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Mechanisms of neutrophil recruitment in septic lung injury

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LUND UNIVERSITY
Faculty of Medicine

Doctoral Thesis

With approval of Medical Faculty at Lund University to be defended on 27th of February 2013 at 9:15 in the Clinical Research Centre (CRC), Entrance 72, Skåne University Hospital, Malmö

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Mechanisms of neutrophil recruitment in septic lung injury

Abstract

Sepsis is one of leading cause of death despite aggressive surgical intervention and antibiotic therapies. Excessive neutrophil recruitment is a major feature in early phase with immune system dysfunction at later phase. Lung is the most vulnerable, critical and sensitive organ during sepsis process.
The aim of thesis was to prevent excessive neutrophil accumulation in lung parenchyma on one hand, and to reinforcement of immune system at later phase of sepsis on other hand.
We hypothesized that CD44 may have a role in mediating pulmonary recruitment of neutrophils along with Rho GTPase subfamily in more than one way for enhancing neutrophil stiffness and migration. Peptides are new evolutionary compound with multifunctional effects, especially during infection and sepsis but potential therapeutic effect of them in polymicrobial sepsis remains elusive. Polymicrobial sepsis was induced by cecal ligation and puncture, purified monoclonal antibody against CD44, Rho kinase inhibitor (Y-27632) and Rac1 inhibitor (NSC23766). Specific TDPs, GKY20 and GKY25 were injected after procedure. Edema formation, bronchoalveolar accumulation of neutrophils, myeloperoxidase activity, and CXC chemokine in lung measured after CLP. We observed that sepsis triggered clear-cut lung damage characterized by edema formation, neutrophil infiltration, and increased levels of MIP-2 in the lung. We demonstrate that immunoneutralization with anti-CD44 reduce neutrophil activation and accumulation as well as edema formation and lung injury. Pretreatment with Y-27632 reduced the CLP-induced pulmonary injury and MPO activity as well as Mac-1 on neutrophils along with clear reduction in F-actin formation. Administration of NSC23766 markedly reduced CLP-triggered neutrophil infiltration, edema formation and tissue damage in the lung. Inhibition of Rac1 decreased CLP-induced neutrophil expression of Mac-1 and pulmonary formation of CXC chemokines. NSC23766 abolished the sepsis-evoked elevation of mRNA levels of CXC chemokines and TNF-α in alveolar macrophages. Moreover, TDPs maintain CD4 T-cells function in spleen by reducing T-cell apoptosis and clear reduction in sepsis-mediated T-regulatory production. TDPs abolished CLP- evoked HMGB1 and IL-6 production. Furthermore TDPs exerts clear cut bacterial clearance in the blood and spleen.
Thus, this work show more details in neutrophil extravagation during sepsis. Our data may dig up the way for establishing more specific and effective treatments of sepsis.

Key words: sepsis, infection, neutrophil, lung, chemokines, apoptosis, CD44, F-actin, peptides

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To my parents

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1. List of papers

The thesis based on the following papers:

1. Hasan Z*, **Palani K***, Rahman M, Thorlacius H. Targeting **CD44 expressed on neutrophils inhibits lung damage in abdominal sepsis**. *Shock*. 2011 Jun; 35(6):567-72.

2. **Palani K**, Rahman M, Hasan Z, Zhang S, Qi Z, Jeppsson B, Thorlacius H. **Rho-kinase regulates adhesive and mechanical mechanisms of pulmonary recruitment of neutrophils in abdominal sepsis**. *Eur J Pharmacol*. 2012 May 5; 682 (1-3):181-7. E pub 2012 Feb 21.

3. Hwaiz R., Hasan Z., Rahman M., Zhang S., **Palani K.**, Syk I., Jeppsson B., and Thorlacius H. **Rac1 signaling regulates sepsis-induced pathological inflammation in the lung via attenuation of Mac-1 expression and CXC chemokine formation**. Submitted to *J innate immunity*.

4. **Palani K**., Rahman M., Hasan Z., Zhang S., Syk I., Schmidtchen A., and Thorlacius H. **Therapeutic effects of human thrombin-derived host defense peptides in polymicrobial sepsis**. Submitted to *Crit Care Med*.

* Equally contributed in the work.

Papers not included in the thesis:

1. Hasan Z, **Palani K**, Rahman M, Zhang S, Syk I, Jeppsson B, Thorlacius H. **Rho-kinase signaling regulates pulmonary infiltration of neutrophils in abdominal sepsis via attenuation of CXC chemokine formation and Mac-1 expression on neutrophils**. *Shock*. 2012 Mar; 37 (3):282-8.

2. Hasan Z, Rahman M, **Palani K**, Syk I, Jeppsson B, Thorlacius H. **Geranylgeranyl transferase regulates CXC chemokine formation in alveolar macrophages and neutrophil recruitment in septic lung injury**. *Am J Physiol Lung Cell Mol Physiol*. 2012 Dec 14.

3. Hasan Z, **Palani K**, Zhang S, Rahman M, Hwaiz R, Lepsenyi Syk I, Jeppsson B and Thorlacius H. **Rho-kinase regulates induction of T-cell immune dysfunction in abdominal sepsis** (Manuscript).

2. Abbreviations

CARS	Compensatory anti-inflammatory response
CD	Cluster of differentiation
CLP	Cecal ligation and puncture
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
i.p.	Intraperitoneally
i.v.	Intravenous
IL	Interleukin
KC	Cytokine-induced neutrophil chemoattractant
LFA-1	Lymphocyte function antigen-1
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
Mac-1	Membrane activate complex-1
MFI	Mean fluorescence intensity
MPO	Myeloperoxidase
PE	Phycoerythrin
PG	Peptidoglycan
PMNL	Polymorphonuclear leukocyte
Rac1	Ras-related C3 botulinum toxin substrate 1
s.c.	Subcutaneous
SIRS	Systemic inflammatory response syndrome

3. Introduction

Sepsis is a potentially life-threatening complication of an infection. The clinical phenomena preceding the development of sepsis and septic shock are highly complex. Paradoxically, persons with a weakened immune system are most likely to develop sepsis, but the detrimental processes that may ultimately lead to the death of the patient are mostly caused by an exaggerated, systemic response to an infection (Van Amersfoort et al, 2003). The mortality rate is still high approximately 30-40% in the elderly and 50% or greater in patients with more severe syndrome, septic shock (Cohen, 2002; Martin et al, 2003). Generally there are different terms under the title of sepsis with strong relation between them (Figure 1) including:

Colonization: microbiological event (presence of bacteria, virus, fungi, etc) with no host defense.

Infection: inflammatory response to microorganism or invasion of normally sterile tissue.

Bacteremia: is the presence of bacteria in the blood.

SIRS: systemic inflammatory response to a variety of severe clinical insults, and can be defined also as ≥ 2 of the following (Bone et al, 1992a):
Fever of more than 38°C (100.4°F) or less than 36°C (96.8°F)
Heart rate of more than 90 beats per minute
Respiratory rate of more than 20 breaths per minute or arterial carbon dioxide tension ($PaCO_2$) of less than 32mm Hg
Abnormal white blood cell count (>12,000/µL or < 4,000/µL or >10% immature [band] forms)

Sepsis: is defined as SIRS with a documented or suspected infection.

Severe sepsis: is defined as sepsis with hypotension and/or organ dysfunction or features of hypotension (lactic acidosis, oliguria and altered mental status).

Septic shock: is severe sepsis with continuous hypotension despite adequate fluid resuscitation and usually associated with hypo-perfusion abnormalities.

MODS: when there is progressive distant organ failure following severe infectious or non infectious insults, characterized by distant organ failure and homeostasis cannot be maintained without interventions.

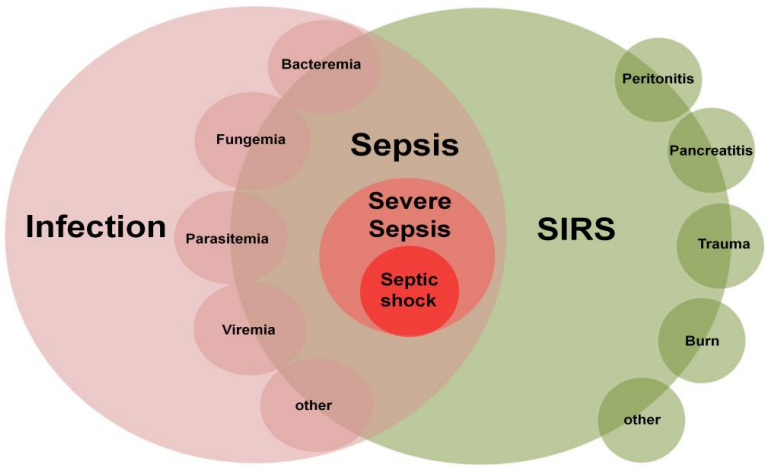

Figure1. Sepsis (adapted from Bone et al1992)

Patients usually develop a single organ failure, lung being the most sensitive and liable organ in sepsis followed by the failure of other organs, for instance, the liver and kidney, resulting in multiple organ dysfunctions (Cohen, 2002).

There are two forms of ALI, Primary ALI which is caused by a direct injury to the lung (e.g., pneumonia) and secondary ALI which is caused by an indirect insult (e.g., peritonitis, pancreatitis).
Lung injury is the most common sequel for the sepsis; there are two forms of lung injury regarding the severity, acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). The committee American-European Consensus Conference (AECC) at 1994 defined ALI and ARDS as an acute syndrome of inflammation and increased lung capillary permeability associated with a number of clinical, radiological and physiological abnormalities that cannot be explained by, but may coexist with heart failure (Table 1). Furthermore, to distinguish between ALI and ARDS, the committee suggested the use of the ratio between arterial oxygen data (PaO2) and the amount of oxygen delivered by ventilator support (FIO2), which is a value usually used to measure arterial blood oxygenation (PaO2/FIO2). The criteria for ALI would be a PaO2/FIO2 ≤ 300mm Hg (40kPa) and for ARDS PaO2/FIO2 ≤ 200mm Hg (26.7kPa) (Bernard et al, 1994).

Table1. AECC criteria for Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS)

	ALI criteria	**ARDS criteria**
Onset	Acute onset	Acute onset
Oxygen saturation	PaO2/FiO2 ≤ **300** mm Hg	PaO2/FiO2 ≤ **200** mm Hg
Chest X-ray	Bilateral infiltrates	Bilateral infiltrates
Pulmonary Artery Wedge Pressure	18 mm Hg or no clinical evidence of left arterial hypertension	18 mm Hg or no clinical evidence of left arterial hypertension

4. Background

4.1. Sepsis

Sepsis and/or SIRS are a serious condition characterized by widespread activation of host innate immune response initiated by gram-negative or gram-positive bacteria or fungi and in rare cases viruses and parasites (Astiz & Rackow, 1998). SIRS can be of non-infectious cause as burn, trauma, pancreatitis or surgery. Sepsis develops when the immune system becomes amplified, and then disorganized and incapable to fight infectious source (Cohen, 2002; Warren, 1997; Zeni et al). Concomitantly sepsis characterized by activation and recruitment of leukocyte to the body organs specially lung which also participate in the complex pathophysiology in the disturbance and injury of pulmonary microvasculature (Doerschuk et al, 1987; Kollef & Schuster, 1995; Kuhnle et al, 1999).

4.2. Pathophysiology of sepsis

In simple terms sepsis can be viewed as an imbalance of inflammation, coagulation, and fibrinolysis. Pathophysiology of sepsis is complex and start when there is an infectious nidus. Consequently, when the surface molecules of microorganisms such as endotoxin, lipoproteins, peptidoglycan as well as exotoxins from gram-positive are recognized as internal danger signals by body immune system (Matzinger, 1994). Recognition of these molecules are sophisticated procedure and mainly mediated through pattern recognition receptors (PRRs) that alert the innate immune response system to the presence of a microbial invader. Toll like receptors (TLRs) are essential members of this family. There are other pattern-recognition molecules such as alternative complement components (Guo & Ward, 2005), mannose-binding lectin (Hibberd et al, 1999), and CD14 (Hoffmann et al, 1999).

Previous study documented that about 60% of the cases are caused by gram-negative bacteria and the rest are caused by gram-positive bacteria (Alberti et al, 2002).

Endotoxin which also called LPS is the main stimulus for initiation of a long and complex signaling pathway for gram- negative microorganisms. LPS molecule is located on the outer membrane of bacterial cell membrane and the lipid A part of this molecule is the most toxic portion and involved in the activation of different host cells (Cohen, 2002; Seydel et al, 2000). CD14/TLR4/MD2 receptor complex of host cells is involved in the recognition of most gram-negative bacteria and their products (Triantafilou, 2005).

Microbial products from gram-positive bacteria including lipoteichoic acid (LTA), peptidoglycan (PG), flagellin, microbial DNA can binds to cell surface receptors and stimulate different cytokine production (Majcherczyk et al, 1999; Morath et al, 2001). CD14/TLR2 receptor complex is involved in binding with these components.

4.3. Immune-inflammatory response in sepsis

Activation of immune system including both innate and adaptive and may be direct or indirect. Moreover previous study has been reported that different toxins from gram-negative or gram-positive organisms activate the host immune system in a totally different way. For example, LPS activate macrophages and stimulate TNF-α production (Ulevitch et al, 1990; Wright et al, 1990), while super-antigens from gram-positive bacteria do not stimulate clear-cut TNF-α production but activate primarily T-lymphocytes casing apoptosis (Renno et al, 1996). However, activation of immune system during polymicrobial sepsis by mixed bacterial flora is much more complex and largely unknown (Parrillo, 1993; Remick, 2007).
Once bacteria or antigen is isolated and recognized by immune system, trigger pan activation of innate immune cells, i.e. neutrophils, macrophages and endothelium, which leading to release different pro-inflammatory mediators like tumor necrosis factor

(TNF), interleukins (IL-1, IL-6), cytokines, prostaglandins (PG), high mobility group box-1 (HMGB1) and platelet activating factor (PAF) to modulate inflammatory response and promote recovery of the tissue. This phase called hyper-inflammatory phase and clinically termed SIRS. The release of the inflammatory mediators starts the coagulation cascade activation and leading to the development of a clot with subsequent septic shock. On the other hand to maintain this clot, inhibitors should release to suppress fibrinolysis or breakdown. This is necessary to have adequate time for the body to destroy the bacteria before the clot is gone. Lastly with the help of pro-inflammatory mediators neutrophils or other phagocytic cells attract bacteria and try to engulf and kill them by producing reactive oxygen species (Aderem & Ulevitch, 2000).

To prevent adverse response of inflammatory reaction from damaging normal tissue, the hyper-inflammatory phase followed by a hypo-inflammatory phase, so the pro-inflammatory mediators is immediately followed by a sustained anti-inflammatory mediator production (Docke et al, 1997; Oberholzer et al, 2001). Anti-inflammatory mediators are including transforming growth factors, interleukins (IL-4, IL-10), IL-1 receptor antagonist and decoy receptor such as IL-1 receptor type II. There is basically a tug of war going on between the pro-inflammatory and anti- inflammatory mediators of the body. The balance between pro-inflammatory and anti-inflammatory mediators restricts the inflammation response to the local site of infection. During hypo-inflammatory phase immune system became weak and incapable to mediate appropriate host-defense responses against infectious agents, this condition named as compensatory anti inflammatory response syndrome (CARS) (Bone et al, 1997; Moore et al, 2011). During CARS there increased strong susceptibility to infections which are the most common cause of death in patients with sepsis (Moore et al, 2011). Moreover, during CARS there is loss of phagocytic activity of phagocytic cells, diminished major histo-compatibility complex type II, loss of delayed type hypersensitivity response with T-cells apoptosis (Ayala et al, 1996; Bommhardt et al, 2004), and increase the regulatory T-cells population which further paralyze the immune system to mediate anti-bacterial responses (Hiraki et al, 2012; Monneret et al, 2003). In sepsis, continued release of pro-inflammatory cytokines overwhelms

the anti-inflammatory cytokines, and when the body is unable to maintain the appropriate balance, the immune response is no longer local but becomes systemic. Inflammation and altered clotting quickly spread through the body.

4.4. Neutrophils and sepsis

Neutrophils play an important role in the innate immune system. Neutrophils sequestration into the pulmonary microvasculature and quick migration into the lung parenchyma are critical steps in the pathogenesis of ALI (Tasaka et al, 2005). Previous studies suggest that the sequestration and Migration of neutrophils into the extravascular space and tissue occurs through at least two sequential events (Doerschuk et al; Kubo et al, 1999; Mizgerd et al, 1997). The first event is a rapid sequestration of neutrophils, followed by subsequent leukocyte recruitment (Figure .2).

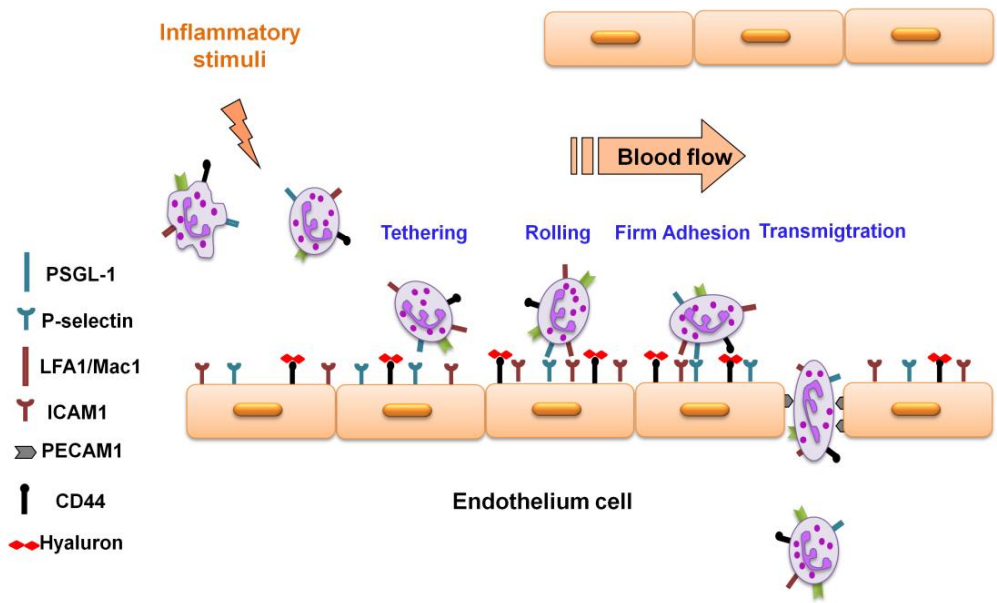

Figure 2. Recruitment process.

4.4.1. Sequestration

The initiating event of ALI is sequestration of PMN in micro-vessels of the lung (Hogg & Doerschuk, 1995), this occurs during the first few minutes after leakage of inflammatory mediators into the blood stream. Sequestration is influenced by the size, deformability and adhesive qualities of PMN and endothelial cells. The discrepancies between the size of PMN and pulmonary capillaries segments dictate the need for PMN to deform to get through pulmonary capillaries (Hogg & Doerschuk, 1995), this related to the fact that the spherical diameter of pulmonary capillaries (6 µm) is smaller than that of neutrophils (7 µm) (Doerschuk et al, 1989). Consequently, most neutrophils must deform to pass through the narrower pulmonary capillaries, which mean that a reduction in their deformability would contribute to increased sequestration and subsequent migration.
Inflammatory stimuli that activate neutrophils can cause a rapid decrease in the deformability of the neutrophils. This decreased deformability of neutrophils is thought to be mediated by a rapid change of soluble G-actin to filamentous F-actin to form an actin-rich rim beneath the plasma membrane, which intern increasing the rigidity and the viscosity of the neutrophils (Downey et al, 1991; Frank, 1990; Worthen et al, 1989). The process of F-actin formation from G-actin called polymerization which is unstable and complex and reversible process take place with the help of ATP and may be followed by depolymerization (Figure .3).
Furthermore, inflammatory stimuli up-regulate adhesive molecules which help the stiffed neutrophils for further consequence steps.

4.4.2. Recruitment

Leukocyte recruitment is a complex multi-step cascade mainly composed of tethering, rolling, adhesion and transmigration (Blankenberg et al, 2003). These inter-dependent steps characterized by close interaction between activated leukocyte in one hand and endothelial cell of post-capillary venules on the other hand with the support of specific adhesion molecules expressed on

the surface of both side, including selectins, integrins and immunoglobulin super-family (Ley, 1996; Smith, 2008).

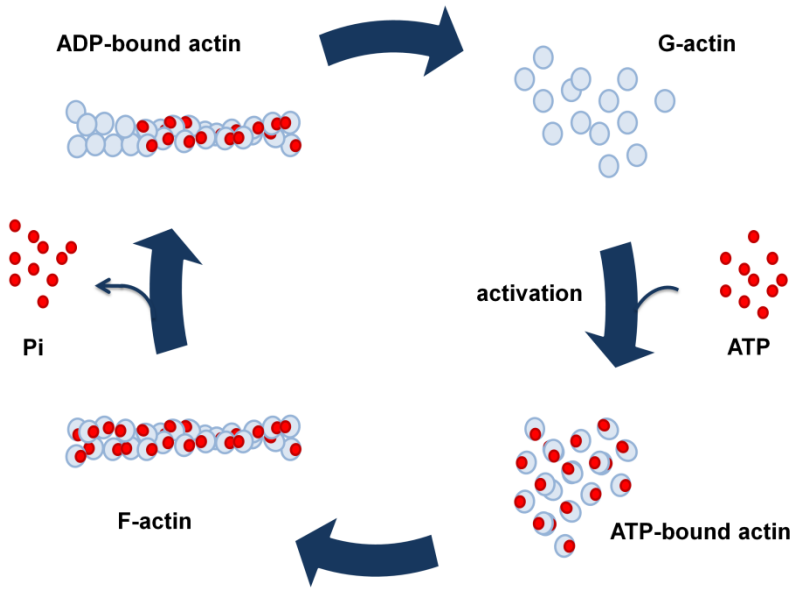

Figure 3. Actin Polymerization

4.4.2.1. Tethering and rolling

Initially, leukocyte rolling is enabled by relatively weak and reversible adhesions and mediated by the selectin family including P- selectin , E-selectin and L-selectin and their ligands and generally considered as the initial step of leukocyte transmigration process (Montoya et al, 1999; Vestweber & Blanks, 1999). Leukocytes are considered to roll when their velocity is approximately 50 times lower than the base line (Tangelder & Arfors, 1991). Many pro-inflammatory agents like TNF-á, histamine, leukotrienes have been reported to regulate rolling by controlling the up-regulation of P-selectin (Klintman et al,

2004; Mansson et al, 2000). However, rolling is not a obligatory for subsequent adhesion and transmigration in narrow lumens, for example, in liver sinusoids, lung capillaries (Fox-Robichaud & Kubes, 2000; Mizgerd et al, 2000).

4.4.2.2. Adhesion

The rolled leukocytes can be adhering tightly to the endothelial lining and shifted to an adhesion stage if there is appropriate chemotactic stimulus. Firm adhesion of leukocytes to endothelial cells is mainly mediated by integrins which binds with their constitutive ligands (Awla et al, 2011a), such as ICAM-1, ICAM-2 and VCAM-1. Integrins are expressed on the surface of leukocyte at a low affinity status but in the presence of adequate stimulus they are activated to mediate firm adhesion (Awla et al, 2011a; Thorlacius et al, 2000). Beta2 (CD18) or “leukocyte” integrins are restricted to leukocytes and as adhesion receptors they are fundamental to both innate and adaptive immunity. Thus, the integrins involved in leukocyte firm adhesion to endothelial cells are mainly of the Beta 2-integrin (CD18) subfamily, such as (LFA-1 or CD11a/CD18); (Mac-1 or CD11b/CD18); (p150, 95 or CD11c/CD18). Moreover, (VLA-4) expressed on lymphocytes and activated neutrophils (Blankenberg et al, 2003).

4.4.4.3. Transmigration

After firm adhesion of leukocytes to the endothelial lining cell, leukocyte try to extravasate to the site of maximal inflammation, which indicated by peak concentration of chemoattractant signals. Migration could occur either para-cellularly or intra-cellularly. Para-cellular pathway which means leukocytes migrate along the junctions between tightly apposed endothelial cells (Hurley & Xeros, 1961; Marchesi, 1961), while intra-cellular means passing through the endothelial cells (Engelhardt & Wolburg, 2004). The process take place by the help of variety of membrane protein located at the edge of endothelial cell membrane. One of the most important

protein molecule which involved in the leukocyte transmigration is platelet endothelial cell adhesion molecule-1 (PECAM-1) (Muller et al, 1993) which also called (CD31) which belongs Ig-like sub-family and It is expressed abundantly on endothelial cells and mainly located at the junctions between adjacent endothelial cells, PECAM-1 is also present on the surface of most other cells as leukocytes and platelets. Other molecules are E-selectin, junction adhesion molecules, ICAMs, vascular cell adhesion molecule -1 and CD99 (Hordijk, 2006; Vestweber, 2007).

5. CD44 and sepsis

The CD44 (cluster of differentiation 44) antigen which also known as phagocytic protein 1, homing cellular adhesion molecule (HCAM), Hermes antigen, and ECMR III (Screaton et al, 1992; Zhou et al, 1989), is a cell-surface glycoprotein present on a variety of cells (Picker et al, 1989). It Comprises more than 40 proteins ranging from 80 to 200 kDa, all coded by a single, highly conserved gene (Screaton et al, 1992). CD44 consists of three portions, an extracellular, which is an amino terminal globular protein domain; a stem structure, a transmembrane part, and a cytoplasmic tail region (Figure .4). Main CD44's extracellular domain ligand is hyaluronan (HA), but the receptor can bind with several components of the ECM e.g. collagen, laminin, fibronectin, and osteopontin. Moreover, CD44 interacts with growth factors, different cytokines, chemokines, and MMPs (Cichy & Puré, 2003; Ponta et al, 2003). A link domain within the extracellular globular domain consists of binding sites for HA as well as 18 other glucosaminoglycans (GAGs). GAG affinity depends on the degree of glycosylation of the extracellular CD44 domain, which is regulated by post-translational modifications (Skelton et al, 1998). These modifications are specific to cell type and growth condition.

Since CD44 has both an extracellular and intracellular domain it is able to participate in several biological processes both extra-cellularly and intra-cellularly. Extracellular amino globular protein domain of CD44 can bind to enzymes and substrates on the cell surface and act as a platform for several molecules e.g. growth factors and MMPs. For example, MMP-9 binds to CD44, degrading collagen IV (Yu & Stamenkovic, 1999) and activating the precursor of transforming growth factor beta (TGF)-β (Yu & Stamenkovic, 2000). Loss of CD44 results in impaired TGF-β activity (Teder et al, 2002). Furthermore, a heparin-sulphate proteoglycan isoform of CD44 including variant exon 3 (CD44v3) binds fibroblast growth factor (FGF) (Jones et al, 2000; Sherman et al, 1998), vascular endothelial growth factor (VEGF) (Jones et al, 2000), and heparin-binding epidermal growth factor (Bennett et al, 1995; Jones et al,

2000). Interestingly, CD44v3 is induced during differentiation of monocytes into macrophages (Jones et al, 2000). Intracellular domain part of CD44 has been shown to be associated with the cell cytoskeleton through the ERM-proteins (ezrin, radixin and moesin) (Tsukita et al, 1994).

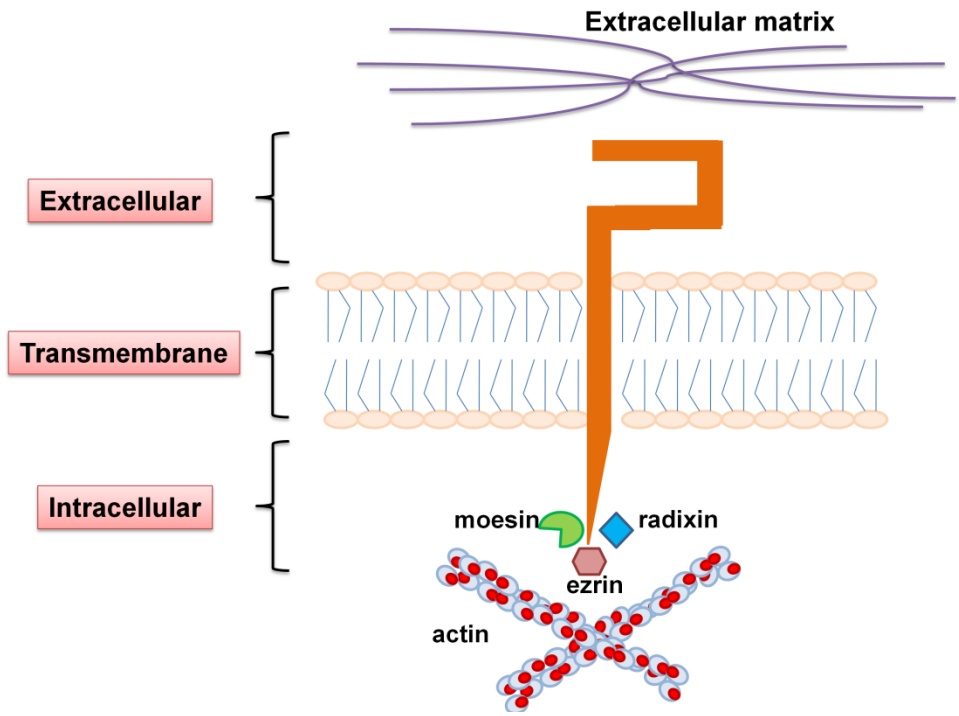

Figure 4. CD44

Recently, several studies documented that CD44 is linked to several biological processes both in pathological, e.g. inflammation and cancer, and healthy conditions (Ponta et al, 2003). Although major functions involve adhesion and migration, other functions affected by CD44 include leukocyte homing and recruitment, phagocytosis, matrix remodeling, proliferation, and apoptosis. Furthermore, CD44 may participate in both arteriogenesis (van Royen et al, 2004) and angiogenesis (Cao et al, 2006).

In cancer, CD44 has been closely linked with metastatic growth in some malignancies. As metastatic growth in a pancreatic cell line was induced by transfection of CD44 encoding cDNA (Günthert et al, 1991) and in another study metastasis formation was inhibited by CD44 antibodies (Seiter et al, 1993).

In inflammation, studies have been shown that CD44 mediate leukocyte rolling in vitro (DeGrendele et al, 1996). In addition, CD44 expression is up-regulated on leukocytes and synovial cells in arthritic joints of both humans and mice (Haynes et al, 1991; Mikecz et al, 1995). Moreover, CD44 has been detected on inflammatory cells in atherosclerotic plaques (Kolodgie et al, 2002).
During inflammation, both inflammatory and vascular cells can respond to different cytokines with altered CD44 expression properties including strength of CD44 expression, expression of vCD44, and altered glycosylation (Krettek et al, 2004; Levesque & Haynes, 1999).
On other hand, CD44 expression may also regulate the production of cytokines, for example; CD44 negatively regulates TLR-mediated cytokine release through a low molecular weight HA-independent mechanism (Kawana et al, 2008). Furthermore, CD44-deficient mice exhibit prolonged and enhanced inflammation following heart infarction, including increased expression of IL-1β, TNF-α, IL-6, IL-10, and osteopontin (OPN) after 6 h but not after 24 h (Huebener et al, 2008). In contrast, CD44 activation in macrophages results in a release of TNF and IL-1 (Noble et al, 1993; Webb et al, 1990).

In ALI, the exact and specific role of CD44 in mediating accumulation of leukocytes in the lung is not well understood and contradictory. For example, one study showed that endotoxin-induced infiltration of neutrophils and macrophages in the lung was markedly reduced in CD44 gene deficient mice (Hollingsworth et al, 2007), whereas another study documented that endotoxin-induced pulmonary accumulation of neutrophils and macrophages was increased in CD44-deficient mice (Liang et al, 2007).

6. Rho GTPase subfamily

GTPases are a large family of hydrolase enzymes that can bind and hydrolyze guanosine triphosphate (GTP) (Scheffzek & Ahmadian, 2005). G proteins which also known as guanine nucleotide-binding proteins, are most prominent among the regulatory GTPases which involved in transmitting chemical signals originating from outside a cell into the inside of the cell. G proteins function as molecular switches. Their activity is regulated by factors that control their ability to bind to and hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP). When they bind GTP, they are 'on', and, when they bind GDP, they are 'off'.

Among G proteins, there is small monomeric protein group which made an important family called Rho GTPase subfamily or small GTPase family. Rho GTPase subfamily discovered for the first time at 1985 and showed homology to Ras (**R**as **ho**mologus) (Madaule & Axel, 1985). Thereafter Rho GTPase family expanded to include 22 members, divided into eight subgroups (Fransson et al, 2003; Ridley, 2001; Wherlock & Mellor, 2002) (Table 2). The most important subgroups are Rho, Rac and Cdc42 (Wennerberg et al, 2005) each with its subtypes. The main focus of this thesis is on Rho and Rac, thus the other members of Rho GTPase subfamily will not be mentioned further.

Table2. Rho GTPase subfamily

Rho GTPase subgroup	***Members***
Cdc42	CdC42, TC10, TCL, Chp and Wrch-1
Rac	Rac1, Rac2, Rac3 and RhoG
Rho	RhoA, RhoB and RhoC
Rnd	Rnd1, Rnd2 and Rnd2
RhoD	RhoD and Rif
Miro	Miro1 and Miro2
RhoH/TTF	RhoH/TTF
RhoBTB	RhoBTB1 and RhoBTB2

6.1 Rho/Rho kinase

Rho is a monomeric, 21-kD GTPase that exists in at least three isoforms in mammals (Rho A, Rho B, and Rho C). Rho play important roles in induction of cell adhesion, stress fibers formation, and cell contraction after thrombin stimulation (Essler et al, 1998; Mackay & Hall, 1998; Ridley & Hall, 1992). In cells other than leukocytes, Rho activity is required for the formation of actin stress fibers (Ridley & Hall, 1992). In human neutrophils, Rho plays an important role in signaling for myosin light chain phosphorylation and cell migration (Niggli, 1999).

Among Rho effectors isolated, the family of Rho-associated coiled-coil–forming protein kinases (ROCK) is implicated in Rho-mediated cell adhesion and smooth muscle contraction (Kimura et al, 1996; Maekawa et al, 1999). Rho-kinases are known to act as molecular switches controlling several critical functions, including, cell adhesion and contraction, migration, reactive oxygen species formation and oncogenic transformation (Alblas et al, 2001; Itoh et al, 1999; Slotta et al, 2006). There are Two ROCK isoforms: ROCK I and ROCK II, Both isoforms are present in the human, rat and mouse tissues although ROCK I is mostly present in the liver, testes and kidney where ROCK II is mainly prominent in the brain and striated muscle (Ishizaki et al, 1996; Leung et al, 1996).

In our study, we have used ROCK inhibitor (Y-27632) which binds to the kinase domain and inhibit both ROCK I and ROCK II with equal potency (Breitenlechner et al, 2003; Ishizaki et al, 1996). The molecular formula of ROCKs is characterized by an N-terminal catalytic kinase domain, a coiled-coil and C-terminal domain (Ishizaki et al, 1996; Shi & Wei, 2007) where the C-terminal-domain acting as a Rho-binding site upon activation (Ishizaki et al, 1996).

At the beginning, the Rho/ROCK pathway was investigated in cardiovascular diseases (Shimokawa & Rashid, 2007; Shimokawa & Takeshita, 2005) since strong correlation between this intracellular pathway with angiotensin II, thrombin and platelet-derived grow-factor (PDGF). For example, pre-treatment with Y27632 in a

coronary occlusion model, reduced the infarction area dose-dependently and attenuated the levels of KC and IL-6 (Bao et al, 2004).

Later on, studies discovered that the Rho-kinase signaling pathway is also involved in inflammatory processes with a particular role in chemokine formation, leukocyte-endothelial cell interaction associated to colonic ischemia-reperfusion (Santen et al, 2010) and LPS-induced platelet capture to the endothelium (Slotta et al, 2006; Slotta et al, 2008; Thorlacius et al, 2006). Clinical trials where patients have been treated with fasudil or Y27632 have shown interesting results in the management of pulmonary hypertension and to prevent cardiovascular injuries (Ishikura et al, 2006; Mohri et al, 2003). Moreover, several studies suggest that Rho-kinase function plays an important role in regulating the integrity of epithelial barriers (Popoff & Geny, 2009). Interestingly, Rho-kinase inhibitors have been reported to protect against tissue fibrosis (Kitamura et al, 2007)**,** cholestasis (Laschke et al, 2010)**,** and pancreatitis (Awla et al, 2011b).

Moreover, there are several lines of evidence for a co-operation between Rho-kinase signaling with F-actin formation. Rho is involved in several processes that require the reorganization of actin, for example migration, morghology, stiffness, cell adhesion and trafficking (Bishop & Hall, 2000; Etienne-Manneville & Hall, 2002; Van Aelst & D'Souza-Schorey, 1997). However, role of Rho-kinase signaling in regulating pulmonary recruitment of neutrophils and tissue damage in abdominal sepsis is not well known. In addition, the influence of Rho-kinase inhibition on adhesive and mechanical aspect of sepsis-induced neutrophil accumulation in the lung remains elusive.

6.2 Rac

Rac which is abbreviation of **Ra**s-related **C**3 botulinum toxin substrate is belongs to the mammalian Rho family of Ras superfamily small GTP-binding proteins. It is second most famous

subtype of Rho GTPase. It is of two distinct isoforms, Rac1 and Rac2.
Shortly, Rac2 is least common, upon GTP loading, a conformational change takes place that allows Rac2 protein to interact with several downstream effectors that ultimately process the information and propagate the signal within the cell, causing changes in the actin cytoskeleton, release of inflammatory modulators and innate immunity.

While Rac1 which we focused mainly in our study, is a small (~21 kDa) signaling G protein. Rac1, in particular, may also be involved in the regulation of exocytic and endocytic pathways, and has a role in activation of the NADPH oxidase enzyme complex (Diekmann et al, 1994; Lamaze et al, 1996). The Rho family GTPase Rac1 is a key regulator of various cellular functions such as cytoskeletal reorganization or cellular growth (Hall, 1998; Van Aelst & D'Souza-Schorey, 1997; Wennerberg & Der, 2004). It is also implicated in different aspects of antibacterial host defense, including leukocyte chemotaxis (Van Aelst & D'Souza-Schorey, 1997)http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3262829/ -pone.0030379-VanAelst1 , pathogen phagocytosis (Lee et al, 2000; Wong et al, 2006), ROS production (Bedard & Krause, 2007), and regulation of TLRs and NOD2 (Arbibe et al, 2000; Eitel et al, 2008; Legrand-Poels et al, 2007; Schmeck et al, 2006).

Various studies have established functional correlation between Rac activity and the regulation of mitogen-activated protein kinases (MAPKs), such as JNK/c-Jun (Coso et al, 1995; Hill et al, 1995) and p38 MAPK (Eom et al, 2001; Uhlik et al, 2003; Woo & Kim, 2002), which are known to be linked in pro-inflammatory activities, such as cytokine formation and leukocyte activation (Asaduzzaman et al, 2008a; Klintman et al, 2005). In point of fact, inhibition of Rac1 has been shown to exert protective effects in models of reperfusion injury in the liver, endotoxemia and acute pancreatitis (Binker et al, 2008; Harada et al, 2003; Yao et al, 2011). However, the potential role of Rac1 signaling in regulating CXC chemokine formation, neutrophil infiltration and lung injury in abdominal sepsis are not well known.

7. Antimicrobial peptides

Antimicrobial peptides (AMPs) which also called host defense peptide are a vital component of the innate immune response, they are fast acting and non-specific (Hancock & Chapple, 1999).They are found widely among all classes of plant and animal including; bacteria, fungi, amoebae, insects, fish, birds, amphibians and mammals (Wang et al, 2009). In invertebrates, AMPs act as the primary defense molecules against infections, when in fact, in mammals they made an important portion in both innate and early adaptive immune responses.

They are either encoded genetically or derived form larger proteins by proteolysis as thrombin.

For first time, AMPs discovered and mentioned by A. Gratia at 1925. A. Gratia noticed that some bacteria will produce special peptide compound to kill surrounding bacteria during competition for space, food and other nutrients. Then at 1950 Hirsch extracts special bactericidal substances from granules from polymorphonuclear leukocytes (PMN) from rabbit (Hirsch, 1956). Later, several researchers have identified different Antimicrobial peptides belonging to different groups from various organisms. Nowadays, there are more than 1500 Antimicrobial peptides (Lai & Gallo, 2009).

There is an immense diversity of sequences, and similarity often is found only within specific groups of host-defense peptides from closely related species. So there are different ways for classifications of AMPs according their size, structure, sequence, polarity (ionic and catatonic), mode of actions,.etc.

Generally, AMPs tend to be shorter than 45 amino acids, cationic, and amphipathic (Hancock, 1997) and have been demonstrated to kill Gram negative and Gram positive bacteria (including strains that are resistant to conventional antibiotics), mycobacteria (including *Mycobacterium tuberculosis*), enveloped viruses and fungi.
There are different hypothesis analyzing mode of action of theses peptides for exerting antimicrobial effects including: disruption of cell

membrane, interfering with metabolism and targeting cytoplasmic components.

In addition to their main direct antimicrobial activity, AMPs can also perform a plenty of functions for example: cell proliferation, angiogenesis, vasculogenesis, exerts cytotoxic effects on cancer cells, wound healing, immune system activation, as anti-inflammatory substance, leukocyte cells recruitment, inhibition of LPS induced pro-inflammatory responses, chemokine production and also activation or blockage of TLRs (Auvynet & Rosenstein, 2009; Bowdish et al, 2005; Easton et al, 2009; Hancock & Sahl, 2006; Pasupuleti et al, 2012) (Figure .5). These sense encouraging the recent and wider definition of Antimicrobial peptides in relation with innate immune system. Antimicrobial peptides has recently been shown to envelop a diverse family of peptides, including proinflammatory and chemotactic chemokines (Cole et al, 2001), neuropeptides (Brogden, 2005), peptide hormones (Kowalska et al, 2002; Mor et al, 1994), growth factors (Malmsten et al, 2007), the anaphylatoxin peptide C3a (Nordahl et al, 2004; Pasupuleti et al, 2007), and kininogen-derived peptides (Frick et al, 2006; Nordahl et al, 2005; Rydengard et al, 2006).

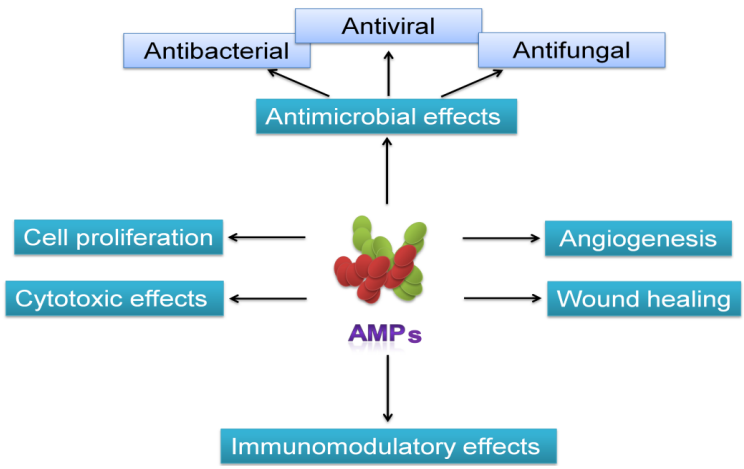

Figure 5. Different actions of Antimicrobial peptides

In mammals, these peptides often have comparably weak antimicrobial activity under normal physiological situations, while their ability to modulate the immune response through different ways and mechanisms may be more critical (Bowdish et al, 2005a; Oppenheim & Yang, 2005). Both functions are an integral part of the process of innate immunity and generally should present with every successful anti-infective therapies. Thereupon cationic peptides are being regard in a certain way as a new generation of antibiotics, as well as innate immune modulators. We refer to these peptides by the group name host-defense peptides, to capture their broader functions in innate immunity and as cationic antimicrobial peptides when they have direct antimicrobial activity under physiologically meaningful conditions.

Recent studies have shown that there are strong relations between sepsis and AMPs, a few antimicrobial peptides (AMPs) also have the potential to neutralize LPS-induced endotoxic effects. They are produced in large quantities at the site of infection and/or inflammation and act rapidly to clear microbes (Zasloff, 2002). Several AMPs prevent LPS-dependent cytokine induction in macrophages and block sepsis in animal models (Giacometti et al, 2002; Gough et al, 1996; Hirata et al, 1995; Larrick et al, 1995; Scott et al, 1999). Despite extensive research, the underlying mechanism and properties of the peptides are not yet clear. Toward meeting this goal we investigated two AMPs and their possible effects in polymicrobial sepsis.

In our study we have used special types of sequenced peptides GKY20 with 20 amino acids and GKY25 with 25 amino acids; they are derived from human thrombin upon proteolysis.

8. Aims of this thesis

The specific aims were:

- To clarify the role of CD44 in sepsis by using murine experimental models.
- To investigate if leukocyte cytoskeleton arrangement are involved in the leukocyte sequestration and migration and to examine the role of Rho kinase on the cytoskeletal arrangement during sepsis.
- To explore the role of Rac1 on systemic activation and pulmonary neutrophil recruitment in polymicrobial sepsis.
- To evaluate the anti-microbial and anti-inflammatory effects of the human thrombin derived peptides in polymicrobial sepsis.

9. Methodological considerations

9.1. Animals

Male C57BL/6 mice (22 +/- 5 g) from (Jackson Laboratory, Bar Harbor, ME, USA) were used. The study protocols were approved by the Ethical Committee at Lund University. The animal care facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The mice were housed in a pathogen-free animal facility with 12:12 hours light-dark cycles at 25°C. Mice were anesthetized intraperitoneally (i.p.) with 75 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per kg body weight.

9.2. Experimental protocol of sepsis model

Polymicrobial Sepsis was induced by CLP. Briefly, mice were anesthetized and shaved; the ventral trunk was prepared with 70% ethanol. A midline incision (~ 1 cm) in the abdomen was made to expose the cecum. The exposed cecum was filled with feces by milking stool backward from the ascending colon. A 5-0 silk suture ligature was placed below the ileocecal valve. The cecum was soaked with phosphate-buffered saline (PBS; pH 7.4) and was then punctured twice with a 21-gauge needle. The cecum was then returned into the peritoneal cavity and the abdominal incision was closed in two layers with 5-0 suture. Animal mortality within 24 h was less than 10% using this protocol. In sham-operated mice, the cecum was located and mobilized as described above but was neither ligated nor punctured. Immediately after the surgery, animals were resuscitated by subcutaneously injecting prewarmed normal saline (0.1 ml/g BW) Analgesia (Temegesic) 0.05 mg/kg is given S.C. directly after operation. The mice were then returned to their cages and provided food and water ad libitum. Animal were treated with different antibodies and drugs according to the project experiments. Then animals re-anesthetized 6 and 24 h after CLP

induction and humanely sacrificed. Blood was obtained from the vena cava for culture, flow cytometric analysis and plasma was acquired by centrifugation and frozen at -20°C for, HMGB1 and IL-6 quantification. Spleen excised for T-cell assessment and culture. In the lung left part of lung was ligated and excised for edema measurement. The right lung was used for collecting bronchoalveolar lavage fluid in which neutrophils were quantified in a Burker chamber. Next, the lung was perfused with PBS, and one part was fixed in formaldehyde for histology, and the remaining lung tissue was weighed, snap-frozen in liquid nitrogen, and stored at -80°C for later enzyme-linked immunosorbent assay (ELISA) and myeloperoxidase (MPO) assays.

9.3. Systemic leukocyte count

Blood was collected from tail vein and was mixed with Turks solution (0.2 mg gentian violet in 1 ml glacial acetic acid, 6.25% v/v) in a 1:20 dilution. Leukocytes were defined as monomorphonuclear (MNLs) and polymorphonuclear (PMNLs) cells in a Bruker champer.

9.4. Bronchoalveolar lavage fluid (BALF)

After exposure of trachea and insertion of angiocatheter, bronchoalveolar lavage fluid was collected by washing five times with 1 ml of PBS containing 5 mM EDTA and was then centrifuged, stained with Turk´s solution; numbers of MNLs and PMNLs were counted in a Burker chamber.

9.5. Lung edema

The left lung was excised, washed in PBS, gently dried with blotting paper and weighed. The tissue was then dried at 60°C for 72 h and re-weighed. The change in the ratio of wet to dry weight was used as an indicator of lung edema formation.

9.6. MPO activity

MPO is an enzyme abundantly present in the azurophilic granules of the neutrophils. For quantitative measurement, frozen lung tissue was thawed and homogenized in 1 mL of 0.5% hexadecyltrimethylammonium bromide. Next, the sample was freeze-thawed, after which the MPO activity of the supernatant was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter 540 nm, 25°C). Values were expressed as MPO unit/g tissue.

9.7. Enzyme linked immunosorbent assay (ELISA)

Levels of macrophage inflammatory protein-2 MIP-2 and KC levels in lung tissue were analyzed by using double antibody Quantikine ELISA kits (R&D Systems). Murine rMIP-2 and rKC were used as standards. Blood samples were collected from inferior vena cava (1:10 acid citrate dextrose), plasma obtained and stored after centrifuging at 14000 for 10 min at -20°C. ELISA kits were used to assess the level IL-6 (R&D system) and high mobility group box-1(HMGB1) (Chonrex, Redmond, WA, USA) according to instruction by manufacture.

9.8. Flow cytometry

To quantify Mac-1 expression on circulating neutrophils, blood was collected into heparinized syringes at 6 h after induction of CLP and incubated (10 min, RT) with an anti-CD16/CD32 antibody blocking Fcγ III/II receptors to reduce nonspecific labeling and then incubated with PE-conjugated anti-Gr-1 (clone RB6–8C5, rat IgG2b; eBioscience, San Diego, CA) and fluorescein isothiocyanate (FITC)-conjugated anti-Mac-1 (clone M1/70, integrin α_M china, rat IgG_{2b}). The mean fluorescence intensity was determined by comparisons with appropriate isotype control (FITC-conjugated rat IgG_{2b}). All antibodies were purchased from BD Biosciences Pharmingen (San Jose, CA) except where indicated. Cells were fixed, erythrocytes

were lysed by BD lysis buffer (Sigma Chemical Co., St. Louis, MO, USA), and then neutrophils were recovered following centrifugation. Flow-cytometric analysis was performed by first gating the neutrophil population of cells based on forward and side scatter characteristics, and then Mac-1 expression was determined on Gr-1^+ in these gated cells on a FACSCalibur flow cytometer (Becton-Dickinson, Mountain View, CA USA). A viable gate was used to exclude dead and fragmented cells.

9.9. Immunoprecipitation and Rac1 activity assay

Rac1 activation assay was performed by using Rac1 activation assay kit as described previously (Wu et al, 2008). Briefly, 50 mg of lung tissue were minced and homogenated in lysis buffer on ice, the samples were centrifuged at 150 000g for 15 min; 10 µl from each supernatant were removed to measure protein content using Pierce BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL, USA) and the rest of the volume was used for the pull-down assay. Supernatant containing equal amount of proteins were then diluted with 2X SDS sample buffer and boiled for 5 min. The protein were separated using SDS-PAGE (10% gel). After transfer to a nitrocellulose membrane (BioRad, Hercules, CA, USA), blots were blocked with 3% bovine serum albumin (BSA) at room temperature for 1 hour, followed by incubation with an anti-Rac1 antibody (1:1000) at 4ºC overnight. Binding of the antibody was visualized using peroxidase-conjugated anti-mouse antibody (1:10000, Pierce Biotechnology) at room temperature for 1 hour and enhanced chemiluminescence method (BioRad, Hercules, CA, USA). â-actin was used as an internal control for total Rac1.

9.10. Histology and histology score

Lung samples were fixed by immersion in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin-embedded. Six µm sections were stained with haematoxylin and eosin. Lung injury was quantified in a blinded manner by using a modified scoring system based on two previous publications

(Carraway et al, 2003; Kostopanagiotou et al, 2009), including alveolar collapse, thickness of alveolar septae, alveolar fibrin deposition and neutrophil infiltration graded on a zero (absent) to four (extensive) scales. In each tissue sample, 5 random areas were scored and mean value was calculated. The histology score is the sum of all 4 parameters.

9.11. Neutrophil isolation and activation

Bone marrow neutrophils were freshly extracted from femurs and tibias of healthy mice by aseptically flushing the bone marrow with complete culture medium RPMI 1640 and then subsequently isolated by using Ficoll-Paque ^{TM} Research Grade (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated neutrophils was higher than 70% as assessed in a haematocytometer. Leukocytes were then re-suspended in PBS to 10×10^6/ml and co-incubated with 300 ng/ml recombinant mouse CXCL2/MIP2 (R&D Systems, Inc., Minneapolis, USA) for 10 min. Cells were stained and fixed for flow cytometric analysis of Mac-1 expression on neutrophils.

9.12. RT-PCR

Gene-expression of MIP-2, KC and IL-6 quantified in alveolar macrophages isolated from sham mice and CLP animals pretreated with vehicle or with Wff25de, GKY20 and GKY25 peptides 30 min after CLP procedure. Alveolar macrophages were isolated from BALF as described in detail (Zhang et al, 2008). Briefly, 30 min after induction of CLP, lungs were flushed three times with 1 ml of PBS supplemented with 0.5 mM EDTA. Alveolar fluid collections were then centrifuged at 1400 RPM, 10 min, 18°C. The cells were then resuspended in RPMI 1640 complete culture medium and incubated at 37°C, 5% CO_2 in 48-well plate. After 2 h, non-adherent cells were washed away by PBS. A total of 2-3 x 10^5 macrophages were obtained per mice and the purity of macrophages was higher than 97%. Total RNA was isolated from the alveolar macrophages using

an RNeasy Mini Kit (Qiagen, West Sussex, UK) following the manufacturer's protocol and treated with RNase-free DNase (DNase I; Amersham Pharmacia Biotech, Sollentuna, Sweden) to remove potential genomic DNA contaminants. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically. Each cDNA was synthesized by reverse transcription from 10 µg of total RNA using the StrataScript First-Strand Synthesis System and random hexamer primers (Stratagene; AH diagnostics, Stockholm, Sweden). Real-time PCR was performed using a Brilliant SYBRgreen QPCR master mix and MX 3000P detection system (Stratagene). The primer sequences of CXCL1, CXCL2, and β-actin were as follows: CXCL1 (forward) 5'-GCC AAT GAG CTG CGC TGT CAA TGC-3', CXCL1 (reverse) 5'-CTT GGG GAC ACC TTT TAG CAT CTT-3'; CXCL2 (forward) 5'-GCT TCC TCG GGC ACT CCA GAC-3', CXCL2 (reverse) 5'-TTA GCC TTG CCT TTG TTC AGT AT-3'; and β-actin (forward) 5'-ATG TTT GAG ACC TTC AAC ACC-3', β-actin (reverse) 5'-TCT CCA GGG AGG AAG AGG AT-3', IL-6 (reverse) 5'-AGC CTC CGA CTT GTG AAG TGG -3'; TNF-á (forward) 5'-CCT CAC ACT CAG ATC ATC TTC TC-3', TNF-á (reverse) 5'-AGA TCC ATG CCG TTG GCC AG-3'and β-actin(forward) 5'-ATG TTT GAG ACC TTC AAC ACC-3', β-actin (reverse) 5'-TCT CCA GGG AGGAAG AGG AT-3'.Standard PCR curves were generated for each PCR product to establish linearity of the RT-PCR reaction. PCR amplifications were performed in a total volume of 50 µl, containing 25 µl of SYBRgreen PCR 2x master mix, 2 µl of 0.15 µM each primer, 0.75 µl of reference dye, and one 1 µl cDNA as a template adjusted up to 50 µl with water. PCR reactions were started with 10 min denaturing temperature of 95°C, followed by a total of 40 cycles (95°C for 30 s and 55°C for 1 min), and 1 min of elongation at 72°C. Cycling time values for the specific target genes were related to that of β-actin in the same sample.

9.13. Adoptive transfer experiment

Bone marrow leukocytes were freshly extracted from healthy mice by using Ficoll-Paque^{TM} Research Grade (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated neutrophils

was higher than 70% as assessed in a haematocytometer. Leukocytes were then resuspended in PBS to 10 X 10^6/ml and co-incubated with 300 ng/ml recombinant mouse CXCL2 for 10 min and 180 min separately, furthermore leukocytes were pre-incubated with anti-CD44 antibody, cytochalasin B 10 µM 30 min and anti-Mac-1 (Purified anti-mouse CD11b, NA/LE, from BD Biosciences) for 15-20 min at room temperature before challenge with CXCL2 co-incubation. Samples were stained with 20 µM Carboxyfluorescein diacetatesuccinimidylester (CFDA-SE; Invitrogen, Paisley, UK) for 1 h at 37°C. Carboxyfluorescein diacetate-succinimidyl ester passively diffuses into cells and is non-fluorescent until its acetate groups are cleaved by intracellular esterases to yield a highly fluorescent ester. Two million labeled neutrophils were injected intravenously into mice immediately before CLP. 4 and 6 h after CLP induction, lungs were harvested, minced, and digested for 1 h at 37°C in buffer containing 20 U/ml collagenase A. Single-cell suspensions were obtained by straining the digested tissue through a 40 µm mesh. Cells were labeled with an APC-labeled anti-Gr-1 antibody and fixed as described above. Finally, cells were analyzed by flow cytometry (FACSCalibur Lung recruitment of transferred neutrophils were quantified by dividing the number of $CFDA^+/Gr\text{-}1^+$ cells by the number of $CFDA^-/Gr\text{-}1^+$ cells in the lung extracts.

9.14. Chemotaxis assay

Neutrophils isolated form bone marrow by use of Ficoll-Paque TM were pre-incubated with different agent accordingly for 30 minutes and 1.5×10^6 neutrophils were placed in the upper chamber of the Transwell inserts (5 µm pore size, Corning Costar, Corning, NY, USA). Inserts were placed in wells containing medium alone (control) or medium plus MIP-2 (100 ng/ml, R & D Systems). After 120 minutes, inserts were removed and migrated neutrophils were stained with Turks solution. Chemotaxis was determined by counting the number of migrated neutrophils in a Burker chamber (Riaz et al, 2004).

9.15. Isolation of splenocytes

Spleen was excised for FACS analysis 24 hours post CLP induction. Single splenocytes suspension was obtained under sterile condition by meshing the spleen and passing it through a 40 µm cell strainer (BD Falcon, Becton Dickinson, Mountain View, CA, USA).ACK lysing buffer (Invitrogen, Carlsbad, CA, USA) used for lyses the red blood cells. The cells washed and resuspended with CLICK's medium (Sigma-Aldrich, Stockholm, Sweden) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 unit/ml) and streptomycin (0.1 mg/ml) (Sigma-Aldrich). Splenocytes were quantified in A Burker chamber after staining with Turk's Solution (Merck).

9.16. T-cell apoptosis

To evaluate apoptosis of CD4 T-cells, splenocytes were fixed and stained by APO-BORDU kit, which labels DNA strand breaks by BrdUTP according to the manufacture's instruction (Phoenix Flow system, San Diego, CA, USA). APC-conjugated anti-CD4 antibody (IgG2b, kappa, clone: GK1.5) was used to indicate CD4 T-cells. Cells were acquired by FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analysed with Cell-Quest Pro software (BD Bioscience, San Jose, CA, USA).

9.17. Regulatory T-cell analysis

Splenocytes were stained with FITC-conjugated anti-CD4 (Rat IgG2a, Clone: RM4-5), APC-conjugated anti-CD25 (Rat IgG1, Clone: PC61.5) and PE-conjugated anti-Foxp3 (Rat IgG2a, Clone: FJK-16s) antibodies. FACSCalibur flow cytometer used for analysis.

9.18. Bacterial cultures

Bacterial loads were determined in blood and spleen. Briefly, the mice were anesthetized at 24 h after CLP. 100 µl of blood from inferior vena cava and spleen taken were placed on ice, blood serially diluted with sterile PBS, spleen was weighed, homogenized

with 1 ml sterile PBS and serially diluted. 30 µl of each diluted samples were placed on trypticase soy agar plates with 5% sheep blood (BD Biosciences, San Diego, CA, USA) separately and incubated at 37°C for 24 h. The numbers of bacterial colonies were then counted and expressed as Log CFU/ml blood and Log CFU/gm spleen respectively.

10. Results and discussion

Paper 1: Neutrophil CD44 regulates sepsis induced Lung injury

Intestinal perforation is a feared condition in which toxins and microbes contaminate the abdominal cavity and evoked local formation of proinflammatory mediators, which are subsequently released into the circulation causing a systemic inflammatory reaction. Abdominal sepsis is a major cause of morbidity and mortality in intensive care units (Cohen, 2002; Simon & Gorbach, 1984). Neutrophil recruitment is a central feature in septic lung injury (Asaduzzaman et al, 2009; Asaduzzaman et al, 2008b). A large body of evidence now suggests that trapping of neutrophil in the pulmonary capillaries and subsequent recruitment is a well known feature of sepsis (Basit et al, 2006; Czermak et al, 1999; Issekutz, 1992 & Reutershan et al, 2005). Initially leukocytes will contact with endothelial cell with help of selectins, followed by rolling on the surface of endothelial wall and integrin-mediated arrest (Carlos & Harlan, 1994; Vestweber & Blanks, 1999). Leukocytes continuously exposed to different stimulatory signals by chemokines, integrins and selectin which help leukocyte extravasations and cytoskeletal rearrangements (Zarbock & Ley, 2008).
Previous studies describe and mention most of the essential receptors and molecules on both leukocytes and capillary endothelial lining. However, there are questions of others in regulating extravasations which may be become a novel finding in management of sepsis.

Here in first study, we demonstrate the role of CD44 in mediating pulmonary recruitment of neutrophils and tissue damage in abdominal sepsis. CD44 is a type I transmembrane glycoprotein expressed on many cell types, including both leukocytes and endothelium (Lesley et al, 1993). The role of CD44 in mediating accumulation of leukocytes and lung injury is not exactly well known and partly conflicting. For example, Hollingsworth et al showed that

endotoxin-induced infiltration of neutrophils and macrophages in the lung was markedly decreased in CD44 Knock-Down mice (Hollingsworth et al, 2007), whereas Liang et al reported that endotoxin-induced pulmonary accumulation of neutrophils and macrophages was increased in CD44- Knock-Down mice (Liang et al, 2007). Based on these considerations, the aim of our study was to define the exact role of CD44 lung injury in a model of polymicrobial sepsis based on intestinal perforation.

In recent study we have used a saturating dose of a monoclonal antibody for complete blocking all murine CD44 receptors in cecal ligation and puncture model. We demonstrate that inhibition of CD44 function effectively decreases neutrophil accumulation in the lung in abdominal sepsis. Moreover, blocking CD44 not only attenuated pulmonary neutrophilia but also protected against sepsis-induced lung edema and tissue injury. We observed that immunoneutralization of CD44 reduced lung levels of MPO in the present study. This inhibitory effect on MPO activity correlated well with our other findings that pretreatment with the anti-CD44 antibody reduced sepsis triggered neutrophil recruitment in the bronchoalveolar space, suggesting that CD44 indeed supports neutrophil accumulation in septic lung injury. Then we asked whether CD44 expressed on neutrophils and/or in the lung mediated neutrophil recruitment and include in septic lung injury, we performed adoptive transfer of labeled neutrophil co-incubated with the anti-CD44 antibody or a control antibody. We found that only blocking neutrophil CD44 can attribute CLP-induced neutropenia.
Hyaluronan is one important ligand of CD44 (Aruffo et al, 1990). Hyaluronic acid is a dominant extracellular matrix component and CD44 -hyaluronic acid interactions are linked to regulate adhesive mechanisms in development of different autoimmune diseases, and tumor metastasis (Aruffo et al, 1990; Gee et al, 2004). Considering our data that neutrophil CD44 appears to control neutrophil recruitment in septic lung injury, we estimate the potential role of hyaluronan in pulmonary infiltration of neutrophils in abdominal sepsis by using hyaluronidase for degradation of hyaluronan from the endothelium. Basis on this result, we declare that CD44-mediated neutrophil infiltration in the lung is independent of hyaluronan in polymicrobial sepsis.

Paper 2: Rho-kinase regulates pulmonary recruitment of neutrophils through both adhesive and mechanical mechanisms

The lung is the most sensitive and clinically important end organ in abdominal sepsis (Bone et al, 1992b). One of the initiating events in the development of acute lung injury is the sequestration of activated neutrophils. Activated neutrophils sequester in microvessels of the lung because of a decrease in their deformability (Hogg & Doerschuk, 1995) and an increase of adhesive qualities of neutrophils and endothelial cells (Carlos & Harlan, 1994). Cytoskeletal rearrangements causing reduction in neutrophil deformability will increase their sequestration in the pulmonary microvasculature.

In our second work we have tried to prevent or reduce neutrophil stiffness on one hand and to down-regulate sepsis mediated adhesive molecule upregulation.

We used a well-known Rho kinase inhibitor Y-27632 30 min before sepsis induction because we hypothesized that Rho-kinase signaling have relation with both neutrophil deformability (mechanical mechanism) and Mac-1 expression (adhesive mechanism) on neutrophil.

Expression of Mac-1 and F-actin formation in neutrophils were quantified by using flow cytometry 6 h after CLP. Mac-1 expression and F-actin formation were also determined in isolated neutrophils up to 3 h after stimulation with CXCL2. Y-27632 reduced the CLP-induced pulmonary injury and MPO activity as well as Mac-1 on neutrophils. Rho-kinase Inhibition decreased CLP-provoked F-actin formation in neutrophils. CXCL2 rapidly increased Mac-1 expression and F-actin formation in neutrophils. Co-incubation with Y-27632 abolished CXCL2-induced Mac-1 up-regulation and formation of F-actin in neutrophils. Adoptive transfer experiments revealed that co-incubation of neutrophils with the anti-Mac-1 antibody or cytochalasin B significantly decreased pulmonary accumulation of neutrophils in septic mice. Overall data show that targeting Rho-kinase effectively reduces neutrophil recruitment and tissue damage in abdominal sepsis. Moreover, these findings demonstrate that Rho-kinase-dependent neutrophil accumulation in septic lung injury is regulated by both adhesive and mechanical mechanisms.

Paper 3: Rac1 signaling regulates sepsis-induced pathological inflammation

Fecal bacteria trigger local production of proinflammatory compounds, which are subsequently released into the circulation causing a systemic inflammatory reaction. It is well known that pulmonary injury is a common feature and the most frequent cause of mortality in patients with systemic inflammation (Babayigit et al, 2005; Wickel et al, 1998).

Treatment of sepsis is largely limited to supportive care. New targets for treating polymicrobial sepsis are urgently needed.
In third study, we hypothesized that Rac1 might play an important role in pulmonary neutrophil accumulation and tissue injury in abdominal sepsis.

Rac1 inhibitor NSC23766 have used before CLP induction. Again tissue sample collected and analyzed. First we found that Rac1 activity was increased in lungs from septic animals and NSC23766 significantly decreased pulmonary activity of Rac1 induced by CLP. NSC23766 decreased CLP-induced neutrophil infiltration and edema formation in the lung. Moreover, Mac-1 expression increased on septic neutrophils, which was significantly attenuated by NSC23766. Moreover, NSC23766 abolished CLP-evoked formation of CXC chemokines in the lung and sepsis-evoked elevation of mRNA levels of CXC chemokines and TNF-α in alveolar macrophages. Rac1 inhibition decreased the CLP-induced increase in plasma levels of HMGB1 and IL-6.

Our data suggest that that Rac1 signaling plays a key role in regulating pulmonary infiltration of neutrophils and tissue injury via regulation of chemokine production in the lung and Mac-1 expression on neutrophils in abdominal sepsis.

Paper 4: TDPs are novel finding for management of sepsis

A fundamentally new strategy for the treatment of sepsis is essential and because of rising antibiotic resistance, substantial research efforts have been devoted to explore new alternative therapies for patients with sepsis. Antimicrobial host defense peptides have been investigated for their potential as a new class of antimicrobial drugs.
It is well known that excessive neutrophil recruitment is a major feature in early phase with immune system dysfunction at later phase. Late profound T-cell dysfunction which explains at least in part of the encouragement of the susceptibility to infections observed in patients with sepsis (Sherwood & Toliver-Kinsky, 2004). T-cell dysfunctions are in form of apoptosis and regulatory T-cell up-regulation.

In last work, we have used two specific kinds of TDPs, GKY20 and GKY25 half hour after sepsis induction. We hypothesized GKY25 or GKY20 might interfere with pulmonary infiltration of neutrophils and/or improve T-cell function in polymicrobial sepsis.
Based on these considerations, we hypothesized GKY25 or GKY20 might interfere with pulmonary infiltration of neutrophils and/or improve T-cell function in polymicrobial sepsis.

Accumulative data shows that, TDPs activity reduced sepsis-induced formation of CXC chemokines and edema as well as neutrophil infiltration in the lung. Moreover, TDPs maintain CD4 T-cells function in spleen by reducing T-cell apoptosis and CLP-induced formation of regulatory T-cells in the spleen was abolished in TDPs-treated animals. Interesting, TDPs abolished the CLP-evoked increase of HMBG1 and IL-6 levels in the plasma. Furthermore TDPs exerts clear cut bacterial clearance in the blood and spleen.

11. Concluding remarks

The conclusions can be summarized as follows:

1. CD44 regulate neutrophil infiltration, edema and pulmonary tissue damage.

2. Rho-kinase inhibitor regulates sepsis-induced pulmonary accumulation of neutrophils, the inhibitory effect of which is related to both blocking actin polymerization, as well as Mac-1 down regulation.

3. Rac1 signaling plays a key role in regulating pulmonary infiltration of neutrophils and tissue injury via regulation of CXC chemokine production in the lung and Mac-1 expression on neutrophils.

4. Natural defense peptides are powerful regulators of pathological inflammation and T-cell immune dysfunction in abdominal sepsis and may have great value as therapeutic tool against sepsis.

All together, these findings will reveal novel therapeutic targets that can improve survival of septic patient.

12. Sammanfattning på svenska

Svår bakteriell infektion inducerar ett systemiskt inflammatoriskt svar som syftar till att eradikera bakterier men kan orsaka vävnadsskada i lungorna. Ett hastighetsbegränsande steg vid inflammatorisk organskada är aktivering och ackumulering av vita blodkroppar i lever och lunga. Syftet med den här avhandlingen var att studera mekanismer som reglerar vita blodkroppars infiltration i lever vid buksepsis. I delarbete I studerades betydelsen av CD44 som är en adhesionsmolekyl uttryckt på många olika typer av celler bla leukocyter och endotelceller. Inhibibering av CD44 minskade infiltrationen av neutrofila granulocyter i lungan vid sepsis. Behandling med hyaluronidas hade ingen effekt indikerande att CD44 utnyttjar annan receptor än hyaluron för ackumulering i lungan. I separata försök kunde det konstateras att det var CD44 på neutrofiler som var viktigast för ackumuleringen av inflammatoriska celler i lungan. I delarbete II studeras betydelsen av Rho-kinase signalering för ackumuleringen av neutrofiler i lungan. Nedströms om Rho-kinase signalering aktiveras små proteiner såsom Rho, Cdc42 och Rac. I delarbete III observerades det att Rac-1 aktiviteten ökade i lungorna vid sepsis. Inhibition av Rac-1 minskade neutrofilackumuleringen samt skyddade mot lungskada vid buksepsis. Antimikrobiella proteiner är naturliga substanser som kan skydda mot baktierier. När man klyver trombin med elastas från neutrofiler bildas bla GKY25 som är en liten peptid med anti-bakteriella effekter. I delarbete IV testades GKY25 som behandling av buksepsis. Det kunde konstateras att GKY25 minskade infiltrationen av neutrofila granulocyter i lungan via hämning av kemokinproduktion i alveolära makrofager i lungan vid buksepsis. Sammantaget har den här avhandlingen lyckats kartlägga nya och viktiga mekanismer som reglerar överdriven aktivitet hos vita blodkroppar i lungan vid buksepsis.

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PAPER 1

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TARGETING CD44 EXPRESSED ON NEUTROPHILS INHIBITS LUNG DAMAGE IN ABDOMINAL SEPSIS

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ABSTRACT—Neutrophil infiltration is an insidious feature in septic lung injury, although the specific adhesive mechanisms regulating pulmonary recruitment of neutrophils in polymicrobial sepsis remain elusive. The aim of this present study was to define the role of CD44 in sepsis-induced neutrophil infiltration and lung damage. Mice were treated with a monoclonal antibody against CD44 before cecal ligation and puncture (CLP) induction. Edema formation, bronchoalveolar accumulation of neutrophils, myeloperoxidase activity, and macrophage inflammatory protein 2 (MIP-2) levels in the lung were determined after CLP. Expression of Mac-1 and CD44 on neutrophils was quantified by using flow cytometry. In separate experiments, fluorescent-labeled neutrophils coincubated with an anti-CD44 antibody were adoptively transferred to CLP mice. Cecal ligation and puncture triggered clear-cut lung damage characterized by edema formation, neutrophil infiltration, and increased levels of MIP-2 in the lung. Notably, immunoneutralization of CD44 reduced CLP-induced pulmonary accumulation of neutrophils. In addition, functional inhibition of CD44 decreased CLP-induced lung damage and edema. However, formation of MIP-2 in the lung and neutrophil expression of Mac-1 were intact in septic mice pretreated with the anti-CD44 antibody. Adoptive transfer experiments revealed that neutrophil rather than lung CD44 mediates neutrophil accumulation in septic lung injury. Moreover, administration of hyaluronidase had no effect on CLP-induced neutrophil recruitment and tissue damage in the lung. Our data demonstrate that CD44 contributes to pulmonary infiltration of neutrophils and lung damage associated with abdominal sepsis. Thus, these novel findings suggest that CD44 may serve as a target to protect against lung injury in polymicrobial sepsis.

KEYWORDS—Adhesion, chemokines, inflammation and leukocyte

INTRODUCTION

Intestinal perforation is a feared condition in which toxins and microbes contaminate the abdominal cavity and trigger local formation of proinflammatory compounds, which are subsequently released into the circulation provoking a systemic inflammatory reaction. Abdominal sepsis is a major cause of morbidity and mortality in intensive care units (1, 2). Acute lung injury is considered to be a critical feature in the clinical course of polymicrobial sepsis. It is widely held that excessive accumulation of neutrophils is a rate-limiting step in septic lung damage (3, 4). Indeed, targeting adhesion molecules, such as P-selectin glycoprotein ligand 1 (PSGL-1) and Mac-1 (CD11b/CD18), not only inhibits pulmonary recruitment of neutrophils but also reduces lung damage in abdominal sepsis (3, 5). Extravascular coordination of neutrophil migration is orchestrated by secreted chemokines, such as macrophage inflammatory protein 2 (MIP-2), belonging to the CXC chemokine family (6, 7). However, the adhesive mechanisms regulating neutrophil infiltration in septic lung injury are incompletely known.

CD44 is a type I transmembrane glycoprotein ubiquitously expressed on many cell types, including fibroblasts, neurons, epithelial cells, and leukocytes (8). CD44 is a major receptor for hyaluronic acid, a dominant extracellular matrix component, and CD44–hyaluronic acid interactions are recognized to regulate adhesive mechanisms in development, autoimmune diseases, and tumor metastasis (9, 10). In addition, CD44 has been reported to be a physiological ligand for E-selectin and supports leukocyte rolling on activated endothelial cells (11). Interestingly, several studies have reported that CD44 also facilitates leukocyte recruitment in inflammatory diseases (12, 13). However, the literature on the specific role of CD44 in mediating accumulation of leukocytes in the lung is complex and partly contradictory. For example, one study showed that endotoxin-induced infiltration of neutrophils and macrophages in the lung was markedly reduced in CD44 gene–deficient mice (14), whereas another study reported that endotoxin-provoked pulmonary accumulation of neutrophils and macrophages was increased in CD44-deficient mice (15). Nonetheless, the role of CD44 in mediating pulmonary recruitment of neutrophils and tissue damage in abdominal sepsis is not known.

Based on the above considerations, the aim of the present study was to define the role of CD44 in regulating neutrophil activation and accumulation as well as edema formation and lung injury in a model of polymicrobial sepsis based on intestinal perforation.

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice 21 to 27 g were housed on a 12-h/12-h light-dark cycle and fed a laboratory diet and water *ad libitum*. All experimental procedures were approved by the ethical committee at Lund University. Mice were anesthetized intraperitoneally with 75 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per kilogram of body weight.

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Experimental protocols

Polymicrobial sepsis in mice was induced by puncture of the cecum as described previously (16, 17). In brief, the abdomen was opened in anesthetized mice, and the exposed cecum was filled with feces by milking stool backward from the ascending colon. A ligature was placed below the ileocecal valve. The cecum was soaked with phosphate-buffered saline (PBS; pH 7.4) and was then punctured twice with a 21-gauge needle. The cecum was then returned into the peritoneal cavity, and the abdominal incision was sutured. Animal mortality within 24 h was less than 10% using this protocol. To determine the functional role of CD44, we used a saturating dose of 4 mg/kg of a monoclonal antibody directed against murine CD44 (clone IM7, rat immunoglobulin G; BD Biosciences Pharmingen, San Jose, Calif) and an isotype-matched control mAb (clone R3-34, rat immunoglobulin G; BD Biosciences Pharmingen) in cecal ligation and puncture (CLP) animals. Antibodies or vehicle (100 μL PBS) was administered intravenously immediately before CLP induction. Sham mice underwent the same surgical procedures, that is, laparotomy and resuscitation, but the cecum was neither ligated nor punctured. The mice were then returned to their cages and provided food and water *ad libitum*. Animals were reanesthetized 6 and 24 h after CLP induction. The left lung was ligated and excised for edema measurement. The right lung was used for collecting bronchoalveolar lavage fluid (BALF) in which neutrophils were quantified in a Burker chamber. Next, the lung was perfused with PBS, and one part was fixed in formaldehyde for histology, and the remaining lung tissue was weighed, snap frozen in liquid nitrogen, and stored at −80°C for later enzyme-linked immunosorbent assay and myeloperoxidase (MPO) assays as described below.

Systemic leukocyte count

Blood was collected from tail vein and was mixed with Turks solution (0.2 mg gentian violet in 1 mL glacial acetic acid, 6.25% vol/vol) in a 1:20 dilution. Leukocytes were defined as monomorphonuclear and polymorphonuclear cells in a hematocytometer.

Lung edema

The left lung was excised, washed in PBS, gently dried by using blotting paper, and weighed. The tissue was then dried at 60°C for 72 h and reweighed. The change in the ratio of wet to dry weight was used as an indicator of lung edema formation.

MPO activity

Myeloperoxidase activity in the lung was determined as described previously (18). In brief, frozen lung tissue was thawed and homogenized in 1 mL of 0.5% hexadecyltrimethylammonium bromide. Next, the sample was freeze thawed, after which the MPO activity of the supernatant was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm; with a reference filter = 540 nm, 25°C). Values were expressed as MPO U/g tissue.

Enzyme-linked immunosorbent assay

Levels of MIP-2 were determined in lung samples, which were thawed and homogenized in PBS. Macrophage inflammatory protein 2 was analyzed by using double-antibody Quantikine enzyme-linked immunosorbent assay kit (R&D Systems) using recombinant murine MIP-2 as standards.

Histology

Lung samples were fixed by immersion in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. Six-micrometer sections were stained with hematoxylin-eosin.

Flow cytometry

To quantify CD44 and Mac-1 expression on neutrophils, blood was collected into heparinized syringes at 24 h after induction of CLP. The blood cells were incubated with an anti-CD16/CD32 antibody blocking Fcγ III/II receptors to reduce nonspecific labeling for 10 min at room temperature and then incubated with a PE-conjugated anti-CD44 (clone IM7) or a fluorescein isothiocyanate–conjugated anti–Mac-1 antibody (clone M1/70). All antibodies were from BD Biosciences Pharmingen. Cells were fixed with 1% formaldehyde solution; erythrocytes were lysed by use of red blood cell lysing buffer (Sigma Chemical Co, St Louis, Mo), and neutrophils were recovered after centrifugation. Flow cytometric analysis of CD44 and Mac-1 expression on Gr-1^+ cells was performed by first gating the neutrophil population of cells based on forward- and side-scatter characteristics on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, Calif). A viable gate was used to exclude dead and fragmented cells.

Adoptive transfer of neutrophils

Bone marrow leukocytes were freshly extracted from healthy mice by using Ficoll-Paque Research Grade (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated neutrophils was higher than 70% as assessed in a hematocytometer. Leukocytes were then resuspended in PBS to 10×10^6/mL and stained with 20 μM carboxyfluorescein diacetate-succinimidyl ester (CFDA-SE; Invitrogen, Paisley, UK) for 1 h at 37°C. Carboxyfluorescein diacetate-succinimidyl ester passively diffuses into cells and is nonfluorescent until its acetate groups are cleaved by intracellular esterases to yield a highly fluorescent ester. Two million labeled neutrophils were injected intravenously into mice immediately before CLP. Six hours after CLP induction, lungs were harvested, minced, and digested for 1 h at 37°C in buffer containing 20 U/mL collagenase A (Sigma Chemical Co). Single-cell suspensions were obtained by straining the digested tissue through a 40-μm mesh. Cells were labeled with an APC-labeled anti–Gr-1 antibody and fixed as described above. Finally, cells were analyzed by flow cytometry (FACSCalibur). Lung recruitment of transferred neutrophils were quantified by dividing the number of $CFDA^+$/Gr-1^+ cells by the number of $CFDA^-$/Gr-1^+ cells in the lung extracts.

Statistics

Data are presented as mean values ± SEM. Statistical evaluations were performed by using Kruskal-Wallis one-way ANOVA on ranks followed by multiple comparisons versus control group (Dunnett method). $P < 0.05$ was considered significant, and n represents the number of animals in each group.

RESULTS

CD44 regulates sepsis-induced neutrophil recruitment

Myeloperoxidase was used as an indicator of neutrophil accumulation into the lung. Cecal ligation and puncture enhanced pulmonary activity of MPO by more than 11-fold, i.e., from 1.1 ± 0.1 to 12.6 ± 0.7 U/g (Fig. 1A, $P < 0.05$ vs. sham, n = 8).

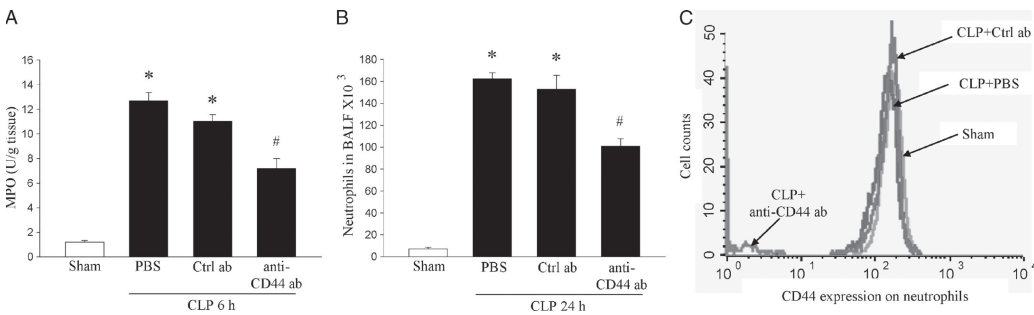

FIG. 1. **Role of CD44 in sepsis-induced neutrophil recruitment in the lung**. A, MPO activity, (B) number of BALF neutrophils in the lung, and (C) neutrophil expression of CD44 24 h after CLP induction. Animals were treated with an anti-CD44 antibody, isotype-matched control antibody (ctrl ab) before CLP induction. Data represent mean ± SEM. *$P < 0.05$ vs. sham and #$P < 0.05$ vs. ctrl ab + CLP, n = 5–8.

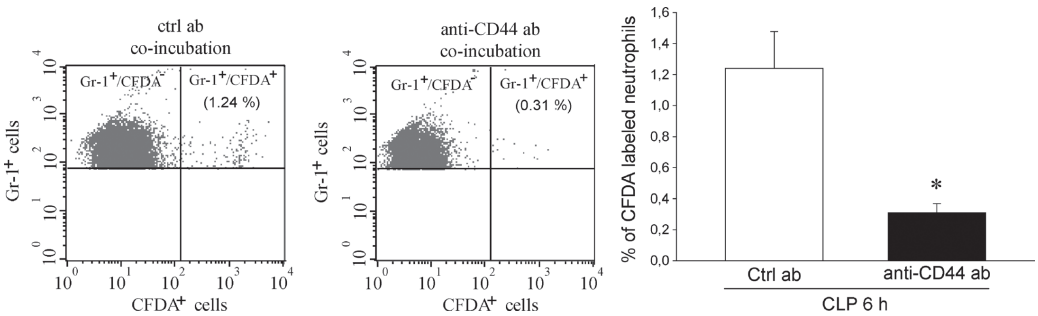

FIG. 2. **Adoptive transfer of CFDA-labeled neutrophils**. Bone marrow neutrophils were labeled with CFDA, and 2×10^6 neutrophils were adoptively transferred to CLP mice. The CFDA-labeled neutrophils were coincubated with an anti-CD44 antibody or an isotype-matched control antibody before cell transfer. Accumulation of transferred neutrophils was determined by quantifying the number of neutrophils (Gr-1$^+$ cells) in the lung labeled with CFDA divided by the total number of neutrophils (Gr-1$^+$ cells) in the lung lacking CFDA. Data represent mean ± SEM. *$P < 0.05$ vs. ctrl ab + CLP, n = 5.

Immunoneutralization of CD44 reduced CLP-induced MPO levels in the lung by more than 47% (Fig. 1A, $P < 0.05$ vs. ctrl ab + CLP, n = 8). In addition, cellular analysis of lavage fluid from the bronchoalveolar compartment revealed that the number of neutrophils increased by more than 22-fold in CLP mice (Fig. 1B, $P < 0.05$ vs. sham, n = 5). Administration of the anti-CD44 antibody decreased CLP-provoked neutrophil accumulation in the bronchoalveolar space by 40% (Fig. 1B, $P < 0.05$ vs. control ab + CLP, n = 5). To determine whether CD44 expressed on neutrophils or in the lung mediated neutrophil recruitment in septic lung injury, we performed adoptive transfer of CFDA-labeled neutrophil coincubated with the anti-CD44 antibody or a control antibody. It was found that homing of CFDA-labeled neutrophils to the lung in CLP mice was markedly reduced when neutrophils were coincubated with the anti-CD44 antibody compared with coincubation with the control antibody (Fig. 2). Notably, flow cytometry revealed that CLP did not alter the expression of CD44 on neutrophils (Fig. 1C). Moreover, the dose of the anti-CD44 antibody was found to completely block CD44 on the surface of neutrophils (Fig. 1C). Importantly, administration of the anti-CD44 antibody attenuated CLP-induced neutropenia in CLP animals (Table 1).

Hyaluronan is not involved in sepsis-induced neutrophil recruitment

Hyaluronan is one important ligand of CD44 (10). Considering our finding that neutrophil CD44 appears to regulate neutrophil recruitment in septic lung injury, we explored the potential role of hyaluronan in pulmonary infiltration of neutrophils in abdominal sepsis by administration of 20 U/g hyaluronidase before CLP. This dose of hyaluronidase has been shown to eliminate hyaluronan from the endothelium *in vivo* (19) and reduce neutrophil accumulation in the liver (13). Nonetheless, we found that elimination of hyaluronan from the vascular endothelium had no effect on MPO activity in the lung and number of BALF neutrophils in CLP mice (Fig. 3, A and B).

TABLE 1. **Systemic leukocyte differential counts**

	MNL	PMNL	Total leukocytes
Sham	4.6 ± 0.4	1.2 ± 0.1	5.8 ± 0.4
PBS + CLP	1.2 ± 0.09*	0.4 ± 0.07*	1.6 ± 0.1*
Ctrl ab + CLP	1.2 ± 0.1*	0.3 ± 0.05*	1.5 ± 0.1*
Anti-CD44 ab + CLP	1.4 ± 0.1*	0.7 ± 0.05*#	2.1 ± 0.1*#

Blood was collected from PBS-, control antibody–, and anti-CD44 antibody–treated mice exposed to CLP for 24 h as well as sham-operated animals. Cells were identified as monomorphonuclear leukocytes (MNLs) and polymorphonuclear leukocytes (PMNLs). Data represent mean ± SEM, 10^6 cells/mL and n = 5. *$P < 0.05$ vs. sham, #$P < 0.05$ vs. ctrl ab + CLP.

MIP-2 chemokine formation in the lung and Mac-1 expression on neutrophils

Cecal ligation and puncture induction enhanced levels of MIP-2 from 3.8 ± 1.1 to 73 ± 12.5 ng/g tissue in the lung (Fig. 4A, $P < 0.05$ vs. sham, n = 5). Inhibition of CD44 had no impact on CLP-provoked formation of MIP-2 in the lung (Fig. 4A, $P > 0.05$ vs. control antibody [ctrl ab] + CLP, n = 5). Knowing that expression of Mac-1 plays a key role in the sepsis-induced neutrophil infiltration in the lung in abdominal sepsis (3), we next asked whether inhibition of CD44 may affect Mac-1 expression on neutrophils. It was found that CLP increased surface expression of Mac-1 on neutrophils (Fig. 4, B and C). However, immunoneutralization of CD44 had no impact on Mac-1 levels on neutrophils in CLP mice (Fig. 4, B and C).

Inhibition of CD44 protects against septic lung injury

Morphologic analysis revealed normal tissue architecture and lack of neutrophil infiltration in lungs from sham mice, whereas CLP triggered destruction of the pulmonary microarchitecture and provoked clear-cut infiltration of neutrophils (Fig. 5). Moreover, CLP caused edema formation in the lung, i.e., wet weight–dry weight ratio increased from 4.5 ± 0.1 to 5.2 ± 0.1 (Fig. 6, $P < 0.05$ vs. sham, n = 5). We observed that administration of the anti-CD44 antibody preserved intact lung tissue architecture in CLP animals (Fig. 5) and decreased edema formation by 88% (Fig. 6, $P < 0.05$ vs. ctrl ab + CLP, n = 5).

DISCUSSION

This study demonstrates an important role of CD44 in the pulmonary recruitment of neutrophils in septic lung damage.

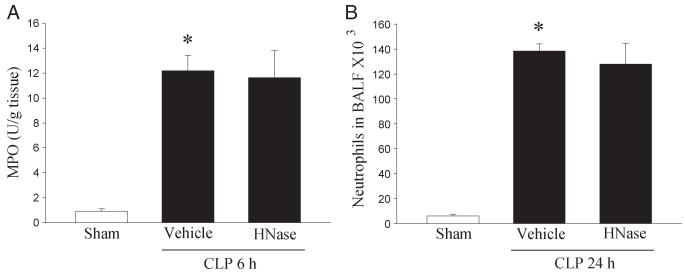

FIG. 3. **Role of hyaluronan in sepsis-induced neutrophil recruitment in the lung**. A, Myeloperoxidase activity, (B) number of BALF neutrophils in the lung at 6 and 24 h after CLP induction. Animals were treated with hyaluronidase or vehicle before CLP induction. Data represent mean ± SEM. $*P < 0.05$ vs. sham and $^{\#}P < 0.05$ vs. vehicle + CLP, n = 5.

Our data show that inhibition of CD44 decreases lung edema and tissue destruction in abdominal sepsis. Moreover, we observed that neutrophil CD44 rather than lung CD44 mediates neutrophil accumulation in septic lung injury. This CD44-dependent infiltration of neutrophils appears to be independent of hyaluronan in the lung.

Polymicrobial sepsis is characterized by widespread activation of the host immune system in which the most insidious component is lung damage and consequently disturbed gas exchange (3, 20). In general, neutrophil recruitment is a central feature in septic lung injury (3, 5), which makes the understanding of the mechanisms regulating pulmonary infiltration of neutrophils an important issue in developing novel and more effective sepsis therapies. We demonstrate for the first time that inhibition of CD44 function effectively decreases neutrophil accumulation in the lung in abdominal sepsis. Moreover, blocking CD44 not only attenuated pulmonary neutrophilia but also protected against sepsis-induced lung edema and tissue injury. Thus, these results suggest that targeting CD44 may be a useful way to ameliorate septic lung injury. The literature on the role of CD44 in regulating neutrophil trafficking is complex and partly contradictory. For example, several studies have shown that inhibition of CD44 reduces neutrophil recruitment to tissues, such as the lung (14), kidney (21), and joints (12), whereas others have reported increased accumulation of neutrophils in the lung (15, 22) and heart (23) in CD44 gene–deficient mice. The reason behind these discrepant findings is not known at present. Nonetheless, we observed that immunoneutralization of CD44 reduced lung levels of MPO in the present study. This inhibitory effect on MPO activity correlated well with our other findings that pretreatment with the anti-CD44 antibody reduced sepsis-triggered neutrophil recruitment in the bronchoalveolar space, suggesting that CD44 indeed supports neutrophil accumulation in septic lung injury. This notion is also in line with a study reporting that endotoxin-induced pulmonary infiltration of neutrophils is dependent on CD44 (14). It should be noted that inhibition of CD44 reduced pulmonary accumulation of neutrophils by less than 50%, suggesting that alternative mechanisms are operating in parallel to CD44-mediated neutrophil accumulation in abdominal sepsis. Indeed, previous studies have shown that adhesion molecules, such as PSGL-1, ICAM-1, Mac-1, and LFA-1, facilitate sepsis-induced neutrophil infiltration in the lung (3, 5, 24). Moreover, knowing that convincing data have shown that leukocyte accumulation in the lung is dependent not only on adhesion molecule-mediated interactions but also on biophysical mechanisms, such as decreased deformability of neutrophils activated in the circulation, which facilitates mechanical trapping of stiff neutrophils in the narrow lung capillaries (25–27). Increased stiffness

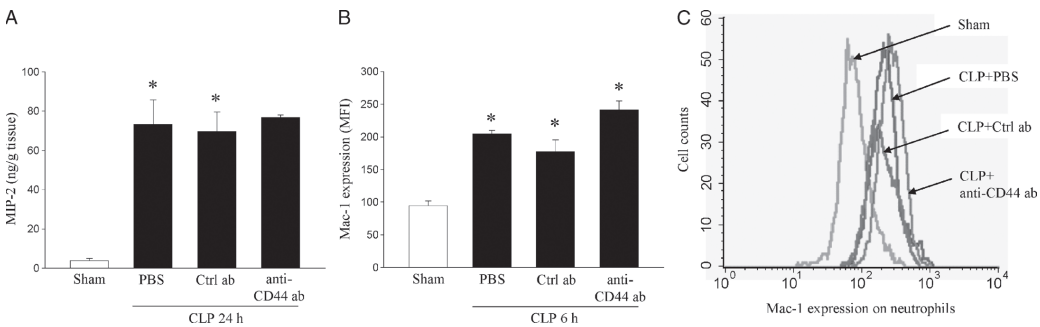

FIG. 4. **CXC chemokine formation in the lung and neutrophil expression of Mac-1**. Enzyme-linked immunosorbent assay was used to quantify the levels of MIP-2 in the lung (A), and flow cytometry was used to determine Mac-1 expression on neutrophils (B, C) 24 h after CLP induction. Animals were treated with PBS, an anti-CD44 antibody, or an isotype-matched control antibody (ctrl ab) before CLP. Data represent mean ± SEM. $*P < 0.05$ vs. sham and $^{\#}P < 0.05$ vs. ctrl ab + CLP, n = 5.

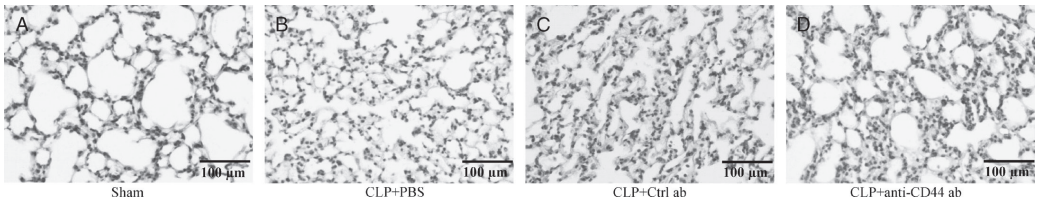

FIG. 5. **Tissue morphology 24 h after CLP induction**. Sham-operated animals served as negative controls (A). Animals were treated with PBS (B), an isotype-matched control antibody (C), or an anti-CD44 antibody (D) before CLP. Representative hematoxylin-eosin sections of lung are shown. Scale bar indicates 100 μm.

(decreased deformability) of circulating neutrophils is caused by chemoattractant-induced polymerization of intracellular F-actin (25). Although the cytoplasmic domain of CD44 does not contain any F-actin–binding sites, CD44 can transmit signals indirectly via specific kinases and regulate F-actin functions in neutrophils (28, 29). Thus, it cannot be excluded that CD44-mediated recruitment of neutrophils in the lung may not only be due to inhibition of adhesive interactions with endothelial cells but may also be related to changes in the cytoskeletal properties of circulating neutrophils in sepsis.

A major ligand of CD44 is hyaluronan (10), and CD44-hyaluronan interactions have been shown to mediate neutrophil trapping in liver sinusoids (13). However, we observed that elimination of hyaluronan on the vascular endothelium by administration of hyaluronidase had no effect on CLP-induced neutrophil recruitment and lung damage, suggesting that CD44-dependent accumulation of neutrophils in the septic lung is independent of hyaluronan. This notion is indirectly supported by a study showing that the expression of hyaluronic acid is relatively low in the lung compared with other tissues (13). Considering that CD44 is expressed both on neutrophils and in the lung (30, 31), we conducted a series of experiments with adoptive transfer of labeled neutrophils, showing that coincubation of neutrophils with the anti-CD44 antibody markedly reduced neutrophil accumulation in the lung, indicating that neutrophil CD44 is mediating neutrophil recruitment in septic lung injury. Certain evidence indicates that CD44 may interfere with proinflammatory signaling in leukocytes (15, 22), and we therefore asked whether blocking CD44 with the anti-CD44 antibody may reduce activation of neutrophils. However, we found that the increase in Mac-1 expression on circulating neutrophils triggered by CLP was intact in neutrophils from septic animals treated with the anti-CD44 antibody, suggesting that neutrophil activation was not negatively affected by immunoneutralization of CD44.

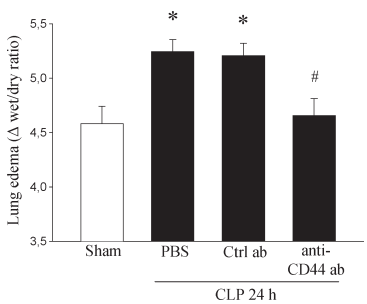

FIG. 6. **Edema formation in the lung**. Edema was determined by measuring changes in wet weight–dry weight ratios of the lung 24 h after CLP induction. Animals were treated with PBS, an anti-CD44 antibody, or an isotype-matched control antibody (ctrl ab) before CLP. Data represent mean ± SEM. $*P < 0.05$ vs. sham and $^{\#}P < 0.05$ vs. ctrl ab + CLP, n = 5.

Secretion of CXC chemokines is known to coordinate neutrophil trafficking in the lung (7). However, we found that CLP-induced formation of MIP-2 was maintained in mice passively immunized against CD44, suggesting that the inhibitory effect of the anti-CD44 antibody on neutrophil infiltration is not related to changes in the pulmonary production of MIP-2. In this context, it is interesting to note that most studies reporting that inhibition of CD44 causes increased accumulation of neutrophils have used CD44 gene-deficient animals. Considering ample data showing that CD44 is a potent negative regulator of macrophages in terms of cytokine and chemokine production (15, 32) and that local tissue macrophages are the main producers of CXC chemokines, such as MIP-2, at the sites of inflammation (33), it may be suggested that the discrepancy between studies on the role of CD44 in neutrophil recruitment mentioned above may be related to the use of antibodies against CD44 or the use of CD44 gene–deficient animals. Thus, administration of an anti-CD44 antibody intravenously mainly targets intravascular CD44 functions and not extravascular functions in macrophages in the alveoli of the lung. In contrast, in gene-deficient mice, all CD44 functions are affected including the potent inhibition of macrophage activation and secretion of proinflammatory compounds. This notion is in part supported by our findings that pulmonary levels of MIP-2 are similar in septic animals receiving the anti-CD44 antibody and the control antibody, whereas MIP-2 levels in the lung are increased in CD44-deficient mice (15, 22). Thus, caution has to be taken when interpreting particular functions of CD44 in terms of neutrophil recruitment at sites of inflammation when using CD44 gene–deficient animals. Another point is that use of the anti-CD44 antibody in sepsis studies more likely resembles future potential therapeutic approaches compared with complete abolition of CD44 in the body.

We provide evidence demonstrating that neutrophil CD44 contributes to neutrophil infiltration in the lung in abdominal sepsis. In addition, our novel findings show that inhibition of CD44-dependent accumulation of neutrophils also protects against edema formation and tissue destruction in septic lung

injury. Notably, we found that this CD44-mediated neutrophil infiltration in the lung is independent of hyaluronan in polymicrobial sepsis. In conclusion, these data suggest that CD44 may be a useful target to attenuate sepsis-triggered inflammation and tissue damage in the lung.

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ERRATUM

Targeting CD44 Expressed on Neutrophils Inhibits Lung Damage in Abdominal Sepsis: Erratum

The first two authors of the article on pages 567–572 of the June issue contributed equally to the article. This information was omitted in error.

Reference:

Hasan Z, Palani K, Rahman M, Thorlacius H: Targeting CD44 Expressed on Neutrophils Inhibits Lung Damage in Abdominal Sepsis. *Shock* 35:567–572, 2011.

PAPER 2

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Immunopharmacology and Inflammation

Rho-kinase regulates adhesive and mechanical mechanisms of pulmonary recruitment of neutrophils in abdominal sepsis

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ABSTRACT

We hypothesized that Rho-kinase signaling plays a role in mechanical and adhesive mechanisms of neutrophil accumulation in lung. Male C57BL/6 mice were treated with the Rho-kinase inhibitor Y-27632 prior to cecal ligation and puncture (CLP). Lung levels of myeloperoxidase (MPO) and histological tissue damage were determined 6 h and 24 h after CLP. Expression of Mac-1 and F-actin formation in neutrophils were quantified by using flow cytometry 6 h after CLP. Mac-1 expression and F-actin formation were also determined in isolated neutrophils up to 3 h after stimulation with CXCL2. Labeled and activated neutrophils co-incubated with Y-27632, an anti-Mac-1 antibody and cytochalasin B were adoptively transferred to CLP mice. Y-27632 reduced the CLP-induced pulmonary injury and MPO activity as well as Mac-1 on neutrophils. Neutrophil F-actin formation peaked at 6 h and returned to baseline levels 24 h after CLP induction. Rho-kinase inhibition decreased CLP-provoked F-actin formation in neutrophils. CXCL2 rapidly increased Mac-1 expression and F-actin formation in neutrophils. Co-incubation with Y-27632 abolished CXCL2-induced Mac-1 up-regulation and formation of F-actin in neutrophils. Notably, co-incubation with cytochalasin B inhibited formation of F-actin but did not reduce Mac-1 expression on activated neutrophils. Adoptive transfer experiments revealed that co-incubation of neutrophils with the anti-Mac-1 antibody or cytochalasin B significantly decreased pulmonary accumulation of neutrophils in septic mice. Our data show that targeting Rho-kinase effectively reduces neutrophil recruitment and tissue damage in abdominal sepsis. Moreover, these findings demonstrate that Rho-kinase-dependent neutrophil accumulation in septic lung injury is regulated by both adhesive and mechanical mechanisms.

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1. Introduction

Intestinal perforation is a major cause of mortality in intensive care units and poses a significant challenge for clinicians (Cohen, 2002; Heyland et al., 2000; Martin et al., 2003). Dissemination of bacteria and their toxins in the abdominal cavity provokes local formation of various pro-inflammatory compounds, which subsequently leak into the circulation, where these mediators activate circulating neutrophils causing a systemic inflammatory response (Aird, 2003). The lung is the most sensitive and clinically important end organ in abdominal sepsis. It is widely held that pulmonary recruitment of neutrophils is a rate-limiting step in septic lung injury. For example, neutrophil depletion or targeting specific adhesion molecules, such as PSGL-1 and Mac-1 are effective ways to protect against sepsis-induced lung damage (Asaduzzaman et al., 2009; Zhang et al., 2011). The recruitment process of neutrophils in the lung is more complex and far less studied than in other organs. Under homeostatic conditions, most neutrophils, which have a diameter larger than that of pulmonary capillaries, must deform in order to pass through the pulmonary microcirculation (Motosugi et al., 1996). Thus, any reduction in their deformability would promote mechanical sequestration of neutrophils in the lung capillaries (Worthen et al., 1989). Considered together, neutrophil accumulation may depend on both adhesive and mechanical factors in the lung. On one hand, adhesion molecules, such as selectins and integrins may support leukocyte–endothelium interactions in the pulmonary microvasculature. On the other hand, activated leukocytes may trigger cytoskeletal changes, including polymerization of F-actin, resulting in cell stiffening and mechanical trapping in the narrow capillaries in the lung. Whether adhesive and mechanical mechanisms of neutrophil accumulation operate in parallel and/or sequentially are not known.

Extracellular stress, including ischemia and infection, activates cellular signaling cascades converging on specific transcription factors regulating gene expression of inflammatory mediators. This intracellular signal transmission is largely regulated by kinases phosphorylating down-stream targets (Itoh et al., 1999). For example, small (~21 kDa)

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guanosine triphosphatases of the Ras-homologus (Rho) family and one of their effectors, Rho-kinase, are known to act as molecular switches controlling several critical functions, including, cell adhesion and contraction, migration, reactive oxygen species formation and oncogenic transformation (Alblas et al., 2001; Itoh et al., 1999; Slotta et al., 2006). Interestingly, Rho-kinase inhibitors have been reported to attenuate ischemia/reperfusion and endotoxemic injury in the liver (Slotta et al., 2008) as well as protecting against tissue fibrosis (Kitamura et al., 2007), cholestasis (Laschke et al., 2010), cerebral and intestinal ischemia (Santen et al., 2010; Shin et al., 2007). However, the role of the Rho-kinase signaling in regulating pulmonary recruitment of neutrophils and tissue damage in abdominal sepsis is not known. Moreover, the influence of Rho-kinase inhibition on adhesive and mechanical aspect of sepsis-induced neutrophil accumulation in the lung remains elusive.

Based on these considerations, the aim of the present study was to define the functional role of Rho-kinase signaling in regulating F-actin polymerization and Mac-1 expression in neutrophils as well as their role in the regulation of pulmonary recruitment of neutrophils and tissue damage in sepsis. For this purpose, we used a model of polymicrobial sepsis based on intestinal perforation in mice.

2. Materials and methods

2.1. Animals

Specific-pathogen-free male C57BL/6 mice (20 to 25 g) were housed in filter-top cages under standardized laboratory conditions. Mice were with a 12 h light/12 h dark diurnal cycle with food and water *ad libitum*. All experimental procedures were performed in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for Animal Experimentation at Lund University, Sweden. Animals were anesthetized by administration of 7.5 mg (i.p.) ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg (i.p.) xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight.

2.2. Experimental protocols

Polymicrobial sepsis was induced by cecal ligation and puncture (CLP) as described previously (Asaduzzaman et al., 2008). Shortly, through laparotomy incision, the exposed cecum was filled with feces by milking stool backward from the ascending colon, and a ligature was placed below the ileocecal valve. The cecum was soaked with phosphate-buffered saline (PBS; pH 7.4) and punctured twice with a 21-gauge needle. The cecum was then pushed back into the abdominal cavity and the incision was sutured. To determine the role of Rho-kinase, vehicle (PBS) or the Rho-kinase inhibitor, Y-27632 {(R)-(+)-N-(4-pyridyl)-4-(1-aminoethyl} cyclohexanecarboxamide; (Calbiochem, San Diego, USA), was given (5.0 mg/kg) i.p. 30 min before CLP induction. The dose of 5 mg/kg was chosen based on a previous study (Awla, et al., 2011). Sham mice underwent the same surgical procedures, but the cecum was neither ligated nor punctured. The mice were then returned to their cages and provided food and water. Animals were re-anesthetized 3, 6, 12 and 24 h after CLP induction. The lung was perfused with PBS, left lung was fixed in formaldehyde for histology, and the remaining lung tissue was weighed, snap-frozen in liquid nitrogen, and stored at −80°C for later myeloperoxidase (MPO) assays as described below.

2.3. Systemic leukocyte count

Blood was collected from tail vein and was mixed with Turk's solution (0.2 mg gentian violet in 1 ml glacial acetic acid; 6.25% vol/vol) in a 1:20 dilution. Leukocytes were identified and counted as monomorphonuclear (MNL) and polymorphonuclear (PMNL) leukocyte cells in a Burker chamber.

2.4. MPO activity

The enzyme MPO is abundant in neutrophils and has been used as reliable marker for detection of neutrophil accumulation in inflamed tissue and was quantified as described previously (Bradley et al., 1982). In brief, frozen lung tissue was thawed and homogenized in 1 ml of 0.5% hexadecyltrimethylammonium bromide. Next, the sample was freeze-thawed, after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter of 540 nm; 25 °C). Values were expressed as MPO units per gram of tissue.

2.5. Histology

Lung samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin-embedded. Six-micrometer sections were stained with hematoxylin and eosin. Lung injury was quantified in a blinded manner by adoption of a pre-existing scoring system as described (Borzone et al., 2007), including size of alveolar spaces , thickness of alveolar septas, alveolar fibrin deposition and neutrophil infiltration, graded on a 1 (absent) to 4 (extensive) scale. The total injury score was expressed as the sum of the four scores.

2.6. Flow cytometry

For detection and analysis of Mac-1 expression on circulating neutrophils, blood was collected into syringes containing 1:10 acid citrate dextrose (22.0 g/L sodium citrate; 7.3 g/L citric acid, anhydrous; and 24.5 g/L dextrose) 3, 6, 12 and 24 h after CLP induction. Blood samples were incubated with an anti-CD16/CD32 antibody (10 min at room temperature) blocking Fcγ III/II receptors to reduce non-specific labeling and then incubated with PE-conjugated anti-Gr-1 (Clone RB6-8C5, rat IgG2b, eBioscience, San Diego, USA), and FITC-conjugated anti-Mac-1 (Clone M1/70, integrin α_M chain, rat IgG2b) antibodies. To determine the distribution of F-actin content within neutrophils, another set of samples was permeabilized by L-lysophosphatidylcholine (LPC, lysolecithin, 1-O-acyl-sn-glycero-3-phophocholine, L-lysophosphatidylcholine-gamma-Oacyl; Sigma, St. Louis , MO, USA) and then stained with BODIPY FL phallicidin (Molecular Probes, Invitrogen, Eugene, USA) and APC-conjugated anti-Gr-1 (Clone RB6-8 C5, rat anti-mouse Ly-6G and Ly-6C) antibodies (all antibodies except those indicated were purchased from BD Biosciences Pharmingen, San Jose, CA, USA). Cells were fixed and erythrocytes were lysed using FACS lysing solution (BD Biosciences Pharmingen, San Jose, CA, USA) and then neutrophils were recovered following centrifugation. Flow cytometric analysis was performed according to standard settings on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and a viable gate was used to exclude dead and fragmented cells.

2.7. In vitro studies

Neutrophils were freshly isolated from healthy mice by aseptically flushing the bone marrow of femurs and tibias from healthy mice by using Ficoll-Paque™ Research Grade (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated neutrophils was more than 70% as assessed in a haematocytometer. Leukocytes were then re-suspended in PBS to 10×10^6/ml and co-incubated with 300 ng/ml recombinant mouse CXCL2/MIP2 (R&D Systems, Inc., Minneapolis, USA) for different time points. Two set of samples were pre-incubated with Y-27632 (100 μM/100 μl) 20 min before activation by CXCL2 for measuring F-actin content within neutrophils and Mac-1 expression.

Cells are permeabilized, stained and fixed as described above. Finally, cells were analyzed by flow cytometry (FACSCalibur). Mac-1 expression on neutrophils and F-actin content within neutrophils were measured in separate experiments. Lastly effects of cytochalasin B 10 μM (cytochalasin B isolated from Drechslera dematioidea; Sigma, Sweden) on F-actin and Mac-1 were checked in two different sets of experiment.

2.8. Adoptive transfer of neutrophils

Again bone marrow leukocytes were freshly extracted from healthy mice by using Ficoll-Paque™ as described above. Leukocytes were then re-suspended in PBS to 10×10^6/ml and co-incubated with 300 ng/ml recombinant mouse CXCL2 for 10 min and 180 min separately; furthermore leukocytes were pre-incubated with cytochalasin B 10 μM 30 min and anti-Mac-1 (Purified anti-mouse CD11b, NA/LE, from BD Biosciences) for 15–20 min at room temperature before challenge with CXCL2 co-incubation. Samples were stained with 20 μM CFDA-SE (carboxyfluorescein diacetate-succinimidyl ester, Invitrogen, Paisley, UK) and for 1 h at 37 °C. CFDA-SE passively diffuses into cells and is non-fluorescent until its acetate groups are cleaved by intracellular esterases to yield a highly fluorescent ester. Two million labeled neutrophils were injected intravenously into mice immediately prior to CLP, then 4 h after CLP induced, lungs were harvested, minced, and digested for 1 h at 37 °C in buffer containing 20 U/ml collagenase A (Sigma). Single-cell suspensions were obtained by straining the digested tissue through a 40-μm mesh. Cells were labeled with an APC-labeled anti-Gr-1 antibody and fixed as described above. Finally, cells were analyzed by flow cytometry (FACSCalibur). Lung recruitment of transferred neutrophils were quantified by dividing the number of $CFDA^+/Gr\text{-}1^+$ cells by the number of $CFDA^-/Gr\text{-}1^+$ cells in the lung extracts.

2.9. Statistics

Data are presented as mean values ± S.E.M. (Standard error of the mean). Statistical evaluations were performed by using Kruskal–Wallis one-way analysis of variance on ranks followed by multiple comparisons versus control group (Dunnett's method). $P<0.05$ was considered significant and n represents the number of animals in each group.

3. Results

3.1. Rho-kinase activity regulates lung tissue damage

CLP caused significant pulmonary damage, characterized by severe destruction of pulmonary tissue microstructure, extensive edema of interstitial tissue, necrosis and massive infiltration of neutrophils (not shown). Quantification of the morphological damage showed that CLP enhanced the lung injury score and that pretreatment with the Rho-kinase inhibitor Y-27632 significantly reduced the lung injury score in CLP mice (Fig. 1A; $P<0.008$ vs. vehicle + CLP, $n=5$). Moreover, leukocytopenia was observed 24 h after CLP induction (Table 1). For example, the number of neutrophils decreased by 53% 24 h after CLP (Table 1; $P<0.05$ vs. sham, $n=5$). Notably, this CLP-induced neutropenia was significantly decreased in mice pretreated with Y-27632 (Table 1; $P<0.05$ vs. vehicle + CLP, $n=5$).

3.2. Mac-1 expression and neutrophil accumulation are regulated by Rho-kinase activity

Accumulation of neutrophils in the lung was quantified by analyzing levels of myeloperoxidase (MPO), an indicator of neutrophils in the lung. It was found that CLP increased MPO activity in the lung by 11-fold (Fig. 1B; $P<0.008$ vs. sham, $n=5$). Notably, Rho-kinase inhibition reduced MPO activity in the lung by 54% in septic mice (Fig. 1B; $P<0.008$ vs. vehicle + CLP, $n=5$). Mac-1 expression was increased on

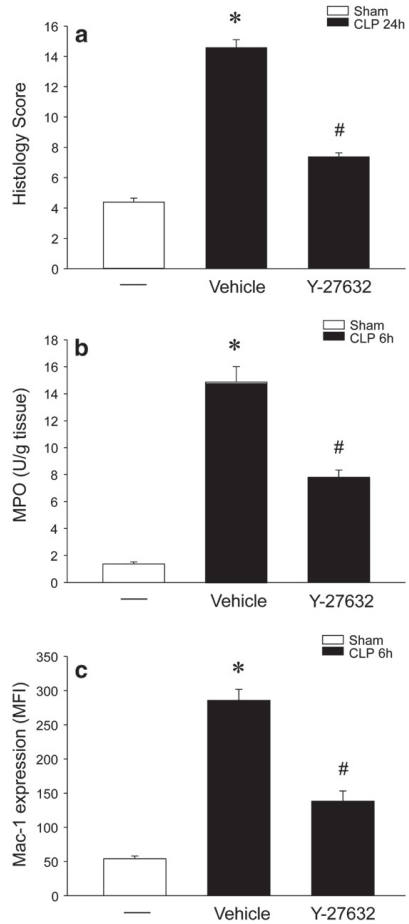

Fig. 1. (A) Evaluation of lung injury at 24 h as described in Materials and methods. (B) Lung MPO activity, a marker of neutrophils in the lung 6 h post-CLP. (C) Rho-kinase activity regulates Mac-1 expression on neutrophils. Sham animals served as a negative control and separate groups of mice were pretreated with vehicle and Y-27632 before operation. Data represents mean ± S.E.M. and $n=5$. *$P<0.05$ vs. sham and #$P<0.05$ vs. vehicle + CLP.

Table 1
Systemic leukocyte differential counts.

	MNL	PMNL	Total
Sham	4.8 ± 0.1	1.5 ± 0.05	6.3 ± 0.1
Vehicle + CLP	1.2 ± 0.1[a]	0.4 ± 0.1[a]	1.6 ± 0.1[a]
Y-27632 + CLP	1.4 ± 0.1[a]	0.9 ± 0.1[a,b]	2.3 ± 0.2[a,b]

Blood was collected from vehicle and Y-27632 treated mice exposed to cecal ligation and puncture (CLP) for 24 h as well as sham-operated animals. Cells were identified as monomorphonuclear leukocytes (MNLs) and polymorphonuclear leukocytes (PMNLs). Data represents mean ± S.E.M. x 10^6 cells/ml and $n=5$.
[a] $P<0.05$ vs. sham.
[b] $P<0.05$ vs. vehicle + CLP.

the surface of neutrophils in CLP animals, indicating that circulating neutrophils were activated in this sepsis model. Inhibition of Rho-kinase activity markedly antagonized CLP-induced up-regulation of Mac-1 on the surface of neutrophils. MFI (mean fluorescence intensity) values of Mac-1 on neutrophils decreased from 285 ± 16 down to 138 ± 15 in CLP mice pretreated with Y-27632, corresponding to a 63% reduction (Fig. 1C; $P<0.029$ vs. vehicle + CLP, $n=5$).

3.3. Rho-kinase activity regulates neutrophil formation of F-actin

We first analyzed the F-actin content of neutrophils at different time points after CLP induction. F-actin formation in neutrophils increased rapidly in septic animals, *i.e.* MFI values of F-actin increased from 122 ± 3 at baseline to 264 ± 24 3 h after CLP (Fig. 2A; $P<0.008$ vs. sham, $n=5$). Maximum levels of F-actin were observed 6 h after induction of CLP when MFI values of F-actin reached 270 ± 26 (Fig. 2A; $P<0.008$ vs. sham, $n=5$). F-actin formation in neutrophils returned to baseline levels 24 h after CLP induction (Fig. 2A). Next we asked whether inhibition of Rho-kinase activity might influence F-actin formation in neutrophils. Administration of Y-27632 decreased the CLP-provoked formation of F-actin in neutrophils (Fig. 2B). Indeed, inhibition of Rho-kinase activity reduced neutrophil formation of F-actin by 68% in septic mice (Fig. 2C; $P<0.001$ vs. vehicle + CLP, $n=5$).

3.4. CXCL2-induced F-actin formation and Mac-1 expression in vitro

F-actin formation and Mac-1 expression were determined in isolated neutrophils after stimulation with CXCL2, which is a potent activator of neutrophils. Challenge with CXCL2 caused a rapid increase in F-actin formation in neutrophils peaking at 10 min and returning to baseline levels after 180 min (Fig. 3A). Neutrophil expression of Mac-1 also peaked at 10 min but remained elevated compared to baseline values 180 min after stimulation with CXCL2 (Fig. 3B). It was found that co-incubation of neutrophils with Y-27632 abolished CXCL2-induced formation of F-actin and expression of Mac-1 in neutrophils (Table 2). Interestingly, co-incubation of neutrophils with cytochalasin B, a well-known inhibitor of F-actin polymerization, also abolished CXCL2 triggered F-actin formation (Table 2; $P<0.008$ vs. vehicle + CXCL2, $n=5$) but had no effect on surface expression of Mac-1 (Table 2) on neutrophils.

3.5. Adhesive and mechanical mechanisms of neutrophil accumulation

In order to discriminate the influence of adhesive and mechanical mechanisms in the accumulation process of neutrophils in septic lung damage, we performed adoptive transfer of CFDA-labeled and CXCL2 activated neutrophils co-incubated with or without the anti-Mac-1 antibody and cytochalasin B. In line with the kinetic experiments showing maximal expression of Mac-1 and F-actin formation in neutrophils 10 min after challenge with CXCL2, we observed that homing of adoptively transferred neutrophils was maximal when the cells had been stimulated with CXCL2 for 10 min (Fig. 4A; $P<0.010$ vs. sham, $n=6$). It was found that homing of CFDA-labeled and activated neutrophils to the lung in CLP mice were markedly reduced when neutrophils were co-incubated with the anti-Mac-1 antibody (Fig. 4B). Notably, co-incubation of labeled and activated neutrophils with cytochalasin B, which had no effect on Mac-1 expression, also significantly decreased pulmonary accumulation of these cells in CLP animals (Fig. 4C; $P<0.001$ vs. vehicle + CXCL2, $n=5$).

4. Discussion

It is widely held that systemic activation and pulmonary accumulation of neutrophils are key features in sepsis. However, the signaling pathways regulating neutrophil activation remain elusive. In the present study, we show that the Rho-kinase inhibitor Y-27632 greatly

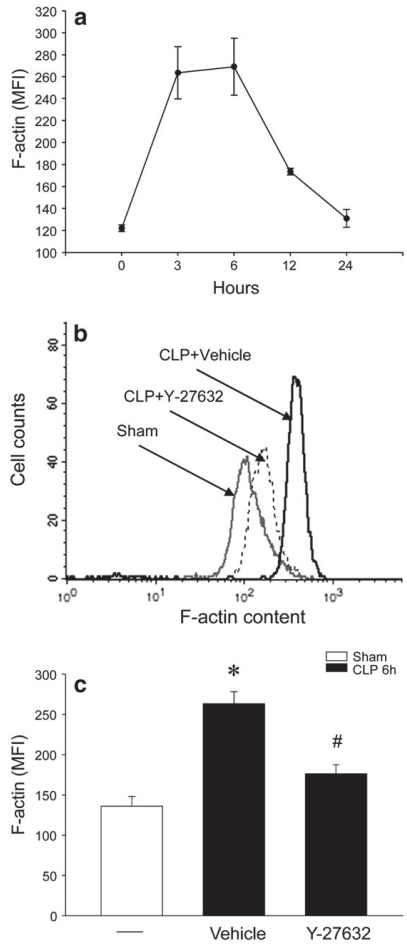

Fig. 2. CLP rapidly increased F-actin content of neutrophils and reached maximum levels 6 h after CLP. F-actin content (MFI) of the neutrophils shown on the Y-axis against time after CLP induction on the X-axis (a). Pre-treatment with Y-27632 significantly reduced F-actin content at 6 h CLP (b) and (c). Data represents mean ± S.E.M. and $n=5$. $^{*}P<0.05$ vs. sham and $^{\#}P<0.05$ vs. vehicle + CLP.

reduced pulmonary levels of MPO, an indicator of neutrophils, suggesting that Rho-kinase controls neutrophil accumulation in septic lung injury. Moreover, inhibition of Rho-kinase activity not only decreased pulmonary neutrophilia but also attenuated lung tissue damage in abdominal sepsis. Considering the close relationship between neutrophil recruitment and tissue damage in septic pulmonary injury (Asaduzzaman et al., 2008) it may be proposed that part of the lung protective effect of inhibiting Rho-kinase signaling is related to the reduced accumulation of neutrophils in the lung. These findings are in line with two previous studies showing that Rho-kinase inhibition can reduce tissue injury in the liver and lung triggered by endotoxin (Lomas-Neira et al., 2006; Thorlacius et al., 2006). Although toxin-based models are frequently used, they are more artificial and do not necessarily resemble the complex events and course in polymicrobial sepsis in terms of

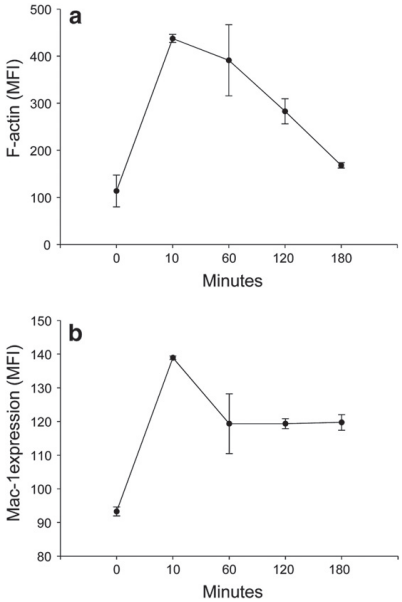

Fig. 3. *In vitro* kinetics of F-actin content (MFI) and Mac-1 expression (MFI). Shown on the Y-axis against time after CXCL2 activation on the X-axis. F-actin peaked at 10 min and reduced at 180 min (a). Mac-1 expression peaked at 10 min again but remained relatively high at 180 min (b). Data represents mean ± S.E.M. and $n = 5$.

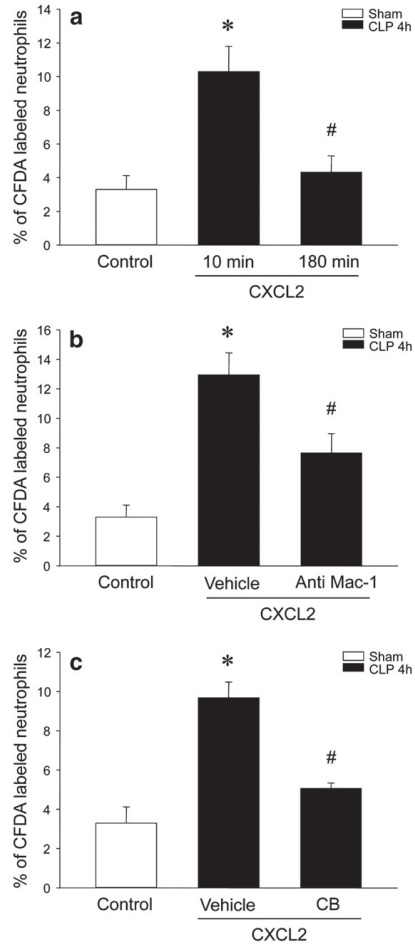

Fig. 4. (a) Adoptive transfer of CFDA-labeled neutrophils. Bone marrow neutrophils were labeled with CFDA and 2×10^6 neutrophils and incubated with CXCL2 for 10 min and 180 min and then adoptively transferred to CLP mice, Data represents mean ± S.E.M. and $n = 6$. *$P < 0.05$ vs. control and #$P < 0.05$ vs. 10 min + CLP. CFDA-labeled neutrophils activated with CXCL2 for 10 min were co-incubated with an anti-Mac-1 (b) or cytochalasin B (c) prior to injection. Accumulation of transferred neutrophils was determined by quantifying the number of neutrophils (Gr-1+ cells) in lung labeled with CFDA divided by the total number of neutrophils (Gr-1+ cells). Data represents mean ± S.E.M. and $n = 5$. *$P < 0.05$ vs. control and #$P < 0.05$ vs. vehicle + CXCL2.

cytokine responses as well as vascular and metabolic changes (Klintman et al., 2004b; Remick et al., 2000; Wichterman et al., 1980). Moreover, different toxins activate the host immune system in a distinctly different manner. For example, LPS has been shown to be a potent activator of macrophages and stimulates TNF-α production (Ulevitch et al., 1990; Wright et al., 1990) whereas superantigens do not provoke clear-cut TNF-α formation and activates primarily T-lymphocytes causing FasL-dependent apoptosis (Klintman et al., 2004a). Nonetheless, considered together with our present findings, it may be forwarded that Rho-kinase signalling is a key feature in acute lung injury.

Leukocyte recruitment in the lung is far less studied and appears to be much more complex than that in other organs. One reason for this is that the spherical diameter of the capillaries in the lung is smaller (6 μm) than that of neutrophils (7 μm) which forces neutrophils to deform and make them prone to mechanical trapping when passing through the narrow lung capillaries (Motosugi et al., 1996). Thus, cytoskeletal rearrangements causing any reduction in neutrophil deformability will increase their sequestration in the pulmonary microvasculature. This concept of F-actin-mediated stiffening and mechanical trapping of neutrophils in the lung has been confirmed in a number of studies (Downey et al., 1991; Frank, 1990; Saito et al., 2002). Therefore, it was of great interest to study F-actin formation in circulating neutrophils in septic mice herein. We observed that F-actin markedly increased in circulating neutrophils in mice with ongoing abdominal sepsis. Moreover, this sespis-induced neutrophil F-actin formation was abolished by administration of Y-27632, indicating that polymerization of F-actin in neutrophils is regulated by Rho-kinase signaling in abdominal sepsis. In contrast, Tasaka et al. (2005) reported that neutrophil formation of F-actin triggered by fMLP, a peptide from *Escherichai coli* bacteria, was insensitive to treatment with Y-27632. The reason behind these apparent discrepancies is not known. However, the mechanisms of neutrophil stiffening in polymicrobial sepsis *in vivo* is likely different from that induced by bacterial proteins *in vitro*. For instance, it is well known that mechanisms of neutrophil stiffening differs signicantly between different stimulus, such as fMLP (Saito et al., 2002) and LPS (Erzurum et al., 1992). In addition, our findings are in line with convincing *in vitro* data showing that the Rho-kinase signaling pathway controls polymerization of F-actin in neutrophils (Chodniewicz and Zhelev, 2003). A common observation in systemic inflammation is a marked decrease in the number of circulating neutrophils due to mechanical sequestration in the lung (Andonegui et al.,

Table 2
Mac-1and F-actin expression in neutrophils.

	Mac-1 expression (MFI)	F-actin expression (MFI)
PBS	395 ± 1.8	176 ± 1.4
CXCL2	1301 ± 11.3[a]	415 ± 3.7[a]
CXCL2 + Y-27632	746 ± 10.4[b]	237 ± 16.2[b]
CXCL2 + CB	1530 ± 10.8	304 ± 14.5[b]

F-actin polymerization and Mac-1 expression in isolated bone marrow neutrophils were determined 10 min after challenge with CXCL2. Neutrophils were co-incubated with Y-27632 and cytochalasin B (CB). Data represent mean ± S.E.M. and $n = 5$.
[a] $P < 0.05$ vs. PBS.
[b] $P < 0.05$ vs. CXCL2.

2003). Indeed, we also found that systemic neutrophil counts were significantly reduced in CLP mice. It is therefore interesting to note that administration of Y-27632 reduced the CLP-induced decrease in circulating neutrophils, which further lends support to the concept that Rho-kinase signaling regulates neutrophil stiffening in abdominal sepsis.

Besides mechanical trapping, leukocytes are also recruited in the lung by specific adhesion molecules expressed on leukocytes and endothelial cells. For example, it has been shown that PSGL-1, LFA-1 and Mac-1 on neutrophils and ICAM-1 on endothelial cells support pulmonary accumulation of neutrophils in abdominal sepsis (Asaduzzaman et al., 2008, 2009; Hildebrand et al., 2005). Herein, we found that expression of Mac-1 was markedly increased on the surface of circulating neutrophils in septic mice. Moreover, administration of Y-27632 abolished sepsis-induced Mac-1 expression on neutrophils, indicating that Rho-kinase regulates Mac-1 up-regulation on neutrophils in abdominal sepsis. Considered together, our findings suggest that Rho-kinase may control sepsis-evoked neutrophil recruitment in the lung via both adhesive and mechanical mechanisms. In order to better define the relative role of adhesive and mechanical mechanisms in this Rho-kinase-dependent neutrophil recruitment in the lung, we stimulated isolated neutrophils with CXCL2 *in vitro*. We observed that CXCL2 increased Mac-1 expression and F-actin formation in neutrophils in a time-dependent manner peaking 10 min after challenge. It was found that Y-27632 reduced CXCL2-induced neutrophil up-regulation of Mac-1 as well as F-actin formation. In contrast, cytochalasin B, a well-known inhibitor of F-actin polymerization (Cooper, 1987), abolished F-actin formation but had no effect on Mac-1 expression on neutrophils in response to CXCL2 stimulation. Next, we adoptively transferred isolated and labeled neutrophils activated with CXCL2 for 10 min to mice with ongoing abdominal sepsis and determined their accumulation in the lung. We found that co-incubation of isolated neutrophils with an antibody directed against Mac-1 markedly reduced pulmonary recruitment of these neutrophils in septic animals. Interestingly, co-incubation of isolated neutrophils with cytochalasin B, which had no effect on Mac-1 expression but abolished F-actin formation, also decreased neutrophil accumulation in the lungs of septic mice. Taken together, our results show for the first time that both adhesive and mechanical mechanisms mediate neutrophil recruitment in the lung. Considering that Y-27632 decreased both Mac-1 up-regulation and F-actin formation in neutrophils, it may be suggested that Rho-kinase signaling regulates both adhesive and mechanical aspects of pulmonary accumulation of neutrophils in abdominal sepsis

5. Conclusion

Our results show that Rho-kinase signaling plays an important role in septic lung damage. Moreover, these findings demonstrate that Rho-kinase-dependent accumulation of neutrophils in lung is composed of both adhesive (Mac-1) and mechanical (F-actin) components in abdominal sepsis. Taken together, our novel data indicate that targeting the Rho-kinase signaling pathway may be a useful strategy to protect against sepsis-induced lung injury.

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PAPER 3

Rac1 signaling regulates sepsis-induced pathological inflammation in the lung via attenuation of Mac-1 expression and CXC chemokine formation

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Abstract

Excessive neutrophil recruitment is a major feature in septic lung damage although the signaling mechanisms behind pulmonary infiltration of neutrophils in sepsis remain elusive. In the present study, we hypothesized that Rac1 might play an important role in pulmonary neutrophil accumulation and tissue injury in abdominal sepsis. Male C57BL/6 mice were treated with Rac1 inhibitor NSC23766 (5 mg/kg) before CLP. BALF and lung tissue were collected for quantification of neutrophil recruitment, as well as edema and CXC chemokine formation. Blood was collected for determination of Mac-1 on neutrophils and pro-inflammatory compounds in plasma. Gene expression of CXC chemokines and TNF-α was determined by qRT-PCR in alveolar macrophages. Rac1 activity was increased in lungs from septic animals and NSC23766 significantly decreased pulmonary activity of Rac1 induced by CLP. Administration of NSC23766 markedly reduced CLP-triggered neutrophil infiltration, edema formation and tissue damage in the lung. Inhibition of Rac1 decreased CLP-induced neutrophil expression of Mac-1 and pulmonary formation of CXC chemokines. Moreover, NSC23766 abolished the sepsis-evoked elevation of mRNA levels of CXC chemokines and TNF-α in alveolar macrophages. Rac1 inhibition decreased the CLP induced increase in plasma levels of HMGB1 and IL-6, indicating a role of Rac1 in systemic inflammation. In conclusion, our results demonstrate that Rac1 signaling plays a key role in regulating pulmonary infiltration of neutrophils and tissue injury via regulation of chemokine production in the lung and Mac-1 expression on neutrophils in abdominal sepsis. Thus, targeting Rac1 activity might be a useful strategy to protect the lung in abdominal sepsis.

Introduction

Polymicrobial sepsis is a significant cause of mortality in intensive care units despite substantial research efforts [1]. Clinical management of sepsis patients is largely limited to supportive care, which is related to an incomplete understanding of the underlying pathophysiology. Intestinal perforation and leakage of bowel contents stimulate massive production of pro-inflammatory substances in the abdominal cavity, which subsequently disseminate into the circulation triggering a systemic inflammatory response syndrome [2]. Activation of the innate immune system, including neutrophils and macrophages, constitute a key feature in systemic inflammation in which the most feared complication is pulmonary damage [3]. Numerous studies have documented that activation of neutrophils constitutes a critical feature in septic lung injury. For example, previous reports have demonstrated that inhibition of pulmonary recruitment of neutrophils protects against pulmonary damage in abdominal sepsis [4]. Sepsis-induced neutrophil accumulation in the lung is mediated by specific adhesion molecules, including PSGL-1 [5] and LFA-1 [6] and co-ordinated by secreted CXC chemokines, such as CXCL1 (KC) and CXCL2 (MIP-2) [6]. Thus, the adhesive mechanisms of pulmonary recruitment of neutrophils are relatively well known whereas the complex signaling cascades orchestrating neutrophil activation and recruitment in the lung in abdominal sepsis are largely unknown.

Recent studies indicate that statins can inhibit neutrophil recruitment and lung damage in abdominal sepsis [7] and clinical data suggest that statins might decrease mortality in patients with severe infections and sepsis [8,9]. Statins regulate cholesterol levels by inhibiting the rate-limiting enzyme, HMG-CoA reductase, in the synthesis of mevalonate [10,11]. Mevalonate is not only a precursor for the formation of cholesterol but also for the generation of isoprenoid pyrophosphates needed for isoprenylation of small G-proteins, including Rac, Cdc42 and Rho, which is critical for their localization at cell membranes [12]. Rac1 is a ubiquitously expressed signal transducer involved in the control of several processes, including, cell adhesion, chemotaxis, vascular permeability and cytoskeletal reorganization [13]. Several investigations have established functional links between Rac activity and the regulation of mitogen-activated protein kinases (MAPKs), such as JNK/c-Jun [14,15] and p38 MAPK [16-18], which are known to be

involved in pro-inflammatory activities, such as cytokine formation and leukocyte activation [19,20]. In fact, inhibition of Rac1 has been shown to exert protective effects in models of reperfusion injury in the liver, endotoxemia and acute pancreatitis [21-23]. However, the potential role of Rac1 signaling in regulating CXC chemokine formation, neutrophil infiltration and lung injury in abdominal sepsis remains elusive.

In the present study, we hypothesized that Rac1 signaling might play an important role in septic lung injury by regulating neutrophil activation and recruitment. For this purpose, we used a model of polymicrobial sepsis induced by cecal ligation and puncture.

Materials and Methods

Animals

Experiments were performed using male C57BL/6 mice weighing 20 to 25 g. All experimental procedures were performed in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for Animal Experimentation at Lund University, Sweden. Animals were anesthetized by administration of 7.5 mg (i.p.) ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg (i.p.) xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight.

Experimental design

Polymicrobial sepsis in mice was induced by puncture of the cecum. Animals were anesthetized, the abdomen was opened, the exposed cecum was filled with feces by milking stool backward from the ascending colon, and a ligature was placed below the ileocecal valve. The cecum was soaked with phosphate-buffered saline (PBS; pH 7.4) and punctured twice with a 21-gauge needle and a small amount of bowel contents was extruded. The cecum was then returned into the peritoneal cavity, and the abdominal incision was sutured. Animals were treated with vehicle (PBS) or with 5 mg/kg of the Rac1 inhibitor, NSC23766 (N6-[2-[[4-(Diethylamino)-1-methylbutyl] amino]-6-methyl- 4-pyrimidinyl]-2 methyl-4, 6-quinolinediamine trihydrochloride, Tocris Bioscience, Bristol, UK) i.p. 30 min before CLP induction. Sham mice underwent the same surgical

procedures, that is, laparotomy and resuscitation, but the cecum was neither ligated nor punctured. The mice were then returned to their cages and provided food and water *ad libitum.* Animals were re-anesthetized 6 and 24 h after CLP induction. The left lung was ligated and excised for edema measurement. The right lung was used for collecting bronchoalveolar lavage fluid (BALF) in which neutrophils were quantified in a Burker chamber. Next, the lung was perfused with PBS, and one part was fixed in formaldehyde for histology, and the remaining lung tissue was snap-frozen in liquid nitrogen, and stored at -80˚C for later enzyme-linked immunosorbent assay (ELISA) and myeloperoxidase (MPO) assays as described subsequently.

Rac1 activity

Rac1 activation assay was performed by using Rac1 activation assay kit as described previously [24]. Briefly, 50 mg of lung tissue were minced and homogenated in lysis buffer on ice, the samples were centrifuged at 150 000 g for 15 min; 10 µl from each supernatant were removed to measure protein content using Pierce BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL, USA) and the rest of the volume was used for the pull-down assay. Supernatant containing equal amount of proteins were then diluted with 2X SDS sample buffer and boiled for 5 min. Proteins were separated using SDS-PAGE (10% gel). After transfer to a nitrocellulose membrane (BioRad, Hercules, CA, USA), blots were blocked with 3% bovine serum albumin at room temperature for 1 h, followed by incubation with an anti-Rac1 antibody (1:1000) at 4ºC overnight. Binding of the antibody was visualized using peroxidase-conjugated anti-mouse antibody (1:10000, Pierce Biotechnology) at room temperature for 1 h and enhanced chemiluminescence method (BioRad, Hercules, CA, USA). β-actin was used as an internal control for total Rac1.

Systemic leukocyte count

Blood was collected from tail vein and was mixed with Turks solution (0.2 mg gentian violet in 1 mL glacial acetic acid; 6.25% vol/vol) in a 1:20 dilution. Leukocytes were counted as monomorphonuclear (MNL) and polymorphonuclear (PMNL) leukocyte cells in a Burker chamber.

BALF

Animals were placed supine and the trachea was exposed by dissection. An angiocatheter was inserted into the trachea. BALF was collected by 5 washes of 1 ml of PBS containing 5 mM EDTA. The numbers of MNL and PMNL cells were counted in a Burker chamber.

Lung edema

The left lung was excised, washed in PBS, gently dried using a blotting paper and weighed. The tissue was then dried at 60°C for 72 h and re-weighed. The change in the ratio of wet weight to dry weight was used as indicator of lung edema formation.

MPO activity

Lung tissue was thawed and homogenized in 1 ml of 0.5% hexadecyltrimethylammonium bromide. Samples were freeze-thawed, after which the MPO activity of the supernatant was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter 540 nm, 25°C). Values were expressed as MPO units per g tissue.

Histology

Lung samples were fixed by immersion in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin-embedded. Six µm sections were stained with haematoxylin and eosin. Lung injury was quantified in a blinded manner by adoption of a modified scoring system [25,26], including size of alveoli, thickness of alveolar septae, alveolar fibrin deposition and neutrophil scoring system infiltration graded on a zero (absent) to four (extensive) scale. In each tissue sample, 5 random areas were scored and mean value was calculated. The histology score is the sum of all four parameters.

ELISA

CXCL1 and CXCL2 levels in lung tissue were analyzed by using double antibody Quantikine ELISA kits (R & D Systems, Europe, Abingdon, Oxon, UK) using recombinant murine CXCL1 and CXCL2 as standards. ELISA kits were used to quantify plasma levels of HMGB1 (Chondrex, Redmond, WA, USA) and IL-6 (R & D Systems)

according to manufacturer's instructions.

Flow cytometry

For analysis of surface expression of Mac-1 on circulating neutrophils, blood was collected (1:10 acid citrate dextrose) 6 h after CLP induction and incubated (10 min at room temperature) with an anti-CD16/CD32 antibody blocking Fcγ III/II receptors to reduce non-specific labeling and then incubated with phycoerythrin (PE)-conjugated anti-Gr-1 (clone RB6-8C5, rat IgG2b, eBioscience, Frankfurt, Germany) and fluorescein isothiocyanate-conjugated anti-Mac-1 (clone M1/70, integrin αM china, rat IgG2b κ, BD Biosciences Pharmingen, San Jose, CA, USA) antibodies. Cells were fixed and erythrocytes were lysed, neutrophils were recovered following centrifugation. Flow-cytometric analysis was performed according to standard settings on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed with Cell-Quest Pro software (BD Biosciences). A viable gate was used to exclude dead and fragmented cells.

In vitro activation of neutrophils

Bone marrow neutrophils were freshly extracted from femurs and tibias of healthy mice by aseptically flushing the bone marrow with complete culture medium RPMI 1640 and then subsequently isolated by using Ficoll-Paque™ Research Grade (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated neutrophils was higher than 70% as assessed in a haematocytometer. Neutrophils were then resuspended in PBS to 10^7 cells per ml and co-incubated with 3 µg/ml recombinant mouse CXCL2 (R & D Systems) for 30 min at 37°C. Neutrophils were pre-incubated with NSC23766 (1 or 10 µM) 20 min before challenge with CXCL2. Cells were stained and fixed for flow cytometric analysis of Mac-1 expression on neutrophils as described above.

Isolation of alveolar macrophages and quantitative RT-PCR

In separate experiments, gene expression of CXCL1, CXCL2 and TNF-α was quantified in alveolar macrophages isolated from sham mice (n = 5) and CLP animals treated with vehicle or 5 mg/kg of NSC23766 i.p. 30 min prior to CLP (n = 5). Alveolar macrophages

were isolated from BALF as described in detail [27]. Briefly, 30 min after induction of CLP, lungs were flushed three times with 1 ml of PBS supplemented with 0.5 mM EDTA. Alveolar fluid collections were then centrifuged at 1400 rpm for 10 min (18ºC). The cells were then resuspended in RPMI 1640 complete culture medium and incubated at 37°C (5% CO_2) in a 48-well plate. After 2 h, non-adherent cells were washed away by PBS. A total of 2-3 x 10^5 macrophages were obtained per mice and the purity of macrophages was higher than 97%. Total RNA was isolated from the alveolar macrophages using an RNeasy Mini Kit (Qiagen; West Sussex, UK) following the manufacturer's protocol and treated with RNase-free DNase (DNase I; Amersham Pharmacia Biotech, Sollentuna, Sweden) to remove potential genomic DNA contaminants. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically. Each cDNA was synthesized by reverse transcription from 10 µg of total RNA using the StrataScript First-Strand Synthesis System and random hexamer primers (Stratagene; AH diagnostics, Stockholm, Sweden). Real-time PCR was performed using a Brilliant SYBRgreen QPCR master mix and MX 3000P detection system (Stratagene). The primer sequences of CXCL1, CXCL2, TNF-α and β-actin were as follows: CXCL1 (forward) 5'-GCC AAT GAG CTG CGC TGT CAA TGC-3', CXCL1 (reverse) 5'-CTT GGG GAC ACC TTT TAG CAT CTT-3'; CXCL2 (forward) 5'-GCT TCC TCG GGC ACT CCA GAC-3', CXCL2 (reverse) 5'-TTA GCC TTG CCT TTG TTC AGT AT-3'; TNF-α (forward) 5'-CCT CAC ACT CAG ATC ATC TTC TC-3', TNF-α (reverse) 5'-AGA TCC ATG CCG TTG GCC AG-3'; and β-actin (forward) 5'-ATG TTT GAG ACC TTC AAC ACC-3', β-actin (reverse) 5'-TCT CCA GGG AGG AAG AGG AT-3'. Standard PCR curves were generated for each PCR product to establish linearity of the RT-PCR reaction. PCR amplifications were performed in a total volume of 50 µl, containing 25 µl of SYBRgreen PCR 2x master mix, 2 µl of 0.15 µM each primer, 0.75 µl of reference dye, and one 1 µl cDNA as a template adjusted up to 50 µl with water. PCR reactions were started with 10 min denaturing temperature of 95°C, followed by a total of 40 cycles (95°C for 30 s and 55°C for 1 min), and 1 min of elongation at 72°C. Cycling time values for the specific target genes were related to that of β-actin in the same sample.

Neutrophil chemotaxis

Neutrophils isolated form bone marrow of healthy mice by use of Ficoll-Paque™ were pre-incubated with NSC23766 (1 or 10 μM) for 30 min and 1.5×10^6 neutrophils were placed in the upper chamber of the transwell inserts (5 μm pore size, Corning Costar, Corning, NY, USA). Inserts were placed in wells containing medium alone (control) or medium plus CXCL2 (100 ng/ml, R & D Systems). After 120 min, inserts were removed and migrated neutrophils were stained with Turks solution. Chemotaxis was determined by counting the number of migrated neutrophils in a Burker chamber.

Statistics

Data were presented as mean values ± standard errors of the means (SEM). Statistical evaluations were performed by using non-parametrical test (Mann-Whitney). $P < 0.05$ was considered significant and *n* represents the total number of mice in each group. Statistical analysis was performed by using SigmaStat® 3.5 software (Systat Software, Chicago, IL, USA).

Results

CLP induces Rac1 activation in the lung

To investigate the activation of Rac1 in the lung by CLP and the effect of NSC23766 on inhibition of Rac1 activity, lungs from sham and CLP mice were harvested for Rac1 activation assay. CLP increased the active form (GTP binding form) of Rac1 (Fig.1). Administration of NSC23766 decreased CLP-induced activation of Rac1 (Fig.1).

Rac1 regulates neutrophil recruitment and lung injury

In order to examine neutrophil infiltration in septic lung damage, we analyzed both, activity of MPO, an indicator of neutrophils, and the number of neutrophils in the BALF. MPO activity and BALF neutrophils in the lung represent early and late phases of neutrophil accumulation, and they peak at 6 and 24 h respectively, in this model (data not shown). CLP enhanced pulmonary levels of MPO by 4-fold (Fig. 2A, $P < 0.05$ vs. Sham, $n = 5$). Treatment with NSC23766 (5 mg/kg), a Rac1 inhibitor, decreased CLP-induced MPO levels in the lung by 58% (Fig. 2A, $P < 0.05$ vs. Vehicle + CLP, $n = 5$).

Moreover, the number of BALF neutrophils increased by 12-fold 24 h after CLP induction (Fig. 2B, $P < 0.05$ vs. Sham, $n = 5$). Rac1 inhibition reduced neutrophil infiltration into the bronchoalveolar space by 94% in CLP animals (Fig. 2B, $P < 0.05$ vs. Vehicle + CLP, $n = 5$). CLP caused significant lung edema formation. More specifically, the lung wet/dry ratio increased from 4.3 ± 0.2 to 6.3 ± 0.1 in septic mice (Fig. 2C, $P < 0.05$ *vs*. Sham, $n = 5$). Administration of NSC23766 decreased the wet/dry ratio by 90% in septic mice (Fig. 2C, $P < 0.05$ *vs*. Vehicle + CLP, $n = 5$). Histological analysis of the lungs showed normal structure in sham animals (Fig.3A). CLP caused destruction of the pulmonary microarchitecture characterized by extensive edema of the interstitial tissue and massive infiltration of neutrophils (Fig. 3B). Treatment with NSC23766 decreased CLP-provoked tissue damage and neutrophil accumulation in the lung (Fig. 3C). Quantification of the morphological injury revealed that CLP increased the lung injury score by more than 3-fold and that Rac1 inhibition attenuated the lung injury score in CLP mice (Fig. 3D, $P < 0.05$ *vs*. Vehicle + CLP, $n = 5$). Moreover, CLP caused leukocytopenia after 24 h (Table 1, $P < 0.05$ vs. Sham, n = 5). The CLP-induced leukocytopenia was decreased in mice pretreated with NSC23766 (Table 1).

Rac1 regulates neutrophil expression of Mac-1

Mac-1 is a key adhesion molecule in mediating neutrophil adhesion to endothelial cells [7]. Mac-1 expression increased on the surface of peripheral blood neutrophils in CLP animals, indicating that circulating neutrophils are activated in this model (Fig. 4A, B, $P < 0.05$ vs. Sham, $n = 5$). Rac1 inhibition reduced CLP-induced expression of Mac-1 on blood neutrophils (Fig. 4A, B, $P < 0.05$ *vs*. Vehicle + CLP, $n = 5$). MFI values of Mac-1 on peripheral blood neutrophils decreased from 110.2 ± 12.9 down to 68.0 ± 5.0 in CLP mice pretreated with NSC23766, corresponding to a 98% reduction (Fig. 4). We next asked whether this inhibitory effect of NSC23766 involved a direct effect on neutrophils and therefore we stimulated bone marrow isolated neutrophils with CXCL2 *in vitro*, which increased Mac-1 expression (Fig. 4C, D). Co-incubation of NSC23766 with CXCL2 significantly decreased neutrophil up-regulation of Mac-1 (Fig. 4C, D), indicating that Rac1 regulates Mac-1 expression in neutrophils.

Rac1 activity controls pulmonary production of CXC chemokines

Extravascular accumulation of neutrophils in the lung is orchestrated by secreted chemokines, such as CXCL1 and CXCL2. Pulmonary levels of CXCL1 and CXCL2 were low but detectable in sham animals (Fig. 5A, B). CLP markedly increased pulmonary generation of CXCL1 and CXCL2 (Fig. 5A, B, $P < 0.05$ *vs*. Sham, $n = 5$). Inhibition of Rac1 by administration of NSC23766 attenuated CLP-provoked formation of CXCL1 and CXCL2 in the lung by more than 86% and 96%, respectively (Fig. 5A, B, $P < 0.05$ *vs*. Vehicle + CLP, $n = 5$). We next isolated alveolar macrophages from the BALF in sham and CLP mice pretreated with or without NSC23766. CLP triggered a clear-cut increase in mRNA levels of CXCL1, CXCL2 and TNF-α in alveolar macrophages (Fig. 6A-C, $P < 0.05$ *vs*. Sham, $n = 5$). Notably, administration of NSC23766 abolished the CLP-induced increase in mRNA levels of CXCL1, CXCL2 and TNF-α in alveolar macrophages (Fig. 6A-C, $P < 0.05$ *vs*. Vehicle + CLP, $n = 5$).

Rac1 regulates plasma levels of HMGB1 and IL-6

Plasma levels of HMGB1 in control animals were low but detectable (Fig. 7A, $n = 5$). CLP enhanced plasma levels of HMGB1 by 6-fold from 8.6 ± 1.1 ng/ml up to 55.0 ± 8.2 ng/ml (Fig. 7A, $P < 0.05$ *vs.* sham, $n = 5$). Pretreatment with NSC23766 reduced CLP-provoked generation of HMGB1 to 17.0 ± 5.5 ng/ml (Fig. 7A, $P < 0.05$ *vs.* PBS+CLP, $n = 5$). In addition, we observed that the plasma levels of IL-6 were increased in CLP compared to sham mice (Fig. 7B, $P < 0.05$ *vs.* sham, $n = 5$). Interestingly, administration of NSC23766 decreased plasma levels of IL-6 from 192.8 ± 26.8 ng/ml down to 38.8 ± 10.6 ng/ml in CLP animals (Fig. 7B, $P < 0.05$ *vs.* PBS + CLP, $n = 5$). Thus, NSC23766 significantly reduced CLP-provoked plasma levels of HMGB1 by 81% and IL-6 by 80%.

Rac1 regulates neutrophil migration in vitro

In order to examine whether NSC23766 might exert a direct effect on neutrophil migration, we examined neutrophil chemotaxis in vitro by using neutrophils isolated from the bone marrow. We found that 100 ng/ml CXCL2 caused a clear-cut increase in neutrophil migration over a time period of 120 min (Fig. 8, $P < 0.05$ *vs*. PBS). Pre-incubation of neutrophils with NSC23766 (1 and 10 μM) dose-dependently reduced

CXCL2-provoked neutrophil migration (Fig. 8, $P < 0.05$ vs. CXCL2 + PBS).

Discussion

Our present study documents a significant role of Rac1 signaling in abdominal sepsis. Thus, inhibition of Rac1 activity reduced sepsis-evoked pulmonary recruitment of neutrophils and tissue damage. In addition, our findings suggest that Rac1 regulates Mac-1 expression on neutrophils and formation of CXC chemokines in lung macrophages in sepsis. Finally, Rac1 inhibition abolished CLP-induced increases of HMGB1 and IL-6 in the plasma, indicating that Rac1 activity regulates systemic inflammation in sepsis.

Management of patients with abdominal sepsis is largely limited to supportive care, which is related to an insufficient understanding of the pathophysiology of sepsis. It is well known that activation of the host innate immune system, including platelets and neutrophils, is a critical step in sepsis, causing lung dysfunction and impaired gaseous exchange [28,29]. However, the signaling pathways regulating neutrophil activation remain elusive. Rac1 signaling is generally considered to regulate cytoskeletal dynamics, including cell contraction and vesicular trafficking, but accumulating data also suggest that Rac1 activity is an important component in inflammatory processes, such as leukocyte chemotaxis, phagocytosis and cytokine formation [30-32]. In the present study, we show that the Rac1 inhibitor NSC23766 not only inhibits Rac1 activity in the lung but also decreases pulmonary edema and tissue damage in abdominal sepsis. Depletion of neutrophils has repeatedly been shown to protect against septic lung injury, illustrating the critical role of neutrophils in septic lung damage [33,34]. Herein, we could document that inhibition of Rac1 decreased pulmonary MPO activity, a marker of neutrophils, by 58% in septic animals. This inhibitory effect was in line with our observation that NSC23766 administration attenuated sepsis-evoked neutrophil recruitment in the bronchoalveolar space by 94%, suggesting that Rac1 is a potent regulator of neutrophil accumulation in septic lung injury. Indeed, this is the first study to show that Rac1 regulates pulmonary infiltration of neutrophils in abdominal sepsis.

Leukocyte infiltration in the lung is a multistep process, including initial microvessel trapping and firm adhesion on the endothelial cells, followed by transendothelial and

transepithelial migration [3]. Recruitment of leukocytes at sites of inflammation is under the control of secreted chemokines [35]. Neutrophils are particularly activated and attracted by CXC chemokines, such as CXCL1 and CXCL2, which are homologues of human IL-8 [36]. Herein, it was observed that administration of NSC23766 reduced CLP-triggered formation of CXCL1 and CXCL2 by 86% and 96%, respectively, suggesting that Rac1 is a key regulator of CXC chemokine generation in septic lung injury. We next asked whether Rac1 might regulate gene expression of CXC chemokines and TNF-α in alveolar macrophages. Indeed, we found that CLP markedly enhanced CXCL1, CXCL2 and TNF-α mRNA levels in alveolar macrophages. Notably, administration of NSC23766 abolished CLP-provoked gene expression of CXC chemokines and and TNF-α in alveolar macrophages, indicating that Rac1 is an important signalling pathway in macrophage production of CXC chemokines and TNF-α in abdominal sepsis. In addition, this finding might also help to explain the inhibitory effect of NSC23766 on sepsis-triggered neutrophil infiltration in the lung. Pulmonary accumulation of neutrophils is mediated by Mac-1 expressed on neutrophils [6]. In the present study, it was observed that Rac1 inhibition reduced CLP-induced expression of Mac-1 on neutrophils. Knowing that CXC chemokines cause Mac-1 up-regulation on neutrophils and our observation that Rac1 is a potent regulator of CXC chemokine formation in the lung it is possible that the reduced Mac-1 expression observed in CLP mice treated with NSC23766 might be an indirect effect of targeting Rac1 activity. It was therefore of interest to study the direct effect of NSC23766 on chemokine-provoked Mac-1 expression on isolated neutrophils. Interestingly, it was found that CXCL2-induced activation of isolated neutrophils was significantly attenuated by the Rac1 inhibitor, suggesting that Rac1 signaling also directly controls neutrophil Mac-1 up-regulation in abdominal sepsis. It is interesting to note that NSC23766 decreased Mac-1 upregulation 97% in vivo and 50% in vitro, suggesting a higher potency of NSC23766 in vivo. The strong inhibiting effect on Mac-1 experiment in vivo might be related to the fact that both direct and indirect (CXC chemokines formation) mechanisms of Mac-1 upregulation are blocked by NSC23766 in vivo. Knowing that CXC chemokines are potent inducers of neutrophil migration [37], it was also of interest to evaluate the role of Rac1 in controlling chemokine-dependent chemotaxis. Indeed, we found that inhibition of Rac1

dose-dependently reduced CXCL2-induced neutrophil migration in vitro. Considered together, these results indicate that Rac1 activity regulates sepsis-induced neutrophil accumulation in the lung at three distinct levels, *i.e.* production of CXC chemokines in the lung, Mac-1 up-regulation on the surface of neutrophils and neutrophil chemotaxis.

HMGB1 is a potent pro-inflammatory cytokine and a late mediator in endotoxemia and sepsis [38] as well a predictor of clinical outcome in patients with severe sepsis [39]. In line with previous studies, we observed that CLP caused a substantial increase in the plasma levels of HMGB1. Notably, NSC23766 treatment decreased HMGB1 levels in the plasma by 81% in septic animals, indicating a potent anti-inflammatory effect of NSC23766 in CLP-induced inflammation. Another indicator of systemic inflammation is IL-6 and a correlation between high IL-6 levels and mortality of septic patients has been demonstrated [40]. Herein, we found that administration of NSC23766 markedly reduced plasma levels of IL-6 in septic animals. Together, these findings suggest that Rac1 is a central regulator of systemic inflammation in abdominal sepsis:

In conclusion, these novel findings indicate that Rac1 is a potent regulator of lung damage in abdominal sepsis. Thus, Rac1 inhibition not only attenuates neutrophil recruitment but also reduces pulmonary edema and tissue destruction. The inhibitory effects of NSC23766 on pulmonary neutrophil appear related to interference with CXC chemokine production in the lung, Mac-1 up-regulation on neutrophils and neutrophil chemotaxis. Taken together, our results suggest that targeting Rac1 signaling might be a way to protect against lung injury in abdominal sepsis.

Acknowledgements

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Figure legends

Figure 1. Six h after CLP challenge and NSC23766 treatment, lungs from control group (−) and CLP group (+) mice were harvested for Rac1 activities. β-actin was used as an internal control for total Rac1, n = 3.

Figure 2. Effects of NSC23766 on CLP-induced pulmonary infiltration of neutrophils. Lung MPO levels at 6 h post-CLP (A), number of BALF neutrophils (B), and edema formation in the lung (C) 24 h after CLP induction. Animals were treated with NSC23766 (5 mg/kg) or vehicle (PBS) before CLP induction. Sham-operated mice served as negative controls. Data represent mean ± SEM and $n = 5$. $^{*}P < 0.05$ *vs.* Sham and $^{\#}P < 0.05$ *vs.* Vehicle + CLP.

Figure 3. Tissue morphology 24 h after CLP induction. Representative haematoxylin and eosin sections of lung are shown. Sham-operated animals served as negative controls (A). Animals were treated with vehicle (PBS) (B) or 5 mg/kg of NSC23766 (C) before CLP induction. Lung injury score as described in Materials and Methods (E). Data represents mean ± SEM and $n = 5$. $^{*}P < 0.05$ *vs.* Sham and $^{\#}P < 0.05$ *vs.* Vehicle + CLP. Scale bar indicates 50 μm.

Figure 4. Rac1 activity regulates Mac-1 expression on neutrophils. Mac-1 expression on circulating neutrophils ($Gr\text{-}1^{+}$ cells) 6 h after CLP induction in sham mice or animals treated with vehicle (PBS) or NSC23766 (5 mg/kg). Representative dot plot and histogram (A) and data in aggregate (B). Data represents mean ± SEM and $n = 5$. $^{*}P < 0.05$ *vs.* Sham and $^{\#}P < 0.05$ *vs.* Vehicle + CLP. Mac-1 expression on isolated bone marrow neutrophils ($Gr\text{-}1^{+}$ cells) incubated with CXCL2 (3 μg/ml) and vehicle (PBS) or NSC23766 (1 or 10 μM). Representative dot plot and histogram (C) and data in aggregate (D). Data represents mean ± SEM and $n = 5$. $^{*}P < 0.05$ *vs.* PBS and $^{\#}P < 0.05$ *vs.* PBS + CXCL2.

Figure 5. Rac1 activity regulates CXC chemokine formation in the lung. ELISA was used to quantify the levels of CXCL1 (A) and CXCL2 (B) in the lung 24 h after CLP

induction. Animals were treated with vehicle (PBS) or NSC23766 (5 mg/kg) before CLP induction. Sham-operated animals served as negative controls. Data represents mean ± SEM and n = 5. *P < 0.05 *vs.* Sham and $^{\#}P$ < 0.05 *vs.* Vehicle + CLP.

Figure 6. Rac1 activity regulates gene expression of CXC chemokines and TNF-α in alveolar macrophages. qRT-PCR was used to determine the levels of mRNA expression of CXCL1 (A) CXCL2 (B) and TNF-α (C) in alveolar macrophages 30 min after CLP induction. Animals were treated with vehicle (PBS) or NSC23766 (5 mg/kg) before CLP induction. Sham-operated animals served as negative controls. Data represents mean ± SEM and n = 5. *P < 0.05 *vs.* Sham and $^{\#}P$ < 0.05 *vs.* Vehicle + CLP.

Figure 7. Rac1 activity regulates systemic inflammation in sepsis. ELISA was used to quantify the levels of HMGB1 (A), IL-6 (B) in the plasma 24 h after CLP induction. Animals were treated with vehicle (PBS) or NSC23766 (5 mg/kg) before CLP induction. Sham-operated animals served as negative controls. Data represents mean ± SEM and n = 5. *P < 0.05 *vs.* Sham and $^{\#}P$ < 0.05 *vs.* Vehicle + CLP.

Figure 8. Rac1 activity regulates neutrophil migration in vitro. Neutrophils isolated from bone marrow were analyzed for their migration in response to PBS (Control), PBS plus CXCL2 (100 ng/ml) with or without pre-incubation of neutrophils with NSC23766 (1 or 10 μM). Data represent mean ± SEM and n= 5. *P < 0.05 *vs.* PBS and $^{\#}P$ < 0.05 *vs.* CXCL2 + PBS.

Table 1: Systemic leukocyte differential counts

	MNL	PMNL	Total
Sham	5.9 ± 0.2	1.4 ± 0.2	7.4 ± 0.4
Vehicle + CLP	0.9 ± 0.1*	0.6 ± 0.2*	1.5 ± 0.2*
NSC23766 + CLP	2.7 ± 0.6$^{\#}$	1.2 ± 0.1$^{\#}$	3.9 ± 0.7$^{\#}$

Blood was collected from vehicle (PBS) and NSC23766 (5 mg/kg) treated mice exposed to CLP for 24 h as well as sham-operated animals. Cells were identified as monomorphonuclear leukocytes (MNLs) and polymorphonuclear leukocytes (PMNLs). Data represents mean ± SEM and 10^6 cells/ml. *P < 0.05 *vs.* Sham, $^{\#}P$ < 0.05 *vs.* Vehicle + CLP and n = 5.

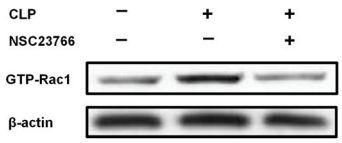
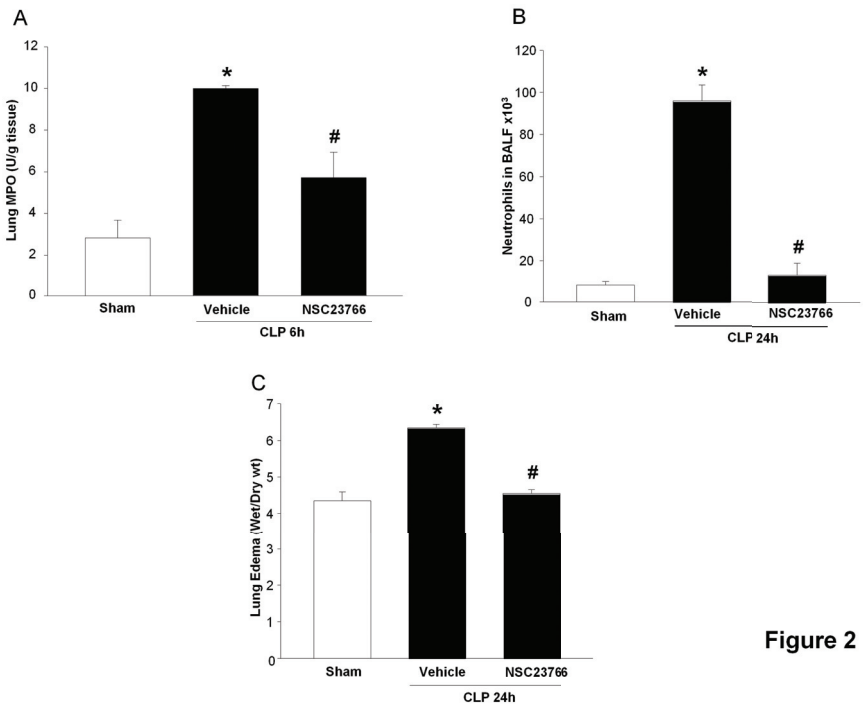


Figure 1



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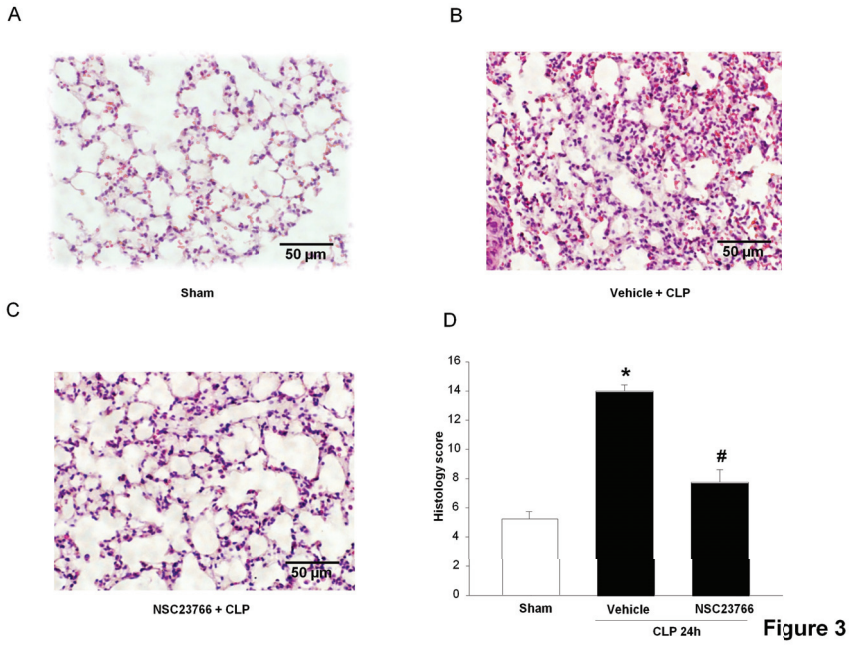

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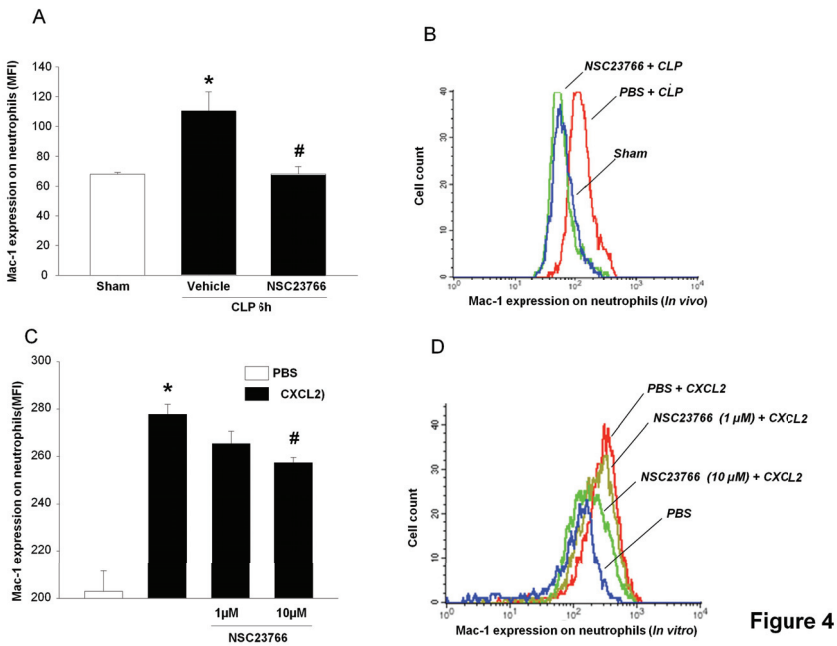

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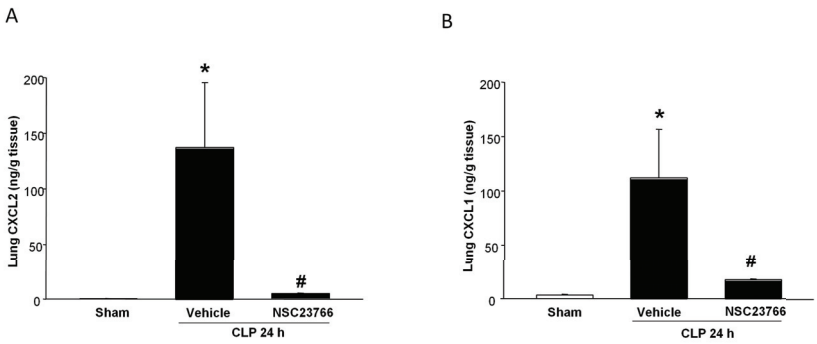

Figure 5

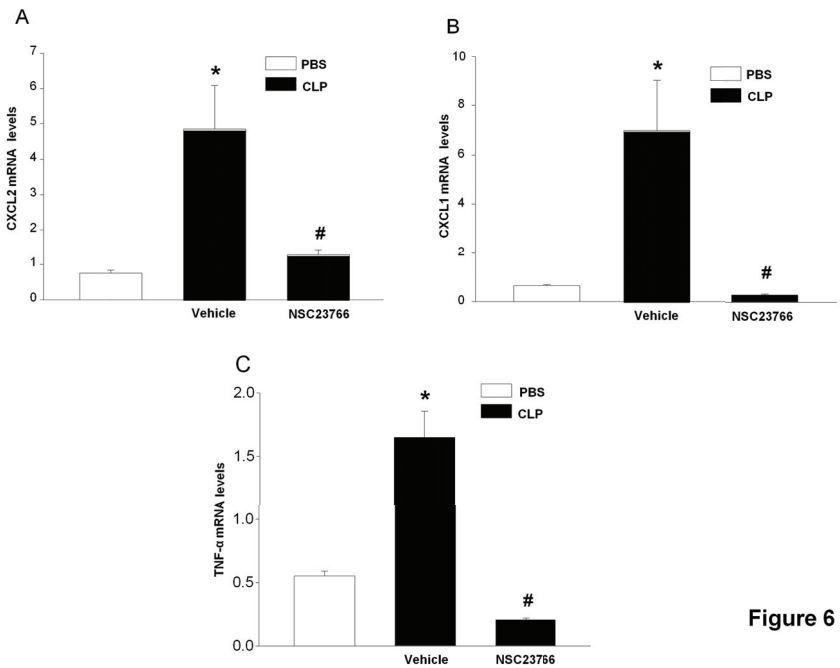

Figure 6

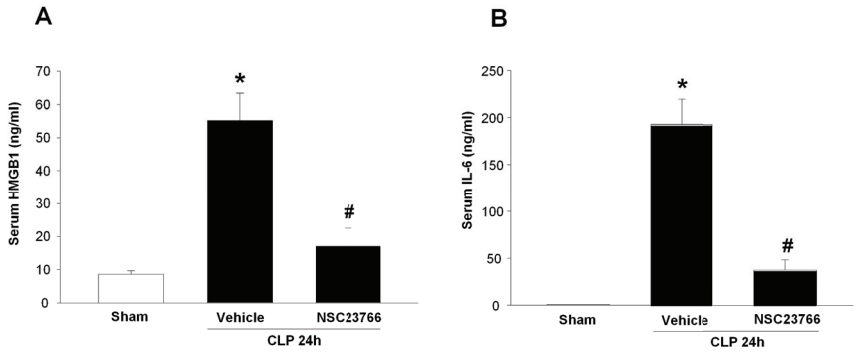

Figure 7

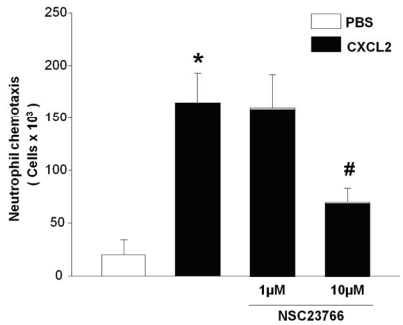

Figure 8

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PAPER 4

Therapeutic effects of human thrombin-derived host defense peptides in polymicrobial sepsis.

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Key words: Adhesion, Chemokines, Inflammation, Leukocyte, Lung and Migration

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Abstract

***Objectives**:* Polymicrobial sepsis triggers pathological systemic inflammation and T-cell dysfunction with subsequent organ failure and increased susceptibility for infections. This study explored the therapeutic potential of thrombin-derived host defense peptides (TDPs) in a model of abdominal sepsis.

Design: Prospective experimental study.

***Setting**:* University research laboratory.

***Subjects**:* Male C57BL/6 mice.

***Interventions**:* Animals were treated with the TDPs GKY20 and GKY25 or a control peptide WFF25 30 min after induction of CLP and samples were harvested 6 and 24 hrs after CLP.

***Measurements and Main Results**:* TDPs decreased the CLP-induced increase in myeloperoxidase activity, neutrophil infiltration, edema and CXC chemokine formation, as well as tissue damage in the lung. Moreover, treatment with TDPs abolished gene expression of CXC chemokines and IL-6 in alveolar macrophages in septic mice. Mac-1 expression on circulating neutrophils and plasma levels of HMGB1 and IL-6 were markedly reduced by administration of TDPs in septic animals. Notably, TDPs inhibited CLP-induced apoptosis and hypo-proliferation in CD4 T-cells as well as decreased formation of regulatory T-cells in the spleen of septic mice. Lastly, it was found that bacterial clearance in the blood and spleen was markedly improved in septic animals treated with TDPs.

Conclusions: Our novel findings demonstrate that TDPs exert therapeutic effects against pathological inflammation and improves T-cell immunity in abdominal sepsis. These results suggest that TDPs might be useful in the management of polymicrobial sepsis.

Introduction

Polymicrobial sepsis is a major cause of morbidity and mortality in intensive care units (1). Organ damage and infectious complications are the most frequent death causes in septic patients (2). The host immune system undergoes two distinct phases in response to a septic insult. Initially, bacterial antigens and toxins trigger local formation of pro-inflammatory compounds, which subsequently disseminate into the circulation and provoke a systemic inflammatory response syndrome (SIRS). SIRS is characterized by widespread activation of innate immune cells, such as neutrophils and macrophages, and is associated with organ damage in patients with sepsis (3). The lung is the most sensitive and important target organ in polymicrobial sepsis. Neutrophil activation and infiltration are key components in septic lung injury and inhibition of pulmonary accumulation of neutrophils by targeting specific adhesion molecules, such as LFA-1, PSGL-1 and CD44, has been shown to protect against septic lung damage (4-6). Later, the septic insult makes the immune system incapable of mounting effective anti-microbial responses due to macrophage and T-cell dysfunction, which is referred to the compensatory anti-inflammatory response syndrome (CARS). CARS is clinically associated with increased susceptibility to infections (3). During this hypo-inflammatory phase, macrophages lose their capacity to present antigens while T-cells undergo apoptosis as well as fail to proliferate (7). Moreover, several studies have reported that the number of regulatory T-cells increase during CARS which further decrease the ability of the immune system to mount anti-microbial responses (8). Improving functional T-cell-mediated immunity during CARS might reduce infectious complications in septic patients.

Current management of patients with sepsis is mainly restricted to antibiotic and supportive therapies. Due to rising antibiotic resistance substantial research efforts have been devoted to explore new alternative therapies for patients with sepsis. One promising group of molecules is antimicrobial peptides, which besides killing bacteria also exert diverse functions, such as chemotaxis, angiogenesis, immunomodulation and tissue regeneration (9-11). This wide diversity of effects has lead to the use of the term ‘‘host defense peptides’’ (HDPs) for this group of molecules (12). Recent findings have shown that neutrophil elastase-mediated proteolysis of thrombin generates novel endogenous HDPs (13). The prototypic C-terminal peptide of thrombin, GKY25, has been shown exert anti-bacterial and anti-endotoxic effects (13). Moreover, amino acid deletions and substitutions at strategic and structurally relevant positions of GKY25 generated a novel peptide variant, GKY20, also with potent anti-microbial and anti-inflammatory effects (12). However, the impact of GKY25 or GKY20 on neutrophil

recruitment in the lung and T-cell dysfunction in abdominal sepsis is not known. In addition, a potential therapeutic effect of these thrombin-derived HDPs (TDPs) in polymicrobial sepsis remains elusive.

Based on these considerations, we hypothesized GKY25 or GKY20 might interfere with pulmonary infiltration of neutrophils and/or improve T-cell function in polymicrobial sepsis. For this purpose, we used a model based on cecal ligation and puncture (CLP) to induce abdominal sepsis in mice.

Materials and Methods

Animals and peptides

Male C57BL/6 mice (22 +/- 5 g, Jackson Laboratory, Bar Harbor, ME) were used for this study. The study protocol was approved by the Ethical Committee at Lund University. The mice were housed in a pathogen-free animal facility with 12:12 h light-dark cycles at 25°C. Mice were anesthetized intraperitoneally (i.p.) with 75 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per kg body weight. The TDPs GKY25 (GKYGFYTHVFRLKKWIQKVIDQFGE) and GKY20 (GKYGFYTHVFRLKKWIQKVI) as well as the control peptide WFF25 (WFFFYYLIIGGGVVTHQQRKKKKDE) were synthesized by Biopeptide Co., (San Diego, USA). The purity (>95%) of these peptides was confirmed by mass spectral analysis (MALDI-ToF Voyager).

Experimental protocol

CLP was used to induce polymicrobial sepsis (14). In brief, animals were anesthetized and the abdomen was opened to exteriorize the cecum, which was gently filled with feces by pushing cecal contents towards the distal cecum on the anti-mesenteric side of the cecum and a ligature was placed below the ileocecal valve. The cecum was soaked in PBS (pH 7.4), punctured twice with a 21-gauge needle and then returned into the peritoneal cavity. The abdominal wall was closed with a suture and mice were resuscitated with one ml of PBS subcutaneously. Animals were treated subcutaneously with 0.5 mg of WFF25, GKY20, GKY25 and vehicle 30 min after CLP induction. Peptides were dissolved in sterile distilled water and diluted just before injection. Sham mice underwent the same surgical procedures, *i.e.*, laparotomy and resuscitation, but the cecum was not ligated or punctured. The mice were then returned to their cages and provided food and water ad libitum. Animals were re-anesthetized at 6 h and 24 h after CLP induction. The left lung was ligated and excised for edema quantification. From the right lung,

bronchoalveolar lavage fluid (BALF) was collected for neutrophils counting. Next, the lung was perfused with PBS and one part was fixed in formaldehyde for histological analysis and the remaining lung tissue was snap-frozen in liquid nitrogen and stored at -80°C for later enzyme-linked immunosorbent assay (ELISA) and myeloperoxidase (MPO) assays as described below.

Systemic leukocyte count

Blood was collected from tail vein and was mixed with Turks solution (0.2 mg gentian violet in 1 ml glacial acetic acid, 6.25% v/v) in a 1:20 dilution. Leukocytes were defined as monomorphonuclear (MNLs) and polymorphonuclear (PMNLs) cells in a haematocytometer.

Lung edema and BALF

The left lung was excised, washed in PBS, gently dried using a blotting paper and weighed. The tissue was then dried at 60°C for 72 h and re-weighed. The change in the ratio of wet weight to dry weight was used as an indicator of lung edema formation. BALF was collected by five washes with 1 ml of PBS containing 5 mM EDTA and then centrifuged; the numbers of MNL and PMNL cells were counted in a Burker chamber.

MPO activity

MPO activity in the lung was determined as described previously. Shortly, frozen lung tissue was thawed and homogenized in one ml of 0.5% hexadecyltrimethylammonium bromide. Next, the sample was freeze-thawed, after which the MPO activity of the supernatant was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter 540 nm, 25°C). Values were expressed as MPO unit/g tissue.

ELISA

Lung levels of CXCL1 and CXCL2 as well as plasma levels of HMGB1 and IL-6 were quantified by use of ELISA kits according to manufacturers' instructions (R&D Systems and Chonrex, Redmond, WA, USA). Murine recombinant CXCL1, CXCL2, HMGB1 and IL-6 were used as standards.

Histology

Lungs samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin-embedded. Six-micrometer sections were stained with haematoxylin and eosin. Lung injury was quantified in a blinded manner by adoption of a pre-existing scoring system as described (15, 16), including alveolar collapse, thickness of alveolar septae, alveolar fibrin deposition and neutrophil infiltration graded on a zero (absent) to four (extensive) scales. In each tissue sample, 5 random areas were scored and mean value was calculated. The histology score is the sum of all 4 parameters.

Flow cytometry

To quantify Mac-1 expression on circulating neutrophils, blood was collected into heparinized syringes at 6 h after induction of CLP and incubated (10 min, RT) with an anti-CD16/CD32 antibody blocking Fcγ III/II receptors to reduce nonspecific labeling and then incubated with PE-conjugated anti-Gr-1 (clone RB6–8C5, rat IgG2b; eBioscience, San Diego, CA) and fluorescein isothiocyanate (FITC)-conjugated anti-Mac-1 (clone M1/70, integrin α_M china, rat IgG_{2b}). The mean fluorescence intensity was determined by comparisons with appropriate isotype control (FITC-conjugated rat IgG_{2b}). All antibodies were purchased from BD Biosciences (San Jose, CA, USA) except where indicated. Cells were fixed, erythrocytes were lysed by BD lysis buffer (Sigma Chemical Co., St. Louis, MO, USA), and then neutrophils were recovered following centrifugation. Flow-cytometric analysis was performed by first gating the neutrophil population of cells based on forward and side scatter characteristics, and then Mac-1 expression was determined on Gr-1^+ in these gated cells on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA USA). A viable gate was used to exclude dead and fragmented cells.

Isolation of splenocytes

The spleen was excised for cell culture and flow cytometric analysis 24 h post CLP induction. Single splenocyte suspension was obtained under sterile condition by smashing the spleen and passing it through a 40 μm cell strainer (BD Falcon, Mountain View, CA, USA). Red blood cells were lysed by use of ACK lysing buffer (Invitrogen, Carlsbad, CA, USA). The cells were washed and resuspended with CLICK's medium (Sigma Chemical Co.) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 unit/ml) and streptomycin (0.1 mg/ml) (Sigma Chemical Co.).

The same medium was used in all experiments described below. Splenocytes were quantified in a Burker chamber staining with Turk's solution.

T-cell apoptosis

To evaluate apoptosis of CD4 T-cells, splenocytes were fixed and stained by APO-BRDU kit, which labels DNA strand breaks by BrdUTP according to the manufacturer's instruction (Phoenix Flow Systems, San Diego, CA, USA). APC-conjugated anti-CD4 antibody (IgG2b, kappa, clone: GK1.5) was used to indicate CD4 T-cells. Splenocytes were acquired by a FACSCalibur flow cytometer (Becton Dickinson) and analyzed with Cell-Quest Pro software (BD Bioscience).

Regulatory T-cell analysis

Splenocytes were stained with FITC-conjugated anti-CD4 (Rat IgG2a, κ, Clone: RM4-5), APC-conjugated anti-CD25 (Rat IgG1, λ, Clone: PC61.5) and PE-conjugated anti-Foxp3 (Rat IgG2a, κ, Clone: FJK-16s) antibodies. Flow cytometric analysis was performed on a FACSCalibur flow cytometer.

In vitro activation of leukocytes

Bone marrow leukocytes were freshly extracted from femurs and tibias of healthy mice by aseptically flushing the bone marrow with complete culture medium RPMI 1640. Leukocytes were resuspended in PBS to 1×10^6 per ml and co-incubated with 100 μM of WFF25, GKY20 or GKY25 for 30 min prior to challenge with 300 ng/ml recombinant mouse CXCL2 (R&D Systems) for 10 min. Leukocytes were incubated with an anti-CD16/CD32 antibody (10 min at RT) blocking Fcγ III/II receptors to reduce non-specific labeling and then incubated with PE-conjugated anti-Gr-1 (clone RB6- 8C5, rat IgG2b, eBioscience), APC-conjugated anti-CD14 (clone Sa14-2, rat IgG2a, Biosite, Täby, Sweden) and FITC-conjugated anti-Mac-1 (clone M1/70, integrin α_M chain, rat IgG2b) antibodies. Neutrophils and monocytes were defined as Gr-1^+/CD14$^-$ and Gr-1^+/CD14$^+$ cells, respectively.

Chemotaxis assay

Neutrophils isolated form bone marrow by use of Ficoll-Paque TM were incubated with 100 µM of WFF25, GKY20 or GKY25 for 30 min. 1.5×10^6 neutrophils were placed in the upper chamber of the Transwell inserts (5 µm pore size, Corning Costar, Corning, NY, USA). Inserts were placed in wells containing medium alone (control) or medium plus CXCL2 (100 ng/ml, R & D Systems). After 120 min, inserts were removed and migrated neutrophils were stained with Turks solution. Chemotaxis was determined by counting the number of migrated neutrophils in a Burker chamber.

Evaluation of bacterial clearance

Bacterial loads were determined in the blood and spleen. Briefly, mice were anesthetized at 24 h after CLP. 100 µl of blood from inferior vena cava and the spleen were placed on ice. The blood serially diluted with sterile PBS. The spleen was weighed, homogenized with sterile PBS and serially diluted. 30 µl of each diluted samples was placed on trypticase soy agar plates with 5% sheep blood (BD Biosciences) separately and incubated at 37°C for 24 h. The numbers of bacterial colonies were then counted and expressed as log CFU/ml blood and log CFU/g spleen respectively.

Isolation of alveolar macrophages and quantitative RT-PCR

Gene-expression of CXCL1, CXCL2 and IL-6 were quantified in alveolar macrophages isolated from sham mice and CLP animals pretreated with vehicle or with WFF25, GKY20 and GKY25 peptides. Alveolar macrophages were isolated from BALF 30 min after CLP as described previously (17). Briefly, lungs were flushed three times with 1 ml of PBS supplemented with 0.5 mM EDTA. Alveolar fluid collections were then centrifuged at 1400 RPM, 10 min, 18°C. The cells were then resuspended in RPMI 1640 complete culture medium and incubated at 37°C, 5% CO_2 in 48-well plate. After 2 h, non-adherent cells were washed away by PBS. A total of 2-3 x 10^5 macrophages were obtained per mice and the purity of macrophages was higher than 97%. Total RNA was isolated from the alveolar macrophages using an RNeasy Mini Kit (Qiagen, West Sussex, UK) following the manufacturer's protocol and treated with RNase-free DNase (DNase I; Amersham Pharmacia Biotech, Sollentuna, Sweden) to remove potential genomic DNA contaminants. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically. Each cDNA was synthesized by reverse transcription from 10 µg of total RNA using the StrataScript First-Strand Synthesis System and

random hexamer primers (Stratagene; AH diagnostics, Stockholm, Sweden). Real-time PCR was performed using a Brilliant SYBRgreen QPCR master mix and MX 3000P detection system (Stratagene). The primer sequences of CXCL1, CXCL2, and β-actin were as follows: CXCL1 (forward) 5'-GCC AAT GAG CTG CGC TGT CAA TGC-3', CXCL1 (reverse) 5'-CTT GGG GAC ACC TTT TAG CAT CTT-3'; CXCL2 (forward) 5'-GCT TCC TCG GGC ACT CCA GAC-3', CXCL2 (reverse) 5'-TTA GCC TTG CCT TTG TTC AGT AT-3'; IL-6 (forward) 5'-ATG CTG GTG ACA ACC ACG GC-3', IL-6 (reverse) 5'-AGC CTC CGA CTT GTG AAG TGG -3'; and β-actin (forward) 5'-ATG TTT GAG ACC TTC AAC ACC-3', β-actin (reverse) 5'-TCT CCA GGG AGG AAG AGG AT-3'. Standard PCR curves were generated for each PCR product to establish linearity of the RT-PCR reaction. PCR amplifications were performed in a total volume of 50 µl, containing 25 µl of SYBRgreen PCR 2x master mix, 2 µl of 0.15 µM each primer, 0.75 µl of reference dye, and one 1 µl cDNA as a template adjusted up to 50 µl with water. PCR reactions were started with 10 min denaturing temperature of 95°C, followed by a total of 40 cycles (95°C for 30 s and 55°C for 1 min), and 1 min of elongation at 72°C. Cycling time values for the specific target genes were related to that of β-actin in the same sample.

Leukocyte binding of fluorescent GKY25

Peritoneal lavage was harvested at 24 h after CLP. Briefly, 5 ml of ice cold PBS (3% FCS) was injected in to the peritoneal cavity. After injection, peritoneum was gently messaged to dislodge adherent cells. Peritoneal fluid was then collected with care and centrifuged at 1500 rpm for 8 min. Cells were washed twice with FACS buffer and counted for further use. For binding of peptides, leukocytes were incubated with TAMRA-labeled GKY25 peptides for 30 min at 37 °C. Then, cells were stained with FITC-conjugated anti-Gr-1 and APC-conjugated anti-CD14 antibodies and flow cytometry was performed as described above. Neutrophils and monocytes were defined as Gr-1$^+$/CD14$^-$ and Gr-1$^+$/CD14$^+$ cells, respectively.

Statistics

Data are presented as mean values ± SEM (standard error of the mean). Statistical evaluations were performed by using Kruskal-Wallis one-way analysis of variance on ranks followed by multiple comparisons versus control group (Dunnett's method). Mann-Whitney rank sum test was used for comparing two groups. $P < 0.05$ was considered significant, and *n* represents the number of animals in each group.

Results

TDPs regulate sepsis-induced neutrophil recruitment in the lung

To investigate the role of GKY20 and GKY25 on neutrophil infiltration in the lung, we assayed the levels of MPO and the number of neutrophils in BALF 6 h and 24 h after CLP, respectively. MPO levels and BALF neutrophils in the lung represent early and late phases of neutrophil accumulation. We observed that MPO levels increased by more than 11-fold after induction of CLP (Figure 1A). Notably, administration of GKY20 and GKY25 decreased pulmonary levels of MPO by more than 60% in septic mice (Figure 1A). Analysis of BALF revealed a 24-fold increase in the number of alveolar neutrophils 24 h after CLP (Figure 1B). Interestingly, treatment with GKY20 and GKY25 reduced CLP-induced recruitment of neutrophils into the bronchoalveolar compartment by 62% and 65%, respectively (Figure 1B). Systemic leukocyte counts decreased after induction of CLP (Table 1). Administration of the GKY20 and GKY25 increased the number of circulating leukocytes in septic animals (Table 1).

TDPs protect against sepsis-induced lung damage

Pulmonary edema was determined as changes in lung wet: dry ratio. It was found that the lung wet:dry ratio increased after CLP (Figure 1C). Notably, treatment with GKY20 and GKY25 decreased the CLP-induced increase in lung wet:dry ratio by more than 60% (Figure 1C). Morphological analysis of lung revealed normal tissue architecture in sham mice and CLP caused severe pulmonary damage, characterized by severe destruction of pulmonary tissue microstructure, extensive edema of interstitial tissue and massive infiltration of neutrophils (Figure 1D). Administration of GKY20 and GKY25 reduced CLP-evoked tissue destruction and neutrophil infiltration in the lung (Figure 1D). Quantification of the morphological damage showed that CLP-induced lung injury was markedly attenuated in TDP-treated animals (Figure 1E).

TDPs inhibit Mac-1 expression on neutrophils

CLP caused a significant upregulation of Mac-1 on neutrophils in the blood (Figure 1F). Treatment with GKY20 and GKY25 markedly reduced Mac-1 expression on neutrophils in septic mice (Figure 1F). Thus, MFI values of Mac-1 on neutrophils decreased from 327 ± 16 to 196 ± 17 and 184 ± 16 in CLP mice treated with GKY20 and GKY25, respectively (Figure 1F and 1G).

TDPs regulate formation of pro-inflammatory mediators

CXC chemokines, such as CXCL1 and CXCL2, are known to co-ordinate neutrophil trafficking in the lung. Baseline levels of CXC chemokines in the lungs of sham animals were low but detectable. In contrast, CLP enhanced CXCL1 and CXCL2 levels in the lung by 43-fold and 69-fold, respectively (Figure 2A and 2B). Administration of GKY20 and GKY25 significantly reduced CLP-provoked production of CXCL1 and CXCL2 (Figure 2A and 2B). CLP increased plasma levels of HMGB1 by 12-fold from 1.8 $\pm$ 0.9 ng/g to 21.8 $\pm$ 3.7 ng/g (Figure 2C). Notably, treatment with GKY20 and GKY25 decreased CLP-induced formation of HMGB1 by more than 82% (Figure 2C). Moreover, plasma levels of IL-6 were markedly enhanced in septic compared to sham mice (Figure 2D). We observed that administration of GKY20 and GKY25 reduced CLP-induced plasma levels of IL-6 by more than 93% (Figure 2D).

TDPs inhibit induction of regulatory T-cells

Regulatory T-cells ($CD4^+CD25^+Foxp3^+$) are known to antagonize immune responses (18). Herein, we found that CLP increased the number of regulatory T-cells in the spleen (Figure 3A and 3B). Treatment with GKY20 and GKY25 decreased the percentage of regulatory T-cells by 53% and 56%, respectively, in septic animals (Figure 3B, $P < 0.05$ vs. WFF25 + CLP, $n = 5$).

TDPs regulate T-cell apoptosis

CLP triggered a significant increase in CD4 T-cell apoptosis in the spleen. The percentage of apoptotic CD4 T-cells was 2.6% in sham and 9.3% in CLP mice (Figure 3C and 3D). Administration of GKY20 and GKY25 decreased the percentage of CD4 T-cell apoptosis by more than 80% in septic animals (Figure 3D).

TDPs improve bacterial clearance

There were no detectable bacteria colonies in the blood or spleen in sham animals. We observed a great increase in bacteria colony formation in the blood and spleen in septic mice (Figure 4A and 4B). Administration of GKY20 and GKY25 significantly decreased bacteria colony formation in the blood and spleen by more than 72% in septic mice (Figure 4A and 4B).

TDPs regulate pro-inflammatory gene expression in alveolar macrophages

We next isolated alveolar macrophages from BALF in sham and CLP animals with or without treatment with WFF25, GKY20 and GKY25. CLP increased mRNA expression of CXCL1, CXCL2 and IL-6 by more than 5-fold (Figure 5). It was found that treatment with GKY20 and GKY25 abolished the CLP-induced increase in mRNA levels of CXCL1, CXCL2 and IL-6 in alveolar macrophages (Figure 5).

TDPs inhibit neutrophil expression of Mac-1 and chemotaxis

We next asked and tested whether TDPs might directly regulate Mac-1 expression on neutrophils and monocytes. Cells were isolated from the bone marrow and stimulated with CXCL2, which markedly increased Mac-1 expression on neutrophils and monocytes (Figure 6). Co-incubation of neutrophils with GKY20 and GKY25 reduced CXCL2-induced expression of Mac-1 (Figure 6A and 6B). Thus, CXCL-2-induced MFI values of Mac-1 on neutrophils in CLP mice decreased by 81% and 91% when co-incubated with GKY20 and GKY25, respectively (Figure 6B). In addition, GKY20 and GKY25 decreased CXCL-2-provoked MFI values of Mac-1 on monocytes by 82% and 95%, respectively (Figure 6C and 6D). It was observed that 100 ng/ml CXCL-2 caused a clear-cut increase in neutrophil migration over a time period of 120 min (Figure 6E). Co-incubation of neutrophils with GKY20 and GKY25 decreased CXCL2-mediated migration of neutrophils by more than 85% (Figure 6E).

GKY25 binds to neutrophils and monocytes

After isolation and counting cells from peritoneal lavage, cells were incubated with with or without TAMRA-labeled GKY25 in a serial concentration (1-100 μM) to assess binding ability of GKY25 to bind to leukocytes. In comparison with control, TAMRA-labeled GKY25 (100 μM) was found to bind to both neutrophils (Fig. 7A and 7B) and monocytes (Fig. 7C and 7D) in a clear-cut manner.

Discussion

Clinical management of patients with septic lung injury and infectious complications is mainly limited to supportive care and poses a major challenge to clinicians. Novel therapeutic options are needed to improve the outcome of patients with abdominal sepsis. The present study demonstrates that HDPs derived from thrombin protects against lung injury in abdominal sepsis via inhibition of neutrophil infiltration. Moreover, we found that TDPs inhibited sepsis-induced T-cell dysfunction. Thus, GKY20 and GKY25 attenuated T-cell apoptosis and T-cell hypoproliferation in septic animals as well decreased sepsis-provoked expansion of regulatory T-cells. Finally, it was found that GKY20 and GKY25 markedly improved bacterial clearance in septic mice. Thus, our results elucidate important protective mechanisms exerted by TDPs in abdominal sepsis and suggest a therapeutic effect of GKY20 and GKY25 in polymicrobial sepsis.

Numerous reports have shown that endogenous antimicrobial proteins exert potent and pleiotropic anti-inflammatory actions besides killing bacteria (13). Sepsis is characterized by a generalized activation of the host immune system in which the most insidious feature is lung injury and impaired gaseous exchange (5, 19). Herein, we show that treatment with two peptides derived from thrombin attenuate pulmonary edema and tissue damage in polymicrobial sepsis. These findings are in line with previous studies reporting that GKY20 and GKY25 inhibit vascular leakage in an endotoxin model of murine lung injury (12, 13). Considering that these TDPs can directly bind endotoxin (12, 13), it is possible that such interactions might be involved in the therapeutic effects of GKY20 and GKY25. However, a potential contribution of endotoxin binding would likely be of minor important since our control peptide WFF25, which also binds endotoxin (20), had no effect on sepsis-induced lung injury, indicating that alternative mechanisms of action mediate the protective effects of TDPs in polymicrobial sepsis. Convincing data have established neutrophil accumulation as a key component in the pathophysiology of septic lung damage (5, 21, 22). In the present study, we could demonstrate that GKY20 and GKY25 reduced pulmonary activity of MPO, a marker of neutrophil infiltration, by more than 60% in abdominal sepsis. This inhibitory effect correlated well with our observation that GKY20 and GKY25 administration decreased sepsis-induced neutrophil accumulation in the bronchoalveolar space by more that 60%, indicating that TDPs effectively attenuate neutrophil recruitment in septic lung injury. Indeed, this is the first study to show that GKY20 or GKY25 can inhibit extravascular infiltration of neutrophils and may, thus, help explain the lung protective effects of TDPs in polymicrobial sepsis.

Pulmonary recruitment of leukocytes is a multistep process, including initial sequestration in microvessels and firm adhesion on the endothelium, followed by transendothelial and transepithelial migration (23). Recruitment of leukocytes at sites of inflammation is under the control of secreted chemokines (24). Neutrophils are particularly activated and attracted by CXC chemokines, comprising CXCL1 and CXCL2 in mice (25). Herein, it was found that treatment with GKY20 and GKY25 decreased CLP-provoked formation of CXCL1 and CXCL2 formation in the lung by more than 90%, suggesting that TDPs are effective inhibitors of CXC chemokine production in septic lung damage. Notably, treatment with GKY20 and GKY25 markedly decreased gene expression of CXCL1 and CXCL2 in alveolar macrophages, suggesting that TDPs directly regulate macrophage production of CXC chemokines in polymicrobial sepsis. Interestingly, we observed that GKY25 bound to isolated macrophages, supporting the notion that TDPs can interact with macrophages in a direct manner. The exact molecular nature of this GKY25 interaction with macrophages is not known at present and warrants further studies. In fact, this is the first study to show that GKY20 and GKY25 can inhibit formation of CXC chemokines. Moreover, this observation might also help to explain the inhibitory effect of TDPs on sepsis-induced neutrophil infiltration in the lung. Pulmonary accumulation of neutrophils has been demonstrated to be mediated by Mac-1 expressed on neutrophils (6). In the present study, it was found that GKY20 and GKY25 reduced CLP-induced expression of Mac-1 on circulating neutrophils, which may help explain the inhibitory effect of TDPs on sepsis-induced pulmonary neutrophilia. However, knowing that CXC chemokines trigger Mac-1 up-regulation on neutrophils and our observation that TDPs are potent regulators of CXC chemokine formation in the lung it is possible that the reduced Mac-1 expression observed in CLP mice treated with GKY20 and GKY25 might be an indirect effect. It was therefore of great interest to examine the direct effect of TDPs on Mac-1 expression on isolated neutrophils. Interestingly, we found that CXCL2-evoked activation of isolated neutrophils was markedly decreased by GKY20 and GKY25, suggesting that TDPs directly controls neutrophil activation and Mac-1 up-regulation in abdominal sepsis. This conclusion is also in line with our finding that GKY20 and GKY25 abolished chemokine-induced migration of isolated neutrophils in vitro. Moreover, in separate experiments, we found that fluorescent-labeled GKY25 bound to isolated neutrophils, which supports direct effects of TDPs on neutrophils. Considered together with the evidence above showing that GKY20 and GKY25 regulate CXC chemokine production, these results suggest that GKY20 and GKY25 regulate sepsis-evoked neutrophil accumulation in the lung at two distinct levels, *i.e.* formation of CXC chemokines in lung macrophages and Mac-1 up-regulation on neutrophils.

Convincing clinical evidence suggest that a major cause of death in septic patients is infectious complications (2). It is generally held that sepsis causes T-cell and macrophage dysfunction, which, at least in part, might help to explain the enhanced susceptibility to infections in patients with sepsis (3). Herein, we found that treatment with GKY20 and GKY25 decreased apoptosis in CD4 T-cells in septic mice. This anti-apoptotic effect of GKY20 and GKY25 might help to increase the number of functional CD4 T-cells able to mount effective anti-bacterial responses. Regulatory T-cells are recognized for their potent capability to control T-cell-dependent immune responses (26). Numerous studies have reported that the number of regulatory T-cells increase in the course of sepsis, which might compromise host defense reactions against microbial infections (27). In the present study, it was found that CLP triggered a clear-cut induction of regulatory T-cells in the spleen. Notably, treatment with GKY20 and GKY25 abolished this increase in regulatory T-cells in septic animals. Considered together, TDPs might improve T-cell-dependent immune responses via several different mechanisms, including increasing the number of functional T-cells and reducing the number of regulatory T-cells. Having shown that TDPs improve T-cell function in sepsis, we next asked whether GKY20 and GKY25 might influence systemic bacteremia. Notably, we found that the levels of bacteria in the blood and spleen were significantly reduced by GKY20 and GKY25 in septic mice. Although it is well known that TDPs exert potent direct anti-bacterial effects in vitro (12) it might be the case that improved T-cell function also contributes to the clearance of bacteria in polymicrobial sepsis.

HMGB1 is a potent pro-inflammatory cytokine and a late mediator in endotoxemia and sepsis (28) as well a predictor of clinical outcome in patients with severe sepsis (29). In line with previous studies, we observed that CLP caused a clear-cut increase in the plasma levels of HMGB1. Notably, treatment with GKY20 and GKY25 reduced HMGB1 levels in the plasma by more than 82% in septic mice, indicating a potent anti-inflammatory effect of TDPs in CLP-induced systemic inflammation. This is the first study showing that GKY20 and GKY25 negatively regulate HMGB1 in sepsis. Interestingly, a recent study reported that inhibition of HMGB1 attenuates tumor cell induction of regulatory T-cells (30). If such a mechanism exists in sepsis it might help explaining the inhibitory impact of TDPs on the formation of regulatory T-cells herein. Another indicator of systemic inflammation is IL-6 and a correlation between high IL-6 levels and mortality of septic patients has been demonstrated (31). IL-6 is a complex cytokine exerting both pro- and anti-inflammatory effects (32), which may be related to different signaling pathways of IL-6 (33, 34). Herein, we found that administration of GKY20 and GKY25 decreased plasma levels of IL-6 in septic animals. The impact of this reduction of IL-6 on T-cell

function is not known at present but this observation also supports the concept that TDPs attenuate the systemic inflammatory response triggered in sepsis.

Conclusions

TDPs exert potent anti-inflammatory effects and improve T-cell function in abdominal sepsis. Our findings indicate that TDPs might be useful in the treatment of patients with polymicrobial sepsis.

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Figure legends

Figure 1. (A) Lung MPO activity 6 h post-CLP. (B) Number of BALF neutrophils and (C) edema formation 24 h after CLP induction. (D) Representative haematoxylin & eosin sections of the lung 24 h post-CLP. (E) Histological lung injury score as described in Materials and Methods. (F) Aggregate data and (G) representative histogram of Mac-1 expression on circulating neutrophils 6 h after CLP. Animals were treated with 0.5 mg of WFF25, GKY20 and GKY25 or PBS 30 min after induction of CLP. Mice treated with PBS served as sham animals. Data are mean ± SEM and $n = 5$. ¤$P < 0.05$ *vs.* Sham and ∗$P < 0.05$ *vs.* WFF25 + CLP.

Figure 2. Lung levels of (A) CXCL1 and (B) CXCL2 as well as plasma levels of (C) HMGB1 and (D) IL-6 24 h after CLP induction. Animals were treated with 0.5 mg of WFF25, GKY20 and GKY25 or PBS 30 min after induction of CLP. Mice treated with PBS served as sham animals. Data are mean ± SEM and $n = 5$. ¤$P < 0.05$ *vs.* Sham and ∗$P < 0.05$ *vs.* WFF25 + CLP.

Figure 3. TDPs inhibit CLP-induced expansion of regulatory T-cells and apoptosis of CD4 T-cells. (A) Representative histograms and (B) aggregate data on regulatory T-cells ($CD4^+CD25^+Foxp3^+$) in the spleen 24 h after CLP induction. (C) Representative histograms and (D) aggregate data on apoptotic CD4 T-cells in the spleen 24 h after CLP induction. Apoptosis was determined by measuring labeling of DNA strand breaks with BrdUTP as described In Materials and Methods. Animals were treated with 0.5 mg of WFF25, GKY20 and GKY25 or PBS 30 min after induction of CLP. Mice treated with PBS served as sham animals. Data are mean ± SEM and $n = 5$. ¤$P < 0.05$ *vs.* Sham and ∗$P < 0.05$ *vs.* WFF25 + CLP.

Figure 4. TDPs regulate bacterial clearance in CLP animals. The number of bacterial colonies was quantified 24 h after CLP in the (A) blood and (B) spleen as described In Materials and Methods. Animals were treated with 0.5 mg of WFF25, GKY20 and GKY25 or PBS 30 min after induction of CLP. Mice treated with PBS served as sham animals. Data are mean ± SEM and $n = 5$. ¤$P < 0.05$ *vs.* Sham and ∗$P < 0.05$ *vs.* WFF25 + CLP.

Figure 5. TDPs regulate gene expression of CXC chemokines and IL-6 in alveolar

macrophages. Quantitative RT-PCR was used to determine mRNA levels of (A) CXCL1, (B) CXCL2 and (C) IL-6 in alveolar macrophages 30 min after CLP induction. Levels of CXCL1, CXCL2 and IL-6 mRNA were normalized to mRNA levels of β-actin. Animals were pretreated with 0.5 mg of WFF25, GKY20 and GKY25 or PBS before CLP induction. Data are mean ± SEM and $n = 5$. ¤$P < 0.05$ *vs.* Sham and ∗$P < 0.05$ *vs.* WFF25 + CLP.

Figure 6. Bone marrow leukocytes were co-incubated with medium alone (Control) as well as 100 μM of WFF25, GKY20 and GKY25 or vehicle 30 min prior to challenge with 300 ng/ml CXCL2. Mac-1 expression was determined 10 min after CXCL2 challenge on (A and B) neutrophils (Gr-1$^+$/CD14$^-$) and (C and D) monocytes (Gr-1$^+$/CD14$^+$). (E) Neutrophil migration was quantified in response to medium alone (Control), medium plus CXCL2 (100 ng/ml) with or without preincubation of neutrophils with 100 μM of WFF25, GKY20 and GKY25 or vehicle. Data are mean ± SEM and $n = 5$. ¤$P < 0.05$ *vs.* Control and ∗$P < 0.05$ *vs.* WFF25 + CXCL2.

Figure 7. GKY25 binds to leukocytes. Leukocytes were harvested by peritoneal lavage 24 h after CLP induction TAMRA-labelled GKY25 (1-100 μM) was incubated with (A and B) neutrophils (Gr-1$^+$/CD14$^-$) and (C and D) monocytes (Gr-1$^+$/CD14$^+$) for 30 min and quantified as described in Materials and Methods. Data are mean ± SEM and $n = 5$. ¤$P < 0.05$ *vs.* Control and ∗$P < 0.05$ *vs.* WFF25 + CXCL2.

Table 1. Systemic leukocyte differential counts

	MNL	PMNL	Total
Sham	5.1 ± 0.3	1.4 ± 0.1	6.6 ± 0.2
PBS + CLP	0.8 ± 0.5[a]	0.6 ± 0.3[a]	1.4 ± 0.8[a]
WFF25 + CLP	0.8 ± 0.7[a]	0.6 ± 0.1[a]	1.4 ± 0.8[a]
GKY20+ CLP	2.4 ± 0.2[a,b]	1.8 ± 0.1[a,b]	4.2 ± 0.3[a,b]
GKY25+ CLP	2.9 ± 0.1[a,b]	1.9 ± 0.1[a,b]	4.8 ± 0.2[a,b]

Blood was collected from sham animals as well as mice treated with 0.5 mg of WFF25, GKY20 and GKY25 or PBS 30 min after CLP. Cells were identified as monomorphonuclear leukocytes (MNLs) and polymorphonuclear leukocytes (PMNLs) 24 h after CLP induction. Data represents mean ± SEM and n = 5. [a]$P < 0.05$ *vs.* Sham and [b] $P < 0.05$ *vs.* WFF25 + CLP.

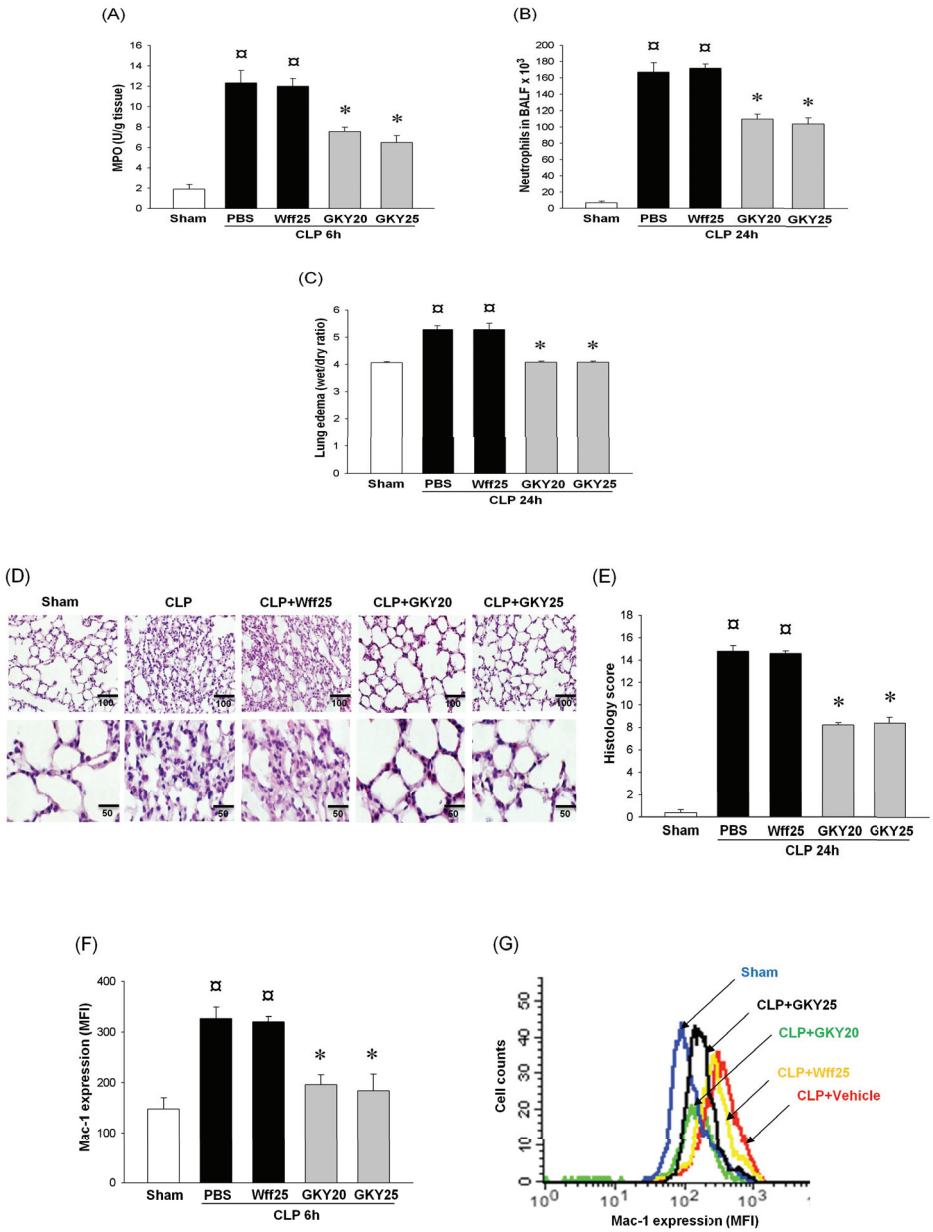

(A)
MPO (U/g tissue)
Sham
PBS
Wff25
GKY20
GKY25
CLP 6h
(B)
Neutrophils in BALF x 10³
CLP 24h
(C)
Lung edema (wet/dry ratio)
CLP 24h
(D)
Sham
CLP
CLP+Wff25
CLP+GKY20
CLP+GKY25
(E)
Histology score
CLP 24h
(F)
Mac-1 expression (MFI)
CLP 6h
(G)
Sham
CLP+GKY25
CLP+GKY20
CLP+Wff25
CLP+Vehicle
Cell counts
Mac-1 expression (MFI)

Figure 1

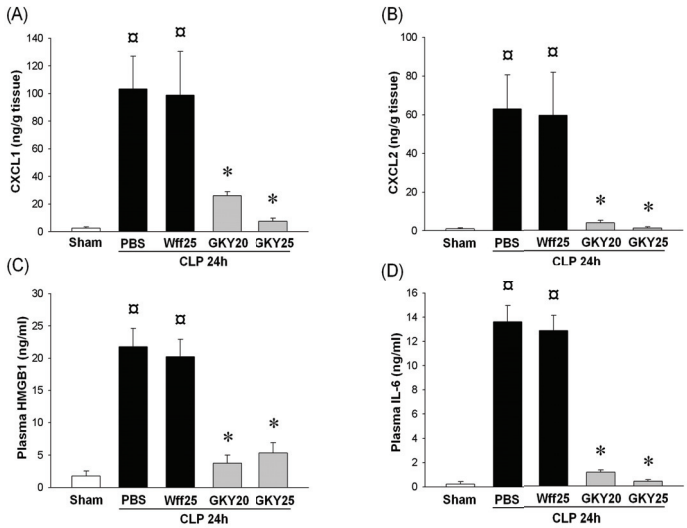


Figure 2

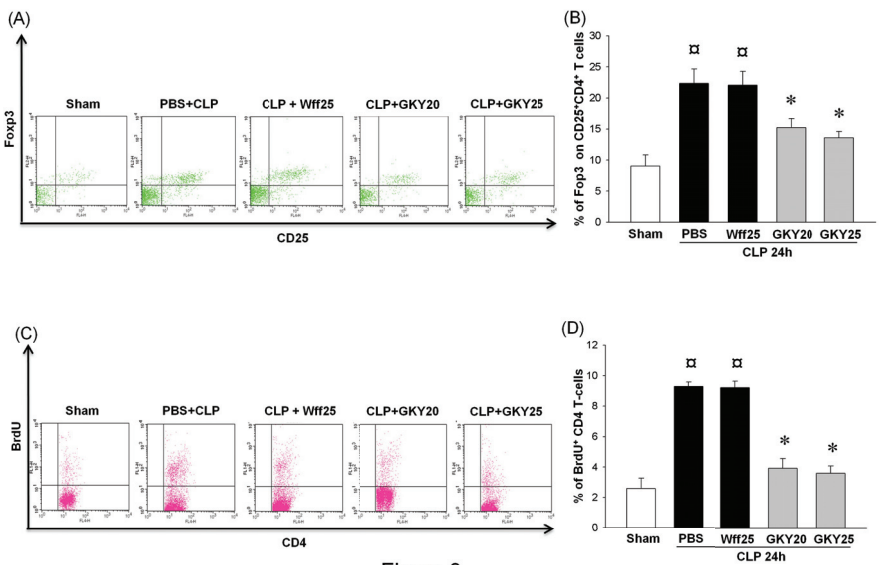


Figure 3

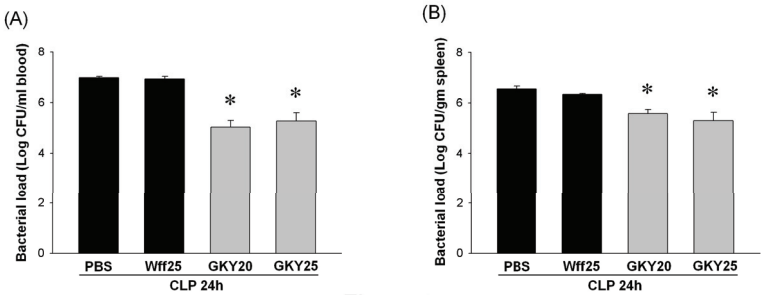


Figure 4

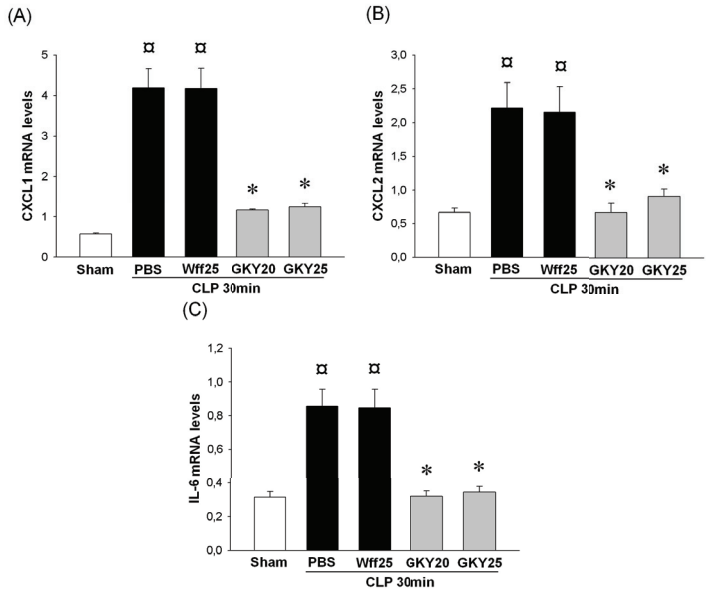
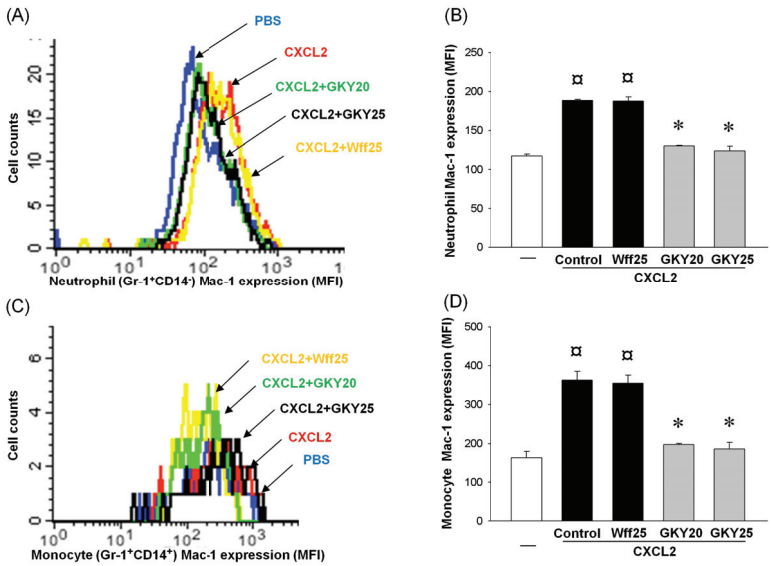


Figure 5


(A)
PBS
CXCL2
CXCL2+GKY20
CXCL2+GKY25
CXCL2+Wff25
Cell counts
Neutrophil (Gr-1+CD14-) Mac-1 expression (MFI)
(B)
Neutrophil Mac-1 expression (MFI)
Control
Wff25
GKY20
GKY25
CXCL2
(C)
CXCL2+Wff25
CXCL2+GKY20
CXCL2+GKY25
CXCL2
PBS
Cell counts
Monocyte (Gr-1+CD14+) Mac-1 expression (MFI)
(D)
Monocyte Mac-1 expression (MFI)
Control
Wff25
GKY20
GKY25
CXCL2

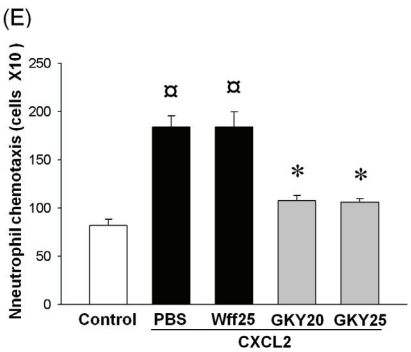

(E)
Neutrophil chemotaxis (cells X10)
Control
PBS
Wff25
GKY20
GKY25
CXCL2

Figure 6

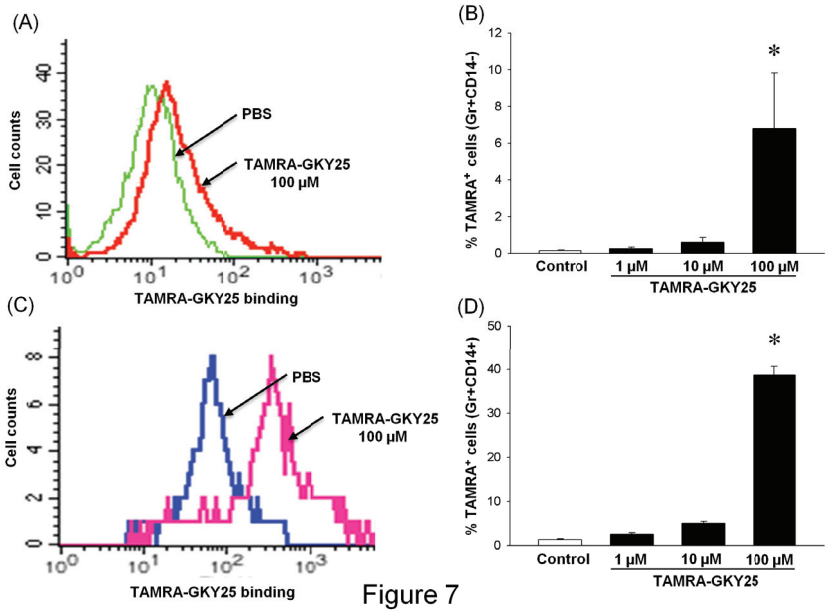

(A)
Cell counts
PBS
TAMRA-GKY25
100 μM
TAMRA-GKY25 binding
(B)
% TAMRA+ cells (Gr+CD14-)
Control
1 μM
10 μM
100 μM
TAMRA-GKY25
(C)
Cell counts
PBS
TAMRA-GKY25
100 μM
TAMRA-GKY25 binding
(D)
% TAMRA+ cells (Gr+CD14+)
Control
1 μM
10 μM
100 μM
TAMRA-GKY25

Figure 7

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