initiated after recognition of different stimuli by receptors on the cell surface. Activation of Raf-MEK-ERK pathway and increased level of cytosolic Ca^{2+} activates NADPH oxidase complex and subsequently generates reactive oxygen species (ROS). Neutrophil elastase and MPO translocate to the nucleus in a ROS-dependent manner. Decondensed chromatins disperse throughout the cytoplasm after breaking down of nuclear envelope. Finally, plasmatic membrane ruptures and web-liked DNA decorated with cytoplasmic proteins and granule toxins are released to extracellular space [14, 110, 111]. In addition, PAD-4 mediated histone citrullination is essential to chromatin decondensation during the suicidal NETosis [112]. On the other hand, in vital NETosis process, neutrophils

Table 1. Comparison of apoptosis, necrosis and NETosis [107-109].

Apoptosis	Necrosis	NETosis
Programmed cell death	Accidental cell death	Programmed cell death
Nuclear and cytoplasmic condensation	Chromatin decondensation and become large round structure	Nuclear delobulation and chromatin decondensation
Cell membrane blebs to membrane-bound fragments or apoptotic bodies, which are taken up by phagocytes and degraded within phagosomes	Cell swells up and cell membrane disintegration Cellular contents are all released into extracellular space	Nuclear envelope disaggregates and nuclear content blends with cytoplasmic content. Cell membrane disrupts Chromatin and granular content are expelled into extracellular space
No inflammation or tissue damage	Can cause inflammation	Can cause inflammation when excessively generated

remain intact and functional after releasing NETs. Oxidant-independent and rapid response are the features of vital NETosis. For example, researchers found that NETs could be generated within 5-60 min in response to *staphylococcus aureus* (*S. aureus*) stimulation [113]. In addition, Clark et al. also reported that NETs was formed within 5 min as a result of LPS-stimulated platelets adhesion to neutrophils [114]. The process of vital NETosis relies on vesicles. The multilobular nucleus are rounded quickly after sensing the stimuli and vesicles bud from nuclear membrane. Then these vesicles in cytoplasm, containing decondensated DNA are fused with plasmatic membrane, and NETs associated with granule proteins are released into extracellular space afterwards [113, 115]. Numbers of evidences indicated that TLR2, TLR4 and complement might play a role in vital NETosis according to different stimuli. LPS-induced TLR4-dependent platelets activation promotes neutrophils activation and NET formation [114], gram-positive bacteria, such as *S. aureus*, induce NETosis in TLR2 and complement-dependent manners [116]. *Candida albicans* was also found to trigger NET release via recognition of β -glucan by complement receptor 3 [117]. In contrast to the conventional ROS-independent vital NETosis, Yousefi et al. reported another type of vital NETosis which is ROS-dependent. In their study, neutrophils were treated with granulocyte-macrophage colony-stimulating factor (GM-CSF) for 20 min and followed with 15 min LPS or

complement factor 5a (C5a) stimulation, mitochondrial DNA instead of nuclear DNA was released in a ROS-dependent manner [118].

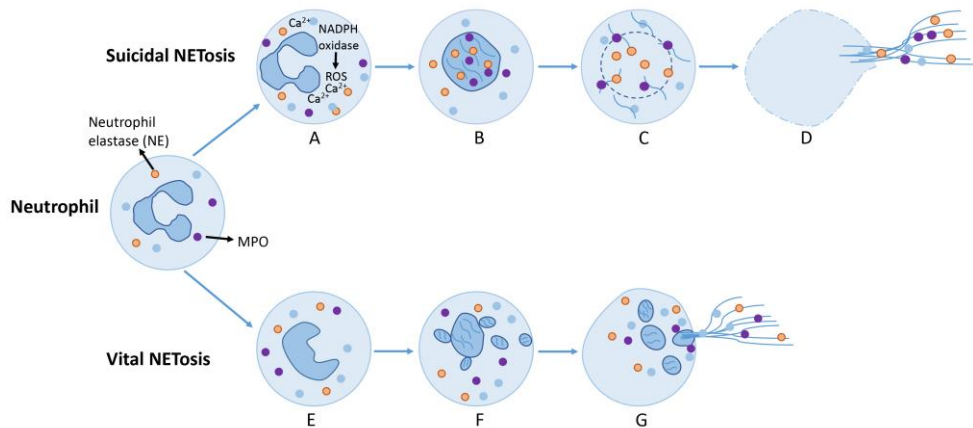


Figure 3. Suicidal NETosis and Vital NETosis. Modified from Rizo et al., Papayannopoulos, and Yang et al. [14, 119, 120]. Suicidal NETosis (A-D) starts with Raf/MEK/ERK pathway activation and Ca²⁺ increase, leading to NADPH oxidase activation and ROS generation. NE and MPO then translocate to nucleus, nuclear delobulation and chromatin decondensation occurred. Nuclear envelope disrupts and decondensed chromatin associated with granule proteins disperse in the cytoplasm. Finally, plasma membrane ruptures and NETs come out in the extracellular space. Vital NETosis (E-G) is a rapid process where nuclear delobulation occurs quickly and vesicles containing DNA filament bleb out of nuclear envelope, then fuse to plasma membrane before releasing as a NETs in the extracellular space.

The structure of NETs

Electron microscopy has shown that the backbones of NETs are smooth fibers structures with diameters of 15-17 nm, globular complexes with 25 nm diameters attached to the fibers and aggregated into larger threads whose diameters could get up to 50 nm [121]. However, a recent study by Atomic Force Microscopy (AFM) described that NETs is not just simply a bundle of thread, they are actually a form of web-like 2D filament structure, containing various sizes of openings. The openings may be the key factors that screen and trap the pathogens according to their sizes regardless of their origins. This mesh structures may also contribute to the NETs ability in promoting clotting and thrombosis. NET-attached proteins play a vital role in regulating the NET stability by maintaining the intact of structure and enhancing the mechanical strength of NETs [122].

NETs: a double-edged sword

Numerous studies have shown that NETs could trap bacteria and have an antimicrobial capacity. For example, NETs were found in the liver sinusoids in LPS-induced endotoxemic mice, and intravascular NETs were found to trap bacteria from bloodstream, thus preventing dissemination of bacteria in septic mice [123]. In line with this discovery, Brinkmann also reported that NETs could disarm both gram-positive and gram-negative bacteria, the proteases on NETs exhibited antimicrobial effect and degraded bacterial virulence factors [121]. A wide range of proteins have been identified on NETs, among which the richest one is histone [124]. NET-bonded MPO was shown to have enzymatic activity and contribute to the killing of *S. aureus* in the presence of hydrogen peroxide (H₂O₂) [125]. Urban and co-workers also confirmed the major role of NET-associated calprotectin in defending against fungal infection [124]. In brief, both NET structure and NET-attached proteases contribute to the bactericidal effectiveness. Although NETs are initially attributed as antimicrobial agent, some molecules laden on NETs can induce inflammatory responses and promote the activation of coagulation [13, 126, 127]. Folco et al. discovered that NETs induced endothelial cells activation and tissue factor production by a concerted action of cathepsin G and IL-1 α [128]. In addition, a study by Saffarzadeh et al. indicated that the cytotoxicity of NET on epithelial and endothelial cells injury is largely dependent on histones, meanwhile, MPO could also contribute to cytotoxicity [129]. In another study, NET-associated serine proteases were found to be involved in the cytotoxicity of NETs and immunodepletion of elastase abrogated major cytotoxicity of NETs [130]. Thereby, aberrant NET generation could also lead to tissue damage and autoimmunity. Indeed, increasing evidences have indicated that excessive accumulation of NET is associated with many different inflammatory diseases, including but not limited to vasculitis, psoriasis, rheumatoid arthritis, systemic lupus erythematosus, sepsis as well as cancer [15, 131, 132]. A recent study detected NET formation in the serum of COVID-19 patients, and the level of NETs in serum might have a relationship with the severity of COVID-19 [133]. In a sepsis study, Colón et al. found NET formation in both LPS-induced infant and adult septic mice. Infant mice were more susceptible to infection and released higher amount of NETs when compared to adult mice. Degradation of NETs could improve the survival rate of septic infant mice. Consistently, they also verified this finding in pediatric sepsis where they observed a higher levels of NETs in patients' serum, which is correlated with the severity of pediatric sepsis [134]. A similar discovery was also reported by Kumar et al., who studied NET formation in 80 sepsis patients. It was found that NET

production was significantly elevated in sepsis patients, and contributed to the severity of sepsis and organ dysfunction [135].

Microparticle complexes

Microparticles (MPs) are extracellular vesicles which are shown to shed from cell membranes. MPs come from activated or apoptotic cells, such as white blood cells, endothelial cells, platelets, and cancer cells in a way of budding and disruption from cell membranes [136, 137]. MPs are produced upon variety of stimuli and express cell surface proteins, antigens and contents of cytoplasm and nucleus according to different cells of origin [138]. Increasing evidences revealed the function of MPs in mediating inflammation, coagulation and thrombosis [138-140]. Circulating MPs, coming from several types of cells, were elevated in plasma of meningococcal septic patients and might promote coagulation, thus exacerbating the pathogenesis of disseminated intravascular coagulation (DIC) in meningococcal sepsis [141]. Leukocyte-MPs could involve in the activation of endothelial cells and interaction between leukocytes and endothelium [142]. In addition, circulating MPs were shown to play a crucial role in regulating endothelial dysfunction, enhancing acute lung injury and respiratory distress syndrome (ARDS) and could be used in predicting the mortality and organ failure in severe sepsis [143]. In one of our previous studies, we have demonstrated neutrophil-derived MPs presence in NETs by electronic microscopy, and found that NET-MP complexes could trigger the generation of thrombin through intrinsic pathway, thereby promoting coagulation in murine abdominal sepsis model [144]. However, the other functions of NET-MP complexes in neutrophil recruitment is still unknown and need to be investigated.

S100A9

S100A9 is a well-known alarmin released by many cell types. It is a member of large S100 protein family, which belongs to the calcium-binding proteins. In neutrophils, S100A9 and S100A8 constitute up to 45% of cytosolic proteins. It was found that extracellular S100A9 play an important role in inflammation, including leukocyte recruitment, cytokine secretion and cell proliferation [145, 146]. Denstaedt and co-workers found that S100A9 gene expression was increased significantly in CLP lung tissue and correlated with CLP. They further confirmed that levels of S100A8/A9 was persistently remained elevated in survived sepsis patients [147]. Similar result was also reported in LPS-treated and CLP-induced

sepsis mice, which showed upregulation of S100A9 in lung tissues. Blockade of S100A9 also shown to attenuate both LPS and CLP-induced lung injury and inflammation response [148, 149]. In addition, S100A9 was found to enhance neutrophilic inflammation by inducing IL-1 β , IL-17 and IFN- γ formation in a model of murine asthma [150]. Numerous evidences also confirmed that S100A9 is a NET attached protein [124, 129, 151], however, the specific role of S100A9 in NET formation is still elusive.

E3 ubiquitin ligase Midline 1 (Mid1)

Mid1 is a microtubular protein that belongs to the tripartite motif family (TRIM), thus also known as TRIM18. It contains one RING domain, two B-Box domains (B-box 1 and B-box 2) and a coiled-coil region and involves in post-translational modification of proteins by ubiquitination [152, 153]. Mid1 has been well studied in malformation syndromes (Opitz BBB/G syndrome (OS)), carcinogenesis, and neurogenic diseases (Alzheimer's disease and Huntington's disease), and also showed to involve in vital function in embryonic development and translational regulation [22, 152]. Although it has been studied in many different diseases, the roles of Mid1 in endothelial cells activation and leukocyte recruitment are not studied yet. Several studies have shown that Mid1 could be regulated by tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) signaling to promote inflammation in different experimental settings [24, 154, 155]. For instance, Collison et. al. discovered upregulation of Mid1 in both mouse and human bronchial epithelial cells when exposed to house dust mite and rhinovirus infection. Mid1 exacerbated allergic airway inflammation and asthma via interacting with TRAIL and catalytic subunit protein phosphatase 2A (PP2Ac) [23]. Mid1 also involves in tissue injury and fibrosis of kidney through Mid1/PTP1B/STAT3 pathway in diabetic mice and knockdown of Mid1 has been shown to attenuate the high glucose-induced renal epithelial-mesenchymal transition, fibrosis and inflammation [156]. A study on cytotoxic lymphocytes (CTLs) uncovered the effects of Mid1 in regulating the exocytosis of lytic granules and Mid1 knockdown found to impair the cytotoxicity of CTLs [25]. Conversely, another study showed that Mid1 could interfere the production of type I interferons (IFN) via interferon regulatory factor 3 (IRF3) ubiquitination and degradation and thus inhibits innate immune activity [157]. In one of our recent studies, Mid1 gene expression was found to be increased in post capillary venules of septic lung [26], however, the specific roles of Mid1 in

endothelial cells activation and leukocyte-endothelial cell interactions are not studied yet.

Aims

- Paper I. To study the impact of PolyPs on the extravasation process of leukocytes and to determine the role of specific adhesion molecules supporting leukocyte-endothelium interactions *in vivo*.
- Paper II. To examine the effect of NET-MP complexes on the leukocyte migration through endothelium and explore the adhesive mechanisms mediating leukocyte-endothelium interactions in response to NET-MP complexes.
- Paper III. To investigate the role of S100A9 in regulating sepsis-provoked formation of NETs and pro-inflammatory function of NET-associated S100A9.
- Paper IV. To determine the role of E3 ubiquitin ligase Mid1 in endothelial ICAM-1 expression and neutrophil adhesion in abdominal sepsis.

Materials & Methods

Animals

All animal experiments were conducted according to the legislation on the protection of animals and were approved by the Regional Ethical Committee for Animal Experimentation at Lund University, Sweden. Male C57BL/6 mice (20-25g) were kept in a pathogen-free facility on a standard 12-h dark/light cycle condition, at a maximum of 5 mice per cage with environment enrichment and given food and water ad libitum. ARRIVE guidelines were followed in studies. For anesthesia, mice were injected intraperitoneally with ketamine hydrochloride (75 mg/kg) and xylazine (25 mg/kg).

Experimental protocol of sepsis

Abdominal sepsis was induced by a procedure called cecal ligation puncture (CLP) in mice. In brief, a midline incision in the abdominal wall was made to expose the cecum outside. Cecum was filled with feces by gently milking feces backward from the ascending colon, then ligated at the 75% of cecum with a 5-0 silk suture. Cecum was then soaked with PBS (pH 7.4) and punctured twice with a 21-gauge needle on the antimesenteric side and then put back to the peritoneal cavity. Abdominal wall was then closed with suture. Sham animals underwent same surgical procedures, but neither punctured nor ligated. Mice were return back to their cages and had free access to food and water. Animals were re-anesthetized after 24h of CLP or sham procedure for sample collection. Blood was collected from inferior vena cava for DNA-histone complex analysis. Lung tissues were collected for transmission and scanning electron microscope examination.

In separate experiment, one group of mice were pretreated with negative control siRNA and in vivo stable Mid1 siRNA (4 mg/kg, Dharmacon, Lafayette, CO, USA) 24h and 2h prior to CLP procedure. Isotype antibody (IgG2b, BioXcell, Labombard

Rd Lebanon, NH USA) and anti-ICAM-1 antibody (YN1/1.7.4, BioXcell) were given intravenously 15 min before CLP induction. Mice were re-anesthetized 4h after CLP to perform lung intravital microscopy (IVM), lung tissues were collected for later assays.

Intravital Microscopy (IVM)

Cremaster IVM

The cremaster muscle of testis was prepared for intravital microscopy. Briefly, skin and fascia were incised from midline over the ventral aspect of the right scrotum. Then, the cremaster muscle sack was exposed by retracting the incised tissues and put it on a transparent pedestal which allows transillumination. After 10-min equilibration, leukocyte recruitment was observed by use of an Olympus microscope (BX50WI; Olympus Optical Co. GmbH, Hamburg, Germany) equipped with water immersion lenses (40/NA 0.75 and 63/NA 0.90). Records were taken in a computer by a charge-coupled device video camera (FK 6990 Cohu; Pieper GmbH, Berlin, Germany) for off-line analysis. Leukocyte rolling flux was measured by counting the number of rolling leukocytes per 20s when passing a reference point in venules (inner diameter between 20 and 40 μm) and expressed as cells/min. Leukocyte rolling velocity was considered as the velocity of the 10 leukocytes rolling along the endothelial cell lining and expressed as $\mu\text{m/s}$. Leukocyte adhesion was quantified as the number of leukocytes which were stationary for 20s in 100- μm -long vascular segments and expressed as number of adherent cells/ mm^2 . Leukocyte migration was determined by the number of extravascular leukocytes in an area of 100 x 70 μm which was immediately adjacent to the venules and expressed as the number of extravascular cells/ mm^2 . Diameters, which was perpendicularly to the vessel path were measured in micrometers. Microvascular hemodynamics were shown by injection of 0.1 ml FITC-dextran (5%, MW 150,000) for contrast enhancement by intravascular staining of plasma. Microvascular of cremaster muscle was visualized by use of a 100-W mercury lamp and filter with blue light (450–490 nm excitation and > 520 nm emission wavelength) epi-illumination. A computer-assisted image analysis system using the line shift method was applied to analyzed the flow velocity. The calculation of the venular wall shear rate was based on the Newtonian definition: wall shear rate = 8 (red blood cell velocity/venular diameter). All analysis of quantification relating to

microhemodynamic parameters in the cremaster microcirculation was performed by use of the computer-assisted image analysis system CapImage (Zeintl, Heidelberg, Germany).

Lung IVM

A pneumothorax was made by incising the diaphragm in a 100 μ l transient stroke volume. A parasternal thoracotomy was performed up to the level of the fourth intercostal space, and the lung tissue was exposed by moving the main part of right thorax wall to the side. Saline (37 °C) was used intermittently to keep the lung surface moisture, great attention was paid to avoid manipulating the lung tissue directly during the preparation. A coverslip was fixed horizontally on the surface of the right lung and horizontal movement of the lung tissue was controlled by adjusting a positive-end expiratory pressure between 5 and 7 cm H₂O. A ventilator (Minivent type 845, Hugo Sachs Elektronik-Harvard Apparatus, March-Hustetten, Germany) was applied to modulate the stroke volume (minimum: 150 μ l) and frequency (minimum: 100 strokes/min). To visualize the leukocytes in lung tissue, the mice were intravenously injected 0.1 ml 0.1% rhodamine 6G (Sigma-Aldrich, Taufkirchen, Germany) for direct staining of leukocytes and 0.1 ml 5% FITC-dextran (MW 150,000; Sigma-Aldrich) for contrast enhancement of plasma. Fluorescence microscopy was performed by use of a modified Olympus microscope (BX50WI, Olympus Optical, Hamburg, Germany), which was equipped with a 100-W mercury lamp and filters with blue (450–490 nm excitation and > 520 nm emission wavelength) and green (530–560 nm excitation; > 580 nm emission) light epi-illumination. Microscopic images of pulmonary post-capillaries were recorded digitally by a charge-coupled device video camera and for off-line analysis. Leukocyte rolling flux was measured by counting the number of rolling leukocytes per 20s when passing a reference point in venules and expressed as cells/min. Leukocyte adhesion was quantified as the number of leukocytes which were stationary for 20s in 100- μ m-long vascular segments and expressed as number of adherent cells/mm². Diameters, which was perpendicularly to the vessel path were measured in micrometers.

Systemic leukocyte count

Blood was harvested from mice tail vein and mixed with Turk's solution (Merk, Darmstadt, Germany) in a 1:20 dilution. The number of MNL and PMNL cells were counted in a Burker chamber (NanoEntek, Seoul, Korea).

NET formation

Neutrophils were isolated from the bone marrow of C57BL/6 mice and stimulated with 500 nM PMA (Sigma-Aldrich, St.Louis, MO, USA) or 500 ng/ml CXCL2 for 3 hours at 37 °C. In indicated experiments, cells were co-incubated with caspase (50 μM, Z-VAD-FMK) and calpain (25 μM, PD150606) inhibitors to generate NETs without MPs. In study III, ABR-238901 (100 μM, a gift from Active Biotech, Lund, Sweden) was applied 1h before NETs collection to block S100A9 on NETs. The mixture of residual neutrophils and NETs were collected through scratching and extensive pipetting. Cellular components were removed by centrifuged at 1400 rpm for 5 min. NET-containing supernatant was collected and further centrifuged at 14000 rpm for 15 min. Then NETs were dissolved in PBS, aliquoted and kept in -20 °C for further use. The quantification of NETs was determined by use of a fluorogenic assay for double stranded DNA (Quant-IT PicoGreen dsDNA kit; Invitrogen, Eugene, USA) according to the manufacture's instruction.

Endothelial cells activation & transfection

The murine endothelioma cell line eEnd.2 (RRID:CVCL_6274) was cultured in DMEM containing 10% fetal calf serum, penicillin, and streptomycin. Cells were seeded into wells for 24h and grown to 70% confluency before transfection and activation.

NET-induced endothelial cells activation

NETs generated as described above were co-incubated with endothelial cells for 0-6h when the confluence of cells is around 90%. Then, cells were washed three times and total RNA was collected for qRT-PCR.

Endothelial cells transfection & TNF- α -induced activation

In study IV, cells were transfected with silencer select negative control siRNA or Mid1 silencer select siRNA (Ambion, CA, USA) when the confluence is about 70% by using TransIT-TKO transfection reagent (Mirus Bio, Madison, Wisconsin, USA) according to the manufacturer's instructions. 24h later, cells were stimulated with 100 ng/ml TNF- α (PeproTech, Rocky Hill, NJ, USA) for 1 or 3h. Cells were then collected for further analysis. In separate experiment, cells were stimulated with 100 ng/ml TNF- α when the confluence is about 90% and then collected for further analysis.

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

Cells or tissues were lysed with Trizol Reagent (Ambion, CA, USA) and total RNA was extracted by use of Direct-Zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The concentration of total RNA was measured by Nanodrop spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) at 260 nm absorbance. Reversed cDNA synthesis was conducted by means of RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) in a final volume of 20 μ l according to the manufacturer's instructions. SYBR Green dye was used to perform real-time PCR procedure, which was in a final volume of 25 μ l. PCR amplifications were processed by Agilent AriaMx Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) and analyzed with $2^{-\Delta\Delta C_t}$ method.

The primer sequences used in study IV:

GAPDH (forward): 5' - CATGTTTCGTCATGGGGTGAACCA -3',

GAPDH (reverse): 5' - AGTGATGGCATGGACTGTGGTCAT -3';

ICAM-1 (forward): 5' - AGCACCTCCCCACCTACTTT -3',

ICAM-1 (reverse): 5' - AGCTTGCACGACCCTTCTAA -3';

Mid1 (forward): 5' - CACTCGCTGAAGGAAAATGACCA -3',

Mid1 (reverse): 5' - AATCCAAGGCAAAAGTGTCAAACG -3'.

Flow cytometry

After stimulation, cells were fixed with 2% formaldehyde and incubated with anti-CD16/CD32 antibody to block Fc γ III/II receptors. Endothelial cells were stained with BV605-conjugated anti-CD31 (clone 390; Biolegend, London, UK) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse ICAM-1 (YN1/1.7.4; Biolegend) antibody was used to detect ICAM-1 expression on endothelial cell surface. For determination of neutrophils activation, blood samples were collected and incubated with anti-CD16/CD32 as described above. Then, cells were stained with phycoerythrin-conjugated anti-Ly6G (clone AL-21, BD Bioscience, San Diego, CA, USA) antibody and FITC-conjugated anti-Mac-1 (clone M1/70, BD

Bioscience, San Diego, CA, USA) antibodies. and erythrocytes were lysed with ACK lysis buffer (Gibco, Dún Laoghaire, Dublin, Ireland). In separate experiment, mice were intraperitoneal injected neutrophil depletion antibody, blood samples were collected and stained with phycoerythrin/Cy7-conjugated anti-mouse Ly-6G (clone 1A8, BD Biosciences), allophycocyanin (APC)-conjugated anti-mouse Ly-6C (clone AL-21, BD Biosciences) to determine the efficiency of neutrophils depletion. For ROS detection, neutrophils were isolated from mice bone marrow, incubated with or without 1 μ M NADPH inhibitor (Diphenyleneiodonium chloride (DPI), D2926, Sigma-Aldrich,), cells were then incubated with 10 μ M dihydrorhodamine 123 (DHR-123, D23806, Thermo Fisher Scientific) and stimulated with 100 ng/ml rS100A9. Flow cytometric analysis was performed by use of CytoFLEX Flow Cytometer (Beckman Coulter, USA) and data were analyzed by Cytexpert 2.0 software (Beckman Coulter) using APC-conjugated anti-mouse Ly-6G (1A8, 127614, Biolegend) to gate neutrophils.

DNA-histone complex assay

Freshly isolated bone marrow neutrophils were stimulated with recombinant mouse S100A9 protein to generate NETs. In separated experiments, isolated neutrophils were incubated with anti-TLR4 or anti-RAGE or ROS inhibitor DPI or S100A9 inhibitor ABR-238901 (a gift from Active Biotech) 30 min before stimulation. NET-containing supernatant was then collected to assay DNA-histone complex formation. Blood samples were harvested and plasma was obtained after centrifugation to determine the DNA-histone complex formation. All samples were detected by use of a Cell Death Detection ELISA kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instruction.

Confocal microscopy

The murine endothelioma cell line eEnd.2 were grown and stimulated with TNF- α as described above. Cells were fixed with formaldehyde, and permeabilized with a 0.1% Triton X-100 solution when needed. Samples were blocked and incubated with primary antibodies overnight at 4 °C and incubated with second antibodies on next day. Then Hoechst 33342 was used to counter stain DNA materials. Coverslips were mounted with Prolong Diamond Antifade Mountant (P-36965, Thermo Fisher

Scientific) on the microscopy slide. Confocal z-stacks images were taken by using LSM 800 confocal (Carl Zeiss, Jena, Germany), and processed by ZEN lite 3.1 (blue edition, Carl Zeiss) software. Orthogonal projection method was applied to create images with all slices for a total height of 10 μm .

Transmission & scanning electron microscope

Lung tissue samples were fixed with 2.5% glutaraldehyde in 0.15 mol/L sodium cacodylate (pH 7.4, cacodylate buffer) for 30 min at room temperature, then washed with cacodylate buffer and dehydrated with an ascending ethanol concentration series from 50% (vol/vol) to absolute ethanol. Subsequently, samples were exposed to carbon dioxide for critical point drying, absolute ethanol was used as intermediate solvent. After mounting on aluminium holders, samples were sputtered with 20 nm palladium/gold and examined by use of a Jeol/FEI XL 30 FEG scanning electron microscope. Ultrathin sectioning and transmission immunoelectron microscopy was used to analyze the location of individual target molecules at high resolution. Samples on coverslips were embedded in Epon 812 and sectioned into 50-nm-thick ultrathin sections by a diamond knife in an ultramicrotome. Sections were incubated with primary antibodies overnight at 4 °C, then the grids were incubated with species-specific, gold-conjugated secondary antibodies (Electron Microscopy Sciences, Fort Washington, MD, USA) and gold-labeled annexin V. The sections were finally post-fixed in 2% glutaraldehyde and post-stained with 2% uranyl acetate and lead citrate. A Jeol/FEI CM100 transmission electron microscope, operated at 80-kV accelerating voltage was applied to observe the samples.

Adhesion assay in vitro

The murine endothelioma cell line eEnd.2 were grown and stimulated with TNF- α as described above. Activated eEnd.2 cells were incubated with freshly isolated neutrophils for 1h at 37 °C. Cells were then washed with PBS and collected for MPO assay. The measurement of neutrophil adhesion to activated endothelial cells was determined in terms of MPO levels (ng/ml). In brief, cells were suspended in 0.02 M Phosphate buffer (PB, pH7.4), centrifuged and resuspended in mixture of 0.05 M PB and 0.5% hexadecyl-trimethylammonium bromide buffer (HTAB), samples were then kept in -20 °C overnight, thawed and sonicated for 90s in water

bath for 2h at 60 °C the next day. MPO levels were detected by a spectrophotometer at 450 nm wavelength, with a reference filter at 540 nm after the reaction with substrate (3,3',5,5'-Tetramethylbenzidine, TMB) (Sigma Aldrich).

Western blot

Pre-treated eEnd.2 cells were collected and lysed with Pierce RIPA buffer (Thermo Fisher Scientific), containing 1% protease inhibitor cocktail (Thermo Fisher Scientific). Supernatant was taken after centrifugation and the concentration of total protein was measured by Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). Protein was then loaded and separated on 8-16% Mini-PROTEAN TGX Stain-free precast gels (Bio-Rad Laboratories, California, USA). Images of total protein gel were taken by Bio-Rad CheMidoc™ MP imaging system (Bio-Rad Laboratories). Then, total protein was transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories) and imaged again to get the picture of total protein on membranes. Membranes were incubated with primary antibodies overnight at 4 °C after blocking in 5% non-fat milk buffer, and incubated with second antibodies the next day for 1h. Membranes were incubated with ECL substrate (Bio-Rad Laboratories) and protein bands were imaged by Bio-Rad CheMidoc™ MP imaging system. Analysis of proteins was performed by use of Image Lab™ software version 6.1 (Bio-Rad Laboratories). The expression of target proteins was calculated by normalization of each band with total protein load in each lane.

Statistics

Data are presented as means \pm standard error of the mean (SEM). N is indicated as number of animals or experiments in each group. Statistical evaluations was performed using Mann-Whitney for two groups comparison and Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's method for multiple comparisons. $P < 0.05$ was considered significant. Graphpad Prism 8 was used for data analysis.

Results & Discussion

PolyP-provoked leukocyte recruitment

PolyPs extensively exist in the biological universe, from microorganisms to mammal cells. The structure of PolyPs is simple, which constitutes a linear polymers of orthophosphate residues that are linked together by ATP-like bonds. The number of orthophosphate residues of PolyPs can be varied from 10 to more than 1000 according to different sources [158], and they can exert different functions depending on their length of chain. Although reports indicate that mast cells and basophils also contain PolyPs, platelets are the main source of PolyPs in mammalian cells and can secrete short PolyP polymers (60–100 residues) into the surrounding [95]. Accumulating lines of evidence report that platelets could promote leukocyte recruitment and vascular permeability at sites of tissue injury [5, 159]. PolyPs are also discovered to play a role in apoptosis, cell proliferation, coagulation, and inflammation [92, 160, 161]. However, the molecular mechanisms regulating PolyP-mediated leukocyte accumulation in tissues are not totally clear.

Leukocyte recruitment is a multistep process, which consists of rolling, adhesion and migration, mediated by specific adhesion molecules expressed on endothelial cells and leukocytes. Different selectins and integrins are involved in the process of leukocyte recruitment according to different organs and disease conditions. Intravital microscopy is a well-developed method to study the dynamic interactions between circulating leukocytes and microvascular endothelium. It has been extensively applied in the study of liver, lung, kidney and brain [162, 163]. Cremaster muscle is a thin transparent layer tissue, and is easily accessible with minor surgery. It is an ideal tissue to observe the interactions between leukocytes and endothelial cells [164]. Herein, we used intravital microscopy to study the PolyP-induced leukocyte recruitment in the model of cremaster muscle. We intrascrotally injected PolyPs (0.1-1 mg, 87 phosphate monomers) 3h before intravital microscopy observation. It was found that local administration of PolyPs significantly increased leukocyte accumulation in vivo (Figure 4), which was

similar as TNF- α -induced leukocyte response. PolyP challenge significantly reduced leukocyte rolling velocity instead of leukocyte rolling flux, since surgical preparation of tissues for intravital microscopy is known to trigger leukocyte rolling [165]. Administration of both 0.5 mg and 1 mg PolyPs markedly enhanced leukocyte adhesion and extravasation (Figure 4). It was observed that 0.5 mg of PolyPs increased firm adhesion of leukocytes by more than 9-fold (Figure 4b). Moreover, the number of extravascular leukocytes increased by more than 4-fold after stimulation with 0.5 mg and 1 mg of PolyPs (Figure 4c). These observations indicate that PolyPs exert potent pro-inflammatory effects in multicellular tissues. Considering the similar effect of 0.5 and 1 mg of PolyPs on leukocyte responses, we used 0.5 mg of PolyPs for the subsequent experiments.

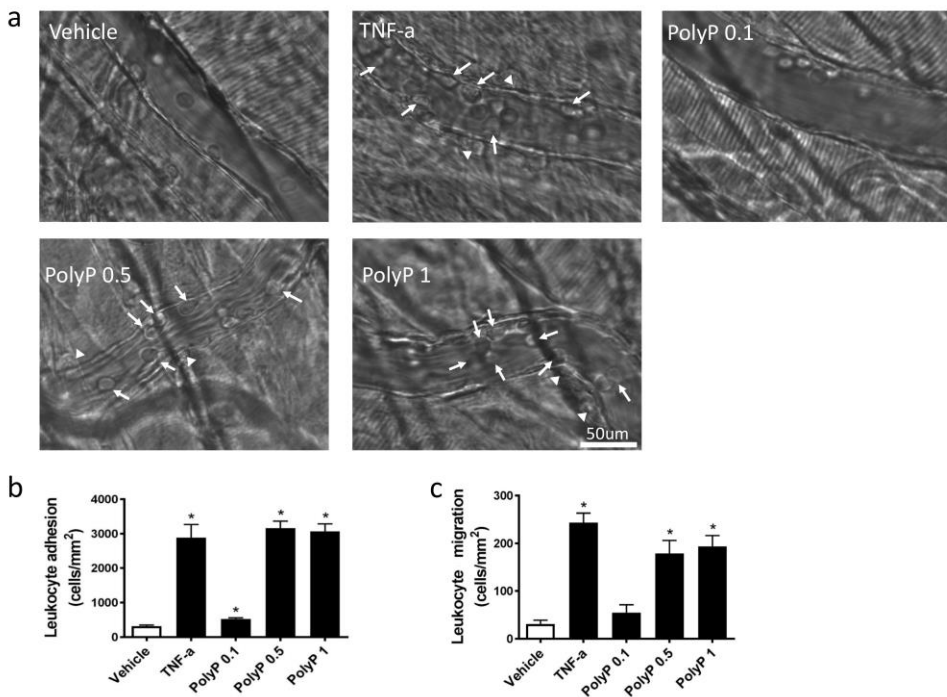


Figure 4. PolyP-induced leukocyte adhesion and migration in vivo. Mice received vehicle, TNF- α , and PolyPs (0.1–1 mg) intrascrotally 3h before checking leukocyte recruitment in cremaster muscle by intravital microscopy. (a) Representative of intravital images of leukocyte adhesion (arrows) and migration (arrowheads). Leukocyte (b) adhesion, and (c) migration in mouse cremaster muscle were calculated. Data are presented as mean \pm SEM. n = 5. * p < .05 vs. vehicle.

Neutrophil is considered as the first and main subpopulation of leukocytes in responding to infections. Herein, we applied a depletion antibody directed against Ly6G of neutrophils to determine whether neutrophil is the main subtype to respond

to PolyP challenge. Neutrophil depletion antibody was administered intraperitoneally 24h before intrascrotal challenge with PolyPs. It was found that the number of circulating neutrophils decreased by 99% after injection of depletion antibody. Interestingly, it was found that neutrophil depletion decreased PolyP-induced leukocyte rolling flux (Figure. 5a), adhesion (Figure 5c), and migration (Figure 5d) by 73, 90, and 67% respectively, indicating that neutrophils are the dominating leukocyte subtype responding to PolyP stimulation. One study reported that platelet-derived PolyPs mediate endotoxin-induced formation of neutrophil-platelet aggregates and thereby contribute to accumulation of neutrophils in the lung [166]. Considered together with our present findings, it might be forwarded that PolyPs can trigger neutrophil recruitment in several distinct ways in vivo.

Leukocyte rolling is mainly mediated by selectin family, which is E-, P-, L-selectin, however, the role of each selectin is different based on different cell types, tissues and experimental settings [167-169]. PSGL-1 is one of the best-characterized selectin counter-receptors, which is initially identified to bind P-selectin [170, 171], but also known to bind with E-selectin and L-selectin [172, 173].

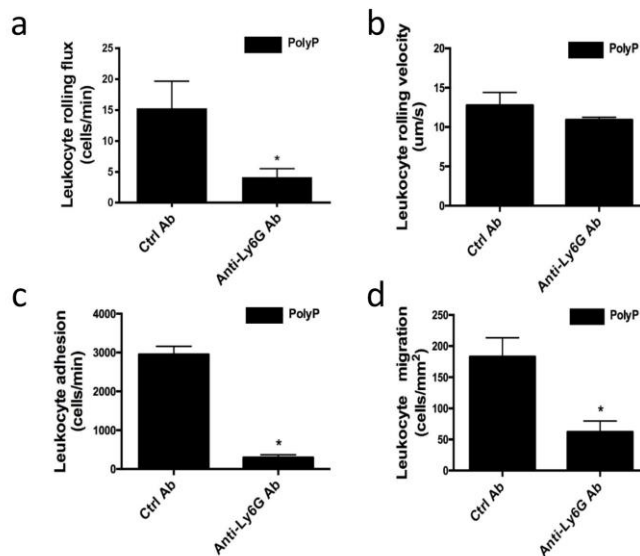


Figure 5. Neutrophil is the main player in PolyP challenge. Mice were treated intraperitoneally with an antibody (Ab) directed against Ly6G (clone 1A8) or a control Ab 24h before intrascrotal challenge with 0.5 mg of PolyPs. Leukocyte (a) rolling flux, (b) rolling velocity, (c) adhesion, and (d) migration were evaluated using IVM. Data are presented as mean \pm SEM. n = 5. * p < .05 vs. Ctrl Ab.

Leukocyte adhesion is largely supported by $\beta 2$ -integrins, such as LFA-1 and Mac-1. Published data suggest that the importance of different $\beta 2$ -integrins can vary depending on the types of inflammatory stimulus and experimental settings [174]. In the present study, we intravenously injected antibodies directed against P-selectin (2 mg/kg), E-selectin (2 mg/kg), PSGL-1 (2 mg/kg), Mac-1 (4 mg/kg), and LFA-1 (4 mg/kg) as well as a control antibody immediately prior to administration of PolyPs, and observed that immunoneutralization of P-selectin or its major counter receptor PSGL-1 reduced PolyP-induced neutrophil rolling by 99% (Figure 6a). Inhibition of P-selectin decreased neutrophil adhesion by 97% and extravasation by 80%. Similarly, blockade of PSGL-1 reduced neutrophil adhesion and migration by 94% and 84%, respectively (Figure 6b and c). However, PolyP-induced leukocyte recruitment in cremaster muscle was not affected by blockade of E-selectin (Figure 6), In present study, our observations indicated that P-selectin/PSGL-1 interactions undertake main roles in PolyP-triggered neutrophil accumulation. In addition, immunoneutralization of Mac-1 and LFA-1 remarkably decreased the number of firmly adherent and migrated neutrophils in PolyP-challenged tissues, whereas, both of them had no impact on neutrophil rolling (Figure 6b and c). Taken together, these adhesion molecules are the main players in mediating PolyP-provoked neutrophil-endothelium interactions in cremaster muscles.

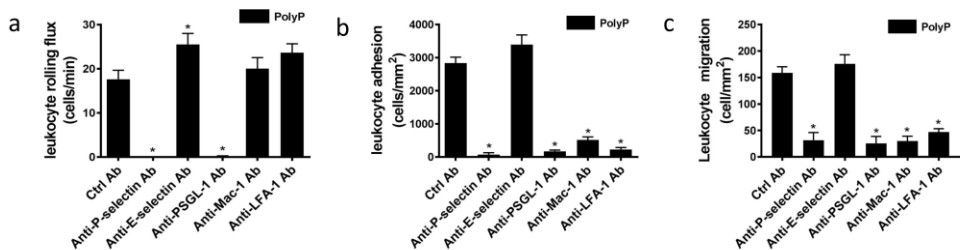


Figure 6. Role of adhesion molecules in PolyP-induced neutrophil recruitment. Mice were pretreated with anti-P-selectin, anti-E-selectin, anti-PSGL-1, anti-Mac-1, anti-LFA-1, and a control antibody (Ab). Leukocyte (a) rolling flux, (b) adhesion, and (c) migration were determined in mouse cremaster muscle 3h after intrascrotal challenge with 0.5 mg of PolyP. Data are presented as mean \pm SEM. n = 5. * p < .05 vs. Ctrl Ab.

In conclusion, our study shows that the short-chain PolyPs are a powerful inducer of neutrophil accumulation in vivo and regulate the basic molecular mechanisms of PolyP-induced interactions between neutrophils and endothelial cells in vivo. Hence, our study revealed new mechanisms and potential target in PolyP-dependent inflammation.

Neutrophil extracellular trap-microparticle complexes in neutrophil recruitment

After activation, neutrophils generate neutrophil extracellular traps (NETs) which is one of the mechanisms that neutrophils use to defend body from infections. However, accumulating data show that excessive accumulation of NETs could cause tissue damage and trigger dysregulated inflammatory responses in many diseases [119, 175]. NETs are composed of web-like DNA structure where proteins are attached with DNA fiber. Microparticles (MPs) are membrane-surrounded sphere-shaped vesicles with size less than 1 μm . They can shed from many types of cells when cells are activated [136]. In our previous study, it was found that MPs were released upon neutrophil activation and can attach to the NETs through histone-phosphatidylserine interactions. These NET-MP complexes formation could mediate thrombin generation via activation of the intrinsic pathway of coagulation [144], however, studies of NET-MPs in regulating inflammatory responses are limited. Herein, we examined the effect of NET-MP complexes on the interactions between leukocyte and endothelium by IVM. In this study, we generated NETs from isolated bone marrow neutrophils by PMA stimulation. Since the effect of surgical preparation is known to affect leukocyte rolling in the model of cremaster muscle [165], leukocyte rolling velocity instead of rolling flux was affected by the NETs challenge. Intrascrotal administration of NETs triggered leukocyte accumulation in vivo and increased leukocyte adhesion in a dose-dependent manner (Figure 7b). 1.5 μg and 4.5 μg NETs enhanced leukocyte migration by more than 8-fold (Figure 7c). Moreover, we examined the effect of NETs generated by CXCL-2 stimulation, and found that CXCL-2-induced NETs exhibit similar effect as of PMA-generated NETs, suggesting that it is a general ability of NETs to induce leukocyte recruitment.

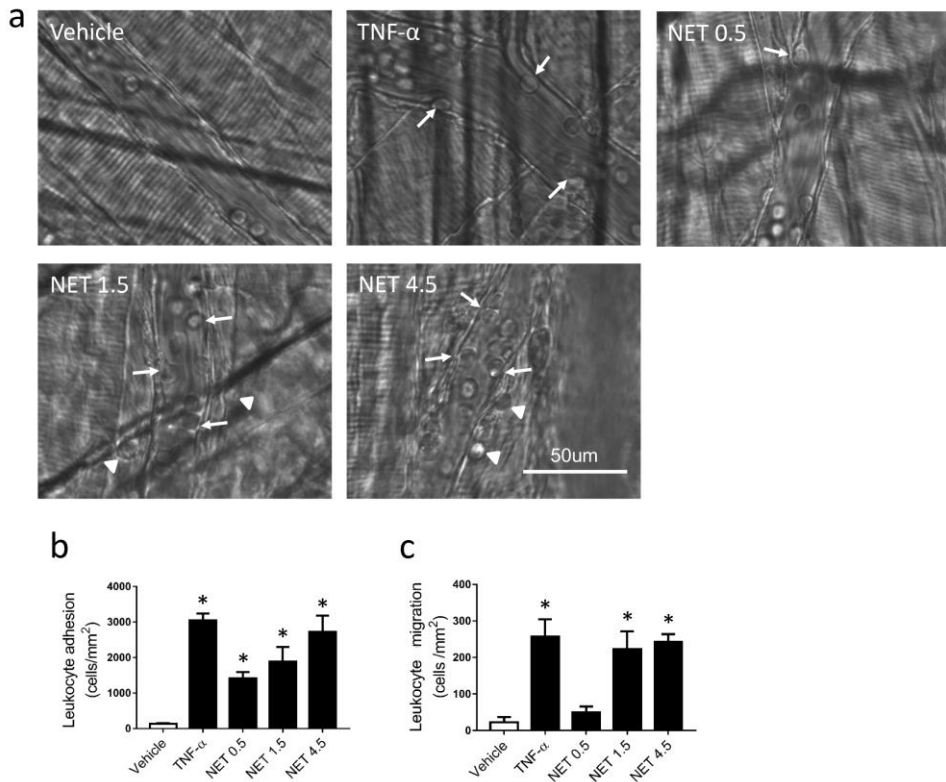


Figure 7. NET-induced leukocyte adhesion and migration in vivo. (a) Representative intravital images of the leukocyte adhesion (arrows) and migration (arrow heads) in response to vehicle, TNF- α (0.5 μ g), and NET (0.5-4.5 μ g) stimulation. Leukocyte (b) adhesion, and (c) migration in mouse cremaster muscle were calculated. Data are mean \pm SEM and n = 5 different animals for each group. *P < .05, significantly different from vehicle.

Depletion of neutrophils by use of an antibody caused an inhibition of leukocytes accumulation induced by NET administration, indicating that the main subpopulation of leukocyte accumulated in response to NET stimulation is neutrophil. Since it is well known that selectins and β 2-integrins mediated leukocyte rolling and adhesion respectively, here we used blocking antibodies of P-selectin, E-selectin, PSGL-1, Mac-1, and LFA-1 to study the potential mechanisms of NET-induced leukocyte recruitment. It was observed that blocking P-selectin and PSGL-1 instead of E-selectin reduced the NET-triggered neutrophil rolling, adhesion and migration all by more than 90%, suggesting the key role of P-selectin-PSGL-1 interactions in mediating neutrophil rolling in NET challenge. Inhibition of Mac-1 and LFA-1 significantly reduced the leukocyte adhesion and migration, while none of them had any effect on rolling.

We then confirmed that NETs generated by PMA stimulation contain MPs by electron microscopy. Numerous studies have shown that disintegration of NETs by DNase or inhibition of NET formation could impact pathological accumulation of inflammatory cells in many diseases [176-178]. Here, we found that neutrophils co-incubated with calpain and caspase inhibitors markedly reduced the level of NET associated MPs, without interfering the expression of other common proteins on NETs, such as MPO and S100A9. These NETs without MPs were less effective in mediating neutrophil accumulation, showing a remarkable reduction on neutrophil adhesion and migration (Figure 8a and b). This reduction is similar to the result of disintegration of NETs with DNase in triggering neutrophil infiltration (Figure 8a and b), indicating that NET-MP complexes are potent stimulator for neutrophil accumulation. These results also provide an implication of NET-MP function in neutrophil-related diseases.

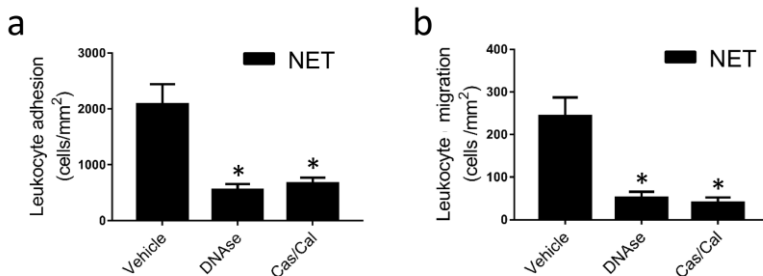


Figure 8. NET-MP complex-induced leukocyte adhesion and migration in vivo. (a) adhesion, and (b) migration in mouse cremaster muscle 3h after intrascrotal challenge with 1.5 μ g NETs, NETs co-incubated with DNase and NETs co-incubated with caspase and calpain inhibitors. Data are mean \pm SEM and n = 5 different animals for each group. *P < .05, significantly different from vehicle.

High mobility group box 1 (HMGB1) not only enhances transcription, but also plays a role in tissue injury, infection, and inflammation via interacting with its receptors, such as receptor for advanced glycation end-products (RAGE), Toll-like receptor 2 (TLR2) and TLR4 [179]. Studies have shown that NET-derived HMGB1 could mediate peritoneal macrophage pyroptosis and HMGB1 in turn could regulate NET formation [180, 181]. One publication discovered that platelet-derived MPs from systemic sclerosis patients express high levels of HMGB1 [182]. Herein, we hypothesized that neutrophil-derived MPs may also contain HMGB1. Indeed, by using transmission electron microscopy (TEM), we found HMGB1 is expressed on the NET-MPs. Inhibition of HMGB1 by anti-HMGB1 antibody could significantly decrease the number of adherent and migrated neutrophils in response to NET challenge (Figure 9a and b). Knowing that TLR2 and TLR4 bind with HMGB1, we

evaluated the role of these two receptors in the NET-induced neutrophil infiltration. It was found that immunoneutralization of TLR2 or TLR4 markedly attenuated neutrophil adhesion and extravasation in response to NET-MPs stimulation (Figure 9c and d). These observations suggest that the interactions between HMGB1 and TLR2/TLR4 signaling play a key role in NET-MP complex-induced neutrophil recruitment.

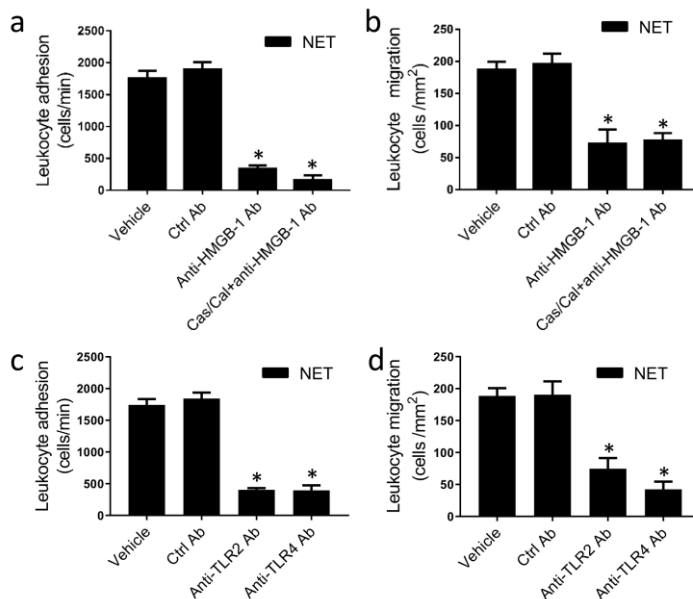


Figure 9. Role of HMGB1, TLR2, and TLR4 in NET-induced neutrophil adhesion and migration. Neutrophil (a, c) adhesion and (b, d) migration in mouse cremaster muscle 3h after intrascrotal challenge with 1.5 μ g NETs. Animals were pretreated with an anti-HMGB-1, anti-TLR2, anti-TLR4 antibody, and a control antibody (Ab). Control animals were received PBS. Data are mean \pm SEM and n = 5 different animals for each group. *P < .05, significantly different from Ctrl Ab. Cas/Cal, caspase and calpain inhibitors.

In summary, neutrophil-derived MPs can bind to NETs and formed NET-MP complexes. These NET-MP complexes are potent inducers of neutrophil recruitment via HMGB1 and TLR2/TLR4 interaction. Thus, our findings provide a new potential target to inhibit NET-associated inflammation and diseases.

Role of S100A9 in NET formation and neutrophil recruitment

S100A9, also known as MRP-14 (myeloid related protein of molecular weight 14 kD), is a calcium binding protein which is known to release by immune cells during inflammation. It belongs to large S100 protein family. Accumulating publications have detected the up-regulation of S100A9 in several diseases, such as in severe

sepsis [183], different types of cancers [184-187], psoriasis [188], and COVID-19 infection [189]. The pro-inflammatory effect of S100A9 has been reported widely. For example, one study discovered that intranasal administration of recombinant S100A9 could significantly increase the number of neutrophils in bronchoalveolar lavage fluid (BALF), and intensive infiltration of inflammatory cells was observed in the peribronchial area along with increased levels of IL-1 β , IL-17 and IFN- γ [150]. This is in line with our previous study, in which we demonstrated that blocking S100A9 could reduce the neutrophil activation and infiltration in the lung of septic mice [149]. These observations give us an implication that S100A9 may play an important role in promoting inflammation responses, however, the role of S100A9 in NET formation is not explored yet.

Increasing publications have verified the pro-inflammatory effect of S100A9 in the pathological process of sepsis [190-193]. In our previous study, we detected the basic role of S100A9 in CLP-induced mouse sepsis models, here in the study III, we further examined the details of S100A9 in sepsis. Mice were pre-treated with ABR-238901, a specific S100A9 inhibitor, 1h before CLP induction, lung tissues were collected after 24h, and NETs formation was examined by using transmission electron microscopy. It was observed that NET is accumulated in the lung of septic mice in compared to the sham group and blocking of S100A9 with ABR-238901 markedly reduced NET formation in septic lungs (Figure 10a-c). Notably, S100A9 was also found in close proximity of NETs in the lung of septic animals, indicating that S100A9 is a NET-associated protein in septic lung. This result is supported by other studies, showing that S100A9 is one of NET-bound components [124, 129, 151]. These observations may help explain the protective effect of blocking S100A9 on lung injury in abdominal sepsis [147, 148]. NET formation was also found to be elevated in the septic plasma, and inhibition of S100A9 could reduce the plasma level of NET generation (Figure 10d), indicating that S100A9 also regulate formation of NETs in plasma of abdominal sepsis.

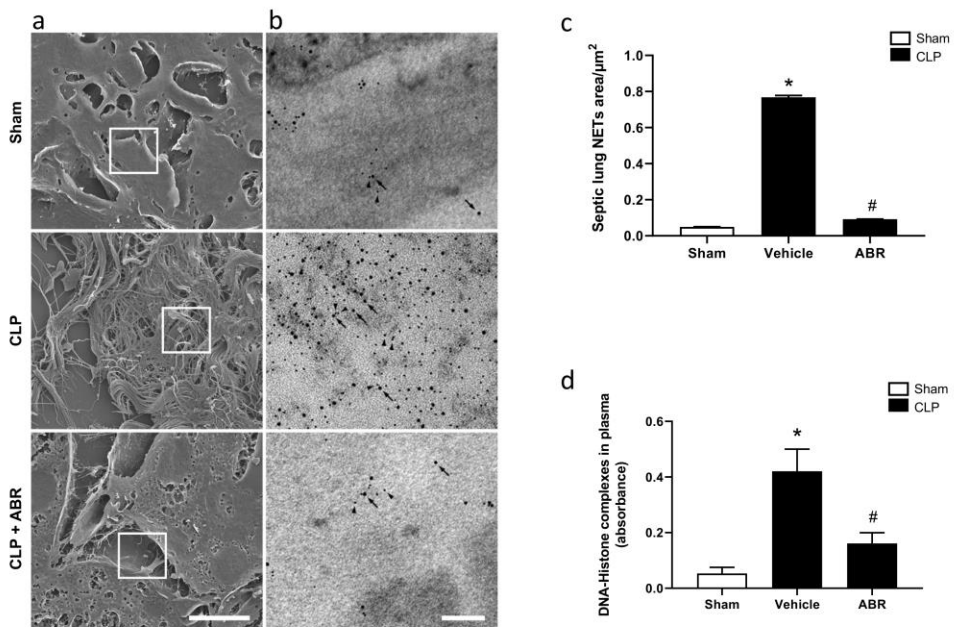


Figure 10. S100A9 regulates NET formation in septic lung tissues. (a) Scanning electron microscopy showing extracellular web-like structures in the mouse lung tissue 24h after induction of sepsis. Scale bar = 10 μm . (b) Transmission electron microscopy of the indicated area of interest from Figure 9a, incubated with gold-labeled anti-citrullinated histone 3 (large gold particles, arrows) and anti-elastase (small gold particles, arrowheads) antibodies. Scale bar = 0.1 μm . (c) Aggregate data of NET formation in the lung tissue. Blood were collected 24 h after CLP and plasma was used to determine the formation of DNA-histone complexes by ELISA (d) DNA-histone complexes in plasma of septic mice. Data are mean \pm SEM and n = 4-5 different animals for each group. *P < .05, significantly different from sham, #P < .05, significantly different from Vehicle.

This notion was supported by our further experiments, in which we explored the mechanisms of S100A9 in NET formation and functions as a pro-inflammation effector. We stimulated bone marrow derived neutrophils with recombinant mouse S100A9 and determined the NET formation by DNA-histone complex ELISA kit. It was found that stimulation with S100A9 markedly increased DNA-histone complex formation. Accumulating data also suggest that TLR4 and RAGE might involve in S100A9-mediated pathological process of diseases, for example, S100A9-TLR4 pathway is shown trigger herpetic neuralgia in a mouse model [194]. Furthermore, it was observed that S100A9-TLR4 pathway could activate integrin β 1/FAK and in turn promote prostate cancer cell invasion [195]. In addition, S100A9-RAGE pathway is shown to contribute to the formation of extracellular vesicle microcalcification [196], pro-inflammatory cytokines formation, and human lung fibroblast cells activation and growth [197]. Hence, we explored the role of TLR4 and RAGE in S100A9 mediated NET formation. We added different doses of TLR4 or RAGE blocking antibodies on neutrophils 30 min prior to S100A9

stimulation, and found that blocking of TLR4 or RAGE receptors decreased S100A9-induced generation of NETs (Figure 11a). These results were further confirmed by confocal microscopy, showing a reduction of S100A9-induced NET formation after blocking with anti-TLR4 or anti-RAGE antibodies (Figure 11b). Considering that ROS generation is a key feature in the process of NETosis, it was of interest to study the role of ROS in S100A9-mediated NET formation. Here, we co-incubated neutrophils with the NADPH inhibitor, called DPI, and then stimulated with S100A9. It was found that both ROS generation and levels of DNA-histone complexes were markedly attenuated when ROS formation pathway was inhibited (Figure 12), indicating a ROS-dependent pathway involvement in S100A9-mediated NET generation.

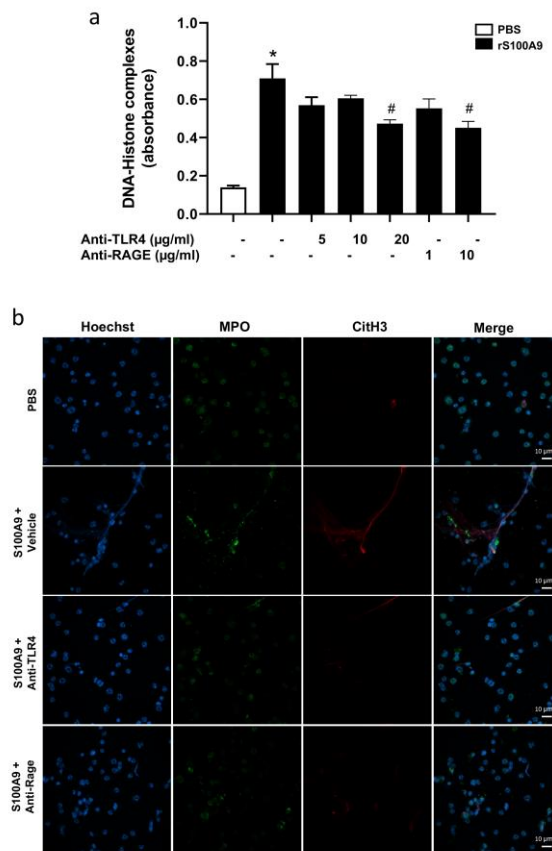


Figure 11. Role of TLR4 and RAGE in S100A9-induced NET formation. Neutrophils were incubated with or without anti-TLR4 or anti-RAGE antibodies 30 min prior to recombinant S100A9 stimulation. NET formation was determined by (a) the formation of DNA-histone complexes and (b) confocal microscopy. Scale bar = 10 μm. Data are mean ± SEM and n = 4 different animals for each group. *P < .05, significantly different from PBS, #P < .05, significantly different from rS100A9.

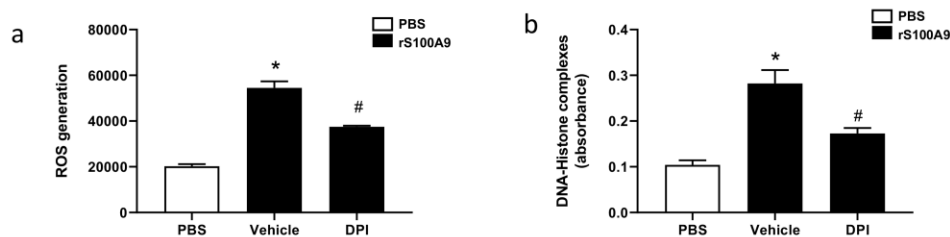


Figure 12. Role of ROS in S100A9-induced NET formation. Neutrophils were stimulated by recombinant mouse S100A9 with or without DPI (1 μ M) for 1h. (a) Aggregate data of ROS generation by FACS, and NET formation was determined by (b) the formation of DNA-histone complexes. Data are mean \pm SEM and n = 4-5 different animals for each group. *P < .05, significantly different from PBS, #P < .05, significantly different from rS100A9.

Since NET is a potent inducer of leukocyte recruitment, and S100A9 could induce neutrophil infiltration in lungs, next we investigated the potential effect of NET-associated S100A9 in leukocyte accumulation. NETs were generated from neutrophils by PMA stimulation in the absence or presence of ABR-238901, then injected intrascrotally in mice 3h prior to IVM observation. We found that local challenge with NETs significantly increased leukocyte adhesion and migration in the cremaster muscle microvasculature, while NETs generated in the presence of ABR-238901 markedly reduced the NET-triggered leukocyte adhesion (Figure 13a) and migration (Figure 13b), indicating that S100A9 expressed on NETs exert an important role in NET-mediated leukocyte recruitment in vivo.

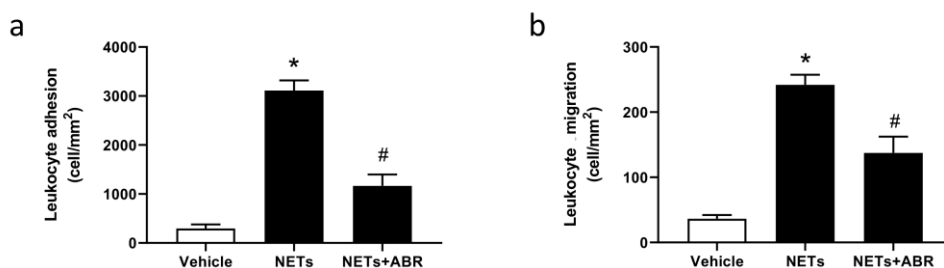


Figure 13. S100A9 regulates NET-induced leukocytes adhesion and migration. NETs were generated by stimulating neutrophils with PMA in the absence or presence of ABR-238901. Vehicle (PBS), NETs or NETs with ABR-238901 were injected intrascrotally in the mice 3h before IVM. Leukocyte (a) adhesion, (b) migration in cremaster muscle microvasculature were recorded and calculated. Data are mean \pm SEM and n = 5 different animals for each group. *P < .05, significantly different from Vehicle, #P < .05, significantly different from NETs.

In conclusion, this study showed that S100A9 regulates NET formation via TLR4 and RAGE signaling in a ROS-dependent manner. Moreover, S100A9 is observed on the NETs and plays a role in NET-induced leukocyte recruitment. Our findings added S100A9 to the list of NET-associated proteins that exhibit important role in

pro-inflammatory responses. Thus, blocking S100A9 function could be a potential therapeutic way to ameliorate inflammation in NET-dependent diseases.

E3 ubiquitin ligase Midline 1 (Mid 1) in sepsis

Mid1, also known as TRIM 18, is a microtubule-associated E3 ubiquitin ligase [198, 199]. Appropriate function of Mid 1 is essential for cell differentiation, migration and organ development during the embryogenesis [200-203]. In addition, it contributes to the pathological process of some diseases, such as cancer, Alzheimer's disease, and diabetic kidney disease [22, 156, 204]. Furthermore, some studies have reported upregulation of Mid1 in allergic airway inflammation, and shown to promote pulmonary fibrosis, activate innate immune system and exacerbate asthma [23, 24, 205]. In study IV, we explored the role of Mid1 in endothelial cells activation and neutrophils recruitment in vitro and in vivo. We first analyzed RNAseq data of our previous work for Mid1 and ICAM-1 expression in the postcapillary venules (PCV) of septic lung. We found that both Mid1 and ICAM-1 were significantly upregulated in the PCV of septic lung (Figure 14a and b). We then stimulated cultured endothelial cell lines (eEnd.2) with TNF- α for 1h and determined the expression of Mid1 and ICAM-1 by RT-qPCR. It was found that TNF- α challenge elevated Mid1 and ICAM-1 expression by more than 5-fold and 6-fold, respectively in the endothelial cells, indicating that the expression of Mid1 and ICAM-1 in eEnd.2 cells in response to TNF- α exposure is similar to sepsis induced Mid1 and ICAM-1 expression in lung PCV.

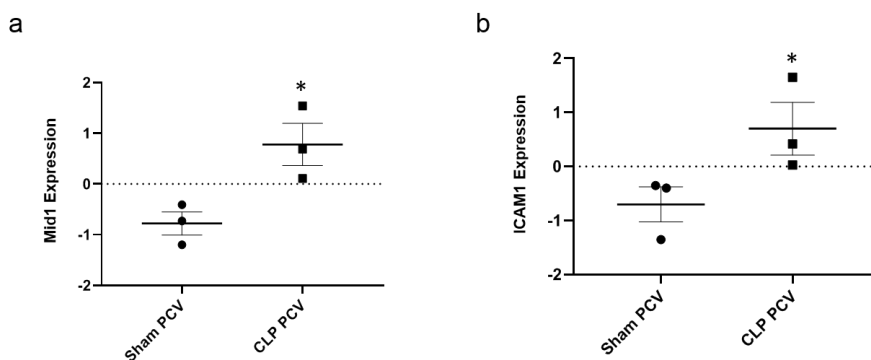


Figure 14. Mid1 and ICAM-1 expression in lung post capillary venules of septic mice. (a) Mid1 expression, (b) ICAM-1 expression in the lung post capillary venules. Expression was analyzed in the RNAseq data using R program (DESeq2). Samples were collected 4 hours after induction of abdominal sepsis and sham mice served as negative control. Data are mean \pm SEM and n = 3. *P < .05, significantly different from Sham PCV.

ICAM-1 is constitutively expressed on endothelial cells and can be quickly up-regulated when stimulated with pro-inflammatory compounds, such as TNF- α [206, 207]. Thus, we investigated the relationship between Mid1 and ICAM-1 expression in endothelial cells. It was found that endothelial cell transfection with Mid1 siRNA significantly reduced TNF- α -provoked ICAM-1 expression in compared to the negative control siRNA group (Figure 15a), suggesting that Mid1 regulates TNF- α -induced ICAM-1 expression in endothelial cells.

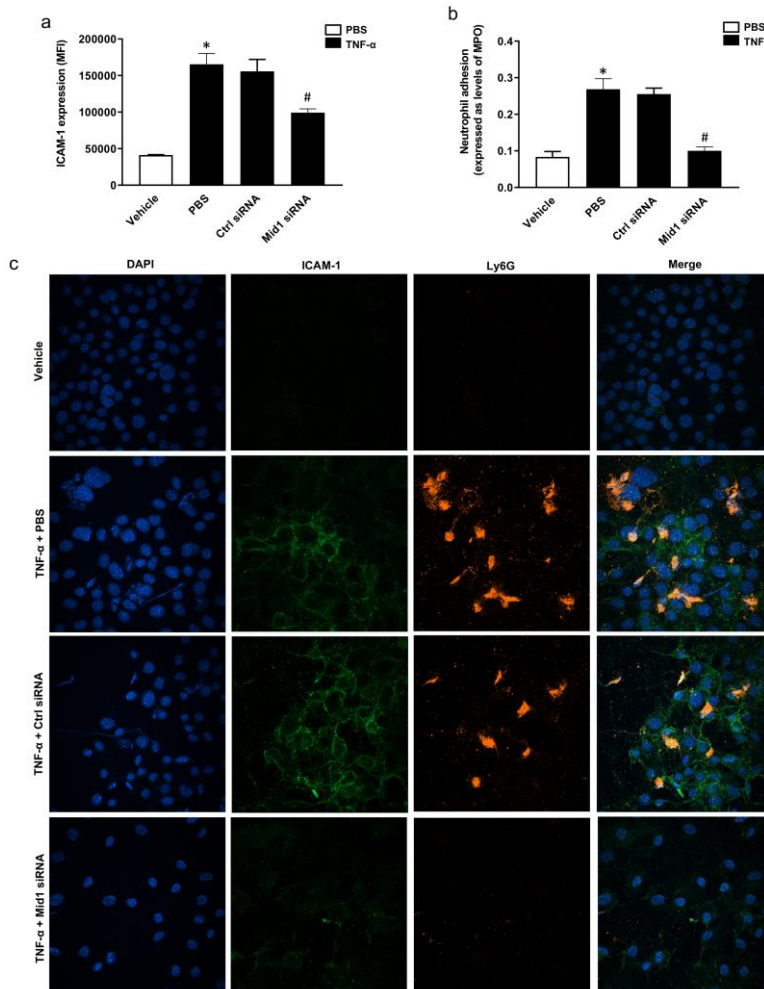


Figure 15. ICAM-1 expression in endothelial cells and neutrophil adhesion in vitro. eEnd.2 cells were transfected with Mid1 siRNA or control siRNA followed by TNF- α stimulation. (a) Aggregate data of ICAM-1 expression examined by FACS. (b) Neutrophil adhesion to endothelial cells in terms of MPO levels. (c) ICAM-1 expression and neutrophil adhesion on eEnd.2 cells were determined by confocal microscopy. Data are mean \pm SEM and n = 4 different animals for each group. *P < .05, significantly different from Vehicle, #P < .05, significantly different from TNF- α + Ctrl siRNA.

Ample amounts of publication have shown that endothelial ICAM-1 mediates leukocyte adhesion via interacting with LFA-1 and Mac-1. Besides, it could also help recruit VCAM-1 to enhance firm adhesion [208-210], thus, targeting of ICAM-1 could decrease leukocyte accumulation and tissue damage. Herein, we performed an *in vitro* adhesion assay to study the role of ICAM-1 and Mid1 in neutrophil adhesion. Endothelial cells were transfected with control siRNA or Mid1 siRNA, followed by stimulation with TNF- α , then co-incubated with neutrophils for 1h. After washing 3 times with PBS, adhesion of neutrophils to endothelial cells were determined as the levels of MPO (ng/ml) in each well. It was observed that MPO levels markedly elevated with TNF- α challenge, while silencing of Mid1 reduced MPO levels by 60% compared to negative control siRNA group (Figure 15b). Additionally, these observations were confirmed by confocal microscopy, in which, we found increase of ICAM-1 expression and neutrophils adhesion to endothelial cells after stimulation with TNF- α . In contrast, ICAM-1 expression and the number of adherent neutrophils remarkably decreased in the Mid1 siRNA treated group of endothelial cell lines (Figure 15c), suggesting the role of Mid1 in regulating ICAM-1 expression in endothelial cells and neutrophil-endothelial cell interactions.

Based on our previous transcriptomic analysis, we found upregulation of Mid1 expression in the post capillary venules of septic lung in mice [26], thereby, we further explored more details here. We intravenously administrated *in vivo* stable Mid1 siRNA to mice before CLP induction and examined leukocyte-endothelium interactions in lungs by use of intravital microscopy. There was a clear increase of leukocyte adhesion in the microvessels of septic lung, and Mid1 silencing significantly attenuated the number of firmly adherent leukocytes in post capillary venules. Furthermore, blocking of ICAM-1 with anti-ICAM-1 antibody reduced leukocyte adhesion to endothelial cells (Figure 16), confirming the role of ICAM-1 in regulating leukocyte accumulation. We then examined the expression of Mid1 and ICAM-1 at mRNA level in septic lung tissues. It was found that the expression of Mid1 and ICAM-1 were both elevated in septic lung tissues, and Mid1 silencing by siRNA down-regulated the expression of both Mid1 and ICAM-1 (Figure 17a and b). Notably, administration of anti-ICAM-1 antibody didn't affect the Mid1 and ICAM-1 expression at mRNA level in lung tissues of CLP mice (Figure 17a and b). Based on these observations, we speculate that Mid1 regulate the leukocyte adhesion by regulating ICAM-1 expression on endothelial cells.

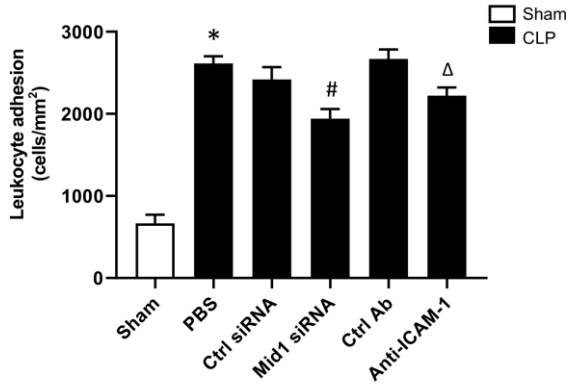


Figure 16. Leukocyte adhesion in septic lung tissues. Mice were pre-treated with Mid1 siRNA or ctrl siRNA. Leukocyte adhesion in lung tissues was observed 4h after CLP induction. Data are mean \pm SEM and n = 5 different animals for each group. *P < .05, significantly different from sham, #P < .05, significantly different from CLP + Ctrl siRNA, Δ p < .05, significantly different from CLP + Ctrl Ab.

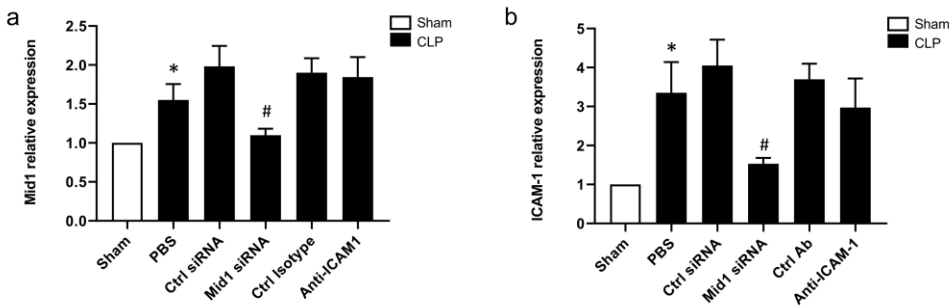


Figure 17. Mid1 and ICAM-1 expression in septic lung tissues. Mice were pretreated with Mid1 siRNA or ctrl siRNA, followed by 4h CLP induction. Lung tissues were collected and total RNA was isolated to determine the (b) Mid1 and (c) ICAM-1 expression. Data are mean \pm SEM and n = 5 different animals for each group. *P < .05, significantly different from sham, #P < .05, significantly different from CLP + Ctrl siRNA

Protein phosphatase 2A (PP2A) complex is widely recognized as target protein of Mid1 and involving in numerous biological activities, such as cell proliferation, motility, cytokinesis, migration, apoptosis and cytoskeleton regulation [211-213]. PP2Ac is the catalytic subunit of PP2A and the expression of PP2Ac could be regulated by Mid1 through ubiquitination. Studies have shown that mutation of Mid1 markedly increased the accumulation of PP2Ac, thus exacerbating the pathological development of Opitz syndrome [214, 215]. Another study found that silencing of Mid1 by siRNA reversed degradation of PP2Ac and reduced airway hyper-reactivity and inflammation in allergic airway inflammation models [23]. Herein, we explored the PP2Ac activity in activated endothelial cells. Cells were

transfected with Mid1 siRNA or control siRNA and then stimulated with TNF- α before the evaluation of PP2Ac levels by western blotting. Calculation of PP2Ac expression was performed by normalization of each band with total protein load of each lane. It was observed that stimulation with TNF- α significantly reduced PP2Ac protein levels, while Mid1 silencing increase the levels of PP2Ac compared to negative control siRNA transfected cells (Figure 18), indicating that Mid1 plays a role in endothelial cells activation via regulating PP2Ac accumulation.

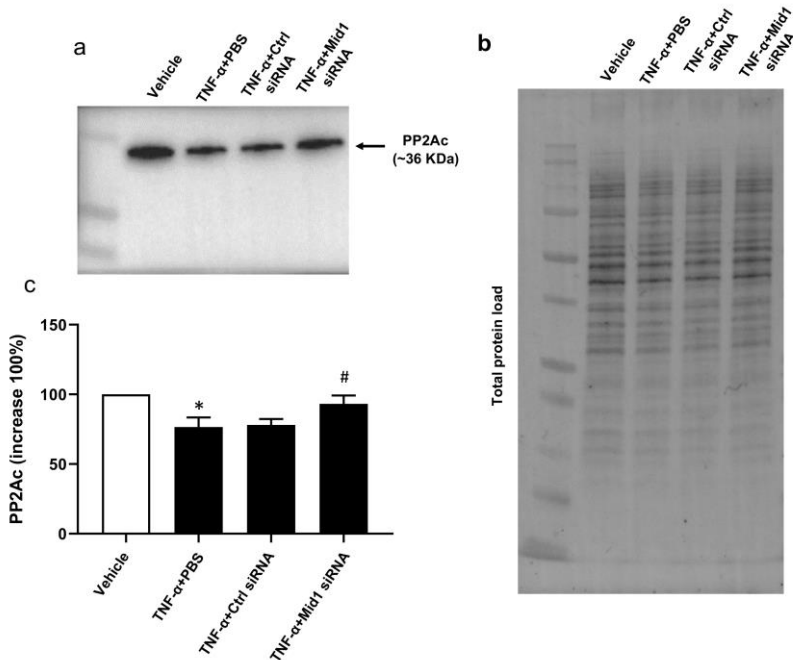


Figure 18. Mid1 regulates PP2Ac expression. eEnd.2 cells were transfected with Mid1 siRNA or control siRNA for 24h, followed by TNF- α stimulation. (a) PP2Ac expression was examined by western blot. (b) Total protein load used to normalize PP2Ac bands. (c) Increase percentage of PP2Ac after normalization with total protein load in each lane. Data are mean \pm SEM and n = 4 different animals for each group. *P < .05, significantly different from Vehicle, #P < .05, significantly different from TNF- α + Ctrl siRNA.

In summary, this study demonstrated that Mid1 might involve in the activation of endothelial cells via regulating expression of PP2Ac protein. It also regulates the process of leukocyte adhesion to endothelial cells through ICAM-1 upregulation both in vitro and in vivo. Thus, targeting Mid1 might be a useful way to reduce lung inflammation in abdominal sepsis.

General discussion and future perspective

It is well known that leukocyte recruitment, on one hand, is a pivotal process to defense against pathogens, while on the other hand, contributes to inflammation and tissue damage. In recent time, much interests have been given on the regulation of leukocyte accumulation in order to control unwanted inflammatory conditions. However, the knowledge on the mechanisms of leukocyte recruitment in different inflammatory conditions is not enough. Increasing number of studies have verified the possibility of blocking leukocyte recruitment to inhibit the inflammatory process in different experimental models [216-218]. Furthermore, some therapeutics targeting leukocyte recruitment have been in evaluation in clinic trails, some even have been employed in treatment [219-222]. For example, several drugs, such as azithromycin, roflumilast, and MK-7123 that target neutrophil infiltration, have been tested in clinical trials, and found to improve neutrophilic inflammation and help to recover lung function of patients with COPD [10].

Selectins and integrins are the major adhesion molecules involved in leukocyte accumulation, thus inhibitors targeting these molecules would be efficient strategies to reduce leukocyte recruitment. Based on different experimental studies, researchers have found that blocking of selectins or PSGL-1 could prevent leukocyte recruitment and regulate inflammatory responses [223-226]. Several monoclonal antibodies or small molecules targeting integrins have been developed and even applied in clinic to control excessive inflammation [227, 228]. In addition, chemoattractants are important molecules for leukocyte activation and recruitment, thus, targeting different chemoattractants or receptors by using antagonists could be an effective way to inhibit inflammatory responses. For instance, a CXCR2 antagonist, SB656933, was found to inhibit neutrophil recruitment to the lung of patients with cystic fibrosis in a clinical study [229]. Besides, signaling cascades regulating the process of leukocyte recruitment could also be a potential therapeutic

target. However, administration of these drugs should be considered carefully since blocking of any inflammatory steps may trigger severe side effects on other physiological processes.

It is well established that platelets support the leukocyte activation and recruitment [230]. Furthermore, evidences also support that platelet-derived mediators, such as cytokines from platelet α -granules, could induce other immune cells activation [231]. Short-chain PolyP is also one of platelet-derived mediators, and researchers have found that it mainly comes from platelets in mammals. To study the mechanism of PolyPs in regulating leukocyte activity, we used IVM to observe the leukocyte recruitment in response to PolyP stimulation locally. We identified that PolyP-mediated leukocyte rolling is mainly mediated by P-selectin-PSGL-1 interactions and firm adhesion is mediated by Mac-1 and LFA-1 binding with their receptors in microvessels. Thus, our study provided new information and potential targets in PolyP-mediated inflammation. However, one study reported a contradictory result concerning the role of selectin. They have shown that upregulation of E-selectin in PolyP-treated endothelial cells support the monocytic THP-1 cells adhesion in vitro [99]. It should be noted that in this study we mainly focus on the neutrophil recruitment in response to PolyPs and we don't exclude the role of E-selectin in regulating monocyte adhesion in other conditions in vivo. Thus, further studies are necessary to explore the role of E-selectin on other conditions. Immune system and hemostasis are intertwined, and PolyPs is also known to regulate both coagulation and inflammation. Whether the process of coagulation is got affected by PolyP challenge is remained to be studied in future.

NET formation is widely investigated in a variety of diseases, thus, there is a possibility that targeting NETs would be a solution to control the process of many diseases. Interestingly, researchers observed that microorganism could develop a way to evade NETs by producing DNase enzyme. For example, Buchanan et al. elucidated that expression of DNase by pathogen group A streptococcus could enable them to evade killing by NETs [232]. Similar results were also reported in other microorganisms. For instance, Palmer et al. detected the activity of DNase from 34 periodontal bacteria and found that DNase is a common mechanism that bacteria employed to defend against NETs, thus promoting the pathological process of diseases [233]. Another study demonstrated the role of DNase-like protein, released by fungus, *Paracoccidioides brasiliensis*, in defending against NETs [234]. Besides, DNase can be produced in many tissues, such as pancreas, parotid glands, kidney etc [235, 236]. By degrading DNA, DNase participates in physiological processes and protects body from harmful conditions. For example, aerosolized

recombinant human DNase I is now in use to treat cystic fibrosis patients. Moreover, NET-attached proteins are vital in NET stability and targeting NET-associated proteins could also be an effective strategy to treat various NET-associated diseases. However, the functions of NET-associated proteins are not well studied. One reason could be the complexity of components loaded on NETs. Our results indicate that NET-associated MPs induces leukocyte recruitment via HMGB1-TLR2/TLR4 axis. Interestingly, we and others found S100 calcium binding protein A9 (S100A9) as a NET attached protein. Considering the existing literatures that NET formation is significantly increased in septic plasma and organs [237], we explored the relations between S100A9 and NET formation in experimental sepsis. We found that blocking S100A9 reduced NET formation both in septic lung tissue and plasma. We also observed that S100A9 regulates NET generation via RAGE and TLR4 receptors in vitro and NET associated with S100A9 is a potent inducer of leukocyte recruitment in vivo. These observations provide an implication that NET-associated MPs and S100A9 are important regulator of neutrophil infiltration during inflammation and targeting those molecules using inhibitors could be a useful way to control excessive inflammatory responses.

It is worth to note that targeting response of endothelial cells during inflammation could be another therapeutic target. One study described that endothelial dysfunction is a common feature in bacterial sepsis and targeting endothelial functions could be a useful way to treat bacterial sepsis [238]. In another study, researchers have shown that blocking of MAdCAM-1 on endothelial cells by a monoclonal antibody, PF-33547659, reduced inflammatory conditions in ulcerative colitis and Crohn's disease. Clinical trials with this antibody showed promising results in ulcerative colitis with a good safety and efficacy [239, 240], while treatment of patients with Crohn's disease failed to improve the conditions. Time frame of therapy and improved assessment should be taken into consideration in the further studies [239]. Herein, we studied the role of endothelial Mid1 in sepsis models and found that Mid1 could regulate the expression of ICAM-1 and enhance leukocyte recruitment in septic lungs, suggesting that Mid1 could be another target to limit excessive leukocyte accumulation during sepsis. It should be noted that Mid1 plays important role in embryonic develop [22], therefore, further preclinical studies are required to confirm the safety and efficacy before any human study.

Considering the complicated and interdependent mechanisms of inflammatory processes in our body, more clinical and preclinical studies should be done before applying the newly revealed knowledge in clinical settings. Nevertheless, the observations in this thesis revealed new mechanisms on interaction between

leukocytes and endothelial cells during inflammation. Thus, these findings might provide some new possibilities for developing new therapies against inflammatory diseases.

Conclusion

- I. P-selectin and PSGL-1 interactions play an important role in neutrophil rolling, which is a prerequisite for subsequent Mac-1 and LFA-1 mediated firmly adhesion and extravascular emigration in response to PolyP challenge. Thus, our observations suggest that PolyP could be a potential target to limit excessive leukocyte migration during inflammation.
- II. Neutrophil-derived MPs could bind NETs and promote NET-induced neutrophil accumulation by expressing HMGB1 and regulating TLR2 and TLR4 signaling. Hence, targeting MPs or blockade of HMGB1, TLR2 or TLR4 may help to ameliorate NET-dependent inflammation.
- III. S100A9 regulates NET formation via TLR4 and RAGE signaling in an ROS-dependent manner. NET-associated S100A9 participates in NET-induced leukocyte recruitment in vivo, indicating that S100A9 might be involved in both the formation and function of NETs in abdominal sepsis.
- IV. Mid1 regulates endothelial ICAM-1 expression and leukocyte recruitment in septic lung via regulating PP2Ac levels, suggesting the role of Mid1-PP2Ac axis in pathological lung inflammation in abdominal sepsis. Thus, targeting Mid1 could be a useful way to ameliorate tissue damage in sepsis.

Populärvetenskaplig sammanfattning

Leukocyter, även kända som vita blodkroppar, skyddar vår kropp från infektioner. Processen för leukocytvandring till inflammationsställena kallas leukocytrekrytering, vilket består av leukocytrullning, adhesion och migration. Trots deras skyddande roll kan överdriven ackumulering av leukocyter orsaka vävnadsskada och förvärra inflammatoriska tillstånd. Att undersöka mekanismerna för leukocytrekrytering är därför mycket viktigt för att kontrollera avvikande inflammatoriska svar. Celladhesionsmolekyler, såsom selektiner och integriner, och kemoattraktanter är de viktigaste molekylerna i processen för cellrekrytering under inflammation. Ändå är andra deltagare, såsom olika stimuli eller sjukdomstillstånd också viktiga faktorer i denna process. Därför är studiet av leukocytrekrytering som svar på olika molekyler eller stimuli mycket viktigt.

I projekt 1 studerade vi rollen av polyfosfater (PolyPs) i leukocytrekrytering in vivo. Blodplättar utsöndrar kortkedjiga PolyPs efter aktivering, som är kända för att reglera rekrytering av leukocyter. Vi utvärderade leukocytrekrytering med intravitalt mikroskop i cremaster muskelmikrokärlen hos möss efter injektion av PolyPs. Intravital mikroskopi (IVM) tillåter forskare att observera leukocyters interaktioner med endotelceller som svar på externa stimuli i levande djur direkt. Blockering av P-selektin eller dess ligand, PSGL-1, förhindrade leukocytrullning och migration. Medan blockering av leukocytreceptorer, Mac-1 och LFA-1, endast hämmade leukocytadhesion men inte rullning, vilket indikerar rollen av dessa adhesionsmolekyler i PolyP-inducerad leukocytrekrytering.

I projekt 2 och 3 undersökte vi bildandet och funktionen av neutrofilers extracellulära fällor (NET) vid inflammation. NET är en nätlignande kromatinstruktur belagd med nukleära och cytoplasmatiska proteiner. NET-bildning är en mekanism för försvar mot patogener av neutrofiler. Det är känt att NET kan fånga invaderande patogener och eliminera dem med de cellulära antimikrobiella komponenterna dekorerade på NET. Överdriven bildning av NET kan utlösa en ny omgång av inflammation om den inte rensas upp ordentligt och i tid. Således studerade vi mekanismerna för NET-bildning och effekterna av vissa komponenter

i NET vid inflammation. Mikropartiklar (MP) är membranvesiklar som visar sig kunna fästa på ytan av NET. Vi injicerade möss med NET och NET utan MP och observerade med IVM. Det visade sig att NET med MP ökade leukocytackumulering i vävnaden, medan NET utan MP är mindre effektiva för att stimulera leukocytackumulering. Förutom MPs är S100A9 en annan komponent som finns på NET, så vi undersökte rollen för S100A9 i NET-bildning och NET-bunden S100A9 vid lokal inflammation. Vi fann att NET-bildning reduceras signifikant hos möss septisk lunga och plasma när de behandlas med S100A9-hämmare. I IVM-experiment observerade vi att NET med blockerad S100A9 visade svagare förmåga att inducera leukocytrekrytering jämfört med NET-stimulering. Dessa resultat betyder att S100A9 deltar i NET-generationen och NET-associerad S100A9 är en stark stimulator av leukocytrekrytering.

Genom att veta att interaktioner mellan leukocyter och endotelceller är en förutsättning för leukocytmigration, undersökte vi i projekt 4 endotelcellers aktiveringsmekanism och interaktion med leukocyter. Midline 1 (Mid1) är ett E3-ligas mikrotubulärt protein involverat i embryonal utveckling och inflammatoriska funktioner. Vi observerade flera gånger ökningen av Mid1 och ICAM-1 i de postkapillära venolerna i mus septisk lunga och i TNF- α -stimulerade endotelcellinjer. TNF- α -stimulering ökade neutrofiladhesion till aktiverade endotelceller in vitro och tystnad av Mid1 av siRNA minskade neutrofiladhesion till aktiverade endotelceller. Dessutom använde vi sepsismodell för att undersöka rollen av Mid1 in vivo, det visade sig att tystnad av Mid1 minskade lungan ICAM-1-uttryck och leukocytadhesion, vilket indikerar att Mid1 kunde bidra till leukocytrekryteringen vid sepsis genom att reglera uttrycket av ICAM-1.

Sammanfattningsvis avslöjade denna avhandling flera nya mekanismer som reglerar leukocyter och endotelcellsinteraktioner i olika inflammatoriska miljöer. Vi föreslår att inriktning på dessa proteiner skulle vara ett effektivt sätt att lindra överdriven leukocytackumulering och vävnadsskada under lokal eller systemisk inflammation.

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