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2015

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Citation for published version (APA):

Hwaiz, R. (2015). *Rac1 signaling regulates platelet-dependent inflammation abdominal sepsis*. [Doctoral Thesis (compilation), Surgery]. Lund University.

Total number of authors:

1

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Rac1 signaling regulates platelet-dependent inflammation in abdominal sepsis

Rundk Hwaiz

Academic Thesis

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Skåne University Hospital, Sweden 2015

Organization LUND UNIVERSITY Department of Clinical Science, Malmö Section for Surgery Skåne University Hospital Lund University, Sweden	Document name DOCTORAL DISSERTATION	
	Date of issue 18th March, 2015	
Author(s) Rundk Hwaiz	Sponsoring organization	
Title and subtitle Rac1 signaling regulates platelet-dependent inflammation in abdominal sepsis		
Abstract <p>Sepsis is a systemic inflammatory response syndrome to a localized or systemic infection that leads to the over production of proinflammatory cytokines and the ultimate failure of multiple organ systems. However, little is known about the potential role of Rac1 in controlling sepsis-induced intracellular signaling pathways. We hypothesized that Rac1 might be involved in sepsis mediated signaling pathways leading to the activation of inflammatory cells.</p> <p>Paper (I), Rac1 signaling plays an important role in polymicrobial sepsis induced by cecal ligation and puncture (CLP). This study shows that Rac1 signaling regulates sepsis-induced inflammation in the lung by reducing chemokine production and Mac-1 expression on neutrophils. Rac1 inhibitor NSC23766 attenuates lung edema, tissue destruction and systemic pro-inflammatory cytokines in septic animals, suggesting that targeting Rac1 may be useful approach to protect against pulmonary injury in abdominal sepsis. Paper (II), in this study we showed that inhibition of Rac1 signaling protect sepsis-induced lung injury through two different mechanisms. First, Rac1 controls surface mobilization of CD40L on activated platelets and second, Rac1 regulates MMP-9 secretion from neutrophils. Our data indicate that inhibition of Rac1 signaling might be a useful target in order to control pathological secretion and shedding of CD40L into the systemic circulation in abdominal sepsis.</p> <p>Paper (III) This study indicates that Rac1 activity is increased in platelets and regulates platelet secretion of CCL5 in abdominal sepsis. Our findings show that CCL5 regulates neutrophil recruitment in septic lung injury via activation of alveolar macrophages leading to local secretion of CXCL2. Thus, our novel data not only elucidates complex mechanisms regulating pulmonary neutrophil trafficking in sepsis but also suggest that targeting Rac1 signaling and platelet-derived CCL5 might be a useful way to control pathological inflammation and tissue damage in the lung in abdominal sepsis. Paper (IV) Rac1 signaling is enhanced in platelets and regulates platelet secretion of CXCL4 in abdominal sepsis. This study indicates that CXCL4 controls neutrophil accumulation via secretion of CXCL2 from alveolar macrophages in septic lung injury. These findings not only delineate complex mechanisms of neutrophil trafficking in sepsis but also suggest that targeting platelet-derived CXCL4 might be an effective way to ameliorate inflammation and tissue damage in septic lung damage.</p>		
Key words: abdominal sepsis, Rac1, platelet, neutrophil, chemokine, inflammation		
Classification system and/or index termes (if any):		
Supplementary bibliographical information:		Language English
ISSN and key title: 1652-8220		ISBN 978-91-7619-118-7
Recipient's notes	Number of pages	Price
	Security classification	

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Rac1 signaling regulates platelet- dependent inflammation in abdominal sepsis

By

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LUND
UNIVERSITY

Faculty of Medicine

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Lund University, Faculty of Medicine, Doctoral Dissertation Series 2015:39

ISSN 1652-8220

ISBN 978-91-7619-118-7

Printed by Media-Tryck, Lund University, Lund, Sweden 2015



KLIMATKOMPENSERAT
PAPPER



To my first country Kurdistan
To my second country Sweden

Live as if you were to die tomorrow. Learn as if you were to live forever.

– **Mahatma Gandhi**

Table of Contents

Abbreviations	9
Original papers	12
Introduction	13
Background	15
Definition of sepsis.....	15
Pathophysiology of sepsis	16
Role of neutrophils in abdominal sepsis	17
Role of platelets in abdominal sepsis	18
Role of macrophage in inflammation	20
Role of cytokines in inflammation	21
Role of chemokines in inflammation	22
Role of CD40L in inflammation	24
Role of Rac1 in inflammation	25
Aims	28
Materials and Methods	29
Animals	29
Experimental model of sepsis	29
Antibodies and biochemical substances	30
Isolation of alveolar macrophages and quantitative RT-PCR	31
Systemic leukocyte counts	32
Myeloperoxidase assay	32
Bronchoalveolar lavage fluid	33
Lung edema	33
Enzyme linked immunosorbent assay	33
Neutrophil chemotaxis	34

Flow cytometry	34
Histology	35
Table 1. Histology scoring system used in study I, III, IV	36
Isolation of platelet	37
Confocal microscopy	37
Pull-down assay and Western blotting	38
Statistics	39
Results and discussion	40
Role of Rac1 in abdominal sepsis	40
Rac1 regulates platelet CD40L shedding	44
Rac1 regulates secretion of platelet-derived CC chemokines	47
Rac1 regulate secretion of platelet-derived CXC chemokines	52
Conclusions	57
Sammanfattning på Svenska	59
Acknowledgements	61
References	63
Paper I	83
Paper II	95
Paper III	107
Paper IV	119
Medicine doctorates in Section of Surgery, Malmö, Lund University	152

Abbreviation

ALI	acute lung injury
AM	alveolar macrophage
APC	allophycocyanin
CCR	CC chemokine receptors
CD	cluster of differentiation
CD40L	CD40 ligand
CLP	cecal ligation and puncture
CXCL1	chemokine (C-X-C motif) ligand 1
CXCL2	chemokine (C-X-C motif) ligand 2
CXCR2	CXCR2 chemokine receptor 2
DAMPs	damage-associated molecular patterns
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
ERKs	extracellular-signal-regulated kinases
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinases
FITC	fluorescein isothiocyanate
fMLP	N-formyl-methionine-leucine-phenylalanine
GDP	guanosine diphosphate
GEFs	guanine nucleotide exchange factors
GTP	guanosine triphosphate
H&E	hematoxylin and eosin
HMGB1	high-mobility group protein B1
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA reductase
i.p.	intraperitoneal
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
IL-6	interleukin 6

INF	interferons
JNK	c-Jun N-terminal kinases
KC/CXCL1	cytokine-induced neutrophil chemoattractant
kD	kilo Dalton
LFA-1	lymphocyte function antigen-1
LPS	lipopolysaccharide
LTA	lipoteichoic acid
Mac-1	macrophage-1 antigen
MAPK	mitogen-activated protein kinases
MCP-1	monocyte chemotactic protein-1
MFI	mean fluorescence intensity
MMPs	matrix metalloproteinases
MOF	multiple organ failure
MPO	myeloperoxidase
NETs	Neutrophil extracellular traps
NF-kB	nuclear factor kappa B
PAK1	p21 protein activated kinase 1
PAMP	spathogen-associated molecular patterns
PAR-4	Protease-activated receptor 4
PBS	Phosphate-buffered saline
PE	phycoerythrin
PF-4/CXCL4	platelet factor-4
PI3K γ	phosphatidylinositol 3 kinase γ
PKB	protein kinase B
PLC	phospholipase C
PMNL	polymorphonuclear leukocyte
PMPs	platelet microbicidal proteins
PRRs	pattern recognition molecular receptors
PSGL-1	P-selectin glycoprotein ligand-1
Pyk-2	Protein tyrosine kinase 2
Rac1	Ras-related C3 botulinum toxin substrate 1

RANTES/CCL5	regulated on activation, normal T cell expressed and secreted
Rap1	Ras-related protein 1
ROS	reactive oxygen species
s.c.	subcutaneous
sCD40L	soluble CD40 ligand
SEM	standard error of the mean
SIRS	systemic inflammatory response syndrome
TGF- β	Transforming growth factor beta
TLR	toll-like receptor
TNF- α	tumor necrosis factor- α
TXA2	thromboxane A2
WBC	white blood cell
VCAM-1	vascular cell adhesion molecule-1

List of original papers:

The thesis is based on the following original papers and will be referred in the text by their sequential numbers:

1. **Hwaiz R**, Hasan Z, Rahman M, Zhang S, Palani K, Syk I, Jeppsson B, Thorlacius H. Rac1 signaling regulates sepsis-induced pathologic inflammation in the lung via attenuation of Mac-1 expression and CXC chemokine formation. *Journal of Surgical Research* 2013, 183: 798–807.
2. **Hwaiz R**, Rahman M, Zhang E, Thorlacius H. Rac1 regulates platelet shedding of CD40L in abdominal sepsis. *Laboratory Investigation* 2014, 94: 1054–1063.
3. **Hwaiz R**, Rahman M, Syk I, Zhang E, Thorlacius H. Rac1-dependent secretion of platelet-derived CCL5 regulates neutrophil recruitment via activation of alveolar macrophages in septic lung injury. *Journal of Leukocyte Biology* 2015 (Epub ahead of print).
4. **Hwaiz R**, Rahman M, Zhang E, Thorlacius H. Platelet secretion of CXCL4 is Rac1 dependent and regulates neutrophil infiltration and tissue damage in septic lung damage. *British Journal of Pharmacology* 2015 (Submitted).

Introduction

Sepsis, or the invasion of microbial pathogens into the bloodstream, is characterized by a systemic proinflammatory response, which can lead to severe sepsis and septic shock [1]. Despite significant advances in intensive care treatment over the last years, sepsis remains associated with high mortality rates [2]. The mortality rate is generally between 30-40% in the elderly and 50% or greater in patient with more severe syndrome such as septic shock [3,4]. Worldwide, thirteen million people are suffering from sepsis every year and among those, around four million die. In the United States alone, every year there are approximately 750000 patients suffering from sepsis resulting in 215000 deaths and frequency is rising. The average annual costs are more than 16.7 billion dollars per year [5]. Similar to the US, in Europe hospitals sepsis is also a common problem [6]. Compared to HIV and cancer, the incidence of severe sepsis captures the number one spot [5]. The major cause of mortality in patient with sepsis is multiple organ failure. Patients usually develop a single organ failure, typically lung injury followed by failure of other organ such as, liver and kidney which resulting in multiple organ dysfunctions [1]. Data from patients hospitalized with severe sepsis revealed that lungs (50-60%) are primary source of infection, followed by abdomen (20-25%), urinary tract (7-10%) and skin (5-10%) [7]. Activation of the innate immune system, including neutrophils, platelets and macrophages, constitutes a key feature in systemic inflammation, in which the most feared complication is pulmonary damage [8,9]. Sepsis-induced neutrophil accumulation in the lung is mediated by specific adhesion molecules, including P-selectin glycoprotein ligand-1 (PSGL-1), lymphocyte function-associated antigen- 1 (LFA-1) and coordinated by secreted CXC chemokines, such as CXCL1 and CXCL2 [10,11]. 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. Platelet factor-4 (PF4, also known as CXCL4) and RANTES (CCL5) are the most abundant chemokine in platelets, which belong to the CXC and CC chemokine family [12,13]. Moreover, several studies have reported that CXCL4 and CCL5 play a role in regulating neutrophil recruitment and tissue damage in complex inflammatory disease models [14,15,16].

The small GTPase Rac1 (Ras-related C3botulinum toxin substrate 1), a member of the Rho family of proteins and a signal transducer involved in the control of several processes including cell cycle progression, cell adhesion, cell migration and cytoskeletal reorganization [17]. Rac1 cycles between a cytosolic soluble GDP-bound inactive state and a membrane associated GTP-bound active state [17]. In previous reports, Rac1 was suggested to play a critical role in pancreatitis and endotoxin-associated lung injury [18,19]. NSC23766 is a molecule arising from rational design, which is capable to inhibit Rac1 activity in several cell types [20,21,22]. Basically, NSC23766 inhibits Rac1 binding and activation by Rac-specific guanine nucleotide exchange factors (GEFs) that catalyses its GTP–GDP exchange [20].

In this thesis, we performed both *in vitro* and *in vivo* experiments to test the ability of Rac1 to regulate neutrophil infiltration and platelet-derived chemokine secretion in polymicrobial sepsis induced by cecal ligation and puncture (CLP).

Background

Definition of sepsis

The word “sepsis” is derived from the word “σηψις,” which in the original Greek means “decomposition” or “putrefaction” [23]. The clinical symptoms of sepsis were already known to Hippocrates (460–377 BC), who showed the term ‘wound putrefaction’. Thereafter, the Persian ‘father of modern medicine’, Ibn Sina (AD 980–1037), observed that septicaemia was usually accompanied by high body temperature. However, it was Louis Pasteur who linked the decay of organic substances to the presence of bacteria and microorganisms in 18th century. In addition, Ignaz Semmelweis observed the significant effect of hygienic measures on decreasing the rate of mortality of women during childbirth. In 1914, Hugo Schottmüller laid the foundations for a modern definition of sepsis and was the first to define that the presence of an infection was an essential component of the sepsis disease. A few hundred years later, Lewis Thomas defined sepsis by popularizing the theory that “it is the host response that makes the disease” [24]. Systemic inflammatory response (SIRS) can result from either infectious or non-infectious conditions. Non-infectious conditions which are associated with SIRS include trauma, burns, pancreatitis, ischemia and hemorrhagic[25] Sepsis, the systemic inflammatory response due to a microbial infection, is an extensive clinical problem with high range of mortality. Sepsis is characterized by low body temperature <36°C or high body temperature >38°C, fast heart rate >90, high respiratory rate >20 breaths/min, abnormal white blood cell count (>12,000 or <4000) and probable or confirmed infection. Severe sepsis occurs when sepsis associated with organ failure, hypotension, organ hypoperfusion, and septic shock arises as a result of extremely low blood pressure that doesn't adequately respond to simple fluid replacement (Figure 1) [26].

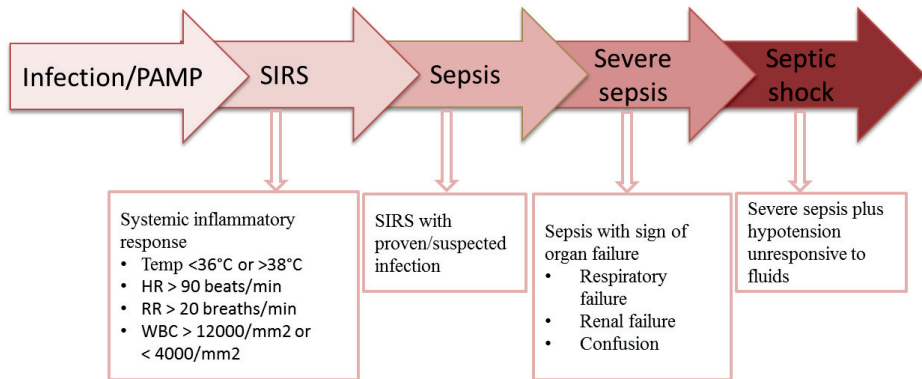


Figure 1: Defining the sepsis continuum.

Pathophysiology of sepsis

Sepsis develops when the initial, appropriate host response to an infection becomes amplified and subsequently dysregulated. In an ideal scenario, once pathogen enters the host the normal immune system should eradicate the microorganism and quickly return the host to normal condition. However, the pathomechanism of sepsis is results from the improper regulation of this normal reaction. The septic response may accelerate due to continuous activation of immune cells for instance, neutrophils and macrophages [27]. The majority of sepsis infections are caused by bacterial agents [28]. However, viruses, parasites and fungi can also cause sepsis [29,30]. Initially, when bacteria enter the host, the innate immune response is triggered, through recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), which interact with receptors residing in cells of the innate immune system, known as pattern recognition receptors (PRRs) [31,32]. The intracellular inflammatory pathway of immune cells is commonly started by the activation of the serine phosphokinase via ligation of exogenous stimuli with pattern recognition protein (PRP), i.e. lipopolysaccharide. In the case of sepsis, over stimulation of monocytes, macrophages, neutrophils, lymphocytes and endothelial cells lead to the enhanced production and systemic release of cytokines,

chemokines, complement-activation products, reactive oxygen species, and other mediators which all are reported to be important for neutrophil recruitment.

Role of neutrophils in abdominal sepsis

Neutrophils are a type of polymorphonuclear leukocyte. They are well recognized as one of the major cells which play an important role during acute inflammation [33,34,35,36,37,38,39,40]. They are usually the first leukocytes to be recruited to an inflammatory site and are able to eliminate pathogens by multiple mechanisms [41]. Neutrophils are strongly implicated in the pathogenesis of acute lung injury [42]. Neutrophils recruitment is a key event in development of ALI resulting in plasma leakage and impairment of oxygenation [43,44,45,46]. In response to inflammatory stimuli, proinflammatory mediators such as CXCL1 and CXCL2 from lung macrophage are released which are able to activate and recruit neutrophils to the site of inflammation [47,48]. Although neutrophils constitute the first line of defense against bacterial invasion [49] but excessive activation and infiltration of neutrophils is a direct cause of tissue damage and organ failure in abdominal sepsis. Neutrophils can damage tissue directly by releasing proteolytic enzymes and reactive oxygen species (ROS) [50] or by formation of neutrophil extracellular traps (NETs), a recently discovered network of extracellular filamentous DNA, which contain histones and neutrophil granular proteins such as elastases, proteases and cathepsin G [51,52]. The importance of neutrophils in ALI is confirmed by studies where lung injury is abolished or reversed by depletion of neutrophils [53,54].

The recruitment of neutrophils is classically defined as a multistep process consisting of leukocyte tethering, rolling, activation, adhesion, and subsequent transmigration, involving cell adhesion molecules and chemokines and their respective receptors by the leukocyte recruitment cascade [55,56,57]. The first contact between a leukocyte and an endothelial cell is called ‘tethering’, while the immediate subsequent interaction is described as ‘rolling’. These steps are mediated predominantly by selectins (E, L, P-selectin) and facilitate by integrins. The most relevant integrins for leukocyte migration are β -2 integrin subfamily macrophage-1 antigen (Mac-1) and Lymphocyte function-associated antigen 1 (LFA-1). The rolling leukocyte can stop only if it receives an

activating signal, such as that provided by a chemokine on the endothelial surface. This signal switches the integrins to a high affinity state. Thereafter, the integrins bind to the endothelial cells receptors vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) and adhere irreversibly. Leukocytes can then transmigrate out of the microvasculature.

In addition to classical neutrophil recruitment, platelets bound to activated endothelial cells can interact with leukocytes, and induce interactions of neutrophils with platelets first, followed by neutrophil-endothelial interaction [58]. Neutrophil rolling on platelets is mostly mediated by platelet P-selectin binding to P-selectin glycoprotein ligand (PSGL)-1 on leukocytes. Platelet depletion reduces neutrophil rolling and adhesion in the brain microvasculature [59]. In addition, platelet depletion reduced leukocyte recruitment into the post-ischemic intestine and lung in models of inflammatory diseases in mice [60].

Role of platelets in abdominal sepsis

Platelets or thrombocytes are small (2-3 μm in diameter) irregular non nucleated cell fragments of cytoplasm which are derived from the bone marrow. After their production, they are released to the circulation by reticuloendothelial system. Platelets are not eukaryotic, but they have many features of them e.g. they contain mitochondria, residual of endoplasmic reticulum, network of actin and myosin filaments. Platelet contains three different forms of granules: dense granules, α -granules and lysosomes. These granules store different proteins and low molecular weight compounds. Some of them act as potent chemotactic factors and other as proinflammatory mediators [61]. Platelets play an essential role in controlling vascular integrity at the site of injury. However, the pathophysiological roles of platelets are broader than regulation of clot formation and homeostasis. Under normal physiological condition, platelets circulate in the blood stream without any interaction with each other or with intact blood vessels [62]. Emerging data indicate that platelets are important mediators of innate immune responses against invading micro-organisms and play an important role in antibacterial host defense and the pathophysiology of sepsis. Platelets have binding sites on their surface glycoproteins (for example, GpIb complex and integrin $\alpha\text{IIb}\beta\text{3}$) for many *Staphylococcus* species of bacteria which are considered to be a “first responder” to bacterial wound

invasion. The interaction between bacteria and platelet leads to platelet activation, secretion and subsequent aggregation, which isolate the microbe and stop the bacterial influx through the damaged blood vessels [63]. Platelets also express TLR2 and TLR4 which bind to specific bacterial PAMPs, such as lipopolysaccharide (LPS) which release a variety of platelet microbicidal proteins (PMPs) [64,65] allowing them to directly sense pathogens that rupture endothelial barriers [65]. Activated platelets directly kill pathogens by releasing platelet microbicidal proteins, including thrombocidines and kinocidins, which are stored in platelet granules [66], and by directly phagocytosing pathogens [67]. Platelets are known to promote neutrophil emigration in various inflammatory models including atherosclerosis [66], kidney failure [68], and ALI [69,70]. Mechanisms underlying platelet-mediated neutrophil recruitment include direct cellular interactions involving integrin α IIB β 3/Mac-1 and P-selectin/PSGL-1 [71] or neutrophil activation through platelet secretory products [68]. During infection, platelets can regulate the function of endothelial cells and leukocytes. The stimulation of TLR2 or TLR4 leads to platelet activation, facilitating platelet binding to endothelial cells alongside with neutrophils and other leukocytes [72]. It has been believed that these interactions are dependent on P-selectin on platelets and PSGL-1 on neutrophils upregulations [73]. Platelets, which adhere to the endothelium after TLR4 stimulation, can trap neutrophils via P-selectin-PSGL-1 interactions, subsequent by secondary interactions between neutrophils and the endothelium via LFA-1. In addition to promoting neutrophil recruitment into infected tissues, platelets also promote the bactericidal activity of neutrophils by increasing levels of TNF- α [74,75,76], secreting IL-1 β [77] and thromboxane A2 (TXA2), which promote the respiratory burst activity of neutrophils [78]. Previous study has demonstrated that blockade of P-selectin by antibody [78], decreases neutrophil infiltration and bacterial clearance in a rat model of CLP [79]. Other important proinflammatory derivative such as CD40 ligand (CD40L), present in the platelet cytosol, is produce from mRNA and release up on activation of platelets. The activation of Platelet during CLP in mice leads to expression of CD40L and P-selectin on platelets, which induces platelet-neutrophil aggregation. During sepsis, platelets are activated and enhance the harmful accumulation of neutrophils in the pulmonary capillaries [80]. Platelets promote neutrophils accumulation directly, through cell-cell

contact, or indirectly through shedding of sCD40L, which increases the expression of neutrophil Mac-1 (CD11b/CD18) [70,80,81,82]. These platelet-neutrophil interactions lead to lung injury in mouse models of sepsis. For example, platelets depletion by antibody [70], or genetic deletion of CD40L decrease lung injury, including lung myeloperoxidase (MPO) content, bronchoalveolar lavage neutrophils (BALF), and the edema formation [81] and decrease the mortality rate in mice [70].

Different platelet-derived chemokines are released after platelet adhesion and activation which modulate function of inflammatory cells such as activation and chemotaxis [83,84]. CXCL4 and CCL5 are stored in α -granule, belong to CXC and CC chemokines family and are potent chemoattractant for leukocyte subtype [85]. CXCL4 have chemotactic effect and play an important role in leukocyte recruitment and adhesion to endothelial cell via LFA-1 upregulation [86]. Moreover, platelet-derived chemokine such as CCL5 (RANTES) have been reported to induce monocyte adhesion [87,88]. Accumulating data suggest that inhibition of CCL5 and CXCL4 can decrease neutrophil infiltrations in the lung in inflammatory diseases [16,89]. A study has found evidence for signaling pathway mediating platelet components storage lesions in which PI3-kinase-dependent Rap1 activation leads to integrin α IIB β 3 activation and platelet degranulation [90]. This mechanism involves two primary actors (i) Rap1, a small GTPase that modulates integrin α IIB β 3 affinity, most likely through effects on the actin cytoskeleton, and (ii) Talin, an adaptor protein that links integrin α IIB β 3 to the actin cytoskeleton [91].

Role of macrophage in inflammation

Macrophages play an important role in the immune system and form in response to an infection. The term macrophage is formed by the combination of the Greek terms "makro" meaning big and "phagein" meaning eat. Macrophages are large, specialized cells that recognize, engulf, and destroy target cells. Alveolar macrophages (AMs) present in the alveoli and the alveolar ducts of the lungs. They consider as a first line defense in the lungs and control the entire inflammatory response [92,93]. Activated alveolar macrophages produce inflammatory mediators such as IL-1, TNF and other potent proinflammatory cytokines during initial phase of pulmonary inflammation. When

there is tissue damage or infection, macrophages have three fundamental function (i) antigen presentation, (ii) phagocytosis and (iii) immunomodulation via production of various cytokines and growth factors [94,95,96,97]. During inflammation macrophages are activated and secrete proinflammatory cytokines but they are deactivated by anti-inflammatory cytokines such as interleukin 10 (IL-10), transforming growth factor β (TGF- β) and cytokine antagonists that are mainly produced by macrophages. The AM is the initial phagocytic cell that comes in contact with inhaled pathogens. In addition, the AM can amplify the pulmonary inflammatory response through the production of various leukocytes chemotactic such as CXCL1 and CXCL2 and activating cytokines. Previous work has provided evidence of abnormal function of AM isolated from animals during the post septic period [98,99]. The most important role of the macrophage in sepsis is the production of a variety of proinflammatory cytokines, such as TNF- α , IL-1 β , and IFN- γ [100] as well as the secretion of chemokines that recruit neutrophils to the site of infection which play an important role in clearing the bacterial infection [101] or by mediating pathologic tissue destruction in many types of human diseases [102]. Macrophages express the CC chemokine receptors such as CCR1 and CCR5 [103]. Several studies showed that these receptors play important roles in leukocyte recruitment in inflammatory diseases [104,105].

Role of cytokines in inflammation

Cytokines are a large family of low molecular weight proteins (16-25 kDa), which regulate the immune response. The innate immune system releases multiple inflammatory cytokines in response to pathogens, and these cytokines directly resist the infectious agent and recruit additional immune responses. However, this cytokine release can become uncontrolled and lead to harmful effects such as leakage from capillaries, tissue edema, organ failure and shock. The expression of most cytokines is modulated by transcription factors such as NF- κ B [106,107,108,109,110].

Over expression of cytokines in the plasma has been reported during abdominal sepsis [111,112]. However, some studies have demonstrated that alveolar macrophages possess ability to produce different types of inflammatory mediators in response to noxious

stimuli. Cytokines which have been shown to be produced by alveolar macrophages are TNF- α , IL-1 β , and IL-6 [113,114,115]. Many investigative studies have shown that TNF- α is the prime mediator of the inflammatory response seen in sepsis and septic shock [116]. Furthermore, a recent study has shown that anti-TNF- α therapy may improve survival in septic patients [117]. Plasma IL-1 β can be detected in a minority of patients with sepsis [118]. Moreover, the primary role of IL-1 β in sepsis syndrome is provided by studies using IL-1ra. Blockade of IL-1ra, the biological activity of exogenous and endogenous IL-1 β decreased the mortality of rabbits treated with endotoxin [119]. In addition, treatment with IL-1ra reduced the production of IL-1 and IL-6 and improved the survival after *E-coli* in fusion in baboons [120]. IL-6 is firmly established as a clinically and experimentally suitable biomarker for sepsis [121,122,123]. It has been observed that high levels of IL-6 is associated with fatal sepsis in patients with meningococcal infection [124]. Moreover, IL-6 blockade during CLP-induced sepsis in mice reduced lung, liver, kidney, and heart injuries and improved survival [125].

These cytokines are responsible for mediation of the initial innate immune system response to injury or infection. These proinflammatory cytokines contribute to activate endothelial cells, attract circulating polymorphonuclear cells (PMNs), and enter the circulatory system. Serum TNF- α , IL-1 β and IL-6 are mediators of inflammation and can be used at the diagnosis and at the evaluation of the therapeutic efficiency in sepsis [126].

Role of chemokines in inflammation

Chemokines are a family of small cytokines (8-10 kDa), act mainly on leukocytes and induce chemotaxis. They perform both beneficial roles in normal host defense against infection and harmful roles in autoimmune diseases [127,128,129]. Chemokines have been shown to mediate inflammatory tissue destruction in a wide variety of human diseases, such as rheumatoid arthritis, myocardial infarction, and acute respiratory distress syndrome [130,131,132,133,134,135,136]. Chemokines have been divided into the two major subfamilies on the basis of the arrangement of the two N-terminal cysteine residues, CXC and CC, depending on whether the first two cysteine residues have an amino acid between them (CXC) or are adjacent (CC) positions. Two other less abundant groups of chemokines have been described which are C and CX3C. Chemokines act

through chemokine receptors (CXCR, CCR, XCR and CX3CR), which are a subfamily of G-protein coupled seven-transmembrane receptors [137,138,139]. Chemokine receptors probably signal through the G $\beta\gamma$ subunit to activate phospholipase C (PLC) and phosphatidylinositol-3 kinase PI3K [137,138,139,140]. This in turn eventually leads to activation of the cytoskeletal regulatory kinases focal adhesion kinases (FAK) and Protein tyrosine kinase 2 (Pyk-2) [141,142] that mediate chemotaxis, and in some cases activation of the mitogen activated protein kinases (MAPKs) proliferative pathway [137,141]. Signaling is initiated after binding of the chemokine to the receptor, which allows the association of guanine triphosphate (GTP) to the G α subunit. This results in dissociation of the heterotrimeric G-protein complex from the receptor and separation into the G α and G $\beta\gamma$ subunits. The G α subunit is able to directly activate the Src family kinases, which then leads to activation of the mitogen activated protein kinases (MAPKs) and protein kinase B (PKB) [143]. Signaling through the G $\beta\gamma$ involves at least three separate pathways. G $\beta\gamma$ can activate protein kinase B (PKB) and the MAPKs through phosphatidylinositol 3 kinase γ (PI3K γ), Protein kinase C (PKC) through phospholipase C (PLC) and Pyk-2 [144]. Activation of PLC induces calcium influx, which activates many cellular processes, including degranulation of neutrophils, basophils, and eosinophils [139].

During inflammation, endothelial cells are stimulated by proinflammatory mediators and synthesize chemokines and present them on the surface [57,145]. The rolling of leukocyte is regulated by combination of chemokines receptors which expressed on their surface and chemokines which express on endothelium [146]. Chemokines are also produced by different tissue cells including activated alveolar macrophage at the site of inflammation [147]. In mouse, CXC chemokines include CXCL1 and CXCL2 which are homologues of humane IL-8 [148], which bind to CXCR2 receptor. Furthermore, expression of CXCR2 has been shown to be reduced on neutrophils of septic shock patients [149]. Genetically modified mice deficient in chemotactic cytokines receptor CXCR2 or pharmacological antagonism with CXCR2 antagonist, have been shown to protect against sepsis [149,150]. Previous study has shown that blockade of CXCR2 by an inhibitor reduced polymorphonuclear infiltration, lung protein leak, and lung tissue content of CXCL1 and CXCL2 [151].

Role of CD40L in inflammation

CD40L is a trimeric, transmembrane protein of the tumor necrosis factor family that was originally identified on cells of the immune system such as activated CD4⁺ T cells and platelets. A study on the cellular distribution of CD40L showed that more than 95% of the circulating CD40L exists in platelets [152]. CD40L is enigmatic in unactivated platelets but is immediately expressed on the platelet surface after platelet stimulation. CD40L is a potent activator of vascular cytokine production and they exert several proinflammatory and procoagulant effect on endothelial cells including upregulation of adhesion molecules (ICAM-1, VCAM-1, E-selectin) [153,154]. Mac-1 is an important mediator of leukocytes adhesion to the activated endothelium during inflammation. CD40L ligation to Mac-1 is regulating leukocyte adhesion and transmigration at the atherosclerotic lesion site and neointimal formation during atherogenesis [155,156]. In addition, a recent study reported that platelet derived CD40L promotes Mac-1 expression on neutrophils and regulate pulmonary accumulation of inflammatory cells in abdominal sepsis [80]. The platelet surface-expressed CD40L is subsequently cleavage by matrix metalloproteinase (MMP-9) and generates a soluble fragment termed soluble CD40L (sCD40L) that remains trimeric [157,158].

MMPs constitute more than 23 members of structurally related zinc and calcium dependent endopeptidases family mediating the cleavage of most extracellular matrix proteins, as well as several non-matrix proteins including chemokines, cytokines, adhesion molecules and surface receptors [159]. Moreover, sCD40L is known to be elevated in the plasmas of individuals with a broad spectrum of inflammatory conditions such as rheumatoid arthritis, cardiovascular diseases, inflammatory bowel diseases and septic shock [160,161,162,163]. A study showed that platelet CD40L enhances both leukocyte and platelet adhesion to the endothelium and regulating the formation of platelets-leukocyte aggregation. Gene deficient mice of CD40L platelet prevented the increase of atherosclerosis and the disruption of T-cell homeostasis [164]. It has been proposed by a study that secretion of sCD40L from platelets and its thrombotic activity appear to be strongly related to the platelet integrin α IIb β 3. Integrin α IIb β 3 is known to be involved in sCD40L production because integrin α IIb β 3 antagonists attenuate the secretion of sCD40L from stimulated platelets *in vitro* [165], whereas other investigators

found that inhibition of MMP-2 and MMP-9 decreased platelet shedding of CD40L [166,167].

Role of Rac1 in inflammation

Rac proteins constitute a subgroup of the Ras superfamily of GTP hydrolases. Although originally implicated in the control of cytoskeletal events, it is currently known that this GTPase coordinate diverse cellular functions, including cell-cell adhesion, vesicular trafficking, the cell cycle and transcriptional dynamics [17,168].

The Rho family of GTPases, including Rho, Rac, and Cdc42, exert major roles in reorganizing the cell actin cytoskeleton in response to external stimuli such as mechanical stress [169]. Most signals transduction is through G-protein coupled receptors, cytokines, tyrosine kinase receptor and adhesion receptors [168,170,171,172]. Under resting conditions, the inactive GDP-bound is localized in the cytosol. Up on stimulation, certain guanine nucleotide exchange factors known as GEFs, induces the GDP for GTP, leading to dissociation of activated GTP-bound and translocate the Rho-GTP to the plasma membrane [173]. Other cytosolic proteins known as GTPase activating proteins (GAPs) stimulate hydrolysis of GTP, thereby returning Rho-GTP to Rho-GDP form [174]. The effect of GEFs and GAPs are enhanced by a third group of Rho-regulatory proteins, called GTPase-dissociation inhibitors (GDIs). GDI form a cytosolic complex with Rho and keep Rho as a resting GDP-bound state. Moreover, they impede the exchange of GDP for GTP and thus finally inhibit Rho activation [175,176,177]. These regulatory steps cause Rho to cycle between an active and inactive state, rapidly turning the signal pathway on and off (Figure 2).

Rac1 is a small (~21 kDa) signaling G protein (more specifically a GTPase), and is a member of the Rac subfamily of the Rho family of GTPases. Rac is prevalent in three isoforms (Rac1, Rac2 and Rac3), Rac1 is ubiquitously expressed and is the most extensively studied isoform. Rac2 is definitely expressed in hematopoietic cells, whereas Rac3 is expressed primarily in the brain during development [178]. Previous study has shown that Rac1 rather than Rac2 or Rac3 is the predominant isoform in platelets [179].

Rac1 play an important role in regulation of mitogen-activated protein kinases, such as JNK/c-Jun and p38 mitogen-activated protein kinase [180,181,182,183,184], which are

known to be involved in proinflammatory activities, such as cytokine formation and leukocyte activation [185]. In fact, inhibition of Rac1 activity by specific Rac1 inhibitor NSC23766, which targets a Rac region involved in the binding to GEFs Trio or Tiam 1 or dominant negative forms of human Rac1 (Ad5N17Rac1), has been shown to exert protective effects in models of acute pancreatitis and reperfusion injury in the liver via regulation of ROS productions [18,186]. In addition, Rac1 plays an important role in the regulation of platelet secretion and aggregation, deficiency of Rac1 GTPase or inhibition of Rac GTPase by NSC23766, a rationally designed specific inhibitor of the Rac–GEF interaction, inhibits activation of Rac1 and p21 protein activated kinase 1 (PAK1), and blocks platelet secretion and aggregation induced by ADP or collagen [187]. Moreover, deficiency or inhibition of Rac1 GTPase results in defective platelet function and consequently a defective hemostatic response in mice which is mediated via the PI3 K and PLC- γ 2 signaling pathway (Figure 2) [188].

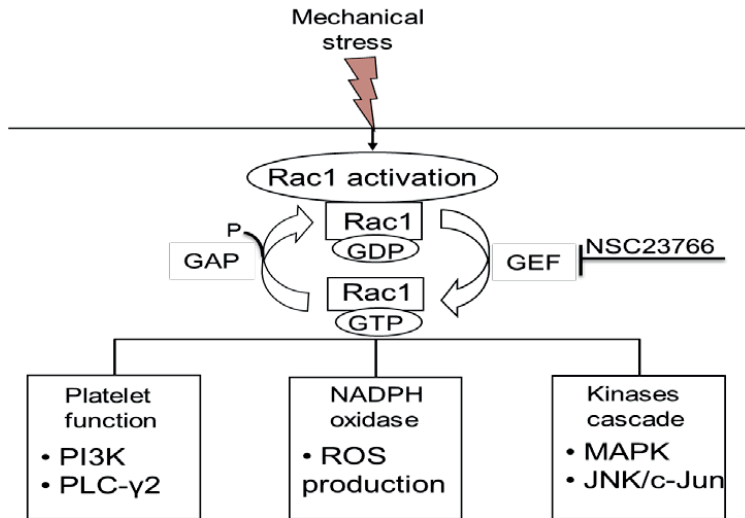


Figure 2. Rac GTPase cycle between an active GTP-bound and an inactive GDP-bound form. This is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Rac1 signaling plays an important role in regulation of mitogen-activated protein kinases, such as JNK/c-Jun and p38 mitogen-activated protein kinase and regulates platelet function via PI3K and PLC- γ 2 as well as NADPH oxidase and ROS formation.

Aims

1. To define the role of Rac1 in regulating pathological inflammation and lung tissue damage in abdominal sepsis.
2. To examine the role of Rac1 signaling in platelet activation and CD40L shedding in septic lung damage.
3. To investigate the role of Rac1 signaling in regulating platelet secretion of CCL5 and alveolar macrophage activation in abdominal sepsis.
4. To define the function of Rac1 in regulating platelet secretion of CXCL4 and neutrophil accumulation in septic lung injury.

Material and methods

Animals

Experiments were performed using male C57BL/6 mice (20-25 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 intraperitoneal (i.p.) administration of 75 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) per kg body weight.

Experimental model of sepsis

The cecal ligation and puncture (CLP) was used to induce septic conditions in mice. This model has many pathophysiological similarities to the clinical situation where bowel perforation-induced peritonitis results from an infection that is caused by mixed intestinal flora. The CLP model is one of the most widely used models of sepsis and is considered as a golden model for sepsis study. This model fulfills the human condition that is clinically relevant. Like in humans, mice that undergo CLP with fluid resuscitation show the first (early) hyperdynamic phase that in time progresses to the second (late) hypodynamic phase [189]. In addition, the CLP-induced sepsis shows a cytokine profile similar to human sepsis [190,191]. However, one major concern of CLP model is its consistency. The underlying inflammatory response and outcome of the CLP depends on several factors i.e. the length of cecum ligated, size of needle used and number of punctures and fluid resuscitation. Thus, a standardized performance of the CLP procedure is ensured to develop a high grade of sepsis by ligating 75% of cecum and puncturing twice with 21-gauge needle in the anti-mesenteric side of the cecum. For the CLP surgery, the mice were anesthetized and the lower quadrant of the abdomen was disinfected with alcohol. The abdominal cavity was opened via a midline laparotomy incision of about 1 cm in an aseptic fashion and the cecum was exposed which was filled with feces by milking stool backwards from the ascending colon and a ligature was

placed below the ileocecal valve. Care was taken not to breach or damage the mesenteric blood vessels. The cecum was soaked with PBS (pH 7.4) and then punctured twice with a 21-gauge needle. The cecum was then returned into the peritoneal cavity and the abdominal wall was closed with a suture. Sham mice underwent the same surgical procedures, i.e., laparotomy and resuscitation, but the cecum was neither ligated nor punctured. The mice were then returned to their cages and provided with food and water *ad libitum*. Animals were re-anesthetized 6 h or 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 counted. 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 western blot, myeloperoxidase (MPO) assays and enzyme-linked immunosorbent assay (ELISA) assays as described below.

Antibodies and biochemical substances

Animals were anaesthetized by intraperitoneal (i.p.) administration of 7.5 mg Ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight. To study the role of Rac1 in abdominal sepsis, 5.0 mg/kg of Rac1 inhibitor, NSC23766 (N6-[2-[[4-(diethylamino)-1-methylbutyl] amino]-6-methyl-4-pyrimidinyl]-2-methyl-4,6-quinolinediamine trihydrochloride; Tocris Bioscience, Bristol, UK) (Figure M1) was administered i.p. 30 min prior to CLP. NSC23766 is a specific and reversible Rac1 inhibitor that competitively inhibits interaction between Rac1 and Rac-specific guanine nucleotide exchange factors. NSC23766 does not affect the activity of related Rho guanosine triphosphatases (GTPases) including Cdc42 or RhoA *in vitro* or *in vivo* [20,192]. In order to deplete platelet, 1.0 mg/kg of a monoclonal antibody directed against murine CD42b (GP1b, rat IgG, Emfret Analytics GmbH & Co. KG, Wurzburg, Germany) was administered i.p. 2 h prior to CLP. A nonfunctional isotype control antibody (clone R3-34; BD Biosciences Pharmingen, San Jose, CA, USA) was administered intraperitoneally before CLP induction. To evaluate the functional importance of CXCL4 and CCL5, 10 μg per mouse antibodies directed against murine CXCL4 (clone 140910, R&D Systems)

and CCL5 (clone 53405, R&D Systems, Minneapolis, MN, USA), and a nonfunctional isotype-matched control antibody (clone 54447, R&D Systems) and (clone 141945 R&D Systems) were administered respectively. Antibodies and PBS (200 μ l) were administered i.p. 30 minutes before CLP induction. Recombinant murine CCL5 or CXCL4 was intratracheally injected to induce CXCL2 secretion in the lung.

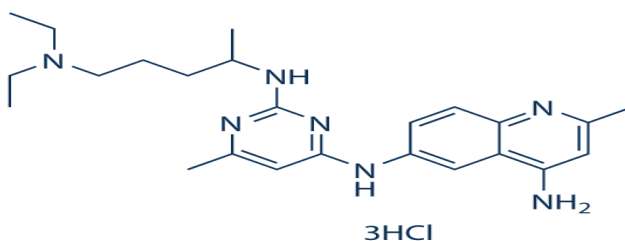


Figure M1. Chemical structure of Rac1 inhibitor NSC23766 (Tocris).

Isolation of alveolar macrophages and quantitative RT-PCR

Gene expression of CXCL1, CXCL2 and TNF- α was quantified in alveolar macrophages isolated from sham mice and CLP animals treated with vehicle or 5 mg/kg of NSC23766 i.p. 30 min prior to CLP. Alveolar macrophages were isolated from BALF as described in detail [193]. 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 (18°C). The cells were then resuspended in RPMI 1640 complete culture medium and incubated at 37°C (5% CO₂) in a 48-well plate. After 2 h, non-adherent cells were washed away by PBS. A total of 2-3 $\times 10^5$ 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.

Systemic leukocyte counts

Blood was collected from the tail vein and mixed with Turks solution (Merck, Darmstadt, Germany) in a 1:20 dilution. Leukocytes were identified as monomorphonuclear (MNL) and polymorphonuclear (PLMN) leukocyte in chamber.

Myeloperoxidase assay

The enzyme MPO is abundant in PMNLs and has been used as a reliable marker for the detection of neutrophil accumulation in inflamed tissue [194,195]. 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 catalysed change in absorbance in the redox reaction of H₂O₂ (450 nm, with a reference filter 540 nm, 25°C). Values were expressed as MPO units per g tissue.

Bronchoalveolar lavage fluid

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 neutrophils were counted in a Burker chamber.

Lung edema

The left lung was excised and then 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.

Enzyme linked immunosorbent assay

For measuring lung chemokines, the lung sample was thawed and homogenized in PBS. CXCL1 and CXCL2 were analyzed by using double antibody Quantikine ELISA kits (R & D Systems) using recombinant murine CXCL1 and CXCL2 as standards. The minimal detectable protein concentrations are less than 0.5 pg/ml. For soluble CD40L analysis, plasma was collected using citrate as anticoagulant and centrifuged for 20 minutes at 2000 x g immediately after collection. An additional centrifugation at 10000 x g for 10 minutes was employed for complete removal of platelets and stored at -20 °C for further use. Plasma samples were then diluted with a sterile buffer (20% fetal calf serum in PBS, pH-7.4) and analyzed by using commercially available ELISA kits (R & D Systems). Plasma levels of CXCL1, CXCL2, CCL5, CXCL4 and MMP-9 in septic animal were analyzed by use of commercially available ELISA kits (Bender MedSystems, Vienna, Austria), using recombinant murine sCD40L CXCL1, CXCL2, CCL5, CXCL4 as standards, respectively.

Neutrophil chemotaxis

Neutrophils isolated from bone marrow by use of Ficoll-Paque™. 1.5 x 10⁶ neutrophils were placed in the upper chamber of the transwell inserts with a pore size of 5 μm (Corning Costar, Corning, NY, USA). Inserts were placed in wells containing medium alone (control) or medium plus CXCL2 (100 ng/ml, R&D Systems), CXCL4 or CCL5 (500 ng/ml). 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 Burkner chamber.

Flow cytometry

Flow cytometry was performed for analysis of the number of Mac-1 expression on circulating neutrophils (I), platelet depletion in paper (II) and for analysis of CCR1, CCR5, CXCR2 (III, IV) on neutrophil. Blood was collected (1:10 acid citrate dextrose) 6 h after CLP induction and incubated with an anti-CD16/CD32 antibody blocking Fcγ III/II receptors to reduce non-specific labeling. Samples were then incubated with phycoerythrin PE-conjugated anti-Gr-1 (clone RB6-8C5, eBioscience, Frankfurt, Germany) and fluorescein isothiocyanate FITC-conjugated anti-Mac-1 (clone M1/70, BD Biosciences Pharmingen, San Jose, CA, USA) antibodies to detect Mac-1 expression on neutrophil. In order to determine the depletion of platelet in mice, blood was incubated with an anti-CD16/CD32 antibody blocking Fcγ III/II receptors and then neutrophils were labeled with PE-conjugated anti-Ly-6G and platelets were labeled with FITC-conjugated anti-CD41 (clone MWReg30, integrin αIIb chain, and rat IgG1) antibodies and plotted as FL1 versus FL2 to determine the percentage of platelet depletion. Cells were fixed with 1% formaldehyde solution; erythrocytes were lysed using red blood cell lysing buffer (Sigma Chemical Co., St. Louis, MO, USA) and neutrophils and/or platelets were recovered following centrifugation. For CCR1, CCR5 and CXCR2 expression analysis, blood were incubated with the anti-CD16/CD32 antibody for 10 min followed by staining with a PerCP-Cy5.5-conjugated anti-mouse CXCR2 antibody (clone TG11/CXCR2; rat IgG2a; Biolegend, San Deigo, CA, USA), a PE-conjugated anti-CCR1 antibody (clone CTC5, R&D Systems) or a PE-conjugated anti-CCR5 antibody (clone 643854, R&D Systems). Cells were fixed and erythrocytes were lysed, neutrophils were recovered following centrifugation. Alveolar

macrophages were isolated as described in detail [193]. Briefly, lungs were flushed ten times with 1 ml of PBS supplemented with 0.5 mM EDTA. Alveolar fluid collections were then centrifuged at 1400 rpm for 10 min (18°C). The cells were then resuspended in FACS buffer and incubated with an anti-CD16/CD32 antibody blocking Fc γ III/II receptors and a PerCP-Cy5.5-conjugated anti-mouse F4/80 antibody (clone BM8, eBioscience, Frankfurt, Germany) and a FITC-conjugated anti-Ly6G (clone 1A8, BD Biosciences) antibody as well as a PE-conjugated anti-CCR1 antibody (clone CTC5) or a PE-conjugated anti-CCR5 antibody (clone 643854). 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.

Histology

Lung samples were fixed by immersion in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin-embedded. Six μ m sections were stained with haematoxylin and eosin. Lung injury was quantified in a blinded manner by adoption of a modified scoring system [196,197], including size of alveoli spaces, 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 (Table 1).

Table 1. Histology scoring system used in study I, III, IV

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

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

The thickness of the alveolar septa: The thickness of the alveolar septa were scored in oil emersion high power field (HPF)

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

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

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

PMN infiltration: Infiltrated PMN were counted in interstitial and intraalveolar spaces in high power field 100X (HPF)

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

Isolation of platelet

Blood was collected in syringes containing 0.1 ml of acid–citrate–dextrose, immediately diluted with equal volumes of modified Tyrode solution (1 $\mu\text{g/ml}$ prostaglandin E1 and 0.1 U/ml apyrase) and centrifuged (100 g, 10 min). Platelet-rich plasma was collected and centrifuged (800 g, 15 min) and pellets were resuspended in modified Tyrode solution. After being washed one more time (10 000 g, 5 min), isolated platelets were used for late western blot or confocal microscopy.

Confocal microscopy

Isolated platelets 0.5×10^6 were seeded on a chamber slide coated with fibrinogen (20 $\mu\text{g/ml}$). Adherent platelets were stimulated with protease-activated receptor 4 (PAR-4) (200 μM , 37°C) with and without NSC23766 (100 μM). Platelets were fixed with 2% paraformaldehyde for 5 min and washed and blocked with 1% goat serum for 45 min. Then platelets were permeabilized with 0.15% Triton X-100 for 15 min followed by washing and incubation with an anti-CD16/CD32 antibody (10 min) blocking Fc γ III/II receptors to reduce non-specific labeling and PE-conjugated anti CD40L in paper (II), a rabbit polyclonal primary antibody against CCL5 (bs-1324R, Bioss, Boston, MA, USA) in paper (III) or a rabbit polyclonal primary antibody against CXCL4 (bs-2548R, Bioss, Boston, MA, USA) in paper (IV) for 1-2 h. Chamber slides were washed and incubated with a FITC-conjugated anti mouse or anti-rabbit secondary antibody (Cell Signaling Technology, Beverly, MA, USA) and a platelet specific PE-conjugated anti-CD41 antibody (clone MWReg30, eBioscience, San Diego, CA, USA) for 1 h. For MMP-9 detection in neutrophils, freshly isolated neutrophils 0.5×10^6 were resuspended in PBS-BSA-CaCl₂ buffer (PBS containing 0.2% BSA and 0.5 mM CaCl₂) and activated with mouse CXCL2 with and without NSC23766 (10 mM). Neutrophils were fixed with 2% paraformaldehyde and washed with PBS-BSA-CaCl₂ followed by permeabilization with PBS-BSA-CaCl₂ saponin buffer (PBS-BSA-CaCl₂ buffer containing 0.01% (w/v) saponin) for 10 min on ice. After permeabilization, cells are incubated with rabbit polyclonal primary antibody against MMP-9 (Abcam) for 1 h on ice. Cells were washed

and incubated with FITC-conjugated anti-rabbit secondary antibody (Cell Signaling Technology, Beverly, MA, USA) and PE-conjugated anti-Ly-6G antibody (clone 1A8, rat IgG2a; BD Pharmingen, San Jose, CA, USA), which selectively binds to neutrophils for 1 h on ice. Chamber slides were washed three times and confocal microscopy was performed using Meta 510 confocal microscopy (Carl Zeiss, Jena, Germany). FITC and PE were excited by 488 nm and 543 nm laser lines and corresponding emission wavelengths of FITC and PE were collected by the filters of 500-530 nm and 560-590 nm, respectively. The pinhole was ~1 airy unit and the scanning frame was 512×512 pixels. The fluorescent intensity was calculated by use of ZEN2009 software.

Pull-down assay and Western blotting

Rac1 activity was determined in the lung tissues in paper (I), neutrophils in paper (II) and platelets in paper (II, III, IV) from sham and CLP mice pretreated with vehicle or NSC23766 by active Rac1 pull-down and detection kit using the protein binding domain of GST-PAK1, which binds with the GTP-bound form of Rac1 (Pierce Biotechnology, Rockford, IL, USA). Briefly, lung tissue or cells were homogenized in lysis buffer on ice and centrifuged (16000 g, 15 min). Ten µl from each lysate were removed to measure protein content using Pierce BCA Protein Assay Reagent (Pierce Biotechnology) and the rest were 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 (10-12% gel). After transferring to a nitrocellulose membrane (BioRad, Hercules, CA, USA), blots were blocked with tris buffer saline/Tween20 containing 3% bovine serum albumin at room temperature for 1 h, followed by incubation with an anti-Rac1 antibody (1:1000) at 4°C overnight. Binding of the antibody was detected using peroxidase-conjugated anti-mouse antibody (1:100000, Pierce Biotechnology) at room temperature for 2 h and developed by Immun-Star WesternC Chemiluminescence Kit (Bio-Rad). Total Rac1 was used as a loading control.

Statistics

Data were presented as mean values \pm 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 SigmaPlot® 10.0 software (Systat Software, Chicago, IL, USA).

Results and discussion

Role of Rac1 in abdominal sepsis

Rac1 is a member of the Rho family of small GTPases that control cells proliferation, differentiation, migration, and inflammation. However, the role of Rac1 signaling in sepsis is not demonstrated yet. In this study, we show for the first time that Rac1 play an important role in lung injury associated with abdominal sepsis. We used Rac1 inhibitor NSC23766 in order to reveal the role of Rac1 in CLP animal. In order to investigate the activation of Rac1 GTPase by CLP, lung from sham, CLP challenge and treatment mice with specific Rac inhibitor were harvested for Rac1 activation assay. CLP challenge increase active form (GTP binding form) of Rac1. Pretreatment with Rac1 inhibitor NSC23766 decreased enhancement of CLP-induced activation of Rac1 (Figure R1).

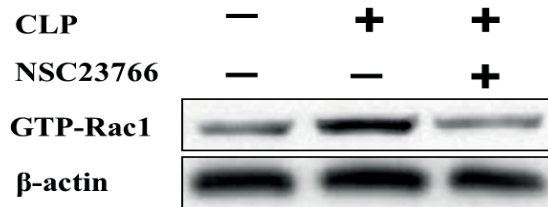


Figure R1. Six hours after CLP challenge and NSC23766 treatment, lungs from control group (-) and CLP group (+) mice were harvested for Rac1 activities. β-actin was used as an internal control $n = 3$.

It is well known that MPO is the most abundantly expressed in neutrophils and it is a marker of infiltrated neutrophils in the inflamed tissue, we asked for effects of NSC23766 on MPO activity, 6 h after CLP challenge resulted in significant increase of MPO activities in lung tissue of CLP group as compared with sham group. However, treatment with NSC23766 at 5 mg/kg markedly decreased the CLP-induced enhancement of MPO activity. We found that the pulmonary levels of MPO and the number of neutrophils in the bronchoalveolar space provoked by CLP was reduced by more than 58% and 94% respectively in mice pretreated with NSC23766, this data suggesting that Rac1 regulate a

significant proportion of neutrophil accumulation in the lung in polymicrobial sepsis. The infiltration of neutrophils occurs mostly depending on their migration, and the migration of neutrophils was triggered by chemokines which activate and recruit neutrophils across the endothelial barrier. CLP increased CXC chemokine production in septic mice; however Rac1 inhibitor NSC23766 significantly attenuated CLP-induced formation of CXCL1 and CXCL2. Macrophages are a potent producer of CXC chemokines in the lung, we isolated alveolar macrophages 30 min after CLP and we found that CLP markedly enhanced CXCL1 and CXCL2 mRNA levels in alveolar macrophages. Notably, administration of NSC23766 abolished CLP-provoked gene expression of CXC chemokines in alveolar macrophages, indicating that Rac1 is an important signaling pathway in macrophage regulation and production of CXC chemokines in septic lung injury and the effect of Rac1 inhibitor on pulmonary infiltration of neutrophils is related to local changes in CXC chemokine production in the lung.

In addition, we found that NSC23766 not only decreased neutrophil recruitment and chemokine formation (Figure R2) but also attenuated sepsis-induced edema formation and tissue destruction in the lung.

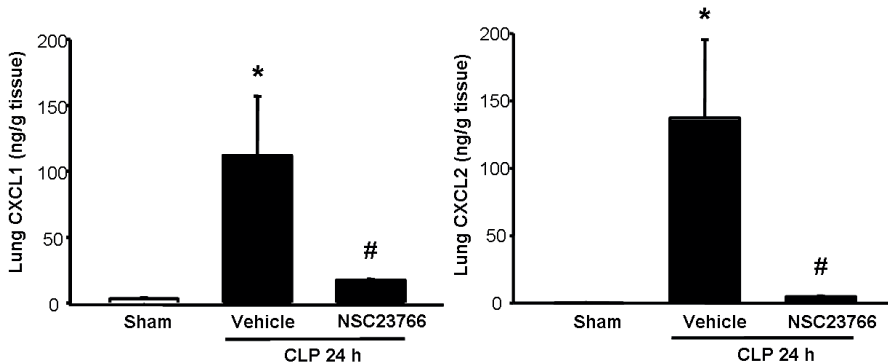


Figure R2. Rac1 activity regulates CXC chemokine formation in the lung. ELISA was used to quantify the levels of CXCL1 and CXCL2 in the lung 24 h after CLP induction. Animals were treated with vehicle (PBS) or NSC23766 (5 mg/kg) before CLP induction. Sham-operated animals served as negative controls. Data represents mean \pm SEM and $n = 5$. * $P < 0.05$ vs. Sham and # $P < 0.05$ vs. Vehicle + CLP.

For example, mice in CLP group exhibited marked increases of neutrophils infiltration in to the lung interstitium, alveolar spaces and exhibited marked thicknesses of alveolar wall and interstitial edema as compared with sham mice. However, in CLP mice treated with NSC23766 markedly attenuated the infiltrations of neutrophils and improved alveolar thicknesses and interstitial edema as compared with CLP challenged mice. These results indicate that targeting Rac1 functions may protect against damage to the lung tissue in abdominal sepsis (Figure R3).

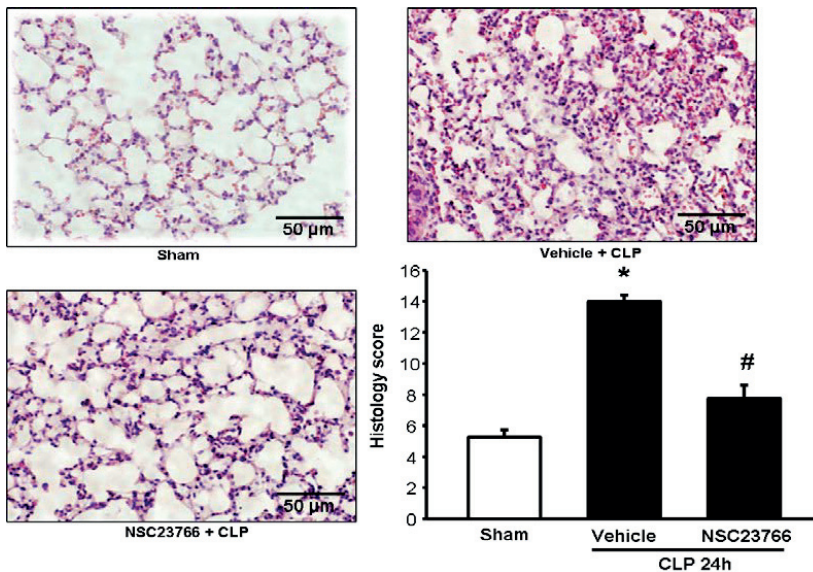


Figure R3. Tissue morphology 24 h after CLP induction. Representative hematoxylin and eosin sections of lung are shown. Lung injury score as described in Materials and Methods section. Data represent mean \pm SEM and $n = 5$. * $P < 0.05$ vs. Sham and # $P < 0.05$ vs. vehicle + CLP. Scale bar indicates 50 μm .

Neutrophils are particularly activated and attracted by chemokines via Mac-1 up-regulation on neutrophils [149] and according to our observation, Rac1 is a potent regulator of chemokines formation in the lung that may explain the reduction of the Mac-1 up-regulation in the CLP mice, pretreated with NCS23766 indicating that Rac1 may play a physiologic role in neutrophil migration *in vivo*. In order to examine the direct effect of Rac1 on Mac-1 expression, as expected CXCL2 caused clear-cut increase of

neutrophils activation by expression of Mac-1. Pre-incubation of activated neutrophils with NSC23766 significantly reduced the Mac-1 expression on neutrophils, suggesting that Rac1 signaling also directly controls neutrophil Mac-1 up-regulation in abdominal sepsis (Figure R4).

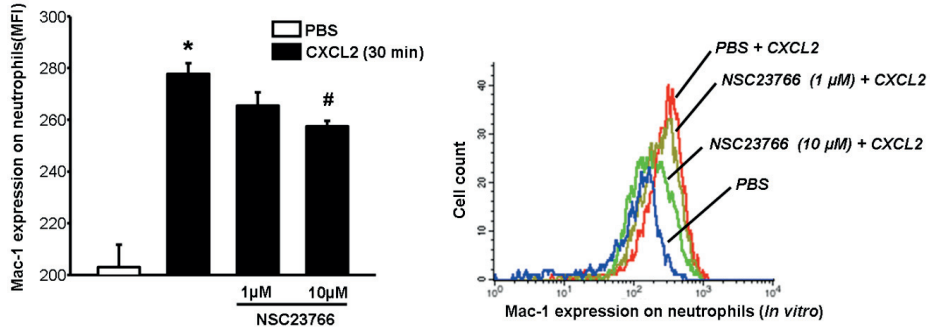


Figure R4. Mac-1 expression on isolated bone marrow neutrophils (Gr-1+ cells) incubated with CXCL2 (0.3 μg/mL) and vehicle (PBS) or NSC23766 (1 or 10 μM). Representative histogram and data in aggregate. Data represent mean ± SEM and $n = 5$. * $P < 0.05$ vs. PBS and # $P < 0.05$ vs. PBS + CXCL2. MFI = mean fluorescence intensity.

The migration of neutrophils can be largely mimicked by using Boyden chamber *in vitro* and was assessed by directed migration (chemotaxis) in response to CXCL2 (100 ng/ml). We found that the migrated cells against CXCL2 stimulation were much more than the negative control and pre-treated cells with NSC23766 significantly decreased neutrophils migration against CXCL2. These findings together indicate a role of Rac1 in interfering with the migration of neutrophils guided by chemokines. This notion is in line with the study reporting that Rac1 gene deletion mice can decrease neutrophil migration toward the chemoattractant fMLP [198].

It has been well known that CLP induces proinflammatory mediators such as IL-6 and HMGB1 which suggested as a marker of systemic inflammation and clinical outcome in patients with severe sepsis [121,122,199,200]. Inhibition of Rac1 by NSC23766

decreased production of HMGB1 and IL-6, which are crucial cytokines for lung inflammation. Thus, Rac1 might be a key molecular link between inflammation and lung injury.

In conclusion, Rac1 signaling plays an important role in polymicrobial sepsis. This study shows that Rac1 signaling regulates sepsis-induced inflammation in the lung by reducing chemokine production and Mac-1 expression on neutrophils. Moreover, Rac1 inhibitor NSC23766 attenuates lung edema, tissue destruction and systemic proinflammatory cytokines in septic animals, suggesting that targeting Rac1 may be useful approach to protect against pulmonary injury in abdominal sepsis.

II. Rac1 regulates platelet CD40L shedding

CD40 ligand (CD40L), identified as a costimulatory molecule expressed on T cells, is also expressed and functional on platelets. CD40L is a trimeric, transmembrane protein of the tumor necrosis factor family that was originally identified on cells of the immune system (activated CD4⁺ cells, mast cells, basophils, eosinophils, and natural killer cells). Studies on the cellular distribution of CD40L indicate that >95% of the circulating CD40L exists in platelets [152]. CD40L is cryptic in unstimulated platelets but is rapidly presented to the platelet surface after platelet stimulation. The surface-expressed CD40L is subsequently cleaved and generating a soluble fragment termed soluble CD40L that remains trimeric [152]. A recent study showed that soluble levels of CD40L increased in plasma of the septic mice [201]. It also believes that platelets-dependent pulmonary accumulation of neutrophils is mediated by soluble CD40L released from platelets in abdominal sepsis [80]. However, a study showed that MMP-9 cleaves off CD40L on platelets [164].

Based on these considerations, we asked whether Rac1 activity might regulate platelet shedding of CD40L. Knowing that Rac1 GTPase is the major Rac isoforms present in platelets [179], In this study, we used CLP model which induce platelet secretion of soluble CD40L also activate Rac1 GTPase, raising the possibility that Rac1 GTPase may be involved in regulation of platelet function. It was found that platelet depletion abolished the sepsis-induced increase of CD40L levels in plasma, suggesting that platelets are the dominating source of soluble CD40L in sepsis, which is in line with our

previous findings [202]. Rac1 inhibitor NSC23766 reduced soluble levels of CD40L in the plasma from septic mice from 2.40 ± 0.30 ng/ml down to 0.145 ± 0.02 ng/ml, corresponding to a 98% reduction, suggesting that Rac1 is an important regulator of platelet shedding of CD40L in abdominal sepsis. It was therefore of great interest to examine the direct effect of Rac1 inhibitor on platelet expression and secretion of CD40L. Interestingly, we found that PAR4 markedly increased surface expression of CD40L on platelets. Notably, pre-incubation with NSC23766 significantly decreased PAR4-induced surface mobilization of CD40L on platelets (Figure R5).

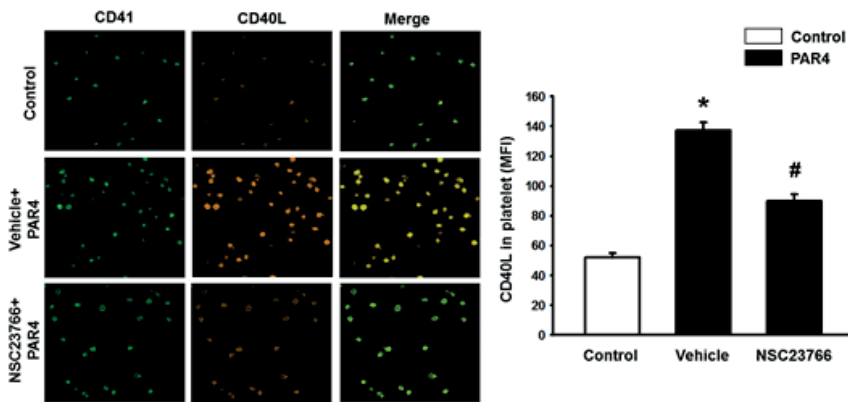


Figure R5. Rac1 regulates platelet shedding of CD40L. Representative image of confocal microscopy showing surface expression of CD40L on isolated platelets. Summarized data showing mean fluorescence intensity (MFI) of CD40L expression on platelet. Non-stimulated platelets served as control. Bars represent mean \pm SEM and $n = 4$. * $P < 0.05$ vs. control, # $P < 0.05$ vs. Vehicle+PAR4.

Moreover, we found that NSC23766 reduced PAR4-induced platelet secretion levels of sCD40L in the supernatant dose dependently (Figure R6) which is in line with confocal analysis of CD40L expressions on platelet, suggesting that Rac1 directly control surface expression of CD40L on platelet.

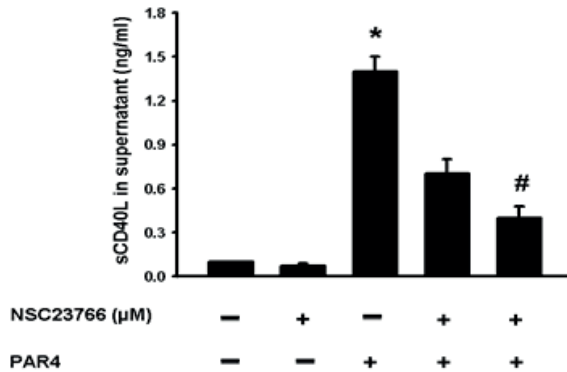


Figure R6. Isolated platelets were activated with protease-activated receptor-4 (PAR4) (200 nM) for 20 min with and without Rac1 inhibitor NSC23766 (10 and 100 μM), and then levels of soluble CD40L were determined in the supernatants by enzyme-linked immunosorbent assay. Nonstimulated platelets served as control. Bars represent mean ± SEM, and $n = 4-5$. * $P < 0.05$ vs control, # $P < 0.05$ vs vehicle ± PAR4.

In fact, several studies have indicated that MMP-9 play an important role in sepsis [157,203,204,205]. However, the role of MMP-9 in controlling CD40L shedding from platelet in sepsis has been studied [157], and it was therefore of interest to examine the role of Rac1 in neutrophil secretion of MMP-9 in sepsis. We found that CLP increased plasma levels of MMP-9 and the administration of NSC23766 decreased CLP-induced plasma levels of MMP-9 by 50%. Knowing that neutrophils are a major source of MMP-9 [206,207], immunostaining revealed abundant levels of MMP-9 in resting neutrophils (Figure. R7). Challenge with CXCL2 rapidly mobilized and decreased intracellular levels of MMP-9 in neutrophils. Notably, pre-incubation with NSC23766 completely inhibited MMP-9 secretion and maintained normal levels of MMP-9 in neutrophils exposed to CXCL2 (Figure R7).

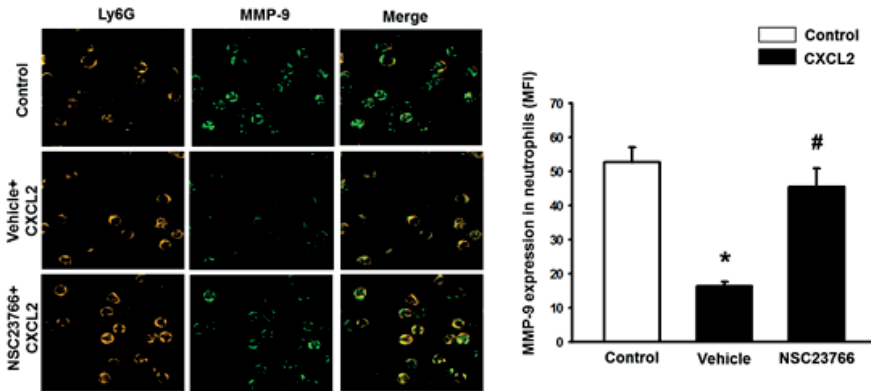


Figure R7. Rac1 regulates neutrophil secretion of MMP-9. Neutrophils were isolated by magnetic beads. Isolated neutrophils were incubated with CXCL2 (0.3 $\mu\text{g/ml}$) then was the level of MMP-9 in permeabilized neutrophils determined by confocal microscopy. Summarized data showing mean fluorescence intensity (MFI) of neutrophils-MMP-9. Bars represent mean \pm SEM and $n = 4$. * $P < 0.05$ vs. Control and # $P < 0.05$ vs. Vehicle+CXCL2.

Taken together, in this study we showed that inhibition of Rac1 signaling protect sepsis-induced lung injury through two different mechanisms. First, Rac1 controls surface mobilization of CD40L on activated platelets and second, Rac1 regulates MMP-9 secretion from neutrophils. Our data indicate that inhibition of Rac1 signaling might be a useful target in order to control pathological secretion and shedding of CD40L into the systemic circulation in abdominal sepsis.

III. *Rac1 regulate secretion of platelet-derived CC chemokines*

Platelets are critical contributors to abdominal sepsis. Several studies have revealed a functional role of platelets in regulating pathological aspects of the inflammatory response in severe infections [80,202]. For example, there are literatures showing that platelets play a key role in regulating neutrophil accumulation in septic lung damage [80]. Platelets are rich sources of different chemokines and platelet activation leads to the release of alpha-granule chemokines such as, CCL5 (RANTES) which attract leukocytes [89]. However, the signaling mechanism in regulating platelet-derived chemokines remains to be study. In this study, we found that Rac1 activity increased in the septic

platelets. Furthermore, we show that platelet depletion is markedly decreased the sepsis-evoked enhancement of CCL5 levels in plasma, indicating that CCL5 is highly abundant in platelets in abdominal sepsis. Moreover, pretreatment with Rac1 inhibitor NSC23766 significantly decreased sepsis-induced enhancement of plasma levels of CCL5, suggesting that Rac1 is an important regulator of CCL5 in sepsis (Figure R8).

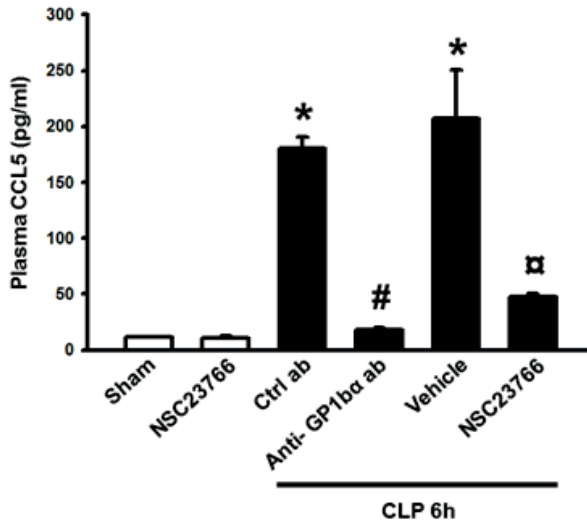


Figure R8. Rac1 regulates platelet secretion of CCL5 in sepsis. Animals were treated with vehicle, NSC23766 (5 mg/kg), a control ab (Ctrl ab) or an anti-GP1b α antibody before CLP induction. A) ELISA was used to quantify the levels of CCL5 in the plasma 6 h after CLP induction. Data represent mean \pm SEM and $n = 5$. * $P < 0.05$ vs. Sham, # $P < 0.05$ vs. Ctrl Ab + CLP, $^{\circ}P < 0.05$ vs. Vehicle + CLP.

The role of Rac1 GTPases in platelet secretion of CCL5 has not been examined so far. Herein, we investigated the possibility that Rac1, in addition to regulating lamellipodia formation and adhesion, may also affect platelet secretion of CCL5. Recent study have shown that Rac1 inhibitor NSC23766 inhibit agonist-induced mobilization of P-selectin in platelets [208] and that P-selectin as well as CCL5 are localized in the platelet α -granules [209]. Previous findings have shown that Rac1 regulates platelet secretion via actin polymerization, a process that seems to be dependent on phospholipase C and phosphatidylinositol 3-kinase signaling but not dependent on nicotinamide adenine dinucleotide phosphate-oxidase activity [210]. We quantified the expression of CCL5 in

isolated platelets, PAR4-induced secretion of CCL5 in isolated platelets *in vitro*. However, Rac1 inhibitor NSC23766 completely inhibited PAR4-triggered secretion of CCL5 in isolated platelets and it is supporting that Rac1 regulates CCL5 secretion from platelets. Thus, Rac1 might be a key regulator of α -granules.

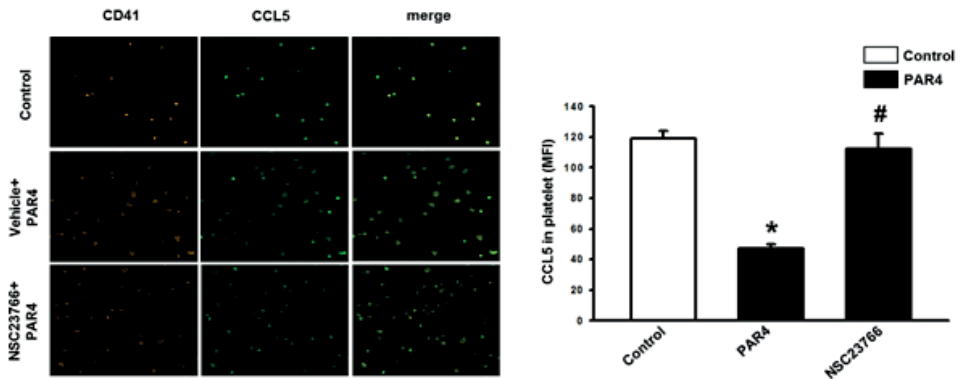


Figure R9. Rac1 regulates platelet secretion of CCL5 in sepsis. Isolated platelets were incubated with or without NSC23766 (10 μ M) and then stimulated with recombinant PAR4 (200 μ M) and the level of CCL5 in permeabilized CD41⁺ platelets was determined by confocal microscopy. Aggregate data showing mean fluorescence intensity of CCL5 in platelets. Non-stimulated platelets served as control. Data represent mean \pm SEM and $n = 5$. * $P < 0.05$ vs. Control, # $P < 0.05$ vs. Vehicle + PAR4.

Polymicrobial sepsis is characterized by widespread activation of the host innate immune system, including neutrophils, platelets and macrophages, which are the most insidious component is lung damage and consequently distributed gas exchange [211,212]. Sepsis induces platelet-derived chemokines which enhance recruitment of various hematopoietic cells to the septic lung [80]. In general, neutrophils recruitment is a central feature in septic lung injury [46]. Herein, we could show that targeting CCL5 function decreased lung levels of MPO, a marker of neutrophils sequestration in the tissue, by more than 47% in septic mice. This inhibitory effect on MPO correlated well with our finding that immunoneutralization of CCL5 reduced sepsis-induced neutrophil infiltration in the bronchoalveolar space by 42%, suggesting that CCL5 is a potent regulator of neutrophil accumulation in septic lung damage. Moreover, targeting CCL5 function by administration of anti-CCL5 antibody not only decreased neutrophilia but also attenuated

lung tissue damage and edema formation in abdominal sepsis. Considering the close relationship between neutrophils recruitment and tissue damage in septic pulmonary injury [11] it may be proposed that part of lung protective effect of inhibiting CCL5 is related to the reduced accumulation of neutrophils in the lung. These findings are in line with previous studies showing that targeting CCL5 function can reduce tissue injury in liver, myocardial reperfusion and lung injuries [14,15,16]. Suggest the role of CCL5 in controlling extravascular trafficking in multiple organs.

Secretion of CXC chemokines such as, CXCL1 and CXCL2 is known to coordinate neutrophils trafficking in the lung [213]. Herein, we found that CLP-induced plasma and lung formation of CXCL1 and CXCL2 were significantly reduced by 87% and 93% respectively in septic mice immunized against CCL5. Knowing that macrophages are important producers of CXC chemokines in the septic lung [214], we next asked whether lung macrophages might be a link between platelet-derived CCL5 and neutrophil recruitment in abdominal sepsis. In our model, CCL5 up-regulated CXCL2 production, consequently affecting the neutrophils recruitment. First, we administered CCL5 locally in the lung by intratracheal infusion and found that local CCL5 challenge significantly increased pulmonary levels of CXCL2 and the number of alveolar neutrophils (Figure C15). However, administration of the CXCR2 antagonist SB225002 abolished CCL5-induced neutrophil accumulation in the lung. In addition, we found that co-incubation of alveolar macrophages with CCL5 caused significant secretion of CXCL2, indicating that CCL5 is a potent stimulator of CXCL2 formation in alveolar macrophages. This notion was in line with our observation demonstrating that intratracheal administration of clodronate not only depleted lungs of alveolar macrophages but also abolished CCL5-evoked generation of CXCL2 in the lung.

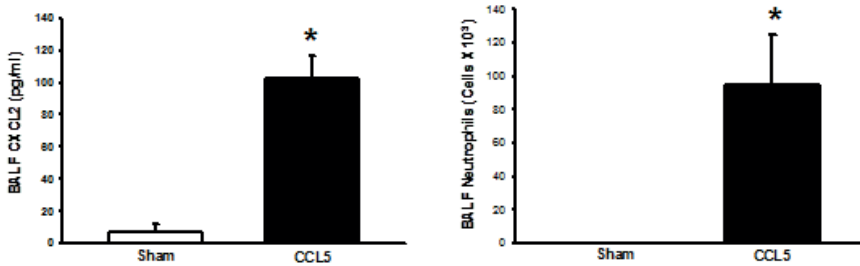


Figure R10. CCL5-induced neutrophil recruitment is dependent on CXCL2 formation. Levels of CXCL2 and number of neutrophils in the lung after intratracheal challenge with CCL5. Data represents mean \pm SEM and $n = 5$. * $P < 0.05$ vs. Sham and # $P < 0.05$ vs. Vehicle + CCL5.

These findings are more supported by an *in vitro* study, we isolated neutrophils from bone marrow of healthy mice and co-incubated with CXCL2 which exerted a strong chemotactic toward neutrophils in contrast, CCL5 had no chemotactic effect toward neutrophils, suggesting that CCL5 play an important role in regulating neutrophils recruitment indirectly in septic lung injury. Interestingly, neutrophils from CLP and sham mice expressed CXCR2 a receptor for CXCL2, but we observed that neutrophils from both sham and CLP animals did not express CCR1 nor CCR5 receptors for CCL5 in contrast, we observed that alveolar macrophages (AM) express the CCL5 receptors, i.e. CCR1 and CCR5 (Figure C11), suggesting that AM are an important target cell of CCL5 in mediating pulmonary formation of CXCL2 and inhibitory effect of anti-CCL5 antibody on neutrophils infiltration is related to changes in the pulmonary production of CXCL2 in the lung.

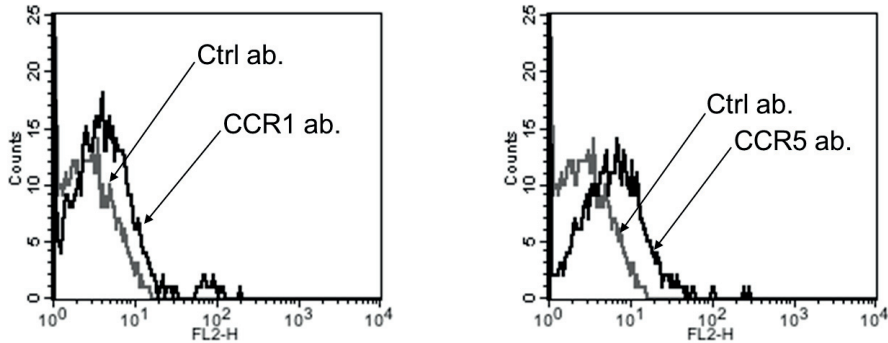


Figure R11. Alveolar macrophage surface expression of CCR1 and CCR5. Isolated alveolar macrophages were stained with antibodies against CCR1 and CCR5 as described in the methods section.

It must be noted that Ness *et al* have previously shown that CCL5 augments the production of CXCL2 and IFN- γ in a CCR1 dependent manner. The novel findings in the present study are, (i) the identification of platelets as a source of CCL5, (ii) determining the importance of Rac1 signaling in CCL5 production and (iii) the delineation of the role of alveolar macrophages as a cellular middleman that translates CCL5 signal into CXCL2 to which neutrophils are responsive.

In conclusion, these results indicate that Rac1 activity is increased in platelets and regulates platelet secretion of CCL5 in abdominal sepsis. In addition, our findings show that CCL5 regulates neutrophil recruitment in septic lung injury via activation of alveolar macrophages leading to local secretion of CXCL2. Thus, our novel data not only elucidates complex mechanisms regulating pulmonary neutrophil trafficking in sepsis but also suggest that targeting Rac1 signaling and platelet-derived CCL5 might be a useful way to control pathological inflammation and tissue damage in the lung in abdominal sepsis

IV. Rac1 regulate secretion of platelet-derived CXC chemokines

An accumulating body of evidence implicates platelets in the pathophysiology of shock. Upon activation, platelets rapidly release high amounts of chemokines [215]. Among these chemokines, chemokine (C-X-C motif) ligand 4 (CXCL4) which also known as

platelet factor 4 [PF4] is stored in micromolar concentrations in the alpha-granules of platelets [86,215,216]. CXCL4, a 70 –amino acid, lysine-rich, 7.8-kDa chemokine, is mainly synthesized by megakaryocytes and comprises 2-3% of the total released protein from mature platelets [216]. Recent studies reported that CXCL4 $-/-$ mice showed a strongly decreased infiltration of neutrophils in models of liver fibrosis [14] and Mesenteric ischemia/ reperfusion injury [217]. However, the signaling mechanism of platelet-derived chemokines CXCL4 secretion is elusive.

In the light of these findings, we hypothesized an important role of Rac1 signaling in regulating the platelet-derived chemokine CXCL4 in abdominal sepsis. Herein, we found that CLP increased the levels of Rac1-GTP in platelets and CXCL4 in plasma. However, administration of Rac1 inhibitor of NSC23766 abolished CLP-evoked Rac1 activation in platelets and significantly decreased plasma level of CXCL4 by 77% in CLP animals (Figure R12), indicating that Rac1 signaling play a critical role in regulating circulating levels of CXCL4 in abdominal sepsis.

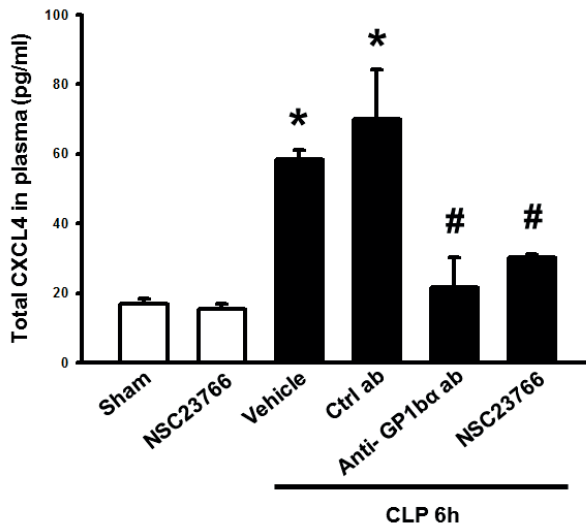


Figure R12. Rac1 regulates platelet secretion of CXCL4 in sepsis. Animals were treated with vehicle, NSC23766 (5 mg/kg), a control ab (Ctrl ab) or an anti-GP1ba antibody before CLP induction. a) ELISA was used to quantify the levels of CXCL4 in the plasma 6 h after CLP induction. Data represent mean \pm SEM and $n = 4$. * $P < 0.05$ vs. Sham or Control, # $P < 0.05$ vs. Ctrl Ab + CLP, ° $P < 0.05$ vs. Vehicle + CLP.

In order to investigate the direct role of Rac1 in regulating platelet-derived secretion of CXCL4, It was found that co-incubation of platelets with NSC23766 abolished PAR4-induced platelet secretion of CXCL4 *in vitro*, showing for the first time that Rac1 regulates CXCL4 secretion from platelets *in vivo* and *in vitro*.

It is well known that depletion of neutrophils protects against septic lung injury, showing that neutrophils accumulation is a critical component in sepsis [10,11,218]. In the present study, we could document that immunoneutralization of CXCL4 by administration of anti-CXCL4 antibody significantly decreased the pulmonary MPO activity by 57% in septic animals, suggesting an important role of CXCL4 in pulmonary neutrophilia in polymicrobial sepsis (Figure R13).

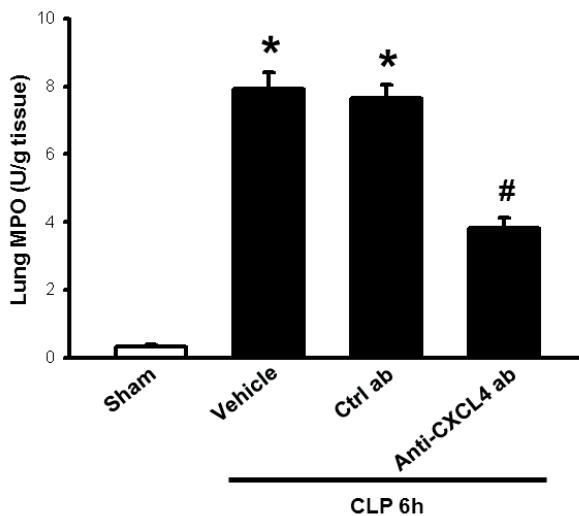


Figure R13. CXCL4 regulates pulmonary recruitment of neutrophils in sepsis. Lung MPO levels at 6 h post-CLP. Data represent mean \pm SEM and $n = 4$. * $P < 0.05$ vs. Sham and # $P < 0.05$ vs. Ctrl ab + CLP.

Neutrophils express constitutively high levels of the integrin Mac-1, which undergo a conformational change to bind to common endothelial cell surface molecule ICAM1. Mac-1 binding to ICAM1 is essential for firm adhesion. However, inhibition of CXCL4 had no effect on neutrophil expression of Mac-1 in septic mice, indicating that CXCL4 is

not implicated in Mac-1 expression on neutrophils. Recruitment of circulating leukocytes to sites of pathogen entry or inflammation involves two separate migration processes, termed extravasations and chemotaxis via CXC chemokines, including CXCL1 and CXCL2. Previous studies have shown that CXC chemokines are potent activator of neutrophils and play an important role in septic lung injury [213]. However, immunoneutralization of CXCL4 abolished plasma and lung levels of CXC chemokines in CLP animals. These results indicate that CXCL4 might control neutrophil accumulation indirectly via generation of CXC chemokines in the septic lung. This conclusion has been confirmed by local intratracheal challenge with CXCL4 which enhanced CXCL2 generation and neutrophils recruitment. That local injection of CXCL4 can provoke neutrophil infiltration in the lung is supported by a previous study showing that local administration of CXCL4 triggers accumulation of neutrophils in the skin [219]. The enhancement of neutrophils in response to CXCL4 challenge in the lung mice may be mediated (at least in part) by increase in CXCL2 in the alveolar space of these animals. In order to support of hypothesis that alveolar macrophages might be a target cell of CXCL4. We found that co-incubation of isolated alveolar macrophage with different dose of CXCL4 enhanced CXCL2 secretion (Figure R14). In this context, it is interesting to note that CXCL4 co-operates with other platelet-derived chemokines, such as CCL5. For example, it has been shown that CXCL4 and CCL5 cooperate to promote respiratory burst in macrophages [220]. Along these lines, we could show herein that combining CXCL4 and CCL5 further increases macrophage secretion of CXCL2, indicating that platelet-derived chemokines might co-operate at multiple stages in the host response to bacterial invasion. These finding suggest for the first time that CXCL4 promote alveolar macrophage secretion of CXCL2.

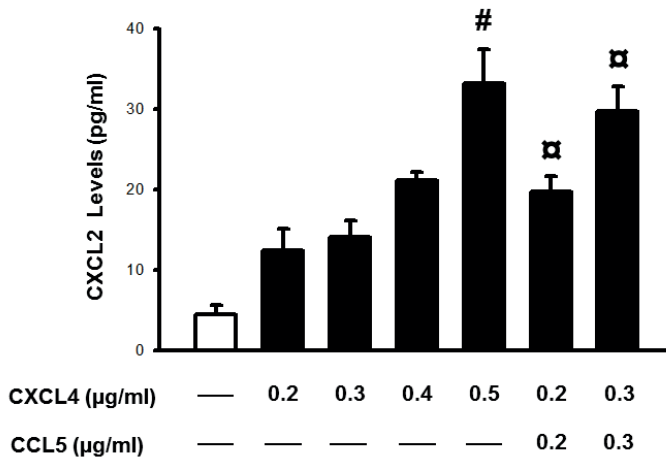


Figure R14. Isolated alveolar macrophages were stimulated with indicated doses of CXCL4 and CCL5 then CXCL2 levels were determined by use of ELISA. Data represents mean \pm SEM and $n = 4$. [#] $P < 0.05$ vs. Control (-), [□] $P < 0.05$ vs. Vehicle + CXCL4 and CCL5.

Thus, our findings suggest that Rac1 signaling is enhanced in platelets and regulates platelet secretion of CXCL4 in polymicrobial sepsis. Moreover, our results indicate that CXCL4 controls neutrophil accumulation via secretion of CXCL2 from alveolar macrophages in septic lung injury. Thus, these findings not only delineate complex mechanisms of neutrophil trafficking in sepsis but also suggest that targeting platelet-derived CXCL4 might be an effective way to ameliorate inflammation and tissue damage in septic lung damage.

Conclusions

1. Rac1 signaling plays a critical role in sepsis by regulating neutrophil infiltration in the lung. Inhibition of Rac1 signaling by using NSC23766 not only ameliorates inflammatory process and CXCL2 formation but also protects against tissue damage.
2. Rac1 plays a critical role in platelet shedding of sCD40L. Rac1 controls surface mobilization of CD40L on activated platelets and MMP-9 secretion from neutrophils.
3. Rac1 controls platelet secretion of CCL5, a potent stimulator for neutrophils accumulation in septic lung damage via generation of CXCL2 in alveolar macrophage in a CCR1 dependent manner.
4. Rac1 signaling enhanced in activated platelet and regulates platelet-derived CXCL4 in polymicrobial sepsis. In addition, CXCL4 controls neutrophil accumulation via secretion of CXCL2 from alveolar macrophages in septic lung injury.

These findings elucidate the mechanistic role of Rac1 signaling in regulating pathological inflammation in the lung during sepsis as summarized in Figure C1.

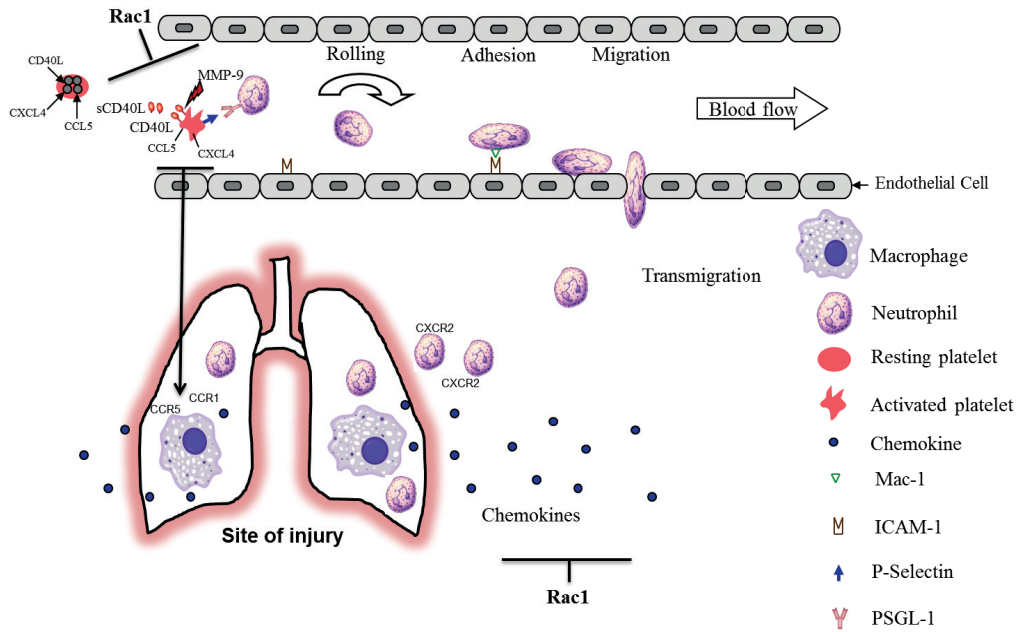


Figure C1. The schematic diagram summarizes the proposed hypothesis behind Rac1 signaling regulates platelet-dependent inflammation in abdominal sepsis. During induction of CLP, alveolar macrophages release chemokines (CXCL1 and CXCL2) which are known to activate endothelial cells and promote neutrophils recruitment. Neutrophil recruit to the site of inflammation by selectin and integrin families. Activated platelet release CD40L on the surface and subsequently cleavage by MMP-9 which is released from activated neutrophils, sCD40L into the circulation leads to further tissue injury. Platelet α -granule also contains CCL5 and CXCL4. Activated platelets release CCL5 and CXCL4 and facilitate neutrophils infiltration into septic lung injury via production of CXC chemokines by macrophages.

Sammanfattning på Svenska

Sepsis, blodförgiftning är en systemisk inflammatorisk reaktion syndrom vid en lokal eller systemisk infektion som leder till överproduktion av proinflammatoriska cytokiner och det ultimata misslyckandet av flera organsystem. Sepsis är ett potentiellt allvarligt och komplicerat kliniskt syndrom samt är en av de vanligaste orsakerna till döden på intensivvårdsavdelningar. Cirka 200 per 100 000 invånare i Sverige drabbas årligen av svår sepsis.

Rac1 är en Rho familjen GTPases, som spelar en viktig roll i celladhesion och motilitet, men lite är känt om en potentiell roll Rac1 i kontrollen sepsis mediterad intracellulära signalvägar. Vi antog att Rac1 skulle vara inblandade i sepsis mediterad signalvägar som leder till aktivering av inflammatoriska celler. Syftet med den här avhandlingen var studera den potentielle betydelsen av Rac1 vid sepsis.

Delarbete (I), Rac1 signalering spelar en viktig roll i polymikrobiella sepsis inducerad med cecal ligation och punktering (CLP). Denna studie visar att Rac1 signalering reglerar sepsisinducerad inflammation i lungan genom att minska kemokin produktion och Mac-1 expression på neutrofiler. Rac1 hämmare (NSC23766 5 mg/kg) sänker lungödem, vävnadsskada och systemisk proinflammatoriska cytokiner i septisk mös, vilket tyder på att målsökning Rac1 kan vara användbart tillvägagångssätt för att skydda mot lungskada i buksepsis. Delarbete (II), i denna studie visar vi att inhibering av Rac1 signalering skydda mot sepsisinducerad lungskada genom två olika mekanismer, först Rac1 kontrollerar ytan mobilisering av CD40L på aktiverade trombocyter och andra, Rac1 reglerar MMP-9 sekret från neutrofiler. Våra resultat visar att hämning av Rac1 signalering kan spela en viktig roll för att kontrollera patologisk sekretion och utsöndring av CD40L i den systemiska cirkulationen i buksepsis. Delarbete (III), denna studie visar att Rac1 aktivitet ökar i trombocyter och reglerar trombocyter sekretion av CCL5 i buksepsis. Våra resultat visar att CCL5 reglerar neutrofila rekrytering i septisk lungskada via aktivering av alveolära makrofager som leder till lokal utsöndring av CXCL2. Således, vårt resultat avslöjar inte bara komplexa mekanismer som reglerar lung neutrofila rekryt i sepsis men också tyder på att hämningen av Rac1 signalering och trombocytdriverad CCL5 kan vara ett bra sätt att kontrollera patologisk inflammation och vävnadsskada i lungan i buksepsis. Delarbete (IV), Rac1 aktivitet ökad i trombocyter

och reglerar trombocyt utsöndring av CXCL4 i buksepsis. Denna studie visar att CXCL4 kontrollerar neutrofil ackumulering via utsöndring av CXCL2 från alveolära makrofager i septisk lungskada. Dessa fynd inte bara beskriva komplexa mekanismer av neutrofila infiltrationen med sepsis, men också tyder på att hämningen av trombocytderiverad CXCL4 kan vara ett effektivt sätt att hindra inflammation och vävnadsskada i septiska lungskador.

Acknowledgements

This thesis is the result of my years of research in Department of Surgery, Malmö, Lund University, Sweden. I would like to express my acknowledgements to everyone who have contributed to this thesis either directly or mentally.

First and foremost, I'm deeply grateful to my supervisor, Professor **Henrik Thorlacius**, for his guidance, patience and support. I consider myself extremely lucky for being able to work with a very considerate and encouraging professor like him. Without his offering to accomplish this research, I would not be able to finish my study at Lund University.

Milladur Rahman, I am extremely grateful for your continuous assistance, suggestions and support throughout my project. Whenever I needed you, I found you beside me. You always advised me to be perfect in all my works. You taught me lessons, which I surely need throughout my research life. Your support and help are highly appreciated.

I am especially grateful to Professor **Bengt Jeppsson**, for his kind support and continuous interest in my work. His vast clinical and scientific knowledge have been invaluable. Also many thanks to his wife **Christina** and thanks a lot for the hospitality.

Anne-Marie Rohrstock, special thanks to you for your great and valuable technical support in the lab and ordering the research material. It is impossible to run any experiment without your kind assistance. Tack så hemskt mycket!

I would like to thank **Zirak Hasan**, the former PhD student who patiently taught me all the basic principles of working in the lab.

It is a pleasure to thank my friend and officemate **Amr Al-Haidari**, it was enjoyable to discuss with you and get fruitful suggestions and technical support. (You are the best!).

I would like to express my sincere thanks to **Pernilla Siming** it was a great pleasure for me to meet you. Your helpfulness is highly appreciated.

The members of our group have contributed immensely to my personal and professional time. I would like to express my gratefulness to my present and past lab member **Yongzhi Wang (Erik)**, **Ling Tao Luo (Tomas)**, **Songen Zhang (Tony)**, **Su Zhang**, **Yousheng Wang**, **Mohammad Yasser Arafat**, **Changhui**, **Johanna Puggi** and former lab member **Karzan Palani** and **Darbaz Awla** and from clinic **Ingrid Palmquist**.

I would like to express my gratitude to all my present and past friends at CRC in particular **Tavga Salim, Taman Mahdi, Sinar Mohammed, Shefaa Al-asfoor, Mahboubeh Daneshpajoo, Lubna Mehdawi and Israa Mohammed.**

I am especially thankful to my dear relatives and friends in Malmö for their great moments and endless support, in particular **Azad, Ariana, Suhaila, Nafih, Rasti, Sirwa, Dilshad, Ivan, Sherin and Amin.** You are all amazing persons million thanks to you again.

I would like to express my gratitude to my children's teachers at Malmö International School and Hålsjöns Förskola, in particular **Zandrina McGowan, Julia Erlandsson, Carina Filbert, Tanja Obradovic, Suzan Fatah, Ann-Louise Christiansson and Lars** for teaching and caring my children (**Sidra and Lawi**).

I am most grateful to my Dad (**Ahmad Hwaiz**) and my Mum (**Atia Mohammed**). They have always loved me and supported my every choice. As I know, they are the happiest and the most proud when seeing their daughter gets this degree, I dedicate this thesis to them. I'm also thankful for the great support brought to me by my sisters **Avin** and **Azhin** and my brothers **Rebin** and **Rassan**.

In particular, I would like to thank my partner and husband, Dr. **Mohammed Merza**, who is always beside me to help and encourage me to go through all the difficulties. None of this would have been possible without his constant support, thanks to God that you, **Sidra** and **Ahmed (lawi)** in my life.

I would like to convey my appreciation and gratitude to **Ministry of Higher Education** and **Hawler Medical University** of Kurdistan Regional government for the financial support. Special thanks to Dr. **Vian Afan Naqshbandi** and Dr. **Dara Meran**.

Finally, I would like to express my apologies to those that I haven't mentioned personally.

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

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ARTICLE INFO

Article history:

Received 15 January 2013

Received in revised form

13 February 2013

Accepted 20 February 2013

Available online 15 March 2013

Keywords:

Adhesion

Inflammation

Leukocytes

Migration and platelets

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 cecal ligation and puncture (CLP). Bronchoalveolar lavage fluid and lung tissue were collected for the quantification of neutrophil recruitment and edema and CXC chemokine formation. Blood was collected for the determination of Mac-1 on neutrophils and proinflammatory compounds in plasma. Gene expression of CXC chemokines and tumor necrosis factor alpha was determined by quantitative reverse transcription–polymerase chain reaction 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 messenger RNA levels of CXC chemokines and tumor necrosis factor alpha in alveolar macrophages. Rac1 inhibition decreased the CLP-induced increase in plasma levels of high mobility group protein B1 and interleukin 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.

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1. 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

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0022-4804/\$ – see front matter © 2013 Elsevier Inc. All rights reserved.
<http://dx.doi.org/10.1016/j.jss.2013.02.045>

production of proinflammatory 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, constitutes a key feature in systemic inflammation, in which the most feared complication is pulmonary damage [3]. Numerous studies have documented that the 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 P-selectin glycoprotein ligand-1 (PSGL-1) [5] and lymphocyte function-associated antigen-1 (LFA-1) [6], and coordinated by secreted CXC chemokines, such as CXCL1 and CXCL2 [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 a precursor not only 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 the Rac activity and regulation of mitogen-activated protein kinases, such as JNK/c-Jun [14,15] and p38 mitogen-activated protein kinase [16–18], which are known to be involved in proinflammatory 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 (CLP).

2. Materials and methods

2.1. Animals

Experiments were performed using male C57BL/6 mice weighing 20–25 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 the administration of 7.5 mg (intraperitoneal [i.p.]) of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg (i.p.) of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight.

2.2. 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. NSC23766 is a specific and reversible Rac1 inhibitor that competitively inhibits interaction between Rac1 and Rac-specific guanine nucleotide exchange factors. NSC23766 does not affect the activity of related Rho guanosine triphosphatases (GTPases) including Cdc42 or RhoA *in vitro* or *in vivo* [24,25]. 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 Burkert chamber. Next, the lung was perfused with PBS, 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 Rac1 activation assay, myeloperoxidase (MPO) assay and enzyme-linked immunosorbent assay (ELISA) as described subsequently.

2.3. Rac1 activity

Rac1 activation assay was performed by using Rac1 activation assay kit (Pierce Biotechnology, Rockford, IL) as described previously [26]. Briefly, 50 mg of lung tissues were minced and homogenated in lysis buffer on ice, and the samples were centrifuged at 15,000 *g* for 15 min; 10 μL from each supernatant was removed to measure protein content using Pierce BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL) and the rest of the volume was used for the pull-down assay. Supernatant containing equal amount of proteins was then diluted with $2\times$ sodium dodecyl sulfate sample buffer and boiled for 5 min. Proteins were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% gel). After transfer to a nitrocellulose membrane (BioRad, Hercules, CA), 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 4°C overnight. Binding of the antibody was visualized using peroxidase-conjugated anti-mouse antibody (1:100,000; Pierce Biotechnology) at room temperature for 1 h and enhanced chemiluminescence method (BioRad). β -Actin was used as an internal control for total Rac1. The expression of internal control β -Actin protein was confirmed by performing western blots on an aliquot taken before protein affinity purification.

2.4. Systemic leukocyte count

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

2.5. BALF

Animals were placed supine, and the trachea was exposed by dissection. An angiocatheter was inserted into the trachea. BALF was collected by five washes of 1 mL of PBS containing 5 mM EDTA. The numbers of monomorphonuclear and polymorphonuclear leukocyte cells were counted in a Burker chamber.

2.6. 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 reweighed. The change in the ratio of wet weight-to-dry weight was used as indicator of lung edema formation.

2.7. 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 gram tissue.

2.8. 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 and eosin. Lung injury was quantified in a blinded manner by adoption of a modified scoring system [27,28], including size of alveoli, thickness of alveolar septae, alveolar fibrin deposition, and neutrophil scoring system infiltration graded on a 0 (absent) to 4 (extensive) scale. In each tissue sample, five random areas were scored, and the mean value was calculated. The histology score is the sum of all four parameters.

2.9. 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 high mobility group protein B1 (HMGB1) (Chondrex, Redmond, WA) and interleukin (IL) 6 (R & D Systems) according to manufacturer's instructions.

2.10. 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 Fc γ III/II receptors to reduce nonspecific labeling and then incubated with phycoerythrin-conjugated anti-Gr-1 (clone RB6-8C5, rat IgG2b; eBioscience, Frankfurt, Germany) and fluorescein isothiocyanate-conjugated anti-Mac-1 (clone M1/70, integrin α M china, rat IgG2b κ ; BD Biosciences Pharmingen, San Jose, CA) 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) and analyzed with Cell-Quest Pro software (BD Biosciences). A viable gate was used to exclude dead and fragmented cells.

2.11. 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 roswell park memorial institute medium (RPMI 1640) 1640 and then subsequently isolated by using Ficoll-Paque Research Grade (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated neutrophils was >70% as assessed in a haematocytometer. Neutrophils were then resuspended in PBS to 10^7 cells/mL and coincubated with 3 μ g/mL of recombinant mouse CXCL2 (R & D Systems) for 30 min at 37°C. Neutrophils were preincubated with NSC23766 (1 or 10 μ M) 20 min before challenge with CXCL2. Cells were stained and fixed for flow cytometric analysis of Mac-1 expression on neutrophils as described previously.

2.12. Isolation of alveolar macrophages and quantitative reverse transcription–polymerase chain reaction

In separate experiments, gene expression of CXCL1, CXCL2, and tumor necrosis factor alpha (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 before CLP ($n = 5$). Alveolar macrophages were isolated from BALF as described in detail [29]. 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 (18°C). 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, nonadherent cells were washed away by PBS. A total of $2\text{--}3 \times 10^5$ macrophages were obtained per mice, and the purity of macrophages was >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 complementary DNA 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 polymerase chain reaction (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'-GCCAATGAGCTGCGCTGCAATG C-3', CXCL1 (reverse) 5'-CTTGGGGACCTTTTAGCATCTT-3'; CXCL2 (forward) 5'-GTTCTCGGGCACTCCAGAC-3', CXCL2 (reverse) 5'-TTAGCCTTGCCCTTTGTTTCAGTAT-3'; TNF-α (forward) 5'-CCTCACACTCAGATCATCTTCTC-3', TNF-α (reverse) 5'-AGAT CCATGCCGTTGGCCAG-3'; and β-actin (forward) 5'-ATGTTTGA-GACCTTCAACACC-3', β-actin (reverse) 5'-TCTCCAGGGAGAA-GAGGAT-3'. Standard PCR curves were generated for each PCR product to establish linearity of the reverse transcription–polymerase chain reaction. PCR amplifications were performed in a total volume of 50 µL, containing 25 µL of SYBR-green PCR 2× master mix, 2 µL of 0.15 µM each primer, 0.75 µL of reference dye, and one 1 µL of complementary DNA as a template adjusted up to 50 µL with water. PCRs 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 those of β-actin in the same sample.

2.13. Neutrophil chemotaxis

Neutrophils isolated from bone marrow of healthy mice by the use of Ficol-Paque were preincubated with NSC23766 (1 or 10 µM) for 30 min, and 1.5 × 10⁶ neutrophils were placed in the upper chamber of the transwell inserts (5-µm pore size; Corning Costar, Corning, NY). 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 the migrated neutrophils were stained with Turks solution. Chemotaxis was determined by counting the number of migrated neutrophils in a Burkner chamber.

2.14. Statistics

Data were presented as mean values ± standard errors of the means. 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).

3. Results

3.1. 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).

3.2. Rac1 regulates neutrophil recruitment and lung injury

To examine neutrophil infiltration in septic lung damage, we analyzed both the activity of MPO, an indicator of neutrophils, and number of neutrophils in the BALF. The 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 four-fold (Fig. 2A, P < 0.05 versus 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 versus vehicle + CLP, n = 5). Moreover, the number of BALF neutrophils increased by 12-fold 24 h after CLP induction (Fig. 2B, P < 0.05 versus Sham, n = 5). Rac1 inhibition reduced neutrophil infiltration into the bronchoalveolar space by 94% in CLP animals (Fig. 2B, P < 0.05 versus vehicle + CLP, n = 5). CLP caused significant lung edema formation. More specifically, the lung wet-to-dry ratio increased from 4.3 ± 0.2 to 6.3 ± 0.1 in septic mice (Fig. 2C, P < 0.05 versus Sham, n = 5). Administration of NSC23766 decreased the wet-to-dry ratio by 90% in septic mice (Fig. 2C, P < 0.05 versus vehicle + CLP, n = 5). Histologic 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 morphologic injury revealed that CLP increased the lung injury score by more than three-fold and that Rac1 inhibition attenuated the lung injury score in CLP mice (Fig. 3D, P < 0.05 versus vehicle + CLP, n = 5). Moreover, CLP caused leukocytopenia after 24 h (Table, P < 0.05 versus Sham, n = 5). The CLP-induced leukocytopenia was decreased in mice pre-treated with NSC23766 (Table).

3.3. 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

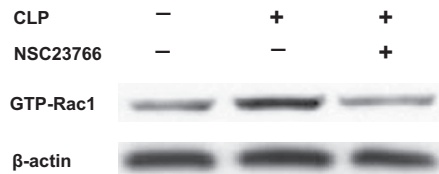


Fig. 1 – Six hours after CLP challenge and NSC23766 treatment, lungs from control group (–) and CLP group (+) mice were harvested for Rac1 activities. β-Actin was used as an internal control for total Rac1, n = 3.

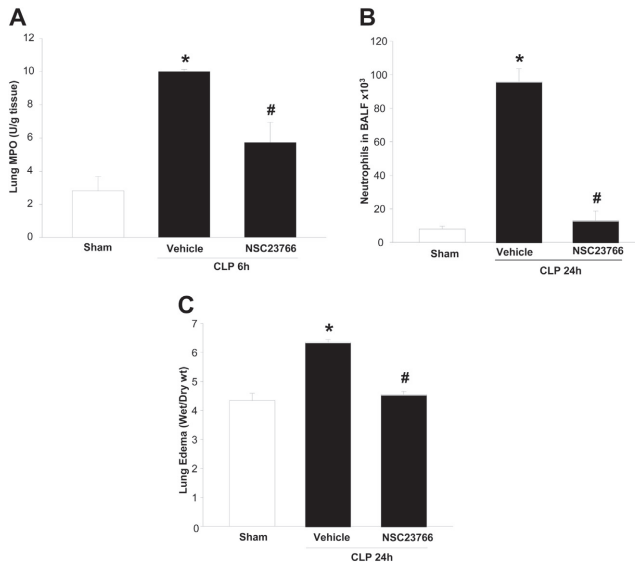


Fig. 2 – Effects of NSC23766 on CLP-induced pulmonary infiltration of neutrophils. (A) Lung MPO levels at 6 h after CLP induction, (B) number of BALF neutrophils, and (C) edema formation in the lung 24 h after CLP induction. Animals were treated with NSC23766 (5 mg/kg) or vehicle (PBS) before CLP induction.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

on the surface of peripheral blood neutrophils in CLP animals, indicating that circulating neutrophils are activated in this model (Fig. 4A and B, $P < 0.05$ versus Sham, $n = 5$). Rac1 inhibition reduced CLP-induced expression of Mac-1 on blood neutrophils (Fig. 4A and B, $P < 0.05$ versus vehicle + CLP, $n = 5$). Mean fluorescence intensity 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 and D). Coincubation of NSC23766 with CXCL2 significantly decreased neutrophil upregulation of Mac-1 (Fig. 4C and D), indicating that Rac1 regulates Mac-1 expression in neutrophils.

3.4. 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. 5). CLP markedly increased pulmonary generation of CXCL1 and CXCL2 (Fig. 5, $P < 0.05$ versus Sham, $n = 5$). Inhibition of Rac1 by administration of NSC23766 attenuated CLP-provoked formation of CXCL1 and CXCL2 in the lung by >86% and 96%, respectively (Fig. 5, $P < 0.05$ versus 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 messenger RNA (mRNA) levels of CXCL1, CXCL2, and TNF- α in alveolar macrophages (Fig. 6, $P < 0.05$ versus 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. 6, $P < 0.05$ versus vehicle + CLP, $n = 5$).

3.5. 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 six-fold from 8.6 ± 1.1 ng/mL up to 55.0 ± 8.2 ng/mL (Fig. 7A, $P < 0.05$ versus sham, $n = 5$). Pretreatment with NSC23766 reduced CLP-provoked generation of HMGB1 to 17.0 ± 5.5 ng/mL (Fig. 7A, $P < 0.05$ versus PBS + CLP, $n = 5$). In addition, we observed that the plasma levels of IL-6 were increased in CLP mice compared with sham mice (Fig. 7B, $P < 0.05$ versus 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, $P < 0.05$ versus PBS + CLP, $n = 5$). Thus, NSC23766 significantly reduced CLP-provoked plasma levels of HMGB1 by 81% and IL-6 by 80%.

3.6. Rac1 regulates neutrophil migration *in vitro*

To examine whether NSC23766 might exert a direct effect on neutrophil migration, we examined neutrophil chemotaxis

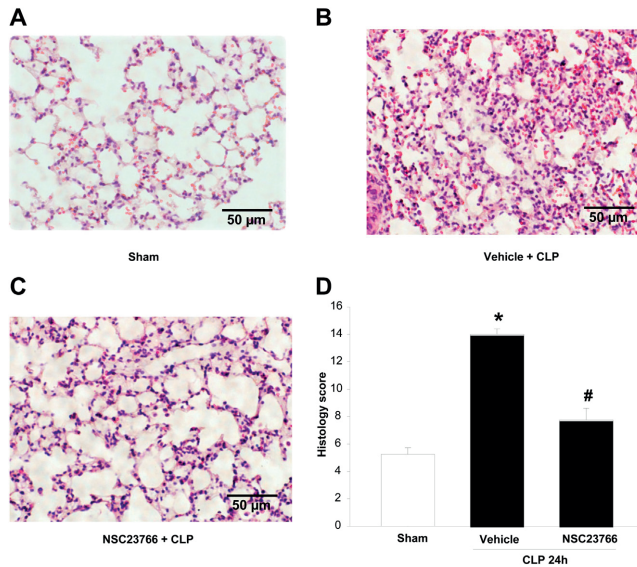


Fig. 3 – Tissue morphology 24 h after CLP induction. Representative hematoxylin and eosin sections of lung are shown. (A) Sham-operated animals served as negative controls. (B) Animals were treated with vehicle (PBS) or (C) 5 mg/kg of NSC23766 before CLP induction. (D) Lung injury score as described in Materials and Methods section. Data represent mean ± standard error of the mean and n = 5. *P < 0.05 versus Sham and #P < 0.05 versus vehicle + CLP. Scale bar indicates 50 μm. (Color version of figure is available online.)

in vitro by using neutrophils isolated from the bone marrow. We found that 100 ng/mL of CXCL2 caused a clear-cut increase in neutrophil migration over a period of 120 min (Fig. 8, P < 0.05 versus PBS). Preincubation of neutrophils with NSC23766 (1 and 10 μM) dose dependently reduced CXCL2-provoked neutrophil migration (Fig. 8, P < 0.05 versus CXCL2 + PBS).

4. 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 [30,31]. 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 [32–34]. 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 [35,36]. Herein, we could document that inhibition of Rac1 decreased pulmonary MPO activity, a marker of neutrophils, by 58% in septic animals. This

Table – 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†

MNL = mononuclear leukocytes; PMNL = polymorphonuclear leukocytes.

Blood was collected from vehicle (PBS)- and NSC23766 (5 mg/kg)-treated mice exposed to CLP for 24 h and sham-operated animals. Cells were identified as MNLs and PMNLs. Data represent mean ± standard error of the mean and 10⁶ cells/mL.

*P < 0.05 versus Sham.

†P < 0.05 versus vehicle + CLP and n = 5.

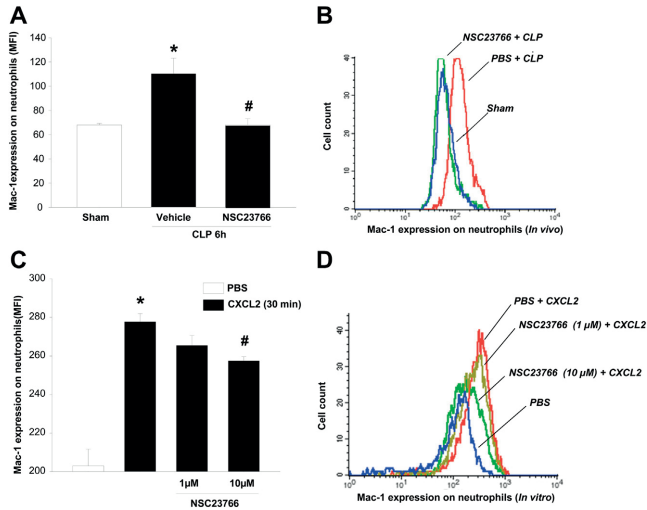


Fig. 4 – Rac1 activity regulates Mac-1 expression on neutrophils. Mac-1 expression on circulating neutrophils (Gr-1⁺ cells) 6 h after CLP induction in sham mice or animals treated with vehicle (PBS) or NSC23766 (5 mg/kg). (A) Representative dot plot and histogram and (B) data in aggregate. Data represent mean ± standard error of the mean and n = 5. *P < 0.05 versus Sham and #P < 0.05 versus vehicle + CLP. Mac-1 expression on isolated bone marrow neutrophils (Gr-1⁺ cells) incubated with CXCL2 (3 μg/mL) and vehicle (PBS) or NSC23766 (1 or 10 μM). (C) Representative dot plot and histogram and (D) data in aggregate. Data represent mean ± standard error of the mean and n = 5. *P < 0.05 versus PBS and #P < 0.05 versus PBS + CXCL2. MFI = mean fluorescence intensity. (Color version of figure is available online.)

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 [37]. Neutrophils are particularly activated and attracted by CXC chemokines, such as CXCL1 and CXCL2, which are homologues of human IL-8 [38]. 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

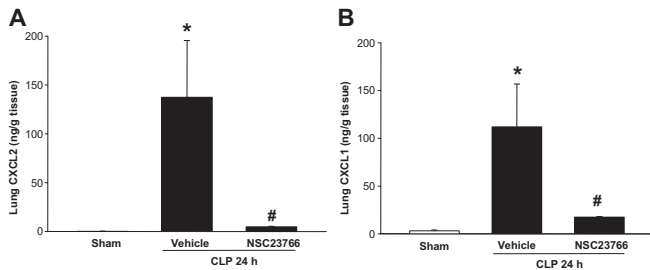


Fig. 5 – Rac1 activity regulates CXC chemokine formation in the lung. ELISA was used to quantify the levels of (A) CXCL2 and (B) CXCL1 in the lung 24 h after CLP induction. Animals were treated with vehicle (PBS) or NSC23766 (5 mg/kg) before CLP induction. Sham-operated animals served as negative controls. Data represent mean ± standard error of the mean and n = 5. *P < 0.05 versus Sham and #P < 0.05 versus vehicle + CLP.

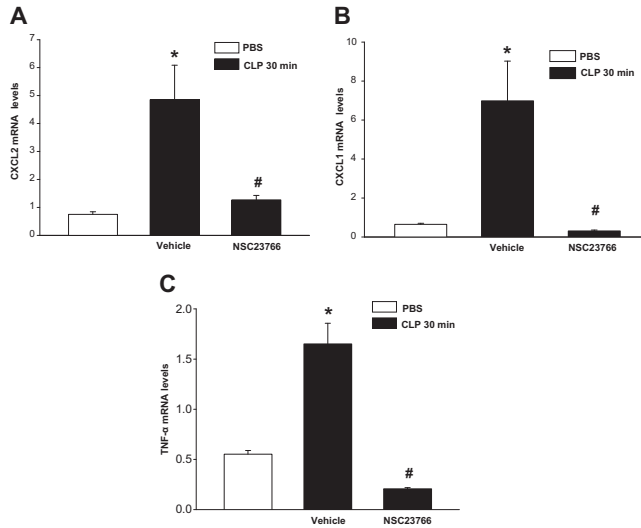


Fig. 6 – Rac1 activity regulates gene expression of CXC chemokines and TNF- α in alveolar macrophages. Quantitative reverse transcription–polymerase chain reaction was used to determine the levels of mRNA expression of (A) CXCL2, (B) CXCL1, (C) and TNF- α in alveolar macrophages 30 min after CLP induction. Animals were treated with vehicle (PBS) or NSC23766 (5 mg/kg) before CLP induction. Sham-operated animals served as negative controls. Data represent mean \pm standard error of the mean and $n = 5$. * $P < 0.05$ versus Sham and # $P < 0.05$ versus vehicle + CLP.

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 TNF- α in alveolar macrophages, indicating that Rac1 is an important signaling 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 upregulation 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

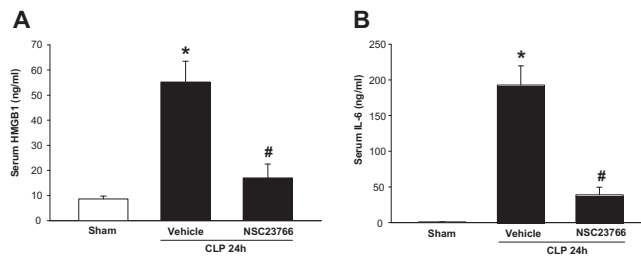


Fig. 7 – Rac1 activity regulates systemic inflammation in sepsis. ELISA was used to quantify the levels of (A) HMGB1 and (B) IL-6 in the plasma 24 h after CLP induction. Animals were treated with vehicle (PBS) or NSC23766 (5 mg/kg) before CLP induction. Sham-operated animals served as negative controls. Data represent mean \pm standard error of the mean and $n = 5$. * $P < 0.05$ versus Sham and # $P < 0.05$ versus vehicle + CLP.

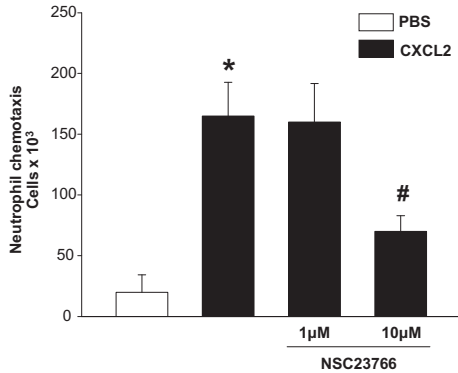


Fig. 8 – Rac1 activity regulates neutrophil migration in vitro. Neutrophils isolated from bone marrow were analyzed for their migration in response to PBS (control), PBS + CXCL2 (100 ng/ml) with or without preincubation of neutrophils with NSC23766 (1 or 10 μM). Data represent mean ± standard error of the mean and $n = 5$. * $P < 0.05$ versus PBS and # $P < 0.05$ versus CXCL2 + PBS.

significantly attenuated by the Rac1 inhibitor, suggesting that Rac1 signaling also directly controls neutrophil Mac-1 upregulation 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 [39], 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, that is, production of CXC chemokines in the lung, Mac-1 upregulation on the surface of neutrophils, and neutrophil chemotaxis.

HMGB1 is a potent proinflammatory cytokine and a late mediator in endotoxemia and sepsis [40] as well a predictor of clinical outcome in patients with severe sepsis [41]. 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 [42]. 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.

5. 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 upregulation 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.

Acknowledgment

This study was supported by the Swedish Medical Research Council (2012-3685), Einar och Inga Nilssons stiftelse, Greta och Johan Kocks stiftelse, Fröken Agnes Nilssons stiftelse, Magnus Bergvalls stiftelse, Mossfelts stiftelse, Nanna Svartz stiftelse, Ruth och Richard Julins stiftelse, Dir. A. Pahlsson's Foundation, Swedish Cancer Foundation, Malmö University Hospital Cancer Foundation, Lundgren's Foundation, Gunnar Nilsson's Foundation and Apotekaren Hedberg's Fond, Malmö University Hospital, and Lund University. R.H. is supported by Hawler Medical University and Kurdistan Regional Government.

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Paper II

Rac1 regulates platelet shedding of CD40L in abdominal sepsis

Rundk Hwaiz¹, Milladur Rahman¹, Enming Zhang² and Henrik Thorlacius¹

Matrix metalloproteinase-9 (MMP-9) regulates platelet shedding of CD40L in abdominal sepsis. However, the signaling mechanisms controlling sepsis-induced shedding of CD40L from activated platelets remain elusive. Rac1 has been reported to regulate diverse functions in platelets; we hypothesized herein that Rac1 might regulate platelet shedding of CD40L in sepsis. The specific Rac1 inhibitor NSC23766 (N6-[2-[[4-(diethylamino)-1-methylbutyl] amino]-6-methyl-4-pyrimidinyl]-2-methyl-4, 6-quinolinediamine trihydrochloride) was administered to mice undergoing cecal ligation and puncture (CLP). Levels of CD40L and MMP-9 in plasma, platelets, and neutrophils were determined by use of ELISA, western blot, and confocal microscopy. Platelet depletion abolished the CLP-induced increase in plasma levels of CD40L. Rac1 activity was significantly increased in platelets from septic animals. Administration of NSC23766 abolished the CLP-induced enhancement of soluble CD40L levels in the plasma. Moreover, Rac1 inhibition completely inhibited proteinase-activated receptor-4-induced surface mobilization and secretion of CD40L in isolated platelets. CLP significantly increased plasma levels of MMP-9 and Rac1 activity in neutrophils. Treatment with NSC23766 markedly attenuated MMP-9 levels in the plasma from septic mice. In addition, Rac1 inhibition abolished chemokine-induced secretion of MMP-9 from isolated neutrophils. Finally, platelet shedding of CD40L was significantly reduced in response to stimulation with supernatants from activated MMP-9-deficient neutrophils compared with supernatants from wild-type neutrophils, indicating a direct role of neutrophil-derived MMP-9 in regulating platelet shedding of CD40L. Our novel data suggest that sepsis-induced platelet shedding of CD40L is dependent on Rac1 signaling. Rac1 controls surface mobilization of CD40L on activated platelets and MMP-9 secretion from neutrophils. Thus, our findings indicate that targeting Rac1 signaling might be a useful way to control pathologic elevations of CD40L in the systemic circulation in abdominal sepsis.

Laboratory Investigation (2014) **94**, 1054–1063; doi:10.1038/labinvest.2014.92; published online 21 July 2014

Management of patients with sepsis poses a major challenge to clinicians and is largely limited to supportive therapies. In fact, sepsis is still a significant cause of mortality in intensive care units, which is related to an incomplete knowledge about the pathophysiology in sepsis.^{1–3} Intestinal perforation contaminates the abdominal cavity with toxins and microbes, evoking local formation of proinflammatory compounds, which can leak into the circulation causing a systemic inflammatory response.^{4,5} The lung is a sensitive and central target organ in polymicrobial sepsis. It is widely held that neutrophil infiltration is a rate-limiting step in septic lung damage. For example, inhibition of pulmonary accumulation of neutrophils by targeting specific adhesion

molecules, such as CD11a, CD44, and CD162, has been shown to protect against septic lung injury.^{4,6,7} Accumulating studies have shown that platelets also have an important role in regulating pulmonary recruitment of neutrophils in abdominal sepsis.^{8,9} For example, it has been reported that platelet-derived CD40L is a potent inducer of neutrophil infiltration in septic lung injury.⁸ Soluble CD40L seems to induce increased plasma levels of CXC chemokines, which are potent stimulators of neutrophils.⁸ We recently showed that matrix metalloproteinase-9 (MMP-9) is an important regulator of CD40L shedding from platelets in abdominal sepsis.¹⁰ However, the detailed signaling mechanisms regulating sepsis-induced platelet secretion of CD40L are not known.

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Received 11 March 2014; revised 20 May 2014; accepted 29 May 2014

Extracellular stress situations, such as ischemia and infection, trigger intracellular signaling cascades converging on specific transcription factors regulating gene expression of inflammatory mediators.^{11,12} This signal transmission is largely regulated by intracellular kinases phosphorylating downstream targets.¹³ For example, small (~21 kDa) guanosine triphosphatases of the Ras-homologous (Rho) family, such as Rho A–C, Cdc42, and Rac1, are known to act as molecular switches regulating numerous important cellular functions.^{14–16} Rac1 is a ubiquitously expressed signal transducer regulating numerous processes related to inflammatory reactions, such as cell adhesion, chemotaxis, vascular permeability, and cytoskeletal reorganization.^{17,18} Rac1 has been shown to be expressed in platelets and recent studies have demonstrated that Rac1 is essential for lamellipodia formation, granule secretion, clot retraction, and phospholipase C γ 2 activation in platelets.^{19–24} Moreover, targeting Rac1 signaling has been demonstrated to exert anti-inflammatory effects in models of reperfusion injury, endotoxemia, acute pancreatitis, and sepsis.^{17,25–27} Based on the findings that Rac1 regulates pleiotropic functions of platelets and that Rac1 appears to have an important role in diverse models of inflammation, we hypothesized in the present study that Rac1 might be involved in the regulation of platelet shedding of CD40L in abdominal sepsis.

MATERIALS AND METHODS

Animals

Experiments were performed using male C57BL/6 mice weighing 20–25 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 (intraperitoneally) ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg (intraperitoneally) xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight.

Experimental Model of Sepsis

Polymicrobial sepsis was provoked by ligating and puncturing the cecum in mice as previously described in detail.²⁸ Briefly, animals were anesthetized and the cecum was exteriorized and a ligature was placed below the ileocecal valve. The cecum was punctured two times with a 21-G needle. The cecum was then returned into the peritoneal cavity and the abdominal wall was closed with a suture. A platelet-depleting antibody directed against murine CD42b (GP1b α , rat IgG, 1.0 mg/kg; Emfret Analytics GmbH KG, Würzburg, Germany) was given intraperitoneally 2 h before cecal ligation and puncture (CLP). A nonfunctional isotype control antibody (clone R3-34; BD Biosciences Pharmingen, San Jose, CA, USA) was administered intraperitoneally before CLP induction. To delineate the role of Rac1 inhibitor,

animals were treated with vehicle (dH₂O) or with 5 mg/kg of Rac1 inhibitor, NSC23766 (N6-[2-[[4-(diethylamino)-1-methylbutyl] amino]-6-methyl-4-pyrimidinyl]-2-methyl-4,6-quinolinediamine trihydrochloride; Tocris Bioscience, Bristol, UK), intraperitoneally 30 min before CLP induction. Sham mice underwent the same surgical procedures except ligation and puncture of the cecum. Animals were reanesthetized at indicated time points after CLP induction.

ELISA

Plasma and supernatant levels of soluble CD40L and plasma level of MMP-9 were assayed 6 h after induction of CLP by use of commercially available ELISA Kits (R&D Systems) using recombinant murine soluble CD40L and MMP-9 as standards.

Platelet Isolation and CD40L Shedding

Blood was collected in 1-ml syringes containing 0.1 ml of acid-citrate-dextrose anticoagulant, immediately diluted with equal volumes of modified Tyrode solution (1 μ g/ml prostaglandin E₁ and 0.1 U/ml apyrase), and centrifuged at 200 g for 5 min at room temperature. Platelet-rich plasma was collected and centrifuged at 800 g for 15 min at room temperature, and pellets were resuspended in modified Tyrode solution. After being washed one more time at 10 000 g for 5 min, platelets were resuspended at a count of 0.5×10^8 platelets per tube in Tyrode solution. Platelets were stimulated with proteinase-activated receptor-4 (PAR4) (200 μ M) (thrombin receptor-activating peptide; Bachem, Weil am Rhein, Germany) at 37 °C with and without NSC23766 (10 and 100 μ M). After stimulation, cells were immediately fixed by the addition of 0.5% paraformaldehyde, samples were centrifuged at 10 000 g for 10 min at 4 °C, and the soluble CD40L that was released was measured in the supernatant by ELISA, according to the manufacturer's protocol. In separate experiment, platelets from sham and CLP mice were isolated and lysed for active Rac1 pull-down assay and western blot was performed to measure the GTP-Rac1 as described below. For platelet confocal microscopy, 0.5×10^6 isolated platelets were seeded onto a chamber slide coated with fibrinogen (20 μ g/ml). Adherent platelets were stimulated with PAR4 (200 μ M) at 37 °C with and without NSC23766 (10 μ M) and fixed with 2% paraformaldehyde for 10 min, and then washed and blocked with 2% bovine serum albumin for 30 min, followed by incubation with a rabbit polyclonal primary antibody against MMP-9 (Abcam, Cambridge, MA, USA) for 1 h at room temperature. Chamber slides were washed and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody (Cell Signaling Technology, Beverly, MA, USA) and platelet-specific phycoerythrin (PE)-conjugated anti-CD41 (clone MWReg30, integrin α IIb chain, and rat IgG1) for 1 h. Chamber slides are washed three times and confocal microscopy was performed using Meta 510 confocal microscopy (Carl Zeiss, Germany) by a $\times 63$ oil immersion objective (NA = 1.25). FITC and PE were excited

by 488 and 543 nm laser lines, corresponding emission wavelengths of FITC and PE were collected by the filters of 500–530 and 560–590 nm, respectively. The pinhole was ~1 airy unit and the scanning frame was 512 × 512 pixels. The fluorescent intensity was calculated by ZEN2009 software.

Neutrophil Isolation and MMP-9 Secretion

Bone marrow neutrophils were freshly extracted from femurs and tibias of healthy mice by aseptically flushing the bone marrow with the complete culture medium RPMI-1640 and then subsequently isolated by using magnetic beads; neutrophils were isolated from bone marrow using Anti-Ly-6G-Biotin and Anti-Biotin MicroBeads (Miltenyi Biotec) as per the manufacturer's instructions (neutrophils purity was over 93% as determined by flow cytometry). About 1×10^6 neutrophils were preincubated with NSC23766 (10 μ M) for 20 min before challenging with 0.3 μ g/ml recombinant mouse CXCL2 (R&D Systems) or PBS as a control for 30 min at 37 °C. Cells were lysed for pull-down assay and western blot was used to detect GTP-Rac1 in neutrophils as described below. For confocal microscopy, freshly isolated 0.5×10^6 neutrophils were resuspended in PBS-BSA-CaCl₂ buffer (PBS containing 0.2% BSA and 0.5 mM CaCl₂) and activated with mouse CXCL2 with and without NSC23766 (10 μ M) as described above. Neutrophils were fixed with 2% paraformaldehyde and washed with PBS-BSA-CaCl₂ followed by permeabilization with PBS-BSA-CaCl₂ Saponin buffer (PBS-BSA-CaCl₂ buffer containing 0.01% (w/v) saponin) for 10 min on ice. After permeabilization, cells are incubated with rabbit polyclonal primary antibody against MMP-9 (Abcam) for 1 h on ice. Cells were washed and incubated with FITC-conjugated anti-rabbit secondary antibody (Cell Signaling Technology, Beverly, MA, USA) and PE-conjugated anti-Ly-6G antibody (clone 1A8, rat IgG2a; BD Pharmingen, San Jose, CA, USA), which selectively binds to neutrophils for 1 h on ice. After labeling, cells were washed three times with saponin buffer and allowed to settle down on chamber slides for confocal imaging as described above.

Pull-Down Assay and Western Blotting

Rac1 activity was determined by active Rac1 pull-down and detection kit using the protein binding domain of GST-PAK1, which binds with the GTP-bound form of Rac1 (Pierce Biotechnology, Rockford, IL, USA). Briefly, cells were resuspended in lysis buffer on ice, and then centrifuged at 16 000 g for 15 min; 10 μ l from each supernatant was removed to measure the protein content using Pierce BCA Protein Assay Reagent (Pierce Biotechnology) and the rest of the volume was used for the pull-down assay. Supernatant containing equal amount of proteins were then diluted with 2 × SDS sample buffer and boiled for 5 min. Proteins were separated using SDS-PAGE (10–12% gel). After transferring to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA),

blots were blocked with Tris-buffered saline/Tween-20 containing 3% bovine serum albumin at room temperature for 1 h, followed by incubation with an anti-Rac1 antibody (1:1000) at 4 °C overnight. Binding of the antibody was detected using peroxidase-conjugated anti-mouse antibody (1:100 000; Pierce Biotechnology) at room temperature for 2 h and developed by Immuno-Star WesternC Chemiluminescence Kit (Bio-Rad). Total Rac1 was used as a loading control. To measure the MMP-9 level in the plasma, 20 μ g of plasma was loaded onto an SDS-polyacrylamide gel for electrophoresis, and then transferred onto immunoblot membranes. The membranes were blocked with Tris-buffered saline/Tween-20 containing 5% non-fat milk for 1 h, and incubated with an anti-MMP-9 antibody (Abcam) at 4 °C over night. The membranes were then washed three times, and incubated with a horseradish peroxidase-coupled secondary antibody (Cell Signaling Technology) for 2 h. Blots were again washed three times, and developed with the Immuno-Star WesternC Chemiluminescence Kit (Bio-Rad). β -Actin was used as a loading control.

Flow Cytometry

For the analysis of platelet depletion, blood was collected into syringes containing 1:10 acid citrate dextrose at 6 h after CLP induction. Immediately after collection, blood samples were incubated with an anti-CD16/CD32 antibody for 10 min at room temperature to reduce non-specific binding of labeled antibody with Fc γ III/II receptors. Neutrophils were labeled with PE-conjugated anti-Ly-6G and platelets were labeled with FITC-conjugated anti-CD41 (clone MWReg30, integrin α IIb chain, and rat IgG1) antibodies and plotted as FL1 versus FL2 to determine the percentage of platelet depletion in the upper-right and lower-right area of the quadrant plot. For the purity of magnetically isolated neutrophils, we were first incubated with an anti-CD16/CD32 antibody for 10 min and then stained with PE-conjugated anti-Ly-6G and FITC-conjugated anti-Mac-1 (clone M1/70, integrin α M chain, and rat IgG2b) antibodies. All antibodies, except where indicated, were purchased from eBioscience (San Diego, CA, USA). 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.

In vitro Shedding of Platelet-Derived CD40L

Neutrophils from wild-type and MMP-9 gene-deficient mice were isolated as described above and a total of 1×10^6 wild-type and MMP-9 gene-deficient neutrophils per tube were incubated with 0.3 μ g/ml recombinant mouse CXCL2 separately (R&D Systems) for 30 min at 37 °C. After stimulation, samples were centrifuged at 10 000 g for 5 min at 4 °C and supernatants were collected for further use. At the same time, platelets from MMP-9 gene-deficient mice were isolated as described above. Platelets were resuspended at a count of 0.5×10^8 platelets per tube in Tyrode solution and stimulated

with 200 μ M of PAR4 and coincubated with equal volume of supernatants derived from wild-type or MMP-9 gene-deficient neutrophils or with PBS at 37 °C for 30 min. After stimulation, cells were immediately fixed by adding 0.5% formaldehyde, where after samples were centrifuged at 10 000 g for 10 min at 4 °C, and soluble CD40L was measured in the supernatant by ELISA.

Statistics

Data were presented as mean values \pm s.e.m. Statistical evaluations were performed by using nonparametric 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 SigmaPlot 10.0 software (Systat Software, Chicago, IL, USA).

RESULTS

Rac1 Activity in Platelets

We first examined Rac1 activity (Rac1-GTP) in platelets. We observed that CLP increased Rac1-GTP levels in septic platelets compared with platelets from sham animals, showing that Rac1 is activated in platelets in septic animals (Figures 1a and b). Notably, administration of the Rac1 inhibitor NSC23766 abolished CLP-induced Rac1 activation in platelets (Figures 1a and b).

Rac1 Regulates Platelet Shedding of CD40L

CLP caused a 26-fold increase in the plasma levels of CD40L, that is, from 0.09 ± 0.02 to 2.40 ± 0.30 ng/ml (Figure 2a). Administration of the anti-GP1b α antibody reduced systemic platelet counts by more than 85% in CLP animals (Figure 2b). Interestingly, we found that platelet depletion decreased plasma levels of CD40L by more than 95% in septic mice (Figure 2a). Administration of NSC23766 in control mice had no effect on plasma levels of soluble CD40L (Figure 2a). In contrast, we found that treatment with NSC23766 reduced soluble levels of CD40L in the plasma from septic mice from 2.40 ± 0.30 down to 0.145 ± 0.02 ng/ml, corresponding to a 98% reduction (Figure 2a). To determine the direct role of Rac1 in regulating platelet expression and secretion of CD40L, isolated platelets were stimulated with PAR4 *in vitro*. It was found that PAR4 markedly increased surface expression of CD40L on platelets (Figures 2c and d). Notably, preincubation with NSC23766 significantly decreased PAR4-induced surface mobilization of CD40L on platelets (Figures 2c and d). Moreover, we found that PAR4 caused substantial secretion of CD40L from platelets and that NSC23766 dose-dependently reduced PAR4-induced platelet secretion of CD40L (Figure 2e). For example, 100 μ M of NSC23766 decreased PAR4-induced platelet secretion of CD40L by 76% (Figure 2e).

Rac1 Activity in Isolated Neutrophils

Neutrophils were isolated from bone marrow as described in Materials and Methods section and Rac1 activity was

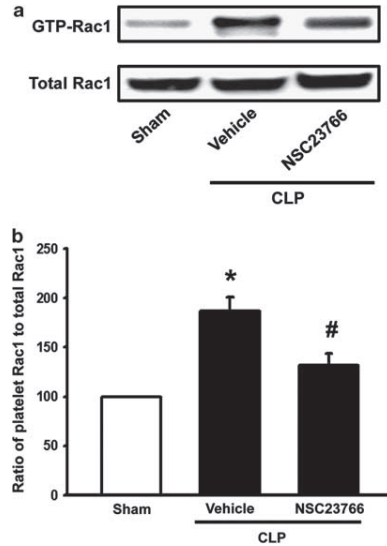
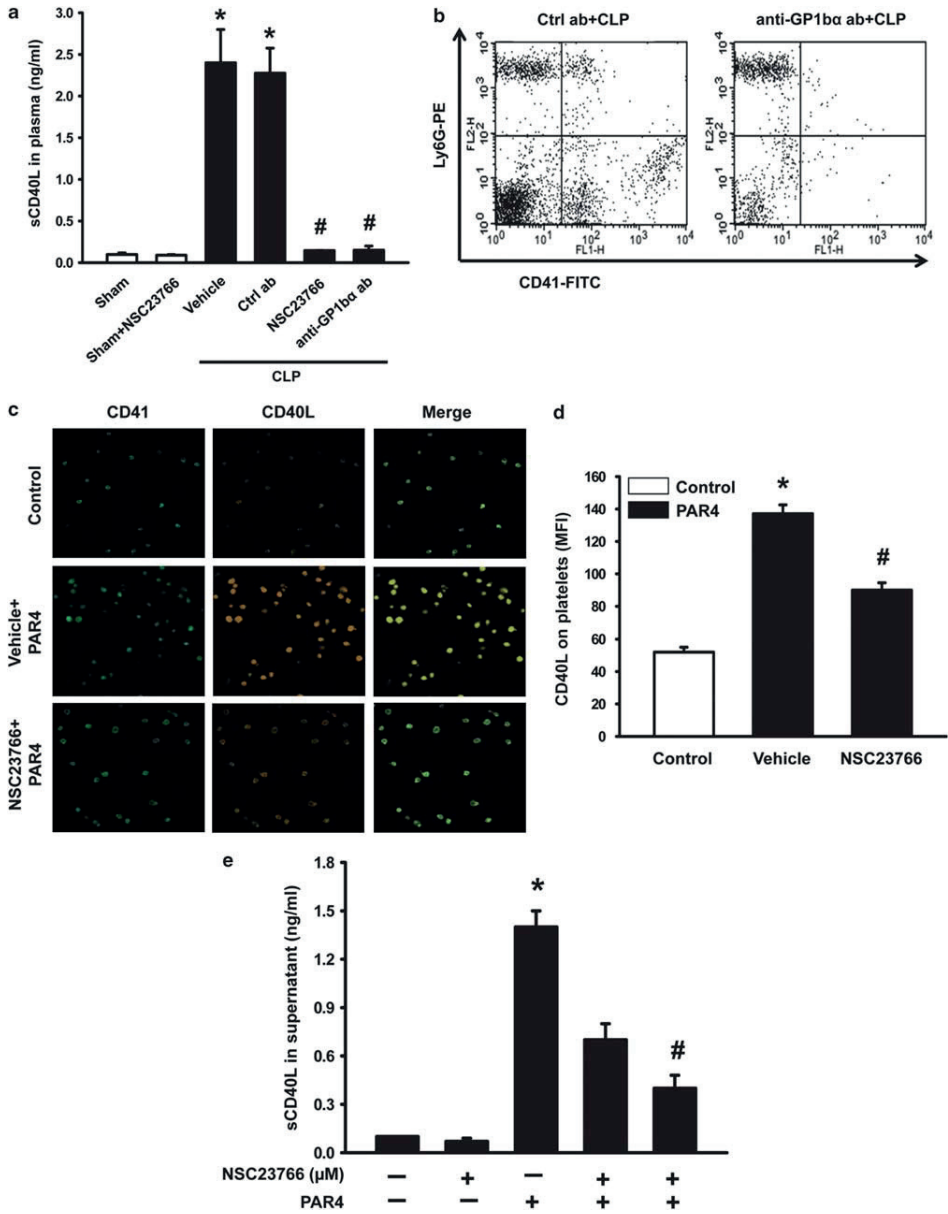


Figure 1 Rac1 activity in platelet lysate. (a) Rac1-GTP was determined by western blotting using GST-PAK pull-down beads 6 h after induction of cecal ligation and puncture (CLP). (b) Band intensities were quantified by densitometry and normalized to total Rac1. Western blots are representative of four independent experiments. Mice were treated with the Rac1 inhibitor NSC23766 (N6-[2-[[4-(diethylamino)-1-methylbutyl]amino]-6-methyl-4-pyrimidinyl]-2-methyl-4,6-quinolinediamine trihydrochloride; 5 mg/kg) or vehicle (dH₂O) 30 min before CLP induction. Sham-operated mice served as negative controls. Bars represent mean \pm s.e.m. and $n = 4$. * $P < 0.05$ vs sham and # $P < 0.05$ vs vehicle + CLP.

analyzed by western blotting. We found that CXCL2 stimulation markedly increased Rac1 activity in neutrophils and that preincubation with NSC23766 abolished the CXCL2-evoked activation of Rac1 in neutrophils (Figures 3a and b).

Rac1 Regulates Neutrophil Secretion of MMP-9

We have recently shown that MMP-9 regulates platelet shedding of CD40L¹⁰ and it was therefore of interest to examine the role of Rac1 in neutrophil secretion of MMP-9 in sepsis. Using ELISA, we found that CLP increased plasma levels of MMP-9 and the administration of NSC23766 decreased CLP-induced plasma levels of MMP-9 by 50% (Figure 4a). Moreover, by use of western blot, it was found that MMP-9 expression in the plasma was significantly enhanced in septic animals (Figures 4b and c). Treatment with NSC23766 markedly reduced sepsis-provoked plasma expression of MMP-9 (Figures 4b and c). Knowing that neutrophils are a major source of MMP-9,²⁹ we next analyzed MMP-9



secretion from neutrophils. Immunostaining revealed abundant levels of MMP-9 in resting neutrophils (Figures 5a and b). Challenge with CXCL2 rapidly mobilized and decreased intracellular levels of MMP-9 in neutrophils (Figures 5a and b). Notably, preincubation with NSC23766 completely inhibited MMP-9 secretion and maintained normal levels of MMP-9 in neutrophils exposed to CXCL2 (Figures 5a and b).

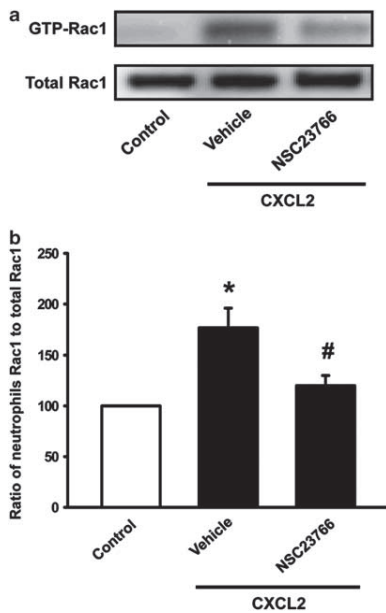


Figure 3 CXCL2 induces Rac1 activity in neutrophils. (a) Neutrophils were isolated by magnetic beads and then 1×10^6 neutrophils were incubated with CXCL2 (0.3 $\mu\text{g}/\text{ml}$) for 20 min with and without Rac1 inhibitor NSC23766 (N6-[2-[[4-(diethylamino)-1-methylbutyl] amino]-6-methyl-4-pyrimidinyl]-2-methyl-4, 6-quinolinediamine trihydrochloride; 10 μM). Active Rac1 protein was pulled down from neutrophil lysates by using GST-PAK beads. Rac1-GTP was detected by western blot. (b) Band intensities were quantified by densitometry and normalized to total Rac1. Bars represent mean \pm s.e.m. and $n = 4$. * $P < 0.05$ vs control and # $P < 0.05$ vs vehicle + CXCL2.

Neutrophil-Derived MMP-9 Regulates Platelet Shedding of CD40L

We used MMP-9 gene-deficient mice to determine the role of neutrophil-derived MMP-9 in regulating platelet shedding of CD40L. Stimulation with PAR4 was used to mobilize CD40L on the surface of MMP-9-deficient platelets. It was found that supernatant from wild-type neutrophils stimulated with CXCL2 increased the shedding of surface expressed CD40L from MMP-9-deficient platelets from 0.02 ± 0.0 up to 1.4 ± 0.08 ng/ml, corresponding to a 53-fold increase compared with control (Figure 6). Incubation of MMP-9-deficient platelets with supernatants from MMP-9 gene-deficient neutrophils stimulated with CXCL2 caused a significantly lower level of CD40L shedding (Figure 6). Stimulation with PAR4 significantly enhanced shedding of CD40L from MMP-9-deficient platelets by 28-fold compared with control (Figure 6).

DISCUSSION

This study demonstrates that sepsis-induced platelet shedding of CD40L is dependent on Rac1 signaling. We found that Rac1 might regulate sepsis-induced plasma levels of CD40L at two distinct levels, that is, on one hand Rac1 controls surface mobilization of CD40L on activated platelets and on the other hand Rac1 regulates MMP-9 secretion from neutrophils. Taken together, our data suggest that Rac1 signaling coordinates multiple events leading to increased plasma levels of soluble CD40L in abdominal sepsis.

Accumulating data suggest that platelets exert multiple effects beyond promoting hemostasis such as proinflammatory actions. For example, it has been reported that platelets have a key role in polymicrobial sepsis by potentiating neutrophil recruitment to the lung in a contact-independent manner.³⁰ Platelets contain numerous proinflammatory substances including chemokines and cytokines.^{31,32} We have demonstrated that platelet-dependent pulmonary infiltration of neutrophils is mediated by soluble CD40L released from platelets in abdominal sepsis.⁸ In support of a role of CD40L in sepsis, elevated plasma levels of soluble CD40L have been observed in patients with sepsis.^{33,34} However, the specific signaling mechanisms regulating platelet shedding of CD40L in sepsis are not known. In the present study, we found that Rac1 activity was increased in platelets from septic mice. We therefore asked whether Rac1 activity might regulate platelet shedding of CD40L. It was found that platelet depletion abolished the sepsis-induced

Figure 2 Rac1 regulates platelet shedding of CD40L. (a) Plasma level of soluble CD40L (sCD40L) from sham and cecal ligation and puncture (CLP) (6 h) animals. (b) A representative plot of flow cytometry showing the platelet depletion. (c) Representative image of confocal microscopy showing surface expression of CD40L on isolated platelets. (d) Summarized data showing mean fluorescence intensity (MFI) of CD40L expression on platelets. (e) Isolated platelets were activated with proteinase-activated receptor-4 (PAR4) (200 μM) for 20 min with and without Rac1 inhibitor NSC23766 (N6-[2-[[4-(diethylamino)-1-methylbutyl] amino]-6-methyl-4-pyrimidinyl]-2-methyl-4, 6-quinolinediamine trihydrochloride; 10 and 100 μM), and then levels of soluble CD40L were determined in the supernatants by enzyme-linked immunosorbent assay. Nonstimulated platelets served as control. Bars represent mean \pm s.e.m. and $n = 4-5$. * $P < 0.05$ vs sham or control, # $P < 0.05$ vs vehicle + CLP or vehicle + PAR4, and $\Delta P < 0.05$ vs Ctrl ab + CLP.

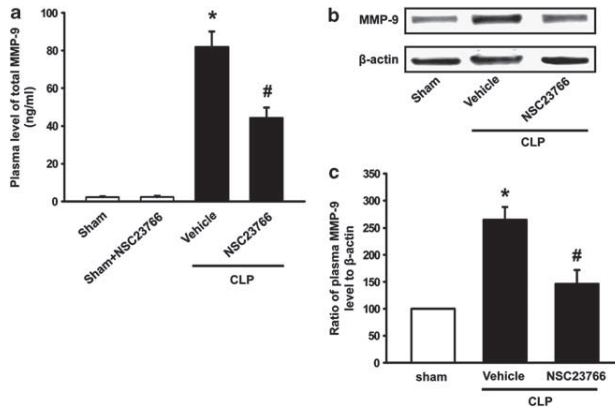


Figure 4 Plasma levels of matrix metalloproteinase-9 (MMP-9) are elevated in cecal ligation and puncture (CLP) mice. (a) Plasma levels of total MMP-9 6 h after CLP. Sham-operated animals served as negative controls. (b) Plasma from sham and 6 h CLP mice were analyzed by western blot for the presence of MMP-9. (c) Band intensities were quantified by densitometry and ratio of plasma MMP-9 to β -actin is shown. Western blots are representative of four independent experiments. Bars represent mean \pm s.e.m. and $n = 4$. * $P < 0.05$ vs sham and # $P < 0.05$ vs vehicle + CLP.

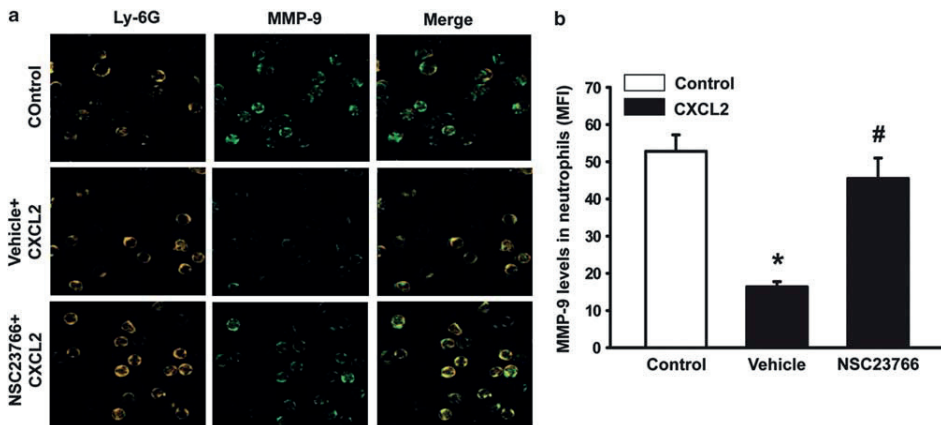


Figure 5 Rac1 regulates neutrophil secretion of matrix metalloproteinase-9 (MMP-9). (a) Neutrophils were isolated by magnetic beads. (a) Isolated neutrophils were incubated with CXCL2 (0.3 μ g/ml) and then was the level of MMP-9 in permeabilized neutrophils determined by confocal microscopy. (b) Summarized data showing mean fluorescence intensity (MFI) of MMP-9 in neutrophils. Bars represent mean \pm s.e.m. and $n = 4$. * $P < 0.05$ vs control and # $P < 0.05$ vs vehicle + CXCL2.

increase of CD40L levels in the plasma, suggesting that platelets are the dominating source of soluble CD40L in sepsis, which is in line with our previous findings.¹⁰ Interestingly, we also found that NSC23766 completely inhibited the CLP-provoked enhancement of plasma levels of CD40L, indicating that Rac1 is an important regulator of platelet shedding of CD40L in abdominal sepsis. More-

over, NSC23766 also abolished PAR4-induced surface expression and shedding of CD40L in isolated platelets *in vitro*, supporting the notion that Rac1 controls CD40L secretion from platelets. In this context, it is interesting to note that NSC23766 was recently shown to inhibit agonist-induced mobilization of P-selectin in platelets.¹⁹ Knowing that both CD40L and P-selectin are localized in the

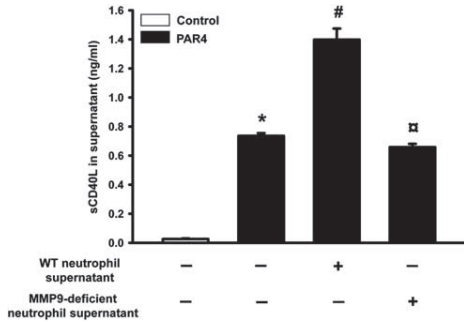


Figure 6 Neutrophil-derived matrix metalloproteinase-9 (MMP-9) regulates platelet shedding of CD40L. Isolated MMP-9 gene-deficient platelets were incubated with proteinase-activated receptor-4 (PAR4) and with supernatants from either wild-type or MMP-9-deficient neutrophils stimulated with CXCL2 for 30 min. Soluble CD40L was determined in the supernatants by enzyme-linked immunosorbent assay. Nonstimulated platelets served as control. Bars represent mean \pm s.e.m. and $n = 4-5$. * $P < 0.05$ vs control, # $P < 0.05$ vs control, and # $P < 0.05$ vs wild-type neutrophil supernatant.

membrane of α -granules in platelets, these findings suggest that Rac1 might be involved in the secretion of α -granules in platelets.

The MMP family of proteases is generally considered to regulate degradation and formation of the extracellular matrix, but overwhelming data in the literature also implicate MMPs in several features of inflammatory reactions by regulating cleavage and shedding of surface molecules.^{35,36} In fact, several studies have indicated that MMP-9 might have an important role in sepsis.^{8,37} One mechanism by which MMP-9 regulate proinflammatory actions in sepsis appears to be related to platelet shedding of CD40L.¹⁰ Using both ELISA and western blot, we observed herein that plasma levels of MMP-9 were increased in abdominal sepsis. This finding is in accordance with other investigations showing enhanced MMP-9 levels in the circulation of sepsis and infectious disease models,³⁷⁻³⁹ as well as in patients with sepsis.^{10,40} Notably, it was observed that administration of NSC23766 significantly decreased the sepsis-induced increase of MMP-9 levels in plasma, indicating that Rac1 might be an important regulator of systemic levels of MMP-9 in abdominal sepsis. It should be mentioned that two studies have reported that MMP-2 might promote platelet release of CD40L *in vitro*,^{41,42} but levels of MMP-2 in the plasma are not elevated in abdominal sepsis.¹⁰ The potential presence of MMP-9 in platelets has been a controversial subject in the literature. Some authors have reported that platelets do not contain significant amount of MMP-9,^{43,44} whereas others have shown that platelets do contain MMP-9.⁴⁵⁻⁴⁷ Nonetheless, it is well accepted that neutrophils are a rich source of MMP-9.^{29,48} We therefore

asked whether Rac1 might have a role in MMP-9 secretion from neutrophils. It was found that stimulation with the neutrophil chemoattractant CXCL2 caused a significant increase in Rac1 activity in neutrophils. Using confocal microscopy, we observed clearcut levels of MMP-9 in isolated neutrophils and that CXCL2 challenge decreased neutrophil content of MMP-9, suggesting that CXCL2 stimulates MMP-9 secretion from neutrophils. Interestingly, we found that administration of NSC23766 completely inhibited CXCL2-induced secretion of MMP-9 in neutrophils, indicating that Rac1 regulates neutrophil secretion of MMP-9. Moreover, we found that supernatant from wild-type neutrophils stimulated with CXCL2 caused significant shedding of CD40L from MMP-9-deficient platelets. In contrast, supernatant from MMP-9 gene-deficient neutrophils stimulated with CXCL2 triggered significantly less CD40L shedding from MMP-9-deficient platelets, indicating that neutrophil-derived MMP-9 is a potent regulator of platelet shedding of CD40L. In this context, it is interesting to note that PAR4 alone caused significant shedding of CD40L from MMP-9-deficient platelets, suggesting that MMP-9-independent mechanisms could also be involved in platelet shedding of CD40L.

It should be mentioned that our present findings are limited to mice and one must be careful when extrapolating findings in experimental sepsis to human sepsis, considering significant differences in age and comorbidity between mice and humans. In addition, Rac1 is an important protein in intracellular signaling⁴⁹⁻⁵¹ and risks of potential side effects should be considered. Therefore, further studies in human materials on the role of Rac1 for MMP-9 secretion and platelet shedding of CD40L are needed.

Taken together, these findings indicate that Rac1 activity is increased in platelets and regulates platelet shedding of CD40L in polymicrobial sepsis. Moreover, these results suggest that Rac1 signaling controls circulating levels of MMP-9 in sepsis and that Rac1 regulate neutrophil secretion of MMP-9. Thus, our data indicate that inhibition of Rac1 signaling might be a useful target to control pathologic secretion and shedding of CD40L into the systemic circulation in abdominal sepsis.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (2012-3685), Crafoord foundation, Einar and Inga Nilssons foundation, Harald and Greta Jaenssons foundation, Greta and Johan Kocks foundation, Agnes Nilssons foundation, Franke and Margareta Bergqvists foundation for promotion of cancer research, Magnus Bergvalls foundation, Mossfelts foundation, Nanna Svartz foundation, Ruth and Richard Julins foundation, Swedish Medical Society fund, Malmö General Hospital foundation for combating cancer, MAS fund, and Skåne University Hospital and Lund University. RH is supported by Hawler Medical University and Kurdistan Regional Government. EZ's position receives funding from the EU Integrated project BetaBat and a collaborative SRC grant, as well as the Swedish Strategic Research EXODIAB. This work was carried out using imaging equipment financed by the Knut and Alice Wallenberg foundation.

AUTHOR CONTRIBUTIONS

RH and MR performed experiments, analyzed data, and wrote the manuscript. EZ performed experiments and wrote the manuscript. HT supervised the project, designed the experiments, and wrote the manuscript.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Paper III

Rac1-dependent secretion of platelet-derived CCL5 regulates neutrophil recruitment via activation of alveolar macrophages in septic lung injury

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RECEIVED DECEMBER 15, 2014; REVISED JANUARY 22, 2015; ACCEPTED JANUARY 29, 2015. DOI: 10.1189/jlb.4A1214-603R

ABSTRACT

Accumulating evidence suggest that platelets play an important role in regulating neutrophil recruitment in septic lung injury. Herein, we hypothesized that platelet-derived CCL5 might facilitate sepsis-induced neutrophil accumulation in the lung. Abdominal sepsis was induced by CLP in C57BL/6 mice. CLP increased plasma levels of CCL5. Platelet depletion and treatment with the Rac1 inhibitor NSC23766 markedly reduced CCL5 in the plasma of septic mice. Moreover, Rac1 inhibition completely inhibited proteasePAR4-induced secretion of CCL5 in isolated platelets. Immunoneutralization of CCL5 decreased CLP-induced neutrophil infiltration, edema formation, and tissue injury in the lung. However, inhibition of CCL5 function had no effect on CLP-induced expression of Mac-1 on neutrophils. The blocking of CCL5 decreased plasma and lung levels of CXCL1 and CXCL2 in septic animals. CCL5 had no effect on neutrophil chemotaxis *in vitro*, suggesting an indirect effect of CCL5 on neutrophil recruitment. Intratracheal challenge with CCL5 increased accumulation of neutrophils and formation of CXCL2 in the lung. Administration of the CXCR2 antagonist SB225002 abolished CCL5-induced pulmonary recruitment of neutrophils. Isolated alveolar macrophages expressed significant levels of the CCL5 receptors CCR1 and CCR5. In addition, CCL5 triggered significant secretion of CXCL2 from isolated alveolar macrophages. Notably, intratracheal administration of clodronate not only depleted mice of alveolar macrophages but also abolished CCL5-induced formation of CXCL2 in the lung. Taken together, our findings suggest that Rac1 regulates platelet secretion of CCL5 and that CCL5 is a potent inducer of neutrophil recruitment in septic lung injury via formation of CXCL2 in alveolar macrophages. *J. Leukoc. Biol.* **97**: 000–000; 2015.

Introduction

Neutrophil activation and recruitment constitute key features in the host response to systemic bacterial infections [1, 2]. Neutrophils are needed for microbial defense, but excessive tissue accumulation of neutrophils can cause organ damage in sepsis. The lung is the most sensitive and critical target organ in sepsis, and neutrophil recruitment constitutes a rate-limiting step in septic lung injury [3–5]. For example, the targeting of specific adhesion molecules, including CD11a, CD44, and CD162, not only decreases pulmonary infiltration of neutrophils but also protects against septic lung damage [3–5]. Interestingly, apart from their well-known role in hemostasis and wound healing [6, 7], a growing body of evidence suggests that platelets exert proinflammatory actions, such as supporting tissue infiltration of leukocytes in septic lung injury [8, 9]. For example, it has been reported that platelet-derived CD40L is a potent inducer of neutrophil infiltration in septic lung injury [10]. However, platelets contain a plethora of potential mediators, including chemokines, capable of stimulating leukocyte activation and recruitment [8–10].

One of the most prevalent chemokine in platelets is CCL5 (RANTES), which belongs to the CC chemokine family and is a potent stimulator of T cells, macrophages, and eosinophils [11–14]. Neutrophils do not normally express the CCL5 receptors, including CCR1 and CCR5 [15–17]. However, it has been reported that activated neutrophils under certain circumstances can up-regulate CCR1 [18, 19]. Moreover, several studies have reported that high CCL5 expression correlates with neutrophil activation in lung disease [20, 21]. Inhibition of CCL5 function has been reported to reduce neutrophil activation and accumulation in models of encephalitis, endotoxemia, stroke, and coronary ischemia, raising the question whether CCL5 might play a potential role in abdominal sepsis [21–24]. The intracellular signaling cascades triggering platelet secretion of CCL5 are not well understood. We have recently observed that Rac1, a member of the Rho family, not only plays an important

Abbreviations: BALF = bronchoalveolar lavage fluid, CD40L = cluster of differentiation 40 ligand, CLP = cecal ligation and puncture, Mac-1 = macrophage-1 antigen, MPO = myeloperoxidase, NSC23766 = N6-[2-[[4-(diethylamino)-1-methylbutyl] amino]-6-methyl-4-pyrimidinyl]-2-methyl-4, 6-quinolinediamine trihydrochloride, PAK = p21-activated kinase 1 protein, PAR4 = protease-activated receptor 4, Rac1 = Ras-related C3 botulinum toxin substrate 1, Rho = Ras homologous

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role in septic lung injury [25] but also regulates platelet secretion of CD40L in sepsis [26]. Moreover, it has been reported that Rac1 is essential for lamellipodia formation, granule secretion, clot retraction, and phospholipase C γ 2 activation in platelets [27–30]. Thus, we hypothesized herein that Rac1 might be involved in the regulation of platelet secretion of CCL5 in abdominal sepsis.

Based on the above considerations, we studied the role of Rac1 in regulating platelet secretion of CCL5, as well as the function of CCL5 in controlling neutrophil recruitment and lung damage in abdominal sepsis. For this purpose, we used a model based on CLP.

MATERIALS AND METHODS

Animals

Experiments were performed with the use of male C57BL/6 mice (20–25 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 i.p. administration of 75 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium)/kg body weight.

Experimental model of sepsis

Polymicrobial sepsis was induced by puncture of the cecum in anesthetized mice. Through a midline incision the cecum was exposed and 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 PBS 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 wall was closed. Animals were treated with vehicle (dH₂O) or with 5 mg/kg of the Rac1 inhibitor, NSC23766 (Tocris Bioscience, Bristol, United Kingdom). A control antibody (clone 54447; R&D Systems, Minneapolis, MN, USA) or a mAb against murine CCL5 (clone 53405; R&D Systems) was injected i.p. (10 μ g/mouse), 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 animals were then returned to their cages and provided food and water ad libitum. Animals were reanesthetized 6 or 24 h after CLP induction. The left lung was ligated and excised for edema measurement. The right lung was used for collecting BALF, in which neutrophils were counted. Next, the lung was perfused with PBS, 1 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 MPO assays and ELISA, as described subsequently.

MPO assay

Lung tissue was thawed and homogenized in 1 ml 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 H₂O₂ (450 nm, with a reference filter 540 nm, 25 $^{\circ}\text{C}$). Values were expressed as MPO U/g tissue.

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 PBS containing 5 mM EDTA. The number of neutrophils was counted in a Burkner chamber.

Histology

Lung samples were fixed by immersion in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. Sections (6 μ m) were

stained with H&E. Lung injury was quantified in a blinded manner by adoption of a modified scoring system [31, 32], including size of alveoli spaces, thickness of alveolar septae, alveolar fibrin deposition, and neutrophil scoring system infiltration graded on a 0 (absent)–4 (extensive) scale. In each tissue sample, 5 random areas were scored, and the mean value was calculated. The histology score is the sum of all 4 parameters.

Lung edema

The left lung was excised and then weighed. The tissue was then dried at 60 $^{\circ}\text{C}$ for 72 h and reweighed. The change in the ratio of wet weight:dry weight was used as an indicator of lung edema formation.

ELISA

CXCL1, CXCL2, and CCL5 levels in lung tissue and plasma were analyzed by use of double antibody Quantikine ELISA kits (R&D Systems). Murine rCXCL1, rCXCL2, and rCCL5 were used as standards.

Flow cytometry

For analysis of surface expression of Mac-1, CCR1, CCR5, and CXCR2 on circulating neutrophils, blood was collected (1:10 acid citrate dextrose), 6 h after CLP induction, and incubated with an anti-CD16/CD32 antibody blocking Fc γ III/II_Rs to reduce nonspecific labeling. Samples were then incubated with PE-conjugated anti-Gr-1 (clone RB6-8C5; eBioscience, Frankfurt, Germany) and FITC-conjugated anti-Mac-1 (clone M1/70; BD Biosciences Pharmingen, San Jose, CA, USA) antibodies. Samples were also incubated with a PerCP-Cy5.5-conjugated anti-mouse CXCR2 antibody (clone TG11/CXCR2, rat IgG2a; BioLegend, San Diego, CA, USA), a PE-conjugated anti-CCR1 antibody (clone CTC5; R&D Systems), or a PE-conjugated anti-CCR5 antibody (clone 643854; R&D Systems). Cells were fixed, erythrocytes were lysed, and neutrophils were recovered following centrifugation. Alveolar macrophages were isolated as described below and incubated with an anti-CD16/CD32 antibody blocking Fc γ III/II_Rs, a PerCP-Cy5.5-conjugated anti-mouse F4/80 antibody (clone BM8; eBioscience), and a FITC-conjugated anti-Ly6G antibody (clone 1A8; BD Biosciences Pharmingen), as well as a PE-conjugated anti-CCR1 antibody (clone CTC5) or a PE-conjugated anti-CCR5 antibody (clone 643854). Flow cytometric analysis was performed according to standard settings on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed with CellQuest Pro software (BD Biosciences Pharmingen). A viable gate was used to exclude dead and fragmented cells.

Alveolar macrophage secretion of CXCL2

BALF was collected as above from healthy mice, and cells were isolated by centrifugation (450 g, 10 min), as described previously [33]. Cells were resuspended in RPMI (Invitrogen, Carlsbad, CA, USA) and adjusted to a concentration of 2×10^5 /ml. Cells were then stained with a PE-conjugated anti-F4/80 and a FITC-conjugated anti-mouse Ly-6G antibody, as described above, and the purity of isolated macrophages was determined by flow cytometry. Macrophages were identified as F4/80⁺/Ly6G⁺ cells. Macrophages were cocultured with murine rCCL5 (500 ng/ml) for 4 h (37 $^{\circ}\text{C}$) and then were CXCL2 levels measured by ELISA.

Intratracheal challenge with CCL5

Through an intratracheal catheter, murine rCCL5 (1 μ g; PeproTech, Neuilly-Sur-Seine, France) or vehicle was administered into the lungs and neutrophils, and CXCL2 levels were quantified in BALF, 4 h later. Animals were treated i.p. with vehicle or a CXCR2 antagonist (SB225002, 4 mg/kg; Calbiochem, Merck, Darmstadt, Germany) before intratracheal challenge with CCL5. In separate experiments, 100 μ l clodronate liposomes or PBS liposomes was administered intratracheally, 24 h before subsequent intratracheal challenge with CCL5. Liposomes were purchased from Encapsula NanoSciences (Brentwood, TN, USA). BALF was collected 4 h after CCL5 administration for quantification of neutrophils and CXCL2 levels.

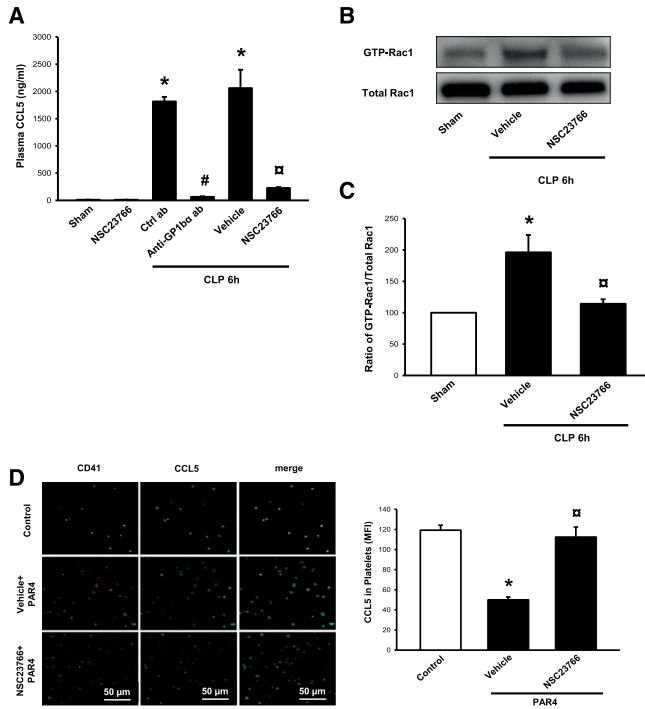


Figure 1. Rac1 regulates platelet secretion of CCL5 in sepsis. Animals were treated with vehicle, NSC23766 (5 mg/kg), a control antibody (Ctrl ab), or an anti-glycoprotein IIb/IIIa antibody (GPIIb/IIIa ab) before CLP induction. (A) ELISA was used to quantify the levels of CCL5 in the plasma, 6 h after CLP induction. (B) Rac1-GTP was determined by Western blotting by use of GST-PAK pull-down beads, 6 h after induction of CLP. (C) Band intensities were quantified in isolated platelets by densitometry and normalized to total Rac1. Western blots are representative of 5 independent experiments. Mice were treated with the Rac1 inhibitor NSC23766 (5 mg/kg) or vehicle before CLP induction. Sham-operated mice served as negative controls. (D) Left, Isolated platelets were incubated with or without NSC23766 (10 μ M) and then stimulated with rPAR4 (200 μ M), and the level of CCL5 in permeabilized CD41⁺ platelets was determined by confocal microscopy; Right, Aggregate data showing mean fluorescence intensity (MFI) of CCL5 in platelets. Nonstimulated platelets served as control. Data represent mean \pm SEM, and $n = 5$. * $P < 0.05$ versus Sham or Control; # $P < 0.05$ versus Ctrl ab + CLP; open circle in box symbol, $P < 0.05$ versus Vehicle + CLP or Vehicle + PAR4.

Neutrophil chemotaxis

Neutrophils were isolated from bone marrow by use of Ficol-Paque. Neutrophils (1.5×10^6) were placed in the upper chamber of the Transwell inserts with a pore size of 5 μ m (Corning Costar, Corning, NY, USA). Inserts were placed in wells containing medium alone (control) or medium plus CXCL2 (100 ng/ml; R&D Systems) or CCL5 (500 ng/ml). After 120 min, inserts were removed, and migrated neutrophils were stained with Turk's solution. Chemotaxis was determined by counting the number of migrated neutrophils in a Burkler chamber.

Platelet isolation and CCL5 secretion

Blood was collected in syringes containing 0.1 ml acid-citrate-dextrose, diluted immediately with equal volumes of modified Tyrode solution (1 μ g/ml PGE₁ and 0.1 U/ml apyrase), and centrifuged (200 g, 5 min). Platelet-rich plasma was collected and centrifuged (800 g, 15 min), and pellets were resuspended in modified Tyrode solution. After being washed 1 more time (10,000 g, 5 min) 0.5×10^6 platelets were seeded on a chamber slide coated with fibrinogen (20 μ g/ml). Adherent platelets were stimulated with PAR4 (200 μ M, 37°C), with and without NSC23766 (100 μ M). Platelets were fixed with 2% paraformaldehyde for 5 min and washed and blocked with 1% goat serum for 45 min. Then, platelets were permeabilized with 0.15% Triton X-100 for 15 min, followed by washing and incubation with an anti-CD16/CD32 antibody (10 min) blocking Fc γ III/IIIRs to reduce nonspecific labeling and a rabbit polyclonal primary antibody against CCL5 (bs-1324R; Bioss, Boston, MA, USA) for 2 h. Chamber slides were washed and incubated with a

FITC-conjugated anti-rabbit secondary antibody (Cell Signaling Technology, Beverly, MA, USA) and a platelet-specific, PE-conjugated anti-CD41 antibody (clone MWR30; eBioscience) for 1 h. Chamber slides are washed 3 times, and confocal microscopy was performed by use of Meta 510 confocal microscopy (Carl Zeiss, Jena, Germany). FITC and PE were excited by 488 nm and 543 nm laser lines, and corresponding emission wavelengths of FITC and PE were collected by the filters of 500–530 nm and 560–590 nm, respectively. The pinhole was ~ 1 airy unit, and the scanning frame was 512 \times 512 pixels. The fluorescent intensity was calculated by use of ZEN 2009 software.

Pull-down assay and Western blotting

Rac1 activity was determined in platelets from sham and CLP mice pretreated with vehicle or NSC23766 by active Rac1 pull-down and detection kit by use of the protein-binding domain of GST-PAK1, which binds with the GTP-bound form of Rac1 (Pierce Biotechnology, Rockford, IL, USA). In brief, platelets were suspended in lysis buffer on ice and centrifuged (16,000 g, 15 min). Ten microliters from each lysate was removed to measure protein content by use of Pierce BCA Protein Assay Reagent (Pierce Biotechnology), and the rest was used for the pull-down assay. Supernatants containing equal amount of proteins were then diluted with 2 \times SDS sample buffer and boiled for 5 min. Proteins were separated by use of SDS-PAGE (10–12% gel). After transferring to a nitrocellulose membrane (BioRad Laboratories, Hercules, CA, USA), blots were blocked with TBS/Tween 20 containing 3% BSA at room temperature for 1 h, followed by incubation with an anti-Rac1 antibody

(1:1000) at 4°C overnight. The binding of the antibody was detected by use of peroxidase-conjugated anti-mouse antibody (1:100,000; Pierce Biotechnology) at room temperature for 2 h and developed by ImmunoStar WesternC Chemiluminescence Kit (Bio-Rad Laboratories). Total Rac1 was used as a loading control.

Statistics

Data were presented as mean values ± SEM. Statistical evaluations were performed by use of nonparametrical 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 use of SigmaPlot 10.0 software (Systat Software, Chicago, IL, USA).

RESULTS

Rac1 regulates platelet secretion of CCL5 in sepsis

CLP increased plasma levels of CCL5 from 12.3 ng/ml in sham mice up to 2075 ng/ml, corresponding to a 169-fold increase (Fig. 1A). We found that depletion of platelets abolished the CLP-induced increase in plasma levels of CCL5 (Fig. 1A), suggesting that platelets are the main source of CCL5 in abdominal sepsis. CLP increased Rac1-GTP levels in platelets, indicating that Rac1 is activated in platelets in septic animals (Fig. 1B and C). Notably, administration of the Rac1 inhibitor NSC23766 completely inhibited CLP-evoked Rac1 activation in platelets (Fig. 1B and C), showing that NSC23766 is an effective inhibitor of Rac1 activation. Administration of NSC23766 in control mice had no effect on plasma levels of CCL5 (Fig. 1A). In contrast, treatment with NSC23766 decreased plasma levels of CCL5 in septic mice from 2075 to 236 ng/ml, corresponding to an 89% reduction (Fig. 1A). To determine the direct role of Rac1 in regulating platelet secretion of CCL5, isolated platelets were stimulated with PAR4 in vitro. We observed that CCL5 was present in resting platelets and that stimulation with PAR4 decreased intracellular levels of CCL5 in platelets (Fig. 1D). Notably, coincubation of platelets with NSC23766 prevented PAR4-induced secretion of CCL5 from platelets (Fig. 1D).

CCL5 regulates lung damage in sepsis

Pulmonary edema was determined as changes in lung wet:dry ratio. It was found that the lung wet:dry ratio increased after CLP (Fig. 2A). Notably, treatment with an antibody directed against CCL5 decreased the CLP-induced increase in lung wet:dry ratio by >65% (Fig. 2A). CLP caused significant lung damage, typified by severe destruction of pulmonary tissue microstructure, extensive edema of interstitial tissue, and massive infiltration of neutrophils (Fig. 2B). Immunoneutralization of CCL5 reduced CLP-evoked tissue destruction and neutrophil infiltration in the lung (Fig. 2B). Quantification of the morphologic damage showed that CLP markedly increased lung injury score and that inhibition of CCL5 significantly decreased CLP-induced tissue damage in the lung (Fig. 2C).

CCL5 regulates pulmonary recruitment of neutrophils in sepsis

MPO is a useful marker of neutrophils. It was observed that CLP increased pulmonary levels of MPO by 24-fold (Fig. 3A). Notably, we found that inhibition of CCL5 function decreased CLP-induced MPO activity in the lung by >47% (Fig. 3A). In addition, CLP induction increased the number of alveolar neutrophils by 21-fold (Fig. 3B). Immunoneutralization of CCL5 reduced the number of alveolar neutrophils by 42% in septic animals (Fig. 3B). Mac-1 is an important adhesion molecule regulating neutrophil adhesion and trafficking [4]. Mac-1 expression was increased on the surface of circulating neutrophils in septic mice (Fig. 3C). However, administration of the anti-CCL5 antibody had no effect of Mac-1 expression on septic neutrophils (Fig. 3C). In contrast to CXCL2, CCL5 exerted no chemotactic effect of isolated neutrophils (Fig. 3D). These findings suggest that CCL5-dependent recruitment of neutrophils is not a direct effect on neutrophils but rather an indirect effect of CCL5. Neutrophil expression of CXCR2 was apparent on neutrophils from sham mice and down-regulated after CLP induction (Fig. 3E). However, we observed that neutrophils from both sham and CLP animals did not express CCR1 nor CCR5 (Fig. 3E).

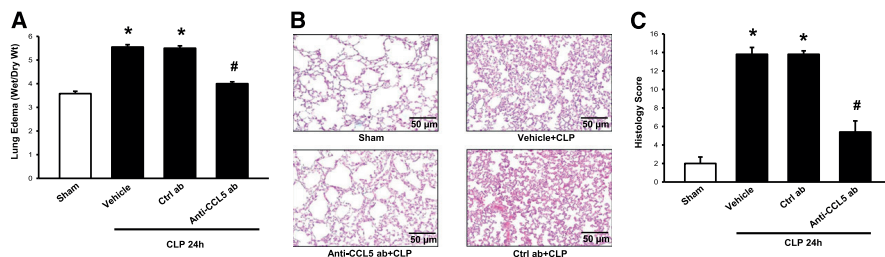


Figure 2. CCL5 regulates lung damage in sepsis. (A) Edema formation in the lung. (B) Representative H&E sections of the lung are shown. Animals were treated with vehicle, a control antibody, or an anti-CCL5 antibody before CLP induction. (C) Lung injury scores, as described in Materials and Methods, 24 h after CLP induction. Sham-operated animals served as negative controls. Data represent mean ± SEM, and *n* = 5. **P* < 0.05 versus Sham; #*P* < 0.05 versus Ctrl ab + CLP.

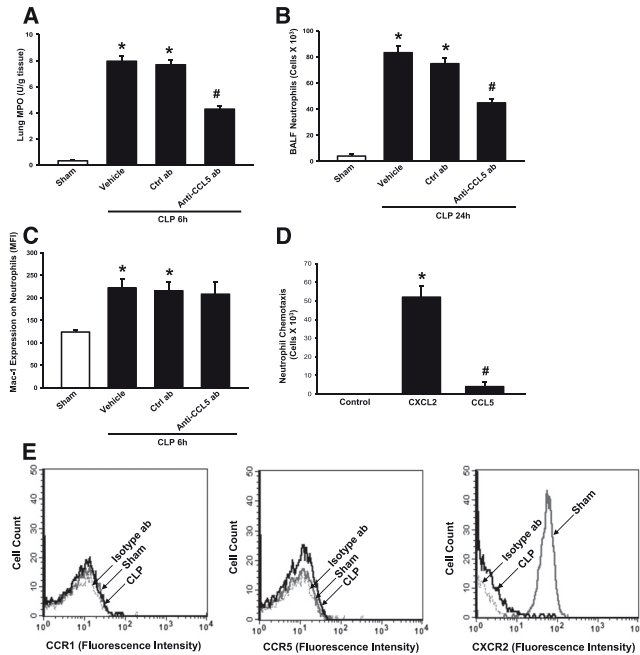


Figure 3. CCL5 regulates pulmonary recruitment of neutrophils in sepsis. (A) Lung MPO levels at 6 h post-CLP. (B) Number of BALF neutrophils, 24 h after CLP induction. (C) Mac-1 expression on circulating neutrophils, 6 h after CLP induction. Animals were treated with vehicle, a control antibody, or an anti-CCL5 antibody before CLP induction. Sham-operated animals served as negative controls. (D) Neutrophils isolated from bone marrow were analyzed for their migration in response to PBS (Control), CXCL2 (100 ng/ml), or CCL5 (500 ng/ml). Nonstimulated neutrophils served as negative control. (E) CCR1, CCR5, and CXCR2 expression on circulating neutrophils in sham and CLP animals. Cells were also stained with an isotype antibody (Isotype ab). Data represent mean \pm SEM, and $n = 5$. * $P < 0.05$ versus Sham or Control; # $P < 0.05$ versus Ctrl ab + CLP or CXCL2.

CCL5 regulates CXC chemokine formation in sepsis

CXCL1 and CXCL2 levels in the plasma and lung were low in sham animals (Fig. 4). CLP markedly increased CXCL1 and CXCL2 levels in the plasma (Fig. 4A and B). Immunoneutralization of CCL5 reduced CLP-evoked plasma levels of CXCL1 by 81% and CXCL2 by 85% (Fig. 4A and B). In addition, CLP enhanced pulmonary levels of CXCL1 and CXCL2 by 76- and 542-fold, respectively (Fig. 4C and D). Inhibition of CCL5 attenuated lung levels of CXCL1 by 87% and CXCL2 by 93% in septic animals (Fig. 4C and D). We next asked whether lung macrophages might be a link between platelet-derived CCL5 and neutrophil recruitment in abdominal sepsis. First, we administered CCL5 locally in the lung by intratracheal infusion and found that local CCL5 challenge significantly increased pulmonary levels of CXCL2 (Fig. 5A) and the number of alveolar neutrophils (Fig. 5B). Moreover, administration of the CXCR2 antagonist SB225002 abolished CCL5-induced neutrophil accumulation in the lung (Fig. 5C). Then, we isolated alveolar macrophages and observed that these cells express the CCL5 receptors, i.e., CCR1 and CCR5 (Fig. 6A). In addition, we found that coinubation of alveolar macrophages with CCL5 triggered a clear-cut increase in CXCL2 formation (Fig. 6B). This finding was repeated in RAW264.7 macrophages showing that CCL5 challenge caused a significant increase in macrophage secretion of CXCL2 (not shown). Finally, intratracheal administration of

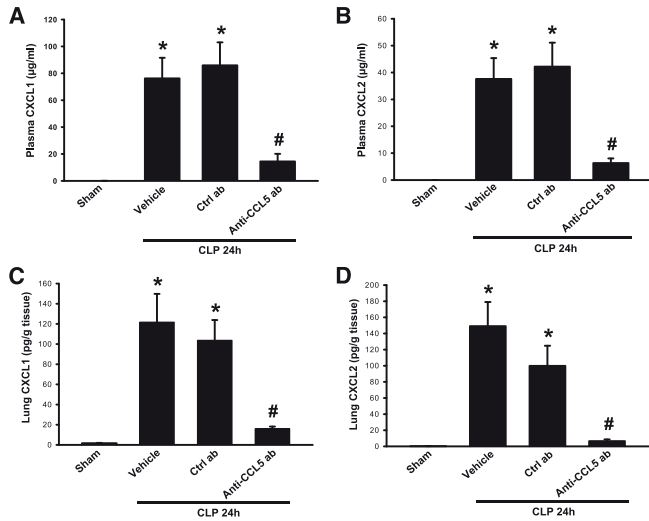
clodronate not only depleted animals of alveolar macrophages (Fig. 6C) but also significantly decreased CCL5-induced formation of CXCL2 in the lung (Fig. 6D).

DISCUSSION

Patients with abdominal sepsis pose a significant challenge to clinicians, which is partly a result of an incomplete understanding of the pathophysiology. This study documents an important role of Rac1-dependent secretion of CCL5 from platelets in sepsis. Moreover, our data also delineate the mechanisms regulating CCL5-mediated neutrophil recruitment in septic lung injury. These novel findings help to clarify the role of platelets in sepsis and suggest that the targeting of Rac1 signaling and/or the function of CCL5 might be useful ways to protect lung function in abdominal sepsis.

Numerous studies have pointed to a functional role of platelets in regulating pathologic aspects of the inflammatory response in severe infections [10, 26, 34]. For example, there is evidence in the literature showing that platelets are important for the development of dysfunctional coagulation in sepsis [35]. Moreover, accumulating evidence has demonstrated that platelets are potent regulators of neutrophil accumulation in septic lung damage [10, 26]. One apparent key mechanism is secretion of potent proinflammatory mediators, such as CD40L, harboring

Figure 4. CCL5 regulates CXC chemokine formation in sepsis. Plasma levels of (A) CXCL1 and (B) CXCL2 and lung levels of (C) CXCL1 and (D) CXCL2 determined 24 h after CLP induction. Animals were treated with vehicle, a control antibody, or an anti-CCL5 antibody before CLP. Sham-operated animals served as negative controls. Data represents mean \pm SEM, and $n = 5$. * $P < 0.05$ versus Sham; and # $P < 0.05$ versus Ctrl ab + CLP.



in platelets [10, 26]. In this context, it is interesting to note that platelets contain numerous other proinflammatory compounds, such as chemokines [36, 37]. However, the most prevalent chemokines in platelets, i.e., CCL5 and CXCL4, mainly activate lymphocytes, macrophages, and eosinophils and have a low or no direct chemotactic effect on neutrophils [15, 17]. Nonetheless, there are reports in the literature indicating that the targeting of CCL5 can decrease neutrophil recruitment in models of inflammatory diseases [21]. Thus, we first wanted to study the signaling mechanisms regulating platelet secretion of CCL5. It was found that Rac1 activity was enhanced in septic platelets. We next asked whether Rac1 activity might control platelet secretion of CCL5. It was observed that depletion of platelets markedly decreased the sepsis-evoked enhancement of CCL5 levels in plasma, indicating that platelets are the dominating source of circulating CCL5 in abdominal sepsis. Notably, administration of the Rac1 inhibitor NSC23766

abolished the sepsis-induced increase of plasma levels of CCL5, suggesting that Rac1 is a key regulator of circulating levels of CCL5 in sepsis. In addition, NSC23766 completely inhibited PAR4-triggered secretion of CCL5 in isolated platelets in vitro, supporting the conclusion that Rac1 regulates CCL5 secretion from platelets. With the consideration that NSC23766 was recently shown to inhibit agonist-induced mobilization of P-selectin in platelets [28, 38] and that P-selectin and CCL5 are localized in the platelet α -granules [28, 39–41], our results indicate that Rac1 could be involved in the mobilization and secretion of α -granules in platelets. In this context, it is interesting to note that we have recently reported that simvastatin treatment decreases sepsis-provoked pulmonary neutrophilia and tissue injury [42]. With the knowledge that statins prevent isoprenylation of Rho proteins, such as Rac1, which is necessary for their function [43], our present findings

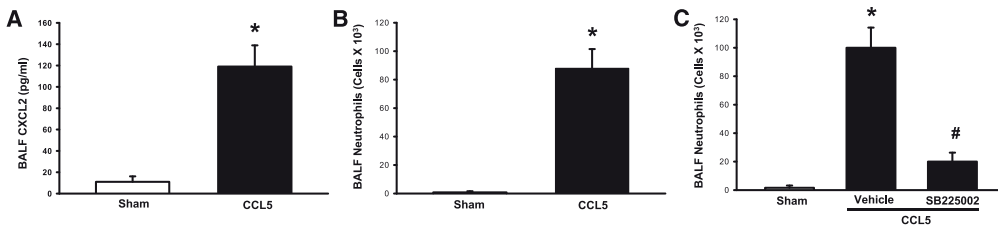


Figure 5. CCL5-induced neutrophil recruitment is dependent on CXCL2 formation. Levels of (A) CXCL2 and (B) number of neutrophils in the lung after intratracheal challenge with CCL5. (C) Neutrophil accumulation in the lungs of animals treated with vehicle or the CXCR2 antagonist SB225002 before intratracheal challenge with CCL5. Data represent mean \pm SEM, and $n = 5$. * $P < 0.05$ versus Sham; # $P < 0.05$ versus Vehicle + CCL5.

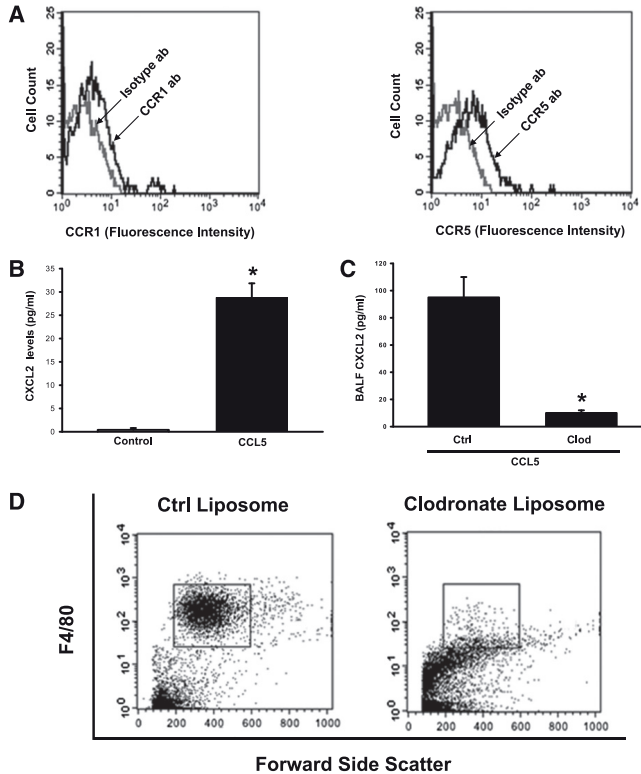


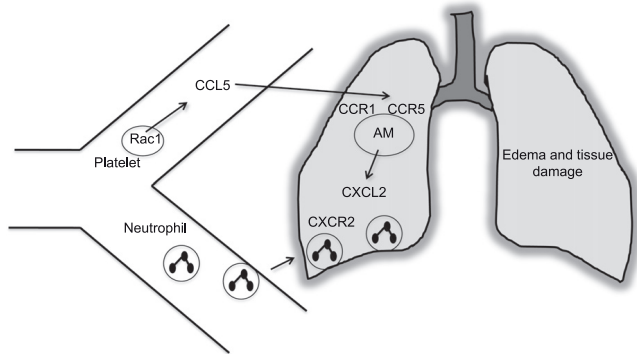
Figure 6. (A) Alveolar macrophage surface expression of CCR1 and CCR5. Isolated alveolar macrophages were stained with antibodies against CCR1 and CCR5, as described in Materials and Methods. (B) Isolated alveolar macrophages were stimulated with CCL5 (500 ng/ml), and the CXCL2 levels in supernatants were determined by use of ELISA. (C) Lung levels of CXCL2 and (D) alveolar macrophages in animals treated intratracheally with a control liposome (Ctrl) or a liposome containing clodronate, as described in Materials and Methods. Data represent mean \pm SEM, and $n = 5$. * $P < 0.05$ versus Control or Vehicle + CCL5.

on the role of Rac1 might help explain the protective effects of simvastatin on lung injury in abdominal sepsis.

Sepsis is typified by a generalized activation of the host innate immune system, including neutrophils and macrophages, causing acute lung injury with impaired gaseous exchange, which is the most insidious feature in patients with abdominal sepsis [9, 44]. Herein, we show that immunoneutralization of CCL5 protects against pulmonary edema and tissue damage in septic animals, indicating that CCL5 plays an important role in septic lung injury. This finding extends on previous studies reporting that CCL5 appears to be critical in diseases, such as encephalitis, endotoxemia, stroke, and coronary ischemia [21–24]. Herein, we could show that the targeting of CCL5 function decreased lung levels of MPO, a marker of neutrophils, by >47% in septic mice. This inhibitory effect on MPO correlated well with our finding that immunoneutralization of CCL5 reduced sepsis-induced neutrophil infiltration in the bronchoalveolar space by 42%, suggesting that CCL5 is a potent regulator of neutrophil accumulation in septic lung damage. With the consideration of the close relationship between

neutrophil recruitment and pulmonary damage, it might be assumed that the protective effect of targeting CCL5 is a result of the inhibition of pulmonary neutrophilia. Several previous studies have reported that inhibition of CCL5 can decrease neutrophil accumulation in the lung [20, 21], heart [45], colon [46], liver [47], and brain [23], suggesting that CCL5 might control extravascular trafficking of neutrophils in multiple organs. Neutrophils are normally unresponsive to CC chemokines [12, 48]. In this context, it is interesting to note that some previous studies reported that neutrophils stimulated with GM-CSF, TNF- α , and IFN- γ can, under certain circumstances, up-regulate CC chemokine receptors, including CCR1 [18, 19, 49]. Thus, we next asked whether neutrophils up-regulate the CCL5 receptors CCR1 and CCR5 in abdominal sepsis. However, we found that neither CCR1 nor CCR5 was expressed on neutrophils in sham or CLP animals, suggesting that CCL5 regulates neutrophil trafficking in septic lung injury in an indirect manner. This notion is also supported by our findings showing that in contrast to CXCL2, CCL5 exerts no direct chemotactic effect on neutrophils.

Figure 7. Proposed model for neutrophil recruitment in septic lung damage mediated by platelet-derived CCL5. Abdominal sepsis triggers Rac1 activation and Rac1-dependent secretion of CCL5 from platelets. CCL5 activates alveolar macrophages (AM) expressing CCR1 and CCR5, leading to CXCL2 secretion in the lung. Increased local concentrations of CXCL2 stimulate neutrophil recruitment to the lung, where they cause tissue edema and injury.



Accumulation of neutrophils at extravascular sites of inflammation is a multistep process facilitated by specific adhesion molecules expressed on neutrophils, including CD162 and Mac-1 [4, 50]. Therefore, we examined whether inhibition of CCL5 might control neutrophil activation and expression of Mac-1. However, immunoneutralization of CCL5 had no effect on Mac-1 up-regulation on neutrophils in septic animals, suggesting that CCL5 is not a regulator of Mac-1 expression on neutrophils. Neutrophil trafficking in the extravascular space is orchestrated by secreted CXC chemokines, such as CXCL1 and CXCL2, which are murine homologs of human IL-8 [51]. Indeed, previous studies have documented a functional role of CXC chemokines in abdominal infections [25, 52]. In the present study, we found that the targeting of CCL5 markedly decreased plasma and pulmonary levels of CXC chemokines in septic mice. These findings suggest that CCL5 might regulate neutrophil recruitment in septic lung injury indirectly via formation of CXC chemokines in the lung. This notion is also supported by our findings showing that local intratracheal administration of CCL5 increased formation of CXCL2 and neutrophil infiltration in the lung. In addition, we observed that inhibition of CXCR2 abolished neutrophil accumulation in the lung, triggered by local intratracheal challenge with CCL5, further supporting the concept that CCL5 promotes sepsis-induced neutrophil recruitment in the lung via formation of CXC chemokines. We next asked if alveolar macrophages might be a target cell of CCL5 in the formation of CXCL2 in the lung. We isolated alveolar macrophages from the murine lung and found that these cells express the CCL5 receptors CCR1 and CCR5, which is in line with previous reports [53–55]. Interestingly, we observed that cocultivation of alveolar macrophages with CCL5 caused significant secretion of CXCL2, indicating that CCL5 is a potent stimulator of CXCL2 formation in alveolar macrophages. This notion is in line with our observation, demonstrating that intratracheal administration of clodronate not only depleted lungs of alveolar macrophages but also abolished CCL5-evoked generation of CXCL2 in the lung, suggesting that alveolar macrophages are an important target cell of CCL5 in mediating pulmonary formation of CXCL2. Thus, these findings

demonstrate how CCL5 indirectly triggers neutrophil recruitment in the lung via alveolar macrophage secretion of CXCL2 in abdominal sepsis.

A schematic representation of the proposed model for neutrophil recruitment in septic lung damage mediated by platelet-derived CCL5 is shown in Fig. 7. In summary, these results indicate that Rac1 activity is increased in platelets and regulates platelet secretion of CCL5 in abdominal sepsis. In addition, our findings show that CCL5 regulates neutrophil recruitment in septic lung injury via activation of alveolar macrophages, leading to local secretion of CXCL2. Thus, our novel data not only elucidate complex mechanisms regulating pulmonary neutrophil trafficking in sepsis but also suggest that the targeting of Rac1 signaling and platelet-derived CCL5 might be a useful way to control pathologic inflammation and tissue damage in the lung in abdominal sepsis.

AUTHORSHIP

R.H., M.R., I.S., and E.Z. performed experiments, analyzed data, and wrote the manuscript. H.T. supervised the project, designed the experiments, and wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (2012-3685), Crafoord Foundation, Einar and Inga Nilssons Foundation, Harald and Greta Jaenssons Foundation, Greta och Johan Kocks Foundation, Agnes Nilssons Foundation, Franke and Margareta Bergqvists Foundation for Promotion of Cancer Research, Magnus Bergvalls Foundation, Mossfelts Foundation, Nanna Svartz Foundation, Ruth och Richard Julins Foundation, Swedish Medical Society Fund, Malmö General Hospital Foundation for Combating Cancer, MAS Fund, Malmö University Hospital, and Lund University. R.H. is supported by Hawler Medical University and the Kurdistan regional government. E.Z.’s position receives funding from the European Union Integrated Project BetaBat and a collaborative SRC Grant,

as well as from the Swedish Strategic Research EXODIAB. This work was done with use of imaging equipment financed by the Knut and Alice Wallenberg Foundation.

DISCLOSURES

The authors have no financial conflicts of interest.

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KEY WORDS:
chemokines · sepsis · CLP · inflammation

Paper IV

Platelet secretion of CXCL4 is Rac1 dependent and regulates neutrophil infiltration and tissue damage in septic lung damage

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Running title: CXCL4 and septic lung damage

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Abbreviations:

BALF= Bronchoalveolar lavage fluid, CCL5= Chemokine (C-C motif) ligand 5, CLP= cecal ligation and puncture, CXCL1= chemokine (C-X-C motif) ligand 1, CXCL2= chemokine (C-X-C motif) ligand 2, CXCR2= CXC chemokine receptor 2, ELISA= The Enzyme-linked immunosorbent assay, FITC= Fluorescein isothiocyanate, Mac-1= Macrophage-1 antigen, MPO= Myeloperoxidase, PAR4= Protease-activated receptor 4, PBS= Phosphate buffered saline, PE= Phycoerythrin, PerCP-Cy5.5= Peridininchlorophyll protein-Cy5.5, Rac1= Ras, related C3 botulinum toxin substrate 1, RPMI= Roswell park memorial institute medium

Abstract

Background and purpose: Platelets are potent regulators of neutrophil accumulation in septic lung damage. In the present study, we hypothesized that platelet-derived CXCL4 might support pulmonary neutrophilia in abdominal sepsis.

Experimental approach: Polymicrobial sepsis was triggered by cecal ligation and puncture (CLP) in C57/Bl6 mice. Platelet secretion of CXCL4 was studied by using confocal microscopy. Plasma and lung levels of CXCL4, CXCL1 and CXCL2 were determined by use of ELISA. Flow cytometry was used to examine surface expression of Mac-1 on neutrophils.

Key results: CLP increased CXCL4 levels in plasma and depletion of platelets markedly reduced plasma levels of CXCL4 in septic animals. Treatment with the Rac1 inhibitor NSC23766 decreased the CLP-induced enhancement of CXCL4 levels in plasma by 77%. In addition, inhibition of Rac1 abolished proteinase-activated receptor-4-induced secretion of CXCL4 from isolated platelets. Inhibition of CXCL4 reduced CLP-evoked neutrophil recruitment, edema formation and tissue damage in the lung. However, immunoneutralization CXCL4 had no effect on CLP-induced expression of Mac-1 on neutrophils. Targeting CXCL4 significantly attenuated plasma and lung levels of CXCL1 and CXCL2 in septic mice. CXCL4 had no effect on neutrophil chemotaxis in vitro, indicating an indirect effect of CXCL4 on pulmonary neutrophilia. Intratracheal administration of CXCL4 enhanced infiltration of neutrophils and formation of CXCL2 in the lung. Treatment with the CXCR2 antagonist SB225002 markedly reduced CXCL4-provoked neutrophil accumulation in the lung. CXCL4 caused clear-cut secretion of CXCL2 from isolated alveolar macrophages.

Conclusions and Implications: These results indicate that Rac1 controls platelet secretion of CXCL4 and that CXCL4 is a potent stimulator of neutrophil accumulation in septic lung damage via generation of CXCL2 in alveolar macrophages. We conclude that targeting platelet-derived CXCL4 might be an effective way to attenuate lung damage in polymicrobial sepsis.

Introduction

Management of patients with sepsis poses a significant challenge to clinicians. One reason is related to the lack of a comprehensive understanding of the underlying pathophysiology in sepsis. It is well-known that the lung is a very sensitive and critical target organ in sepsis (Parrillo, 1993). Although neutrophil recruitment constitutes a central component in the host response to bacterial infections (Gorbach *et al.*, 1974; Reutershan *et al.*, 2005), excessive neutrophil responses are known to cause organ damage in sepsis (Issekutz *et al.*, 1992; Reutershan *et al.*, 2005; Basit *et al.*, 2006; Asaduzzaman *et al.*, 2008). Based on experiments blocking neutrophil recruitment several studies have shown that neutrophil accumulation constitutes a rate-limiting step in septic lung damage (Asaduzzaman *et al.*, 2008; Asaduzzaman *et al.*, 2009b; Hasan *et al.*, 2011). It is interesting to note that accumulating data implicate platelets in the development of septic lung injury. For example, several studies have shown that platelets promote neutrophil activation and recruitment to the lung in sepsis (Zarbock *et al.*, 2007; Asaduzzaman *et al.*, 2009a). One study has shown that platelet-derived CD40L is a potent inducer of neutrophil infiltration in septic lung injury (Rahman *et al.*, 2009). However, platelets harbor a wide-spectrum of different pro-inflammatory compounds, such as chemokines, which could mediate platelet-dependent accumulation of neutrophils in septic lung damage.

CXCL4 is one of the most abundant chemokine in platelets, which belong to the CXC chemokine family although it lacks an ELR sequence needed for binding to chemotactic CXCRs on neutrophils (Clark-Lewis *et al.*, 1993). The literature on the chemotactic activity of CXCL4 is complex and contradictory. For example, one early study reported that CXCL4 exerts chemotactic activity toward neutrophils (Deuel *et al.*, 1981). However, these findings could not be confirmed in later studies and the early findings may have been caused by contamination with other chemokines (Petersen *et al.*, 1996). The studies on the role of CXCL4 have also been complicated due to the absence of clearly defined CXCL4 receptors. CXCL4 binds avidly to glycosaminoglycans, e.i. a chondroitin sulphate proteoglycan on neutrophils (Petersen *et al.*, 1998). In addition, CXCL4 has been shown to bind to CXCR3B, which is a splice variant of CXCR3 expressed in humans but not in mice (Lasagni *et al.*, 2003; Kowalska *et al.*, 2010). Nonetheless, experimental evidence have shown that CXCL4 play a role in regulating neutrophil recruitment and tissue damage in complex inflammatory disease models, such liver fibrosis

(Zaldivar *et al.*, 2010) and intestinal reperfusion injury (Lapchak *et al.*, 2012). One study reported that plasma levels of CXCL4 are elevated in patients with sepsis (Lorenz *et al.*, 1988), raising the question whether CXCL4 can regulate neutrophil-dependent tissue damage in abdominal sepsis.

The intracellular signaling cascades triggering platelet secretion of CXCL4 are not well understood. We recently reported that Rac1, a member of the Ras-homologus (Rho) family, not only plays key function septic lung damage (Hwaiz *et al.*, 2013), but also controls platelet secretion of CD40L in sepsis (Hwaiz *et al.*, 2014). Other studies have identified an essential role Rac1 in lamellipodia formation, phospholipase C γ 2 activation, granule secretion and clot retraction in platelets (McCarty *et al.*, 2005; Akbar *et al.*, 2007; Pleines *et al.*, 2009). Thus, we asked whether Rac1 might be involved in the secretion of CXCL4 from platelets in the present study.

Thus, one aim of the present study was to examine the function of Rac1 in regulating platelet secretion of CXCL4. Moreover, we wanted to investigate the role of CXCL4 in regulating pulmonary neutrophilia and tissue injury in polymicrobial sepsis.

Materials and Methods

Animals

All experiments were performed using male C57Bl/6 mice (20-25 g) 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 intraperitoneal (i.p.) administration of 75 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) per kg body weight.

Experimental model of sepsis

Abdominal sepsis was induced in anesthetized mice by puncture of the cecum. First, the abdomen was opened and the cecum was filled with feces by milking stool backward from the ascending colon. Then a ligature was placed below the ileocecal valve and the cecum was soaked with phosphate-buffered saline (PBS; pH 7.4). The cecum was 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 wall was closed. Animals were treated with vehicle (dH₂O) 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). A control antibody (clone 141945 R&D Systems, Minneapolis, MN, USA) or a monoclonal antibody against murine CXCL4 (clone 140910, R&D Systems) was injected i.p. (10 µg per mouse) 30 min before CLP induction. Sham mice underwent the same surgical procedures, i.e. laparotomy and resuscitation, but the cecum was neither ligated nor punctured. The animals were then returned to their cages and provided food and water *ad libitum*. Animals were re-anesthetized 6 h 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 counted. 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.

MPO assay

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 H₂O₂ (450 nm, with a reference filter 540 nm, 25°C). Values were expressed as MPO units per g tissue.

BALF and lung edema

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 Burkner chamber. The left lung was excised and then 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.

Intratracheal challenge with CXCL4

500 ng of CXCL4 was administered intratracheally. Negative control mice underwent the same surgical procedures but received only PBS. 4 h after CXCL4 challenge, BALF was collected for analysis of CXCL2 and neutrophils. In certain experiments the CXCR2 antagonist SB225002 administered prior to intratracheal injection of CXCL4.

Histology

Lung tissue was fixed 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 use of a modified scoring system (Carraway *et al.*, 2003; Borzone *et al.*, 2007), 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.

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 Fc γ 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 α M china, rat IgG2b κ , BD Biosciences Pharmingen, San Jose, CA, USA) antibodies. Alveolar macrophages were isolated as described below and incubated with an anti-CD16/CD32 antibody blocking Fc γ III/II receptors and a PerCP-Cy5.5-conjugated anti-mouse F4/80 antibody (clone BM8, eBioscience, Frankfurt, Germany) and a FITC-conjugated anti-Ly6G (clone 1A8, BD Biosciences) antibody. 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.

ELISA

CXCL1, CXCL2 and CXCL4 levels in lung tissue and plasma were analyzed by using double antibody Quantikine ELISA kits (R & D Systems, Europe, Abingdon, Oxon, UK) using recombinant murine CXCL1, CXCL2 and CXCL4 as standards.

Neutrophil chemotaxis

Neutrophils were isolated from bone marrow by use of Ficoll-Paque™. 1.5 x 10⁶ neutrophils were placed in the upper chamber of the transwell inserts (5 μ m 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) or CXCL4 (500 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 Burkner chamber.

Alveolar macrophage secretion of CXCL2

BALF was collected as above from healthy mice and cells were isolated by centrifugation (450 g, 10 min) and resuspended in RPMI1644. Purity was determined by staining cells with anti-F4/80 and anti-Ly6G antibodies as described above and macrophages were defined as F4/80⁺/Ly6G⁻ cells. Alveolar macrophages were co-incubated with recombinant murine CXCL4 (500 ng/ml) for 4 h (37°C) and then were CXCL2 levels measured by ELISA.

Platelet isolation and CXCL4 secretion

Blood was collected in syringes containing 0.1 ml of acid-citrate-dextrose, immediately diluted with equal volumes of modified Tyrode solution (1 µg/ml prostaglandin E₁ and 0.1 U/ml apyrase) and centrifuged (200 g, 5 min). Platelet-rich plasma was collected and centrifuged (800 g, 15 min) and pellets were resuspended in modified Tyrode solution. After being washed one more time (10 000 g, 5 min) 0.5 x 10⁶ platelets were seeded on a chamber slide coated with fibrinogen (20 µg/ml). Adherent platelets were stimulated with protease-activated receptor 4 (PAR4) (200 µM, 37°C) with and without NSC23766 (100 µM). Platelets were fixed with 2% paraformaldehyde for 5 min and washed and blocked with 1% goat serum for 45 min. Then platelets were permeabilized with 0.15% Triton X-100 for 15 min followed by washing and incubation with an anti-CD16/CD32 antibody (10 min) blocking Fcγ III/II receptors to reduce non-specific labeling and a rabbit polyclonal primary antibody against CXCL4 (bs-2548R, Bioss, Boston, MA, USA) for 2 h. Chamber slides were washed and incubated with FITC-conjugated anti-rabbit secondary antibody (Cell Signaling Technology, Beverly, MA, USA) and platelet specific PE-conjugated anti-CD41 (clone MWReg30, eBioscience, San Diego, CA, USA) for 1 h. Chamber slides are washed three times and confocal microscopy was performed using Meta 510 confocal microscopy (Carl Zeiss, Jena, Germany). FITC and PE were excited by 488 nm and 543 nm laser lines and corresponding emission wavelengths of FITC and PE were collected by the filters of 500-530 nm and 560-590 nm, respectively. The pinhole was ~1 airy unit and the scanning frame was 512×512 pixels. The fluorescent intensity was calculated by use of ZEN2009 software.

Pull-down assay and Western blotting

Rac1 activity was determined in platelets from sham and CLP mice pretreated with vehicle or NSC23766 by active Rac1 pull-down and detection kit using the protein binding domain of GST-PAK1, which binds with the GTP-bound form of Rac1 (Pierce Biotechnology, Rockford, IL, USA). Briefly, platelets were suspended in lysis buffer on ice and centrifuged (16000 g, 15 min). Ten μ l from each lysate were removed to measure protein content using Pierce BCA Protein Assay Reagent (Pierce Biotechnology) and the rest 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 (10-12% gel). After transferring to a nitrocellulose membrane (BioRad, Hercules, CA, USA), blots were blocked with tris buffer saline/Tween20 containing 3% bovine serum albumin at room temperature for 1 h, followed by incubation with an anti-Rac1 antibody (1:1000) at 4°C overnight. Binding of the antibody was detected using peroxidase-conjugated anti-mouse antibody (1:100000, Pierce Biotechnology) at room temperature for 2 h and developed by Immuno-Star WesternC Chemiluminescence Kit (BioRad). Total Rac1 was used as a loading control.

Statistics

Data were presented as mean values \pm 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 SigmaPlot® 10.0 software (Systat Software, Chicago, IL, USA).

Results

Rac1 regulates platelet secretion of CXCL4

Plasma levels of CXCL4 were low but detectable in sham animals (Fig. 1a). CLP increased CXCL4 levels in plasma by 41-fold (Fig. 1a). To deplete animals of platelets, an anti-CD41 antibody was administered before CLP induction. It was observed that platelet depletion reduced plasma levels of CXCL4 by 87% in septic animals (Fig. 1a), indicating that platelets are the dominating source of CXCL4 in abdominal sepsis. Treatment with the Rac1 inhibitor NSC23766 had no effect on CXCL4 levels in the plasma of control animals (Fig. 1a). However, administration of NSC23766 reduced plasma levels of CXCL4 by 77% in CLP animals (Fig. 1a). Moreover, we found that CLP increased Rac1-GTP levels in platelets, indicating that Rac1 is activated in platelets in septic animals (Fig. 1b and 1c). Administration of NSC23766 abolished CLP-evoked Rac1 activation in platelets (Fig. 1b and 1c), demonstrating that NSC23766 is an effective inhibitor of Rac1 activation. We stimulated isolated platelets with PAR4 in order to determine the direct role of Rac1 in regulating platelet secretion of CXCL4. It was found that CXCL4 was present in unstimulated platelets and that co-incubation with PAR4 reduced intracellular levels of CXCL4 in platelets (Fig. 1d and 1e). Notably, co-incubation with NSC23766 markedly decreased PAR-induced secretion of CXCL4 from the platelets (Fig. 1d and 1e).

CXCL4 regulates septic lung injury

It was found that CLP increased lung edema and immunoneutralization of CXCL4 reduced edema formation in the lung by 65% in septic animals (Fig. 2a). The lung injury in septic mice was characterized by severe destruction of tissue microarchitecture, extensive edema of and massive infiltration of neutrophils (Fig. 2b). Inhibition of CXCL4 attenuated CLP-induced tissue damage and neutrophil accumulation in the lung (Fig. 2b). Quantification of the morphological damage revealed that CLP significantly enhanced lung damage score and that immunoneutralization of CXCL4 decreased CLP-evoked tissue injury in the lung by 67% (Fig. 2c).

CXCL4 regulates neutrophil infiltration in septic lung injury

CLP increased pulmonary levels of MPO by 24-fold and immunoneutralization of CXCL4 reduced MPO activity in the lung by 57% in septic mice (Fig. 3a). CLP increased the number of alveolar neutrophils by 19-fold and administration of the anti-CXCL4 antibody decreased the number of alveolar neutrophils by 42% in the inflamed lungs (Fig. 3b). Neutrophil expression of Mac-1 was increased in septic mice (Fig. 3c). Inhibition of CXCL4 function had no effect on neutrophil expression of Mac-1 (Fig. 3c). In vitro, CXCL2 triggered robust migration of isolated neutrophils whereas CXCL4 exerted no chemotactic effect on neutrophils (Fig. 3d), suggesting that the CXCL4-dependent accumulation of neutrophils is not a direct action on neutrophils but rather an indirect effect of CXCL4.

CXCL4 regulates CXC chemokine formation in sepsis

Plasma and lung levels of CXCL1 and CXCL2 were low but detectable in sham mice (Fig. 4). We found that CXCL1 and CXCL2 levels in the plasma increased by 1183-fold and 1153-fold, respectively, in septic animals (Fig. 4a and 4b). Inhibition CXCL4 decreased CLP-induced plasma levels of CXCL1 by 94% and CXCL2 by 95% (Fig 4a and 4b). Moreover, CLP increased lung levels of CXCL1 by 144-fold and CXCL2 by 581-fold (Fig. 4c and 4d). Immunoneutralization of CXCL4 decreased CLP-induced increases of CXCL1 and CXCL2 by 85% and 95% respectively (Fig. 4c and 4d). We next studied direct effects of CXCL4 on neutrophil accumulation in the lung. Intratracheal administration of CXCL4 enhanced pulmonary levels of CXCL2 (Fig. 5a) and the number of alveolar neutrophils (Fig. 5b). In addition, treatment with the CXCR2 antagonist SB225002 reduced CXCL4-triggered recruitment of neutrophils by 82% in the lung (Fig. 5c). We next isolated alveolar macrophages and co-incubated them with CXCL4 and found that CXCL4 increased CXCL2 formation (Fig. 5d). This finding was repeated in RAW264.7 cells showing that CXCL4 enhanced secretion of CXCL2 from macrophages (not shown). Moreover, another platelet-derived chemokine, i.e. CCL5 also dose-dependently increased macrophage secretion of CXCL2. Notably, we found that CXCL4 and CCL5 cooperate to increase macrophage secretion of CXCL2 (Fig. 5d).

Discussion

Our present findings indicate an important function of Rac1-mediated secretion of CXCL4 from platelets in sepsis. In addition, this study reveals key mechanisms controlling CXCL4-dependent pulmonary accumulation of neutrophils in abdominal sepsis. These results point to an important role of platelets in sepsis and implicate that inhibition of Rac1 signaling and/or CXCL4 function might be useful strategies to ameliorate septic lung damage.

Platelets are not only critical in wound healing and thrombosis but also exert numerous pro-inflammatory functions in the host response to bacterial invasion (Rahman *et al.*, 2009; Rahman *et al.*, 2013; Hwaiz *et al.*, 2014). For example, data have shown that platelets regulate numerous aspects of leukocyte responses to severe infections (Asaduzzaman *et al.*, 2008; Asaduzzaman *et al.*, 2009a). One such key aspect of the inflammatory response is neutrophil activation and accumulation at sites of microbial invasion. For example, platelet-derived CD40L has been reported to regulate sepsis-evoked neutrophil activation and tissue recruitment (Rahman *et al.*, 2009; Rahman *et al.*, 2013). Notably, platelets harbor several other pro-inflammatory substances, including a plethora of different chemokines (Yan *et al.*, 1994; Flad *et al.*, 2010). The most abundant chemokines in platelets are CCL5 and CXCL4 but these chemokines are poor stimulators of neutrophil migration (Petersen *et al.*, 1996; Hartl *et al.*, 2008). Instead, CCL5 and CXCL4 are potent activators lymphocytes, macrophages and eosinophils (McCull *et al.*, 1993; Hartl *et al.*, 2008). Anyhow studies in the literature have reported that targeting CXCL4 can decrease neutrophil recruitment in models of inflammatory diseases (Zaldivar *et al.*, 2010; Grommes *et al.*, 2012; Lapchak *et al.*, 2012). Interestingly, we found that platelet depletion greatly reduced plasma levels of CXCL4 in CLP animals, suggesting that platelets are a dominating source of circulating CXCL4 in polymicrobial sepsis. Since the mechanisms of platelet secretion of CXCL4 are not known, we wanted first examine the signaling mechanisms controlling platelet secretion of CXCL4. We observed that platelets from septic mice exhibited increased Rac1 activity. In addition, treatment with the Rac1 inhibitor NSC23766 prior to CLP induction markedly decreased plasma levels of CXCL4 in septic animals, indicating that Rac1 signaling is a critical component in regulating circulating levels of CXCL4 in abdominal sepsis. Next, we wanted to determine the direct role of Rac1 in controlling platelet secretion of CXCL4. It was found that co-incubation of platelets with NSC23766 abolished PAR4-induced platelet

secretion of CXCL4 *in vitro*, showing for the first time that Rac1 regulates CXCL4 secretion from platelets. In this context, it is interesting to note that Rac1 was demonstrated to be involved in agonist-evoked mobilization of P-selectin and PDGF on platelets (Akbar *et al.*, 2007; Dwivedi *et al.*, 2010) and considering that P-selectin, PDGF and CXCL4 are localized in the platelet α -granules (Akbar *et al.*, 2007; Galkina *et al.*, 2007; Gleissner *et al.*, 2008; Blair *et al.*, 2009), these present findings suggest that Rac1 is a potential regulator of α -granule secretion in platelets.

Activation of innate immune cells is a landmark in the host response to severe infections and is an important cause of acute lung injury characterized by disturbed gaseous exchange (Remick, 2007; Asaduzzaman *et al.*, 2009a). In the present study, we demonstrate that inhibition of CXCL4 reduces edema formation and tissue damage in the lungs of septic mice, suggesting that CXCL4 exerts a causative role in the development of acute lung injury in polymicrobial sepsis. This notion is in line with recent observations reporting a key role of CXCL4 in inflammatory diseases, such as atherosclerosis and liver fibrosis (Zaldivar *et al.*, 2010; Karshovska *et al.*, 2014). Our data showed that immunoneutralization of CXCL4 reduced pulmonary MPO activity, a marker of neutrophil accumulation, by 57% in septic animals, which correlated well with the 42% reduction of neutrophil numbers in the bronchoalveolar space. These findings suggest that CXCL4 is an important regulator of pulmonary neutrophilia in polymicrobial sepsis. This notion is also supported by a recent study showing that neutrophil accumulation in the lung in a model of mesenteric ischemia and reperfusion is decreased in mice lacking CXCL4 (Lapchak *et al.*, 2012). Knowing that neutrophil infiltration is a rate-limiting step in septic lung injury, our data suggest that the protective effect of inhibiting CXCL4 is related to the reduction in neutrophil recruitment in the lung.

Neutrophil accumulation at extravascular sites of tissue injury and inflammation is a multistep process mediated by specific adhesion molecules on neutrophils, such as CD162 and Mac-1 (Asaduzzaman *et al.*, 2008; Zhang *et al.*, 2013). Herein, we studied whether targeting of CXCL4 could reduce neutrophil up-regulation of Mac-1. However, inhibition of CXCL4 had no effect on neutrophil expression of Mac-1 in septic mice, indicating that CXCL4 is not involved in Mac-1 expression on neutrophils. Tissue trafficking of neutrophils are co-ordinated by secreted CXC chemokines, including, CXCL1 and CXCL2, which are murine homologues of human interleukin-8 (Tekamp-Olson *et al.*, 1990). Previous reports have shown that CXC chemokines plays an important role in septic lung injury (Hasan *et al.*, 2013; Hwaiz *et al.*, 2013).

Herein, it was observed that immunoneutralization of CXCL4 abolished plasma and lung levels of CXC chemokines in CLP animals. These results indicate that CXCL4 might control neutrophil accumulation indirectly via generation of CXC chemokines in the septic lung. This conclusion is also in line with our present results demonstrating that local intratracheal challenge with CXCL4 enhanced generation of CXCL2 and neutrophil recruitment in the lung. That local injection of CXCL4 can provoke neutrophil infiltration in the lung is supported by a previous study showing that local administration of CXCL4 triggers accumulation of neutrophils in the skin (Sharpe *et al.*, 1991). Moreover, we observed that blocking CXCR2 function markedly decreased pulmonary neutrophilia induced by intratracheal administration of CXCL4, which further supports the conclusion that CXCL4 triggers neutrophil accumulation in the lung via generation of CXC chemokines in the inflamed lung. We next asked if alveolar macrophages might be a target cell of CXCL4. We found that co-incubation of isolated alveolar macrophages with CXCL4 dose-dependently increased CXCL2 secretion, suggesting that CXCL4 is a potent inducer of CXCL2 secretion in alveolar macrophages. In fact, this is the first time CXCL4 has been demonstrated to promote macrophage secretion of CXCL2 although this observation is in line with findings showing that CXCL4 stimulates macrophage phagocytosis and oxidative burst (Pervushina *et al.*, 2004). In this context, it is interesting to note that CXCL4 co-operates with other platelet-derived chemokines, such as CCL5. For example, it has been shown that CXCL4 and CCL5 cooperate to promote respiratory burst in macrophages (Pervushina *et al.*, 2004). Along these lines, we could show herein that combining CXCL4 and CCL5 further increases macrophage secretion of CXCL2, indicating that platelet-derived chemokines might co-operate at multiple stages in the host response to bacterial invasion.

Figure 6 shows a schematic representation of how platelet-derived CXCL4 cause neutrophil accumulation in septic lung injury. Thus, our findings suggest that Rac1 signaling is enhanced in platelets and regulates platelet secretion of CXCL4 in polymicrobial sepsis. Moreover, our results indicate that CXCL4 controls neutrophil accumulation via secretion of CXCL from alveolar macrophages in septic lung injury. Thus, these findings not only delineate complex mechanisms of neutrophil trafficking in sepsis but also suggest that targeting platelet-derived CXCL4 might be an effective way to ameliorate inflammation and tissue damage in septic lung damage.

Acknowledgements

This work was supported by grants from the Swedish Medical Research Council (2012-3685), Crafoord foundation, Einar and Inga Nilssons foundation, Harald and Greta Jaenssons foundation, Greta och Johan Kocks foundation, Agnes Nilssons foundation, Franke and Margareta Bergqvists foundation for promotion of cancer research, Magnus Bergvalls foundation, Mossfelts foundation, Nanna Svartz foundation, Ruth och Richard Julins foundation, Swedish Medical Society fund, Malmö General Hospital foundation for combating cancer, MAS fund, Malmö University Hospital and Lund University. RH is supported by Hawler Medical University and Kurdistan Regional Government. EZ's position receives funding from the EU Integrated project BetaBat and a collaborative SRC grant, as well as the Swedish Strategic Research EXODIAB. This work was done using imaging equipment financed by the Knut and Alice Wallenberg foundation.

Contribution of authors

R. Hwaiz, M. Rahman and E. Zhang performed experiments, analyzed data and wrote the manuscript. H. Thorlacius supervised the project, designed the experiments and wrote the manuscript

Disclosure

Authors have no financial conflicts of interest.

Figure Legends

Figure 1. Rac1 regulates platelet secretion of CXCL4 in sepsis. Animals were treated with vehicle, NSC23766 (5 mg/kg), a control ab (Ctrl ab) or an anti-GPIIb/IIIa antibody before CLP induction. a) ELISA was used to quantify the levels of CXCL4 in the plasma 6 h after CLP induction. b) Rac1-GTP was determined by western blotting using GST-PAK pull-down beads 6 h after induction of CLP. c) Band intensities were quantified in isolated platelets by densitometry and normalized to total Rac1. Western blots are representative of four independent experiments. Mice were treated with the Rac1 inhibitor NSC23766 (5 mg/kg) or vehicle prior to CLP induction. Sham operated mice served as negative controls. d) Isolated platelets were incubated with or without NSC23766 (10 μ M) and then stimulated with recombinant PAR4 (200 μ M) and the level of CXCL4 in permeabilized CD41⁺ platelets was determined by confocal microscopy. e) Aggregate data showing mean fluorescence intensity of CXCL4 in platelets. Non-stimulated platelets served as control. Data represent mean \pm SEM and $n = 4$. * $P < 0.05$ vs. Sham or Control, # $P < 0.05$ vs. Ctrl Ab + CLP, ^{ns} $P < 0.05$ vs. Vehicle + CLP or Vehicle + PAR4.

Figure 2. CXCL4 controls lung damage in sepsis. a) Edema formation in the lung. b) Representative haematoxylin and eosin sections of lung are shown. Animals were treated with vehicle, a control ab (Ctrl ab) or an anti-CXCL4 antibody before CLP induction. c) Lung injury scores as described in Materials and Methods 24 h after CLP induction. Sham-operated animals served as negative controls. Data represent mean \pm SEM and $n = 4$. * $P < 0.05$ vs. Sham, # $P < 0.05$ vs. Ctrl ab + CLP.

Figure 3. CXCL4 regulates pulmonary recruitment of neutrophils in sepsis. a) Lung MPO levels at 6 h post-CLP. b) Number of BALF neutrophils 24h after CLP induction. c) Mac-1 expression on circulating neutrophils 6 h after CLP induction. Animals were treated with vehicle, a control antibody (Ctrl ab) or an anti-CXCL4 ab before CLP induction. Sham-operated animals served as negative controls. d) Neutrophils isolated from bone marrow were analyzed for their migration in response to PBS (Control), CXCL2 (100 ng/ml) or CXCL4 (500 ng/ml). Non-stimulated neutrophils served as negative control. Data represent mean \pm SEM and $n = 4$. * $P < 0.05$ vs. Sham or Control and # $P < 0.05$ vs. Ctrl ab + CLP or CXCL2.

Figure 4. CXCL4 controls CXC chemokine formation in sepsis. Plasma levels of a) CXCL1 and b) CXCL2 and lung levels of c) CXCL1 and D) CXCL2 determined 24 h after CLP induction. Animals were treated with vehicle, a control antibody (Ctrl ab) or an anti-CXCL4 ab before CLP. Sham-operated animals served as negative controls. Data represents mean \pm SEM and $n = 4$. * $P < 0.05$ vs. Sham and $^{\#}P < 0.05$ vs. Ctrl ab + CLP.

Figure 5. CXCL4-induced neutrophil recruitment is dependent on CXCL2 formation. Levels of a) CXCL2 and b) number of neutrophils in the lung after intracheal challenge with CXCL4. c) Neutrophil accumulation in the lungs of animals treated with vehicle or the CXCR2 antagonist SB225002 prior to intracheal challenge with CXCL4. d) Isolated alveolar macrophages were stimulated with indicated doses of CXCL4 and CCL5 then CXCL2 levels were determined by use of ELISA. Data represents mean \pm SEM and $n = 4$. * $P < 0.05$ vs. Sham, $^{\#}P < 0.05$ vs. Vehicle + CXCL4 and $^{\alpha}P < 0.05$ vs. Vehicle + CXCL4 and CCL5.

Figure 6. Proposed model for neutrophil recruitment in septic lung damage mediated by platelet-derived CXCL4. Abdominal sepsis triggers Rac1 activation and Rac1-dependent secretion of CXCL4 from platelets. CXCL4 activates alveolar macrophages (AM) leading to CXCL2 secretion in the lung. Increased local concentrations of CXCL2 stimulate neutrophil recruitment to the lung where they cause tissue edema and injury.

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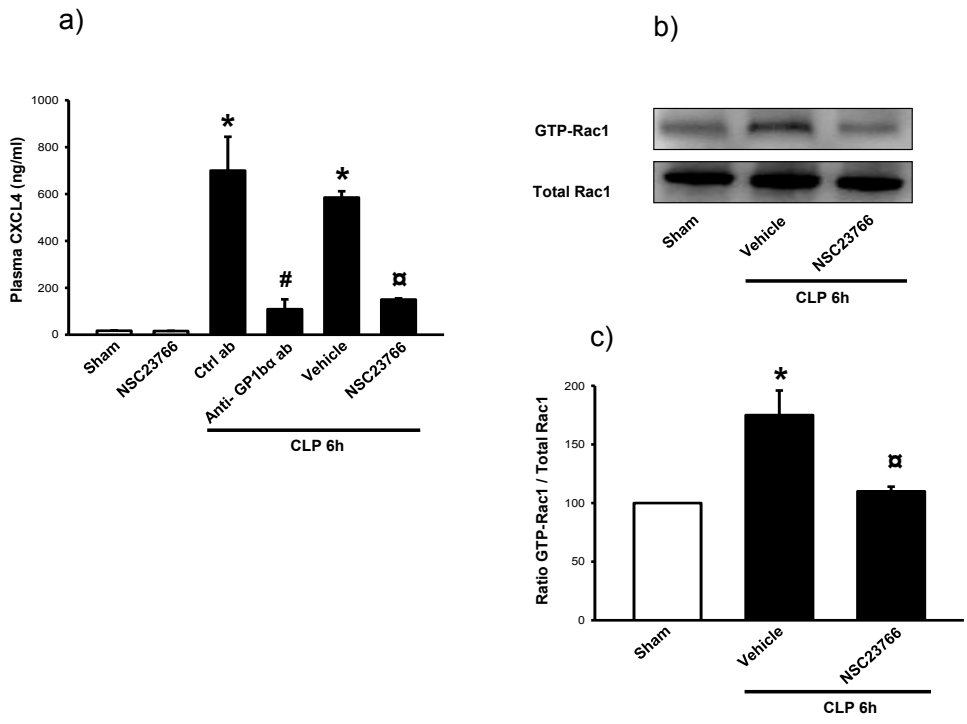


Figure 1

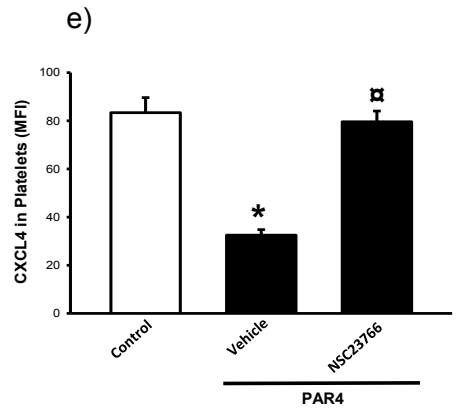
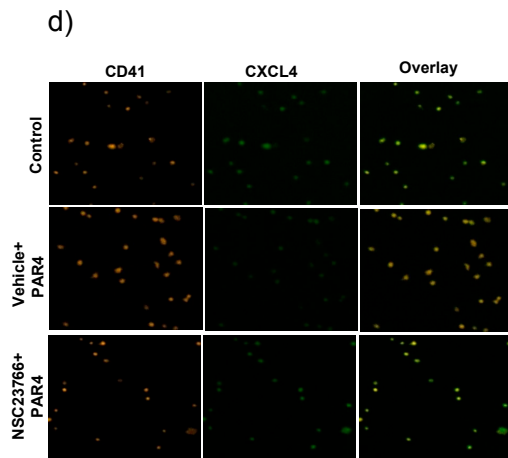


Figure 2

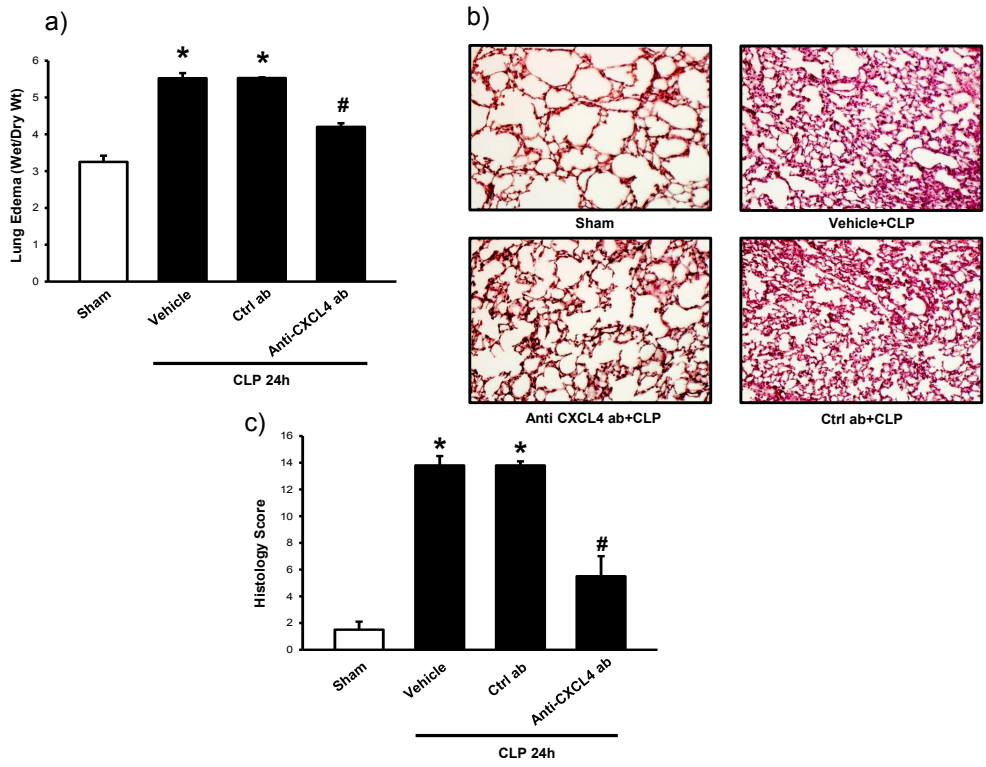


Figure 3

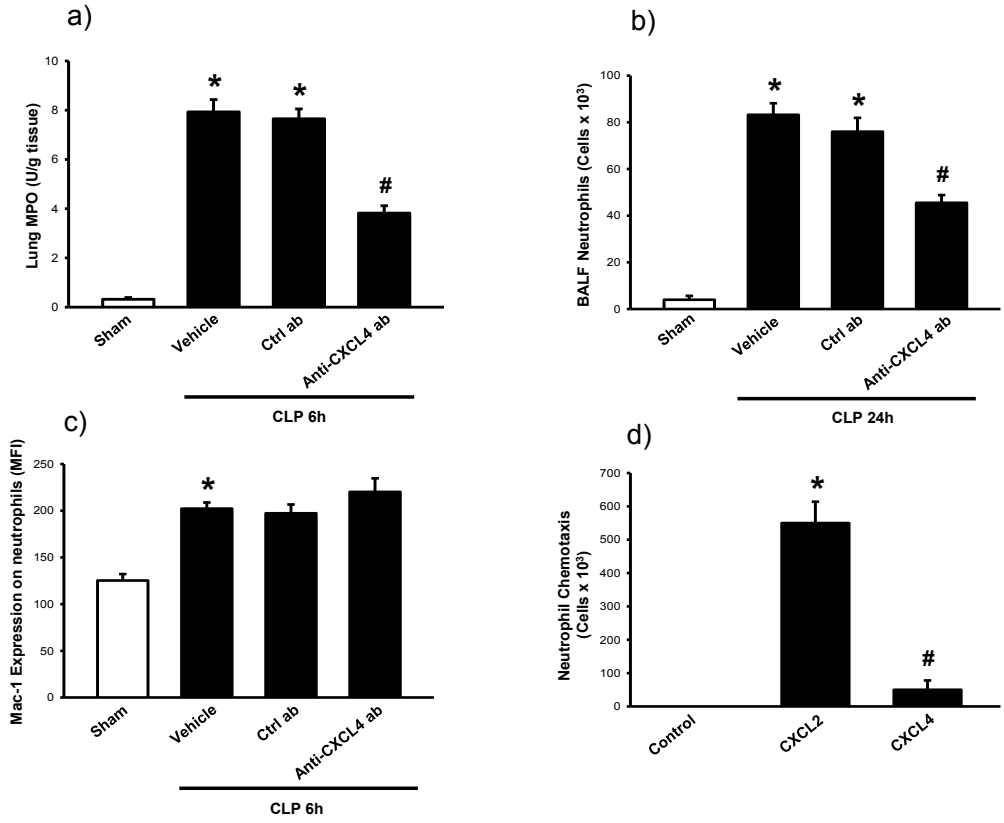


Figure 4

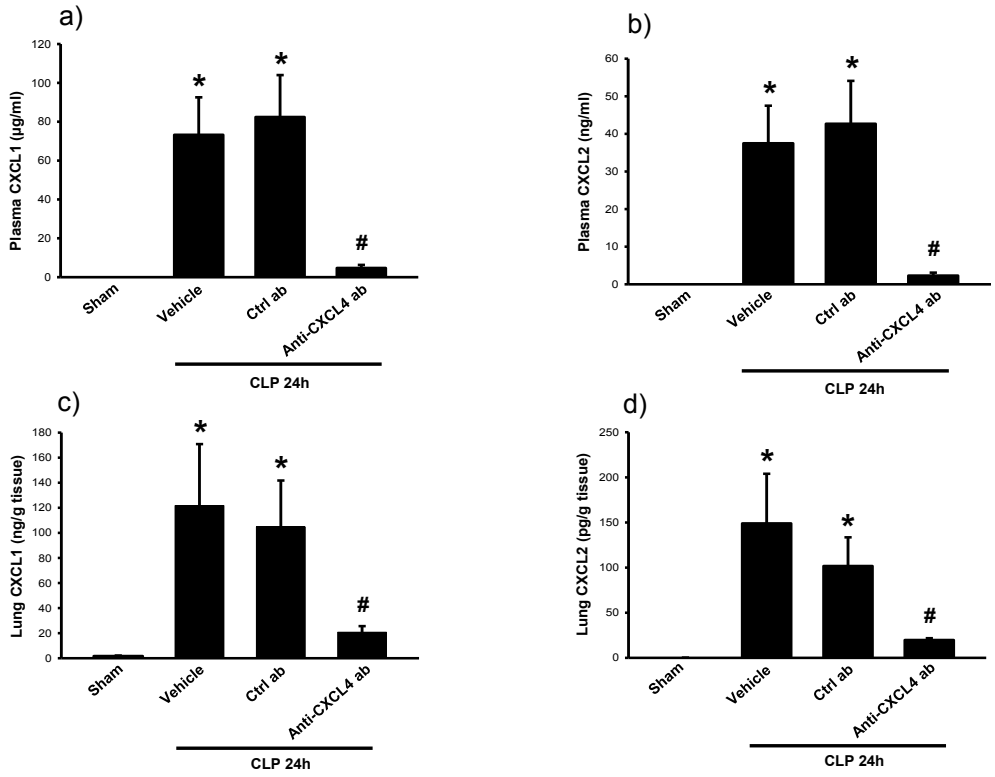


Figure 5

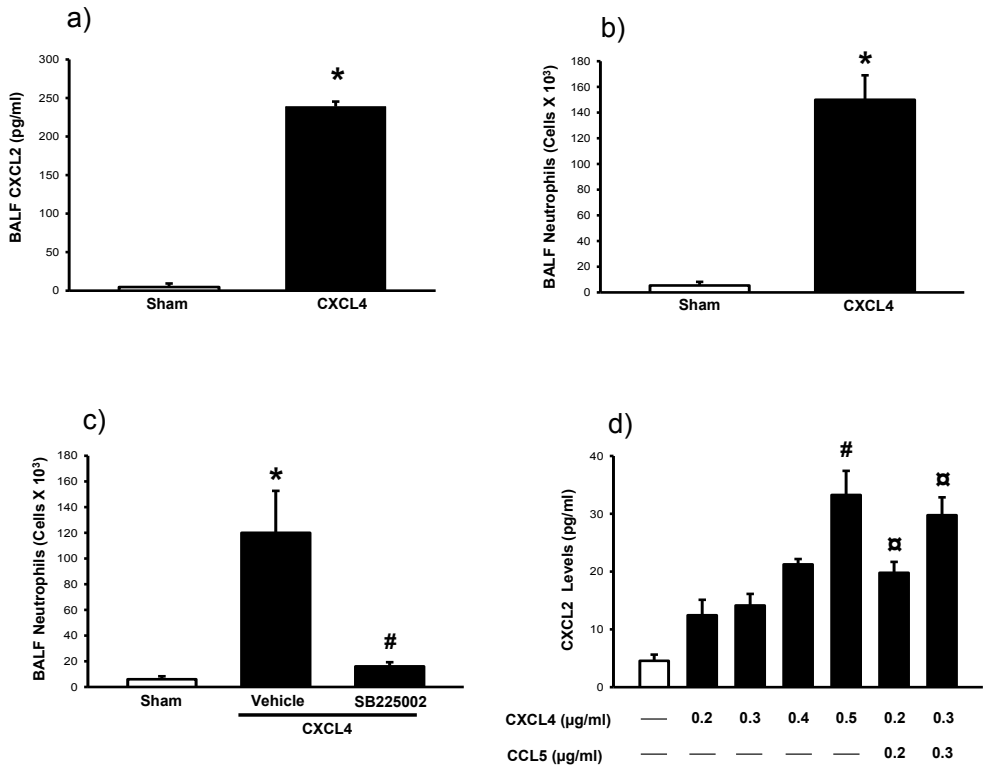


Figure 6

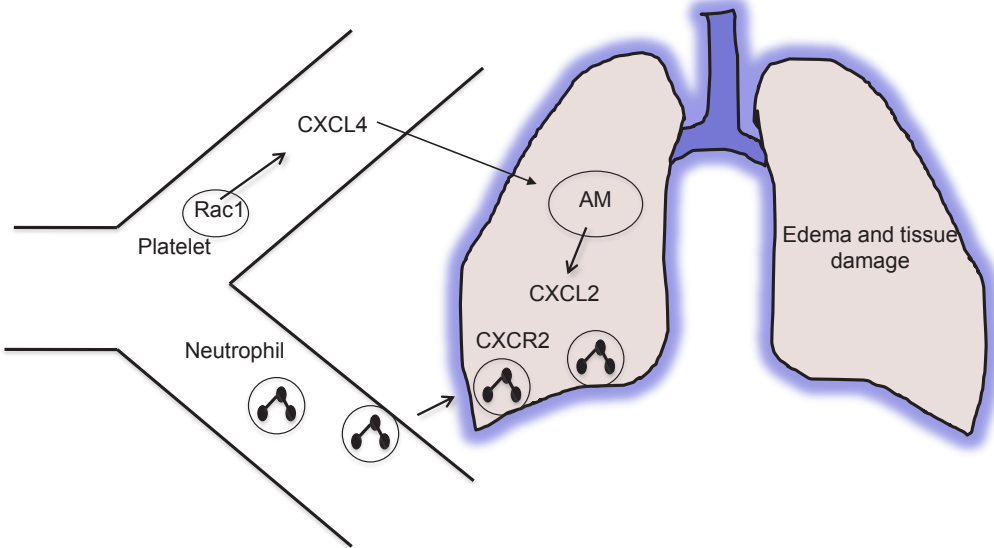


Figure 7

Medicine doctorates in Section of Surgery, Malmö, Lund University

1951	Arne Malm	1980	Göran Balldin	1991	Jan Berglund	2002	Thomas Sandgren
1953	Erik Akerlund	1981	Stefhan Brandsedt	1991	Hans Olof Håkansson	2002	Max Nyström
1953	Nils P Berg	1982	Tomas Lindhagen	1992	Henrik Bengtsson	2002	Rene Schramm
1953	Nils Carstam	1982	Harald Ljungner	1992	Thomas Troeng	2002	Ervin Töhl
1955	Anders Weneckert	1982	Carlos Esquivel	1992	Michael Hartmann	2002	Daniel Klintman
1955	Lars G Hallen	1982	Igor Niechajev	1992	Peter Björk	2002	Ake Mellström
1957	Lawe Svaneberg	1982	Einar Vernersson	1992	Anita Ringberg	2002	Amjid Kiaz
1958	Torsten Widén	1983	Svend Borup Christensen	1992	Henrik Weibull	2002	Matthias Corbascio
1959	Ivar Borg	1983	Afzal Vazery	1992	Erney Mattsson	2003	Nina Kvarning
1959	Arne Wether	1983	Johan Sällström	1992	Thorvaldur Jonsson	2003	Gudmundur Danielsson
1959	Knut Haeger	1983	Jan-Bertil Wieslander	1992	Magnus Bergenfäldt	2003	Fritz Berndsen
1960	Stig Borgström	1983	Bill Marks	1993	Anders Lundell	2003	Salathiel Mzezewa
1961	Karl-Fredrik Atonson	1983	Bengt Lindblad	1993	Baimeng Zhang	2004	Marianne Starck
1961	Oddvar Eiken	1984	Anders Larsson	1994	Stefan Matthiasson	2004	Li Xiang
1961	Carl-Fredrik Liedberg	1984	Peter Blomquist	1994	Stafåan Weiber	2004	Karl Malm
1962	Bertil Olow	1984	Ake Larsson	1994	Björn Sönesson	2004	Glaes Jansen
1963	Claes-Göran Backström	1984	Claes-Göran Björck	1995	Jan Stewenius	2004	Peter Danielsson
1963	Thorsten Stenberg	1984	Staffan Källero	1995	Björn Arnljots	2004	Lisa Rydén
1967	Slen Jacobsson	1984	Per Almqvist	1995	Jan Holst	2005	Ann-Cathrin Moberg
1970	Bengt Lindskog	1984	Anne-Greth Bondeson	1995	Leif Israelsson	2005	Anders Holmström
1971	Bertil Robertsson	1984	Peter Konrad	1995	Per Jonsson	2005	Helene Malm
1971	Björn F Ericsson	1984	Magnus Grahe	1996	Norman Jensen	2005	Carolin Freccero
1971	Erik G Ohlsson	1985	Anders Lindhagen	1996	Jens Peter Game	2005	Nishman Dizayi
1971	Sune Isacson	1985	Stefan Arvidsson	1996	Hans Bohe	2005	Cecilia Osterholm Corbascio
1972	Jörgen Gundersen	1985	Kent Jonsson	1997	Wayne Hawthorne	2005	Saad Elzanaty
1973	Bo Phil	1985	Hans Hedlund	1997	Öyvind Östraat	2005	Björn Lindkvist
1973	Bo Husberg	1985	Måns Bohe	1997	Yllei Mato	2006	Louis Banka Johnson
1974	Lars Janzon	1986	Henty Svensson	1998	Diya Adawi	2006	Henrik Dyhre
1974	Sigvard Olsson	1987	Heitti Teder	1998	Liselotte Frost-Arner	2006	Erik Almqvist
1974	Jerzy Senyck	1987	Hans Högström	1998	Martin Malina	2006	Yusheng Wang
1974	Göran Ekelund	1988	Per Uden	1998	Thomas Björk	2007	Peter Mangell
1975	Bengt Pallin	1988	Erik Svarholm	1998	Mats Hedberg	2007	Martin Persson
1975	Sven Kristersson	1988	Per-Anders Abrahamsson	1998	Håkan Bronson	2008	Sara Regnier
1976	Rabbe Takolander	1988	Toste Länne	1998	Magnus Becker	2008	Stefan Santén
1976	Nils T Johansson	1990	Bengt Hjelmqvist	1999	Zhongquan Qi	2008	Asadzaman Muhammad
1976	Svarker Hellsten	1990	Nils H Persson	1999	Stefan Appelros	2008	Mattias Lascchese
1977	Pal Svedman	1990	Henrik Åkesson	1999	Göran Ahlgren	2009	Martin Almqvist
1977	Anders Henriksson	1990	Thomas Matzsch	1999	Håkan Weiber	2009	Farokh Collander Farzaneh
1977	Sune Wetterlin	1990	Magnus Delshammar	2000	Ingar Syk	2010	Dorthe Johansen
1977	Sven Genell	1990	Anders Törnqvist	2001	Xiao Wei Zhang	2010	Björn Schönmeier
1978	Bo Lindell	1990	Magnus Erlansson	2001	Christer Svedman	2010	Fredrik Jörgren
1978	Olof Lannerstad	1990	Jan Brunkwall	2001	Ulf Petersson	2010	Patrik Velander
1978	Magnus Åberg	1991	Johan Ottosson	2001	Mats Bläckberg	2010	Andrada Mihăescu
1978	Allan Edde-land	1991	Ragnar Källén	2001	Peter Månsson	2011	Salma Butt
1978	Hasse Jiborn	1991	Lars Salemark	2001	Tor Svensjö	2011	Emma Hansson
1979	Anders Bergström	1991	Claes Forsell	2001	Ursula Miraschehijnski	2011	Aree Omar Abdulla
1980	Ingrid Tengrup	1991	Agneta Montgomery	2001	Torbjörn Söderström	2011	Darbaz Awla

