SIGNALING MECHANISMS IN SEPSIS-INDUCED IMMUNE DYSFUNCTION

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Academic Thesis

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SIGNALING MECHANISMS IN SEPSIS INDUCED IMMUNE DYSFUNCTION

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In memory of my father

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Abbreviations

ALI	acute lung injury
AMs	alveolar macrophages
APC	allophycocyanin
ARDS	acute respiratory distress syndrome
BALF	bronchoalveolar lavage fluid
CD	cluster of differentiation
CARS	compensatory anti-inflammatory response syndrome
CFSE	carboxyfluorescein diacetate succinimydul ester
CLP	cecal ligation and puncture
DAMPs	damage-associated molecular patterns
ECM	extracellular matrix
EDTA	ethelenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
Foxp3	forkhead box P3
H&E	hematoxylin and eosin
GGT	geranylgeranyl transferase
GGTI	geranylgeranyl transferase inhibitor
HMG-CoA	3-Hydroxy-3-methylglutary coenzyme A
HMGB1	high-mobility group box-1
i.p.	intraperitoneal
i.v.	intravenous
ICAM	intercellular adhesion molecule
ICU	intensive care unit
IFN	interferon
IL	interleukin
JAMs	junctional adhesion molecules
KC/CXCL1	cytokine-induced neutrophils chemoattractant
LFA-1	lymphocyte function antigen-1
LPS	lipopolysaccharide
LTA	lipoteichoic acid
mAb	monoclonal antibody
Mac-1	membrane activated antigen-1
MAPK	mitrogen-activated protein kinase
MFI	mean fluorescence intensity
MIP-2/CXCL2	macrophage inflammatory protein-2
MMPs	matrix metalloproteinases
MNL	monomorphonuclear leukocyte

MOF	multiple organ failure
MPO	myeloperoxidase
NF-κB	nuclear factor κB
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
NLRs	NOD-like receptors
PBS	phosphate buffered saline
PAMPs	pathogen associated molecular patterns
PE	polyethylene
PI	propidium iodide
PG	peptidoglycan
PMNL	polymorphonuclear leukocyte
PRRs	pattern-recognition receptors
PSGL-1	p-selectin glycoprotein ligand-1
ROCK	Rho-associated coiled-coil protein kinase
ROS	reactive oxygen species
RLRs	RIG-like receptors
SD	standard deviation
SEM	standard error of mean
SIRS	systemic inflammatory response syndrome
s.c.	subcutaneously
sCD40L	soluble CD40 ligand
Th	T helper cells
TLR	toll-like receptor
TNF	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule-1

List of original papers

I. **Hasan Z*,** Palani K*, Rahman M, Thorlacius H. Targeting CD44 expressed on neutrophils inhibits lung damage in abdominal sepsis. *Shock* 36: 431-431, 2011.

II. **Hasan Z,** Rahman M, Palani K, Syk I, Jeppsson B, Thorlacius H. Geranylgeranyl transferase regulates CXC chemokine formation in alveolar macrophages and neutrophil recruitment in septic lung injury. In press Am. J. Physiol., 2013.

III. **Hasan Z,** Palani K, Rahman M, Zhang S, Syk I, Jeppsson B, Thorlacius H. Rho-kinase signaling regulates pulmonary infiltration of neutrophils in abdominal sepsis via attenuation of cxc chemokine formation and Mac-1 expression on neutrophils. *Shock* 37: 282-288, 2012.

IV. **Hasan Z,** Palani K, Zhang S, Rahman M, Lepsenyi M, Hwaiz R, Syk I, Jeppsson B, Thorlacius H. Rho-kinase regulates induction of T-cell immune dysfunction in abdominal sepsis. Submitted to Infection and Immunity, 2013.

* Equally contributed

Introduction

Sepsis is a devastating and complex clinical syndrome in which every year approximately 18 million individuals suffering from it internationally [1]. Only in the United States approximately 751,000 cases with severe sepsis are hospitalized per year, resulting in 215,000 deaths [2]. Sepsis is the leading cause of death in non-coronary intensive care units and is the tenth leading cause of death overall in the United States. The mortality rate of septic patient ranges from 20-65% despite substantial investigative efforts and management is largely limited to supportive care [2, 3].

The most common causes of sepsis are respiratory infection (35%), intra-abdominal infection (21%), genitourinary (13%), blood stream infections or unknown primary site (16%), other causes (8%) and wound infection (7%) [4]. Gastrointestinal tract perforations are the most common cause of the intra-abdominal infection mostly due to perforated appendix, perforated gastric and duodenal ulcer and perforated colon [5]. When intra-abdominal sepsis is associated with perforation, bowel contents and fecal bacteria directly contaminate abdominal cavity and the resulting peritonitis is almost always polymicrobial, comprising both aerobic and anaerobic; gram positive and gram negative bacteria which depends on the site of perforation. For instance upper gastrointestinal tract contains relatively few amount and mostly gram positive bacteria while lower gastrointestinal tract contains large amount of bacterial species predominantly gram negative bacteria [6, 7]. Fecal bacteria and their toxins stimulate local production of pro-inflammatory compounds, which are released into the systemic circulation. Moreover, disruption of gut barrier leads to direct systemic spread of gut derived bacteria resulting in systemic bacteremia [8].

Sepsis develops largely as a result of amplified and dys-regulated host immune response to invading microorganism and their toxins [9]. This hyper-inflammatory phase is followed by a prolonged anti-inflammatory response leads to immunosuppressive state and failure to clear infection known as compensatory anti-inflammatory response syndrome (CARS) [10]. The pathophysiology of sepsis is complex and is not driven by single mediator, system or pathway. The central component is host response to an infectious insult which is mediated by inflammatory cells such as neutrophils, macrophages and platelets [11]. Leukocytes are attributed to play a dominant role in systemic inflammatory response and physiological alteration in sepsis. Leukocytes interact with platelets and endothelial cells through cell mediators and a sequence of receptor-ligand interaction allowing them to leave the circulation as a result of increased vascular

permeability [11, 12]. The increased vascular permeability and loss of endothelial integrity affect microvascular blood flow which is responsible for global tissue hypoxia and organ dysfunction, the hallmark of sepsis [12].

Lung is the most important and sensitive end organ in the body [13]. It is widely held that neutrophil infiltration is a key feature in the pathophysiology of septic lung damage [14, 15], however, the signaling mechanisms behind neutrophil infiltration in the lung and immune dysfunction in abdominal sepsis remain elusive. A more thorough understanding and ability to control these mechanisms can help to identify potential targets for more specific treatments in septic patient. Therefore, in the present study we investigate the signaling mechanisms of pulmonary neutrophil recruitment and immune dysfunction in abdominal sepsis. Furthermore, we want to define the role of platelets and CXC chemokines in this process.

Background

Sepsis

Sepsis represents systemic inflammatory response syndrome (SIRS) to host microbial invasion. SIRS is the systemic inflammatory reaction to a wide range of severe clinical insults and is diagnosed when alteration in two or more of SIRS criteria are present, including temperature, heart rate, respiratory rate and leukocytes [16] (Table 1).

Table 1**. SIRS criteria**

Two or more of the following criteria are present

1. Core temperature > 38° (fever) or < 36° (hypothermia)
2. Tachycardia (heart rate > 90 beats per minute)
3. Tachypnea (respiratory rate > 20 breaths per minute) or hypocapnea (a $PaCO_2$ < 32 mm Hg) or a need for mechanical ventilation
4. Leukocyte count > 12000/mm^3 (leukocytosis) or < 4000/mm^3 (leukopenia) or > 10% immature bands (bandemia)

Severe sepsis is defined as sepsis associated with single or multiple organ dysfunctions such as renal, liver, cardiac failure as well as coagulation abnormalities and altered mental status. Septic shock occurs when sepsis is complicated by hypotension and hypo perfusion despite of adequate fluid resuscitation. Lactic acidosis, oliguria, hypoxia and altered mental status are indicative of hypo perfusion and evolution to septic shock [17]. Table 2. Diagnostic criteria for sepsis.

Table 2. **Diagnostic criteria for sepsis** [17]

-Infection, documented or suspected, plus some of the following
-General criteria Fever, hypothermia, tachycardia, tachypnea, altered mental status, significant edema or positive fluid balance (>20 ml/kg over 24 h), hyperglycemia (plasma glucose >120mg/dl or 7.7 mM/l) in the absence of diabetes
-Inflammatory criteria Leukocytosis, leukocytopenia, Bandemia, plasma C-reactive protein >2SD above the normal value, plasma procalcitonin >2SD above the normal value
-Hemodynamic criteria Arterial hypotention (systolic blood pressure <90 mmHg, mean arterial pressure <70, or a systolic blood pressure decrease >40 mmHg in adults or <2 SD below normal for age), mixed venous oxygen saturation >70%, cadiac index >3.5 L min^{-1} m^{-2}
-Organ dysfunction criteria Arterial hypoxia (PaO_2/FIO_2 <300), acute olyguria (urine output <0.5ml kg^{-1} hr^{-1} or 45 mmol/L for at least 24 hrs), creatinine increase ≥0.5 mg/dl, Coagulation abnormalities (INR >1.5 or aPPT >60 secs), Ileus (absent bowel sounds), thrombocytopenia (platelet count <100,000/μL^{-1}), hyperbilirubinemia (plasma total bilirubin >4 mg/dL or 70 mmol/L)
-Tissue perfusion criteria Hyperlactatemia (>3 mmol/L), decreased capillary refill or mottling

Pathogenesis of sepsis

Microbial pathogen

Over time significant changes have occurred in the frequency of microbial pathogens which are responsible for initiating the septic process. Gram-negative bacteria were the predominant micro-organisms since the 1960s, however, from the late 1980s; the incidence of gram-positive sepsis has increased. A large study from the United States showed that Gram-negative bacteria account for 37%, Gram-positive 52% and fungi 4.6% [18]. Staphylococcus aureus and streptococcus pneumonia are the most common Gram-positive whereas *E-coli*, *Klebsella* species and *pseudomonas aerogenosa* are the most common Gram-negative bacteria which are commonly isolated from septic patients [19].

Microbial toxins and host's response to them are widely considered to be the principal component in the pathogenesis of sepsis. One crucially important bacterial toxin is lipopolysaccaride (LPS). LPS is an essential component of the outer membrane of Gram-negative bacteria and it is required for bacterial growth and viability [20]. LPS is a highly anionic macromolecule with variable hydrophobic and hydrophilic regions and because of unique place in microbial physiology and in the pathogenesis of sepsis, LPS is often referred to as endotoxin. LPS is responsible for septic shock that accompanies severe Gram-negative infections. The toxicity of LPS is related to harmful host response during infections other wise LPS has no intrinsic toxicity by itself [21].

Gram-positive bacteria also can cause sepsis and septic shock but it is not mediated through LPS. Gram-positive bacteria secrete exotoxins such as lipoteichoic acid and peptidoglycans which can induce shock state for example toxic shock syndrome that caused by *Staphylococcus aureus* or *Streptococcus pyogenes* infectuions [22]. Candedemia and septic shock has increased relatively in immunocompramised patients and it is associated with multiple organ failure and higher mortality rate, however, no toxins have been found to be responsible for fungemic shock state. Fungal proteins activate immune system like LPS and they interact with TLR-4 to induce the production of pro-inflammatory compounds [23].

Pathogen recognition

Cells of innate immunity such as neutrophils, monocytes and macrophages constitute the first line of host defense against invading microbial pathogens. Microbial components such as LPS, lipoteichoic acid,

peptidoglycan, lipopeptide, flagellin and double-stranded RNA are known as pathogen-associated molecular patterns (PAMPs) and are recognized by cells of innate immunity via pattern-recognition receptors (PRRs) on these cells [24]. In addition, to these exogenous- derived ligands, PRRs can recognize endogenous mediators released during injurious processes, thereby warning the host of danger. Such endogenous mediators termed as alarmins or danger-associated molecular patterns (DAMPs) such as; hyaluronic acid, high-mobility group box-1 (HMGB-1) and heat-shock proteins (HSPs), which cause further amplification of host inflammatory response [25]. There are three families of PRRs which are involved in detection of both PAMPs and DAMPs during sepsis and tissue injury including; Toll-like receptors (TLRs), Nucleotide-binding oligodimerisation domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene (RIG)-I(RIG-I) like receptors (RLRs) [26].

The Toll family receptors of PRRs have a pivotal role in the recognition of microbes and initiation of cellular innate immune responses [24]. TLRs are single-spanning transmembrane glycoproteins and are expressed on the cell surface (TLRs 1, 2,4,5,6 and 10) and within the cytoplasm in particular within the lysosomes and endosomes (TLRs 3, 7, 8 and9). To date, TLRs 1-13 have been identified. TLRs family can detect microbial components from bacteria (TLR 2, 4, 6 and 9), viruses (TLR 3, 7, 8 and 9), fungi and protozoa and thereby activate immune cells to produce pro-inflammatory cytokines [27, 28]. Upon recognition and ligation of TLRs with PAMPs or DAMPs, various TLR domain–containing adaptors such as myeloid differentiation primary-response protein (My88), Toll/interleukin-1 receptor (TIR) domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor protein-inducing IFN-β (TRIF) and TRIF related-adaptor molecule (TRAM) become activated and recruited. The recruitment of these adaptors triggers the activation of NF-κB and cytokine promoter genes, resulting in production of various pro-inflammatory cytokines and chemokines [29, 30].

NOD proteins such as NLRs are cytoplasmic PRRs which contribute to the detection of microbial components that invade the cytosol [31]. However the role of NLRs in sepsis pathophysiology is not clear. RLRs serve as intracellular PRRs which have role in the recognition of viruses by the cells of innate immunity [32].

Cells of adaptive immune system, T-cells and B-cells, have the ability to produce highly specific responses against presented pathogens and to establish protective immunity against re-infection by the same microorganism. Phagocytic cells, macrophages and dendritic cells ingest invading pathogens, then present cellular components of these pathogens on

their surface to the cells of adaptive immunity. When T-cells recognize foreign antigen, they are activated, allowing them to release cytokines and further augment immune response. T-cells are involved in a wide variety of activities, and are thought to regulate inflammatory response. Some T-lymphocytes directly invade infected cells and induce the death of the cell (CD8+ cytotoxic T-cells); while others direct and regulate immune response (CD4+ T helper cells) [33]. CD4+ T helper 1 (Th1) cells release interferon-gamma (IFN-γ) and tumor necrosis factor-αlpha (TNF-α), which increase anti-microbial activity of macrophages, enabling them to destroy intracellular pathogens. T helper 2 (Th2) cells secrete IL-10 and IL-4 and stimulate B-cells to produce killing antibodies, thus producing humoral immunity against extracellular pathogens [10].

Inflammatory response in sepsis

Immune response in sepsis has been postulated to represent the interplay of an early systemic inflammatory response or hyper-inflammatory status, characterized by excessive production of pro-inflammatory compounds and a compensatory anti-inflammatory response or hypo-inflammatory status characterized by releasing large number of anti-inflammatory mediators, increase apoptosis, T-cell inactivation and de-activation of monocytes [10, 17].

Following pathogen recognition there is widespread activation of the innate immune response involving both humoral and cellular components, the aim of which is to coordinate defensive responses against invading microorganisms. The initial steps are caused by cells of innate immunity in particular mononuclear cells which release classic inflammatory mediators IL-1, IL-6 and TNF-α [9]. These inflammatory mediators are the prototypic inflammatory cytokines and they are critically involved in the pathogenesis of septic shock [34]. They are released into systemic circulation 30-90 min after exposure to microbial pathogens lead to a uniform syndrome called SIRS by activation a second level of inflammatory cascade including cytokines, lipid mediators, reactive oxygen species (ROS), as well as up-regulation of cell adhesion molecules resulting in the initiation of inflammatory cell migration into tissues. Under normal condition, harmful pathogens are successfully eliminated by immune cells with out any tissue damage. However, the amplified and dys-regulated host immune response during sepsis can cause tissue damage, organ injury

which eventually leads to multiple-organ disorder and multiple-organ failure (MOF) [9].

Although the inflammatory response is essential for the initial success of the immune system, the adequate control and resolution of pro-inflammatory signals are equally important for survival of affected individuals. This over-inflammation can be avoided if counter-regulatory response comes at right time which leads to complete restoration of host. When anti-inflammatory response prolonged or too pronounced may lead to immunosuppressive state and failure to clear infection known as compensatory anti-inflammatory response syndrome (CARS) [10].

CARS is characterized by T-cells hypo-responsiveness and excessive lymphocyte apoptosis which might be the cause of sepsis and progressive organ failure due to inadequate host defense against infection [9]. Lymphocytes play an important role in modulating sepsis response because they have the ability to interact with the innate and adaptive immunity as well as they can regulate, increase and decrease the inflammatory responses. CD4+ T-lymphocyte is subdivided into Th1 and Th2 based on functional activities and pattern of cytokine production. Th1 cells predominate immune response in the initial stages of pathogen recognition, characterized by secretion of IFN-γ, TNF-α and IL-12 to coordinate the adaptive immune response and prevent damage to the host. However, in sepsis immune response appears to shift toward Th2 cell-mediated immune response characterized by the secretion of IL-4 and IL-10, resulting in immunoparalysis and inability to combat invading microbial agents [10, 33].

In this state extensive apoptosis of lymphocytes, suppression of proliferation and IFN formation are seen, resulting in an inadequate host defense against infection and hence increased risk of developing nosocomial infections [35, 36]. Regulatory-T cells are another subgroup of T-cells which limit and suppress the immune system and controlling immune responses to self antigens. Many studies have shown that the number of regulatory T-cells is enhanced in the course of sepsis, which might compromise anti-bacterial defense capability [37-39]. Moreover, the increased regulatory T-cells are involved in the defect in cytokines release by Th1 cells in CLP mice [40]. It has been also shown that there is a positive correlation between number of regulatory T-cells and levels of anti-inflammatory cytokine, IL-10, and transforming growth factor beta (TGF-β) in the serum both in septic patients and CLP induced animals and blocking of IL-10 reduces regulatory T-cells and mortality [41]. In addition, during hypo-inflammatory status of sepsis monocytes from septic patient

decrease proliferation, secrete fewer cytokines in response to microbial challenges and decrease antigen presenting capacity [42].

Organ dysfunction

Organ dysfunction and organ failure occur frequently in septic patients and MOF is a major cause of morbidity and mortality in intensive care units [43]. There is direct correlation between number of organ systems failed and mortality. The more organ failure the greater risk of death, mortality is 9% in septic patient with no organ failure, 22% in one, 38% in two, 69% in three and 83% in four and more organ failure [44]. The mortality is also influenced by severity of organ dysfunction on admission to intensive care unit [45] and by the duration of organ dysfunction [46].

The pathogenesis of organ failure in patients with severe sepsis is multi-factorial and incompletely understood. Alterations in microvascular blood flow and tissue oxygenation are dominant factors [9]. Excessive production of inflammatory mediators induces leukocyte/endothelial activation, increasing vascular permeability and polymorphonuclear leukocyte migration which lead to widespread endothelial and parenchymal cell injury resulting in compromised organ function [47]. The order of organ failure may vary due to pre-existing disease. The organs that show dysfunction are respiratory, heart, renal, hepatic, gastrointestinal and hematological system as well as endocrine and central nervous system [47, 48]. The lung is the most sensitive and critical organ for the inflammatory response in sepsis [49]. Clinically lung is the first organ to fail and acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) usually present in several hours to three days after initial insult [47].

Acute Lung injury/ acute respiratory distress syndrome (ALI/ARDS)

ALI and ARDS are acute inflammatory disorders characterized by increased pulmonary microvascular permeability and widespread inflammation of the lung, resulting in destruction of alveolar epithelial and pulmonary capillary endothelial cells with subsequent hypoxemia and respiratory failure [50]. Physiologically when partial arterial pressure of oxygen is $\leq$300 and $\leq$200 the condition defined as ALI and ARDS respectively. While radiologically both ALI and ARDS are defined as

bilateral lung field infiltrates [51, 52]. The ALI/ARDS may occur as a consequence of direct injury to lung (55% Pulmonary) such as pneumonia, toxic inhalation, aspiration, or lung contusion, while indirect mechanism (extrapulmonary) can be seen in patients with sepsis, burn, pancreatitis, trauma and massive blood transfusion [53]. About 7% of ICU patients are affected by ALI/ARDS and more than half of these develop fully ARDS within 24 h [2].

Severe sepsis is the most infectious and inflammatory disorder associated with the development of ALI and ARDS. Approximately 30% of patients with severe sepsis develop pulmonary dysfunction which is associated with high morbidity and mortality [49]. However, mortality from ARDS has declined from 70% at eighteenth to 30-40% at present, as a result of the implantation of new protective methods and drug therapies [52]. The Pathophysiology of acute lung injury includes endothelial activation, inflammatory and haemostatic changes and vascular alteration. In severe sepsis, the systemic inflammatory response characterized by excessive production of pro-inflammatory compounds and concomitant activation of endothelial cells and circulating immune cells especially leukocytes. Acute lung injury begins with a massive cellular inflammatory infiltration of neutrophils, monocytes and lymphocytes [54].

Leukocyte mediated Lung injury

Polymorphonuclear leukocytes play a crucial role in pathgenesis of sepsis induced ALI [55, 56]. They are essentially the first host defense response against invading pathogens. Neutrophil response to injury is initiated when chemoattractant signals such as IL-1, IL-8 and TNF-α, from lung macrophages, direct and recruit neutrophils to the site of inflammation [3, 47]. Neutrophils cross the endothelium, in response to proinflammatory cytokines, and gain access to the alveolar space and airways. Upon recruitment to the site of infection or inflammation, neutrophils can damage tissue directly by releasing proteolytic enzymes and reactive oxygen species (ROS) [54].

Neutrophils accumulation in the lung parenchyma and bronchoalveolar lavage fluid (BALF) in animals with severe lung inflammation indicates that these cells play a pivotal role in the development of ALI [15, 55]. Patients with ARDS have abundant neutrophils in BALF which correlate with physiological abnormalities that occur [57, 58]. Moreover, sustained high numbers of BALF neutrophils in patients with ARDS following sepsis is associated with a higher mortality

[59]. On the other hand, activated neutrophils have a progressive decrease in apoptosis due to delayed phagocytosis by macrophages [55]. Decreased neutrophil apoptosis appears to be related to the severity of sepsis in the septic patient. This prolonged survival allows neutrophils to accumulate at the local site of injury and inflammation, resulting in further activation of other proinflammatory cytokines [55, 60].

Leukocyte recruitment

Leukocytes infiltration from the blood stream into the surrounding tissue is a key feature in the pathogenesis of inflammatory and autoimmune diseases. The emigration process is a complex and multistep process that involves initial leukocyte sequestration in microvessels, tethering, rolling, adhesion and finally trans-endothelial trans-epithelial migration Fig.1 [61-63]. Each of these steps appears to be critical for leukocyte recruitment. Because blocking any of them can significantly reduce leukocyte accumulation in the tissue.

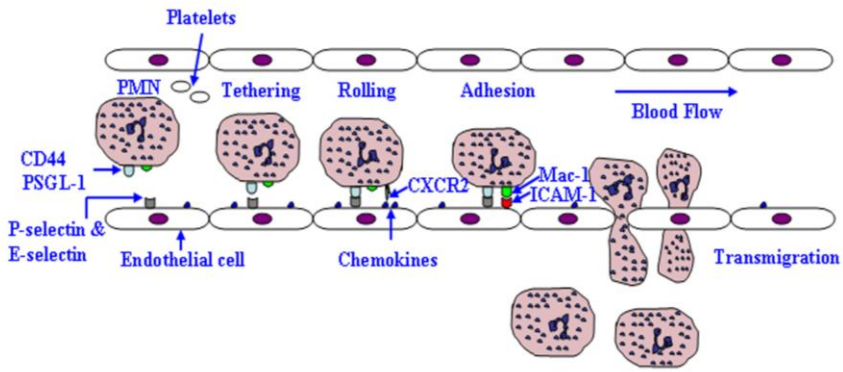

Figure.1 Leukocyte recruitment. Leukocyte recruitment is a complex and multistep process, which involves leukocyte tethering, rolling, adhesion and finally trans-endothelial trans-epithelial migration.

Leukocytes are considered to roll when the high velocity moving leukocytes that normally move through micro-vessels, slow down 40-50 times and tend to move into a position close to the endothelial surface due to hemodynamic factors in the microcirculation [64]. The rolling phase of leukocyte recruitment is predominantly mediated by the selectin family of

adhesion molecules, which consists of three closely related members of calcium-dependent glycoproteins, i.e. E-selectin (ELAM-1, CD62E), L-selectin (LAM-1, CD62L), and P-selectin (PADGEM, CD62P) and their corresponding ligands [65].

Leukocyte rolling increases the possibility of interactions between the leukocyte and endothelium with subsequent leukocyte activation. Rolling leukocytes on surface of microvascular endothelium are not always committed to firm adhesion; Leukocytes frequently detach from vessel wall and return to circulation. However, in the presence of appropriate chemotactic stimulus, the rolling phase can be shifted to an irreversible firm adhesion which is predominantly mediated by integrins [14, 66].

In inflamed organ, pro-inflammatory mediators stimulate endothelial cells to synthesize chemokines and transport them to their luminal surface [67]. When leukocytes roll on the endothelium, they are further activated by the interaction of endothelial selectins with leukocytic PSGL-1 on the one hand and the interaction of chemokine receptors on the leukocyte surface with the secreted chemokines of the endothelium on the other hand [68]. This results in up-regulation or increase the avidity of integrins within minutes which mediate firm leukocyte adhesion [69].

Integrins are a large family of heterodimeric type I transmembrane glycoproteins (24 heterodimers) [68]. The most relevant integrins for leukocyte migration are the beta-2 (β2) integrin subfamily which are composed of a common β-subunit (CD18) and one α-subunit including CD11a (CD11a/CD18 or LFA1), CD11b (CD11b/CD18 or MAC-1) CD11c (CD11c/CD18 or P150,95) and CD11d (CD11d/CD18 or α2β2) [70]. The β2 integrins are expressed on leukocyte surface which upon activation lead to increased expression or avidity for their endothelial ligands, thereby promoting strong adhesive interactions and firm leukocyte arrest [66, 71]. Lymphocyte function antigen-1 (LFA-1) and membrane activated antigen-1 (MAC-1) are suggested to be the primary integrins that mediate firm leukocyte adhesion in the inflammatory process by interacting with members of the immunoglobulin superfamily expressed on the endothelial cell surface including intercellular adhesion molecule (ICAM-1- ICAM-5), junctional adhesion molecules (JAMs) and vascular cell adhesion molecule-1 (VCAM-1) [68].

The last step in the process of leukocyte recruitment into the inflamed tissue is transmigration. Extravasation of adherent leukocytes occurs through the venular walls, most frequently at endothelial intercellular junctions namely paracellular pathway [72, 73]. Although, a trans-cellular route has also been proposed, i.e. by crossing the endothelial

cells either by trans-cytotic migration or via pre-existing holes and it contributes only for 10-30% [73-75]. Moreover, paracellular pathway is of greater relevance under physiological conditions because many of endothelial membrane proteins that are involved in leukocyte transmigration have been found to be mainly localized at endothelial junctions [75].

Chemokine mediated leukocyte activation

Chemokines are a group of low molecular-weight chemotactic cytokines (8-12 KDa) that are involved in leukocyte activation and chemotaxis [76, 77]. Chemokines are proteins that are subdivided into four subfamilies: C, CC, CXC and CX3C chemokines, based on the number and spacing of the N-terminal cysteine residues [78]. CXC and CC chemokines are the main two groups and most studied in sepsis. The CXC chemokines including cytokine induced neutrophil chemo-attractant (KC or CXCL1) and macrophage inflammatory protein-2 (MIP-2 or CXCR-2) are functional homologues of human IL-8 in mice [79, 80]. Mouse MIP-2 and KC are involved in all steps of leukocyte recruitment, including rolling, adhesion and transmigration [81]. CXC chemokines are considered to attract predominantely neutrophils in response to tissue injury and infection [82], and they have been shown to modulate vascular permeablility [83], which might serve to facilitate leukocyte extravasation. Moreover, increased MIP-2 and KC level has been shown to be associated with neutrophil recruitment in many inflammatory conditions [84-87].

Chemokines activate and regulate leukocyte recruitment via G-protein coupled receptors, chemokine receptors [77, 80]. CXCR1 and CXCR2 are two receptors for CXC chemokines and are expressed in both humans and mice leukocytes. CXCR2 is the high affinity receptor for both MIP-2 and KC and is essential for neutrophil infiltration into the lung during bacterial infection [88, 89]. It has been shown that neutrophil expression of CXCR2 is down-regulated in sepsis due to internalization [90], but plama and lung levels are elevated in sepsis [89]. Deficiency of CXCR2 or inhibition with CXCR2 specific inhibitor appears to protect against pulmonary neutrophil infiltration and septic lung injury in mice [15, 89, 91].

Role of alveolar macrophages in ALI

Alveolar macrophages (AMs) are long-lived cells that serve as the first line of defense in the lungs and control the entire inflammatory response [54, 92]. Systemic immune response possibly can be regulated by regulating macrophage responses [54]. They are reported to be the principle mediators in the pathogenesis of septic shock [93]. AMs are present in the alveoli and the alveolar ducts of the lungs. They are phagocytic cells which actively phagocytize and kill invading pathogens. These microbial components (bacteria or endotoxin) stimulate alveolar macrophages to produce inflammatory mediators such as IL-1, TNF and other potent proinflammatory cytokines during initial phase of pulmonary inflammation. Many studies reported that AMs play a pivotal role in the regulation of both pro and anti- inflammatory responses in sepsis-induced ALI [94, 95]. Pro-inflammatory cytokines induce migration of circulatory inflammatory cells especially neutrophils to the site of infection and release lysosomal enzymes and ROS. Moreover, neutrophils stimulate release of anti-inflammatory cytokine, IL-10, after phagocytosis by alveolar macrophages which subsequently inhibit the additional cytokine production and pulmonary inflammation. However, during sepsis percentage of AM apoptosis increases which results in a significant reduction of AM numbers 20 h post CLP with subsequent decrease the antimicrobial effect in the lung in sepsis [96, 97]. Suppression of either neutrophils or macrophages in septic mice following hemorrhage reduces pulmonary inflammation [98]. Thus, both neutrophils and macrophages are essential for development of ALI in sepsis.

Platelets in inflammation

Although platelets play an essential role in hemostasis and thrombosis, more recent data also suggested their important role in inflammation and tissue injury as well [99]. Some studies have shown that platelets are involved in the process of leukocyte recruitment [100, 101]. Accordingly, depletion of platelets leads to reduce pulmonary neutrophil recruitment in a murine model of allergic inflammation [102, 103] and ischemia-reperfusion injury [100] and protects against sepsis- and hydrochloric acid induced lung damage [104, 105]. A potential role of platelets in pulmonary leukocyte recruitment in abdominal sepsis has been reported via up-regulation of Mac-1 expression on neutrophils [105]. Platelets express large numbers of adhesion molecules on their surface such as PSGL-1, P-selectin, ICAM-2 and JAM-A, allowing them to interact with leukocytes on the one hand and with endothelium on the other hand and enable them to support leukocyte

recruitment into inflamed tissue[99]. Moreover, platelets may activate leukocytes by releasing of several secretary products and pro inflammatory mediators such as platelet factor-4, platelet derived growth factor, CD40L and IL-1β [99].

CD40 ligand (CD40L, CD154) is a trimeric 33 kD, transmembrane protein of tumor necrosis factor (TNF) family. It was first identified on cells of the immune system (Activated CD4+ cells) [106]. Subsequently, Henn and collaborators showed that CD40L and CD40 are also present in platelets [107, 108]. CD40L provides a link between immune and coagulation systems. Platelets carry a bulk of CD40L in the blood and they contain >95% of the circulating CD40L [109]. Platelets express small amount of CD40L on their surface and much of them are cryptic and appears localized to granule membranes. On platelet stimulation, CD40L is rapidly expressed on the platelet surface where it is cleaved by metalloproteinases, forming a soluble CD40L (sCD40L) [106, 110]. Platelet-derived CD40L, sCD40L and surface expressed, can exert various inflammatory response, including synthesis of chemokines (IL-1, MCP-1 and IL-8), expression of various adhesion molecules on endothelium (ICAM, E-selectin) and up-regulation of tissue factors [107, 111]. Moreover, platelet derived CD40L is involved in leukocyte recruitment and pulmonary damage via regulation of chemokine production and expression of adhesion molecules in a murine model of abdominal sepsis [56].

CD44

CD44 is a cell surface transmembrane glycoprotein widely expressed on most cell types, including hematopoietic stem cells, leukocytes, fibroblastoid, neural, muscle cells, as well as epithelial and endothelial cells [112]. CD44 is encoded by a single gene but it has more than 40 isoforms which are generated by alternative splicing and/or post translational modification. CD44 consists of an extracellular amino-terminal globular protein domain, a stem structure, a transmembrane region, and a cytoplasmic-tail region Fig.2 [113].

Hyaluronic acid is one of major ligands of CD44 (including hyaluronan, collagen, laminin, fibrinogen and glycosaminoglycansand) [114, 115]. All CD44 isoforms contain hyaluronan binding site (N-terminal globular domain of CD44) and CD44-hyaluronic acid interactions play an

essential role in many biological processes including immune response development, autoimmune diseases and tumor metastasis [113, 115, 116]. CD44 has hyaluronan –dependet and –independent functions. For example neutrophil trapping in the liver sinusoids is mediated by both CD44- and hyaluronic acid [117], whereas, lymphocyte infiltration into the dermis and epidermis of inflamed skin is independent of hyaluronan [118].

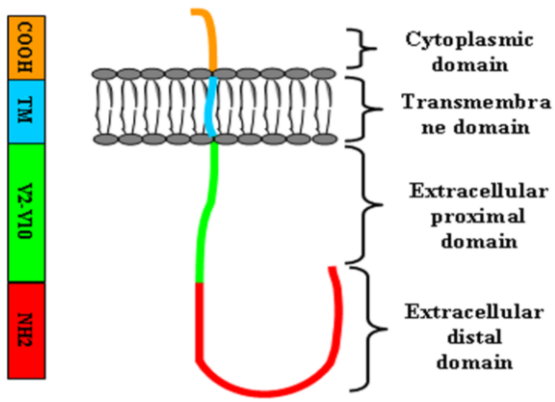

Figure.2 CD44 Structure. CD44 comprises a large distal extracellular ligand-binding domain (red), the membrane proximal domain (green), is the site of alternative splicing of CD44 to form numerous CD44 variants, the transmembrane domain (blue) and the cytoplasmic domain (brown), which contains protein motifs responsible for intracellular signaling.

As a multifunctional adhesion molecule, CD44 isoforms are involved in many physiologic and pathologic processes, such as cell-cell and cell-matrix interaction, leukocyte extravasation, cytokine and growth factor presentation, cell motility, differentiation and cell trafficking [119-121]. Since major functions involve adhesion and migration, many studies have shown that CD44 is also involved in leukocyte homing and recruitment [117, 122]. Moreover, endotoxins and inflammatory cytokines can modulate CD44-hyaluronan binding which has profound effect on inflammatory cell migration and development of immune responses [116].

Many studies have revealed the role of CD44 in leukocyte recruitment for example anti-CD44 has decreased neutrophil infiltration

and inflammatory response in murine models of arthritis [123] and central nervous system infection [124]. The role of CD44 in mediating pulmonary leukocyte recruitment is contradictory. For example, one study reported increased in endotoxin-induced pulmonary leukocyte recruitment in CD44-gene deficient mice [125], whereas, another study showed decreased in endotoxin-induced leukocyte accumulation in the lung in CD44-gene deficient mice [126]. However, the role of CD44 in regulating neutrophil activation and pulmonary leukocyte infiltration in abdomenl sepsis is not known.

HMG-CoA reductase-dependent signaling

3-hydroxy-3-methylglutaryl (HMG) coenzyme A CoA) reductase is the rate limiting enzyme in the mevalonate pathway. Mevalonate is a precursor not only for the formation of lipids but also for the formation of isoprenoids, which are critical in protein isoprenylation [127]. Prenylation is one of the recently discovered post translational lipid modifications of proteins with the 15 carbon moiety farnesyl pyrophosphate, farnesylation, or the 20 carbon moiety geranylgeranyl pyrophosphate, geranylgeranylation.

Farnesylation is the addition of the farnesyl pyrophosphate to cysteine residues in the CAAX motif at the carboxyl terminus of proteins (where C is cystein, A is commonly an aliphatic acid, and X is any amino acid), catalyzed by farnesyl transferase. Farnesylation is involved in regulation of several protein functions including maturation, membrane localization and protein-protein interaction [128]. It has been shown that inhibition of farnesyl transferase with the use of farnesyl transferase inhibitor (FTI) exerts anti-inflammatory activities, for instance inhibition of NF-κB and Ras activation [129, 130]. Moreover, recently has been shown that farnesyl transferase is involved in streptococcal M1 protein-induced formation of CXC chemokines in alveolar macrophages and neutrophil infiltration of the Lungs [131].

Geranylgeranylation is the addition of the geranylgeranyl pyrophosphate to cysteine residues in the CAAX motif at the carboxyl terminus of Rho family proteins, catalyzed by geranylgeranyltransferase type-1 (GGT-1) [132]. Geranylgeranylation is crucial for the membrane targeting and proper function of Rho proteins. Geranylgeranylation facilitates Rho protein localization to cell membranes where they can interact with downstream signalling effectors [133, 134]. Geranylgeranylation of Rho GTPase is also important for inflammatory cell functions; migration into inflamed tissue and chemokine production [135,

136]. Moreover, clinical data have shown that geranylgeranyl transferase seems to be essential in many inflammatory diseases such as viral infection [137], rheumatoid arthritis [138] and glaucoma [139]. Consequently, inhibiting geranylgeranyltranferase signaling has been proposed as an effective way to treat above and many other inflammatory disorders [128, 140].

Statins, the generic names for a group of cholesterol-lowering drugs, are HMG-CoA reductase inhibitors and statins have been shown to mediate anti-inflammatory and immunomedulatory effects such as chemokine formation and expression of adhesion molecules [141, 142]. Statins reduce mortality in patients with severe infections and sepsis [143, 144] and in our group it has recently been reported that simvastatin treatment decreases pulmonary neutrophil infiltration and improve T-cell function in abdominal sepsis [15, 145], however, the protective mechanisms of statins remain elusive. Knowing that statins mediate their biological effects at least in parts through isoprenoids [146], inhibiting GGTase-I, to mediate Rho protein geranylgeranylation, might help to explain certain anti inflammatory effects of statins in abdominal sepsis Fig.3.

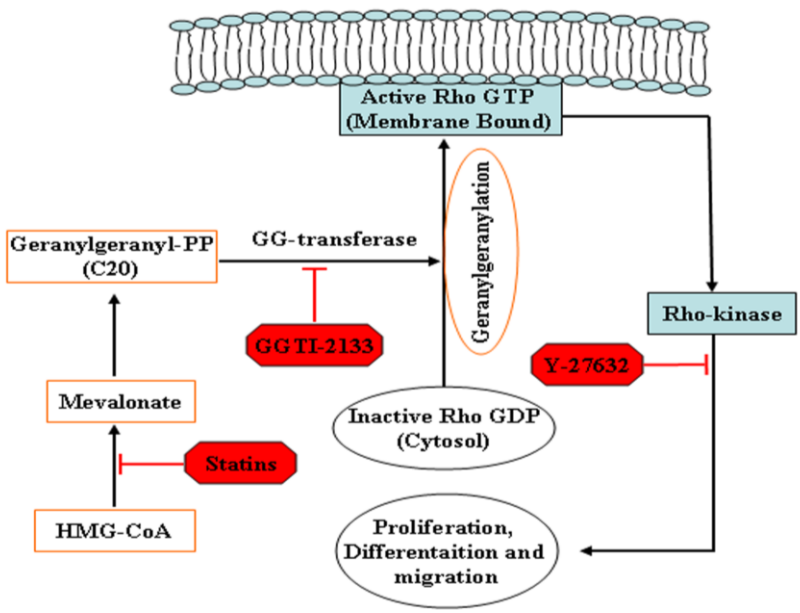

Figure.3 Signaling mechanisms in CLP-induced immune dysfunction

Small GTPases of the Rho family are essential regulators of fundamental cellular functions including cell motility, adhesion, proliferation, differentiation and apoptosis [147, 148]. Rho GTPase family of protein is considered as the most important member of the Rho family group, includes Rho (A-C), Rac (1 and 2) and Cdc42. Indeed, Rho A, Rac1 and Cdc42 are the most common members in the Rho family [149, 150]. Under basal conditions the proteins of Rho GTPase family exist in an inactive GDP-bound form. Various intra- and extra cellular stimuli can activate the Rho proteins pathway and upon activation these proteins undergo prenylation and become an active GTP-bound form [151]. These stimuli act via binding to their receptors, mainly G-coupled receptors [152]. Upon stimulation, these receptors activate GTPases, a group of cytoplasmic GTP-cleaving enzymes, which regulate the degree of activation of their downstream cytoplasmic molecules Rho, Rac and Cdc42 [153]. Activated Rho interacts with its downstream effectors and Rho kinases are the most abundantly studied and recognizable effectors [151].

Rho-kinase which is also known as Rho-associated protein kinase or Rho-associated coiled-coil containing protein kinase (ROCk) is a downstream effector protein of the small GTPase Rho. Two ROCK isoforms have been identified with great similarity, ROCK1 (Rho-kinase B^3 or ROKβ^4) and ROCK2 (Rho-kinase α^3 ROKα^4). The two Rho-kinase isoforms are expressed ubiquitously in almost all human, rat and mouse tissues; although, ROCK1 expression is more abundant in the liver, testis, lungs, spleen and kidneys, whereas ROCK2 is mostly expressed in the brain, heart and striated muscle cells. Rho-kinase molecular structure consist of three compositions including N-terminal catalytic kinase domain, a coiled coil central domain (C-C) which acting as a Rho-kinase binding site (RBS) upon activation and a C-terminal pleckstrin homology domain which contains a cystein-rich region Fig.4 [154, 155]

The Rho-kinase signaling pathway has been identified as an important regulator of different cellular functions, such as smooth muscle contraction, cytoskeleton organization, vesicle trafficking, cell adhesion and motility and gene expression [152, 156, 157]. The role of Rho/ROCK pathway has been intensively investigated in cardiovascular diseases because this specific intracellular signaling pathway is closely related with angiotensin II, thrombin and platelet-derived grow-factor [157].

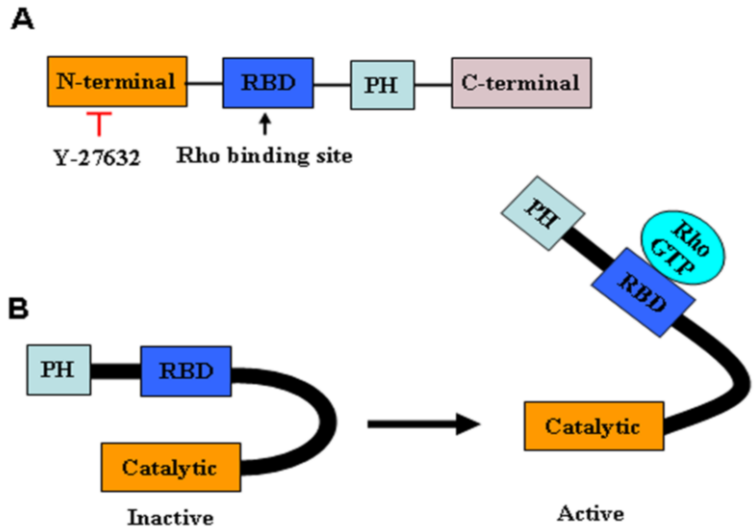

Figure.4 Structure of ROCK (A) and regulation of ROCK function (B). The plectrin homology (PH) domain and the Rho-binding domain (RBD) of Rho-kinase folds back onto catalytic (kinase or amino terminal) domain of the protein, forming an auto-inhibitory loop that maintains the enzyme in the resting state, inactive form. In response to extracellular signaling, GTP Rho binds to the RBD of Rho-kinase resulting in activation of the enzyme.

Accumulating data also suggest that Rho-kinase activity is an important component in inflammatory processes, such as leukocyte chemotaxis, phagocytosis and cytokine formation [158-160]. Moreover, Rho-kinase signaling pathway is involved in renal diseases, malignant diseases and metastasis [161, 162]. Considering that Rho/ROCK pathway might be an important therapeutic target in many diseases; therefore, several inhibitors of ROCKs have been developed. Fasudil and Y-27632 are the oldest and most widely used specific Rho-kinase inhibitors. Y-27632 is a potent selective inhibitor of both ROCK1 and ROCK2 and it mediates its inhibitory effect by binding to the N-terminal doman of Rho-kinases [163]. Notably, Rho-kinase inhibitors have been demonstrated to attenuate reperfusion and endotoxemic injury in the liver [164] as well as protecting against tissue fibrosis [165], obstructive cholestasis [166], cerebral and

intestinal ischemia [167, 168], acute pancreatitis [158] and pulmonary hypertension [169]. However, the role of the Rho-kinase signaling in regulating leukocyte recruitment and immune dysfunction in abdominal sepsis remains elusive. Thus, based on above considerations, we hypothesized herein that Rho-kinase signaling might play an important role in abdominal sepsis.

The mitogen-activated protein kinase (MAPK) signaling pathways are among the major intracellular transduction mechanisms of eukaryotic cell regulation and they constitute major inflammatory signalling pathways from the cell surface to the nucleus. P38MAPK is a member in MAPK signaling pathway which intermediates between Rho proteins and actin structures as well as gene expression. P38 MAPK signaling involved in many cellular functions such as migration, proliferation and differentiation [170]. P38MAPK activity in inflammation has been extensively investigated by using selective inhibitors of P38, SB203580 and SB239063 [171, 172]. P38MAPK has an important role in the production of pro-inflammatory mediators, TNF- and other cytokine as well as enzyme induction and expression of adhesion molecules [173]. In addition, it has been shown that inhibition of P38MAPk protects against sepsis- and streptococcal M1 protein-induced lung injury as well as ischemic reperfusion-induced inflammation in the colon [174-176].

The extracellular signal-regulated kinasee (ERK) signalling pathway is also another member of MAP kinase and is an important regulator of a number of cellular functions including growth, proliferation, and survival. ERK are involved in most cellular responses to extracellular signals; growth factor, cytokines and stress signals. Activtion of ERK occurs through different membrane receptors, but the most recognized pathway is binding of growth factor to receptor tyrosine kinase [177, 178]. However, the main function of ERK signaling pathway related to cell growth and proliferation; it is clear now that ERK activation involves in several inflammatory processes and ERK activation is essential for T cell activation [179].

Aims

l- To investigate the function of CD44 in sepsis-induced neutrophil recruitment and lund damage.

2- To define the function of geranylgeranyl transferase in sepsis-induced neutrophil infiltration and tissue damage in the lung

3- To define the role of Rho-kinase signaling on systemic activation and recruitment of neutrophils into the lung in a murine model of polymicrobial sepsis.

4- To analyze the role of Rho-kinase signaling pathway in regulating T-cell immune dysfunction in abdominal sepsis.

Materials and Methods

Animals

Male C57BL/6 wild type mice (21-27 g body weight, 8-10 weeks) were housed on an animal facility 12-12 h light dark cycle at 22°C, and fed a laboratory diet and water *ad libitum*. All experimental procedures were performed in accordance with the legislation on the protection of animals and were approved by the Regional ethical committee for Animal Experimentation at Lund University, Sweden.

Experimental protocol

Polymicrobial sepsis was induced by puncture of the cecum. In brief, the abdomen was opened and the exposed cecum was filled with feces by milking stool backward from the ascending colon. A ligature was placed below the ileocecal valve (by ligating 75% of cecum). The cecum was soaked with phosphate-buffered saline (PBS, pH 7.4) and punctured twice with a 21-gauge needle. This cecal ligation and puncture (CLP) protocol is associated with less than 10% mortality within 24 h. The cecum was then pushed back into the abdominal cavity and the abdominal incision was sutured. Sham mice underwent the identical laparotomy and resuscitation procedures, but the cecum was neither ligated nor punctured. The mice were then returned to their cages and provided food and water *ad libitum*. Animals were re-anesthetized 30 min, 6 and 24 h after CLP or sham operation. Blood was collected from inferior vena cava for later flow cytometric analysis and plasma was acquired by centrifugation and frozen at -20°C for CXCL1, CXCL2, CCL2, TNF-α, sCD40L, MMP-9, HMGB1 and IL-6 quantification. The left lung was ligated and excised for edema measurement. The right lung was used for collecting bronchoalveolar lavage fluid (BALF) to quantify neutrophils in a haematocytometer. Next, the lung was perfused with PBS, and one part was fixed in formaldehyde for histology, and the remaining lung tissue was weighed, snap-frozen in liquid nitrogen, and stored at -80°C for later enzyme-linked immunosorbent assay (ELISA) and myeloperoxidase (MPO) assays as described below.

Antibodies and biochemical substances

Animals were anesthetized intraperitoneally (i.p.) with 75 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per kg body weight. One ml of PBS mixed with buprenorfin hydrochloride (0.05 mg/kg body weight, Schering-plough Corporation, New Jersey, USA) was admisntered subcutaneously (s.c) as analgesia and for resuscitation.

To determine the functional role of CD44, we used a saturating dose of 4 mg/kg of a monoclonal antibody directed against murine CD44 (clone IM7, rat immunoglobulin G; BD Biosciences Pharmingen, San Jose, CA, USA) and an isotype-matched control mAb (clone R3-34, rat immunoglobulin G; BD Biosciences Pharmingen) in CLP animals. Antibodies or vehicle (100 μl PBS) was administered intravenously immediately prior to CLP induction.

Vehicle (PBS) or Rho-kinase inhibitor, Y-27632 (0.5-5.0 mg/kg), [(R)-(+)-N-(4-pyridyl)-4-(1-aminoethyl) cyclohexanecarcarboxamide; Calbiochem, San Diego, USA] was administered i.p. 30 min before cecal ligation and puncture, to delineate the role or Rho-kinase. These doses of Y-27632 were chosen based on our previous studies and other published papers.

To delineate the role of geranylgeranyl transferase, vehicle (dimethyl sulfoxide), or the geranylgeranyl transferase inhibitor, GGTI-2133 N-[[4-(Imidazol-4-yl)methylamino]-2-(naphthyl)benzoyl]leucine trifluoroacetate salt, G5294, Sigma Aldrich, St. Louis, MO, USA], was given (1.0 or 10 mg/kg) i.p. 30 min before CLP induction.

A mixture of 0.5μg of CXCL1 and 0.5μg CXCL2 was administered into the lungs via the trachea immediately after CLP in mice pretreated with 10 mg/kg of GGTI-2133 to delineate the role of chemokines in sepsis induced pulmonary neutrophil recruitment.

Systemic leukocyte counts

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

Lung edema and Bronchoalveolar lavage fluid (BALF)

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

Myeloperoxidase activity (MPO)

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 as previously. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter 540 nm, 25°C). Values were expressed as MPO units per g tissue.

Enzyme-linked immunosorbent assay (ELISA)

Lung homogenate levels of CXCL1, CXCL2, TNF-α and CCL2 were analyzed by using commercially available ELISA kits (R & D Systems, Abingdon, Oxon, UK). Lung samples were thawed and homogenized in PBS. Recombinant CXCL1, CXCL2, TNF-α and CCL2 diluted in a specific dilutent provided by the ELISA kit manufacturer were used to make standard curves.

Plasma levels of CD40L, MMP-9, CXCL1, CXCL2, TNF-α, CCL2, IL-6 and HMGB1 were analyzed. Blood samples were collected from the vena cava (1:10 acid citrate dextrose) and centrifuged at 14,000 RPM for 10 min at 4°C and stored at -20°C until further use. ELISA kits were used to quantify plasma levels of CXCL1, CXCL2, TNF-α, CCL2, IL-6 (R & D Systems, Abingdon, Oxon, UK) and HMGB1 (Chondrex, Redmond, WA, USA). Total MMP-9 was analyzed in heparinized plasma according to the manufacturer's protocol. For soluble CD40L analysis, plasma was collected on ice using citrate as anticoagulant and centrifuged for 20 min at 2000 x g immediately after collection. An additional centrifugation at 10000 x g for

10 min at 4°C was conducted for removal of platelets and stored at -20°C until further use. Plasma samples were then diluted 10 times with a sterile buffer (10% fetal calf serum in PBS, pH 7.4) to overcome the matrix effects and analyzed as per the protocols provided (R & D system). Recombinant CD40L, MMP-9, CXCL1, CXCL2, TNF-α, CCL2, IL-6 and HMGB1 diluted in a specific diluent provided by the ELISA kit manufacturer were used to make standard curves.

Flow cytometry

For analysis of surface CD40L expression on platelets as well as CD44, Mac-1 and CXCR2 expression on circulating neutrophils, blood was collected into syringes containing 1:10 acid citrate dextrose 6 h after CLP induction. Blood samples were incubated with an anti-CD16/CD32 antibody (10 min at RT) blocking Fcγ III/II receptors to reduce non-specific labeling and then incubated with PE-conjugated anti-Gr-1 (clone RB6-8C5, rat IgG2b, eBioscience, San Diego, CA, USA), APC-conjugated anti-CD14 (Sa14-2, rat IgG2a, Biosite, Täby, Sweden) and FITC-conjugated anti-Mac-1 (clone M1/70, integrin α_M chain, rat IgG_{2b}) or PerCP Cy5.5-conjugated anti-mouse CD182 (CXCR2) antibody (clone TG11/CXCR2, rat IgG2a, Biolegend, San Diego, CA, USA) or PE-conjugated anti-CD44 (clone IM7) antibodies. Another set of samples was stained with FITC-conjugated anti-CD41 (clone MWReg30, integrin α_{IIb} chain, rat IgG1) and PE-conjugated anti-CD40L (clone MR1, hamster IgG, eBioscience) antibodies (all antibodies except those indicated were purchased from BD Biosciences Pharmingen, San Jose, CA, USA). Cells were fixed with 1% formaldehyde solution; erythrocytes were lysed using FACS lysing solution (BD Biosciences Pharmingen, San Jose, CA, USA) and then neutrophils and platelets were recovered following centrifugation. Flow cytometric analysis was performed according to standard settings on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and a viable gate was used to exclude dead and fragmented cells. Neutrophils were defined as Gr-1+/CD14- cells. A PE-conjugated anti-mouse F4/80 (clone BM8, Biolegend, London, UK) was used to identify macrophages isolated from the lung.

Platelet isolation and CD40L shedding

Blood was collected in syringes containing 1:10 acid-citrate-dextrose anticoagulant and diluted with equal volumes of modified Tyrode solution (1 µg/ml prostaglandin E1 and 0.1U/mL apyrase) and centrifuged at 200 x g for 5 min at room temperature (RT). Platelet rich plasma (PRP) was collected and centrifuged at 800 x g for 15 min at RT, and pellets were resuspended in Tyrode solution. After washing once, platelets were resuspended at a count of 0.5 × 108 platelets/tube in Tyrode solution. Platelets were pre-incubated with vehicle or 1-100 µM of GGTI-2133 and stimulated with 200 µM of AYPGKF (thrombin receptor activating peptide, Bachem, Weil am Rhein, Germany) for 15 min at 37°C. After stimulation, cells were immediately fixed by adding 0.5% formaldehyde where after samples were centrifuged at 10000 x g for 10 min at 4°C. Platelets were incubated with fluorescent-labeled antibodies and surface expression of CD40L was analyzed using flow cytometry as described below.

Neutrophil isolation

Bone marrow Neutrophils were freshly extracted from healthy mice by using Ficoll-Paque TM Research Grade (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated neutrophils was higher than 70% as assessed in a haematocytometer. Neutrophils were then resuspended in PBS to 10 x 10^6/ml and used in Adoptive Transfer, in vitro activation and chemotaxis.

Adoptive transfer of neutrophils

Isolated bone marrow neutrophils were labeled with 20 µM CFDA-SE (carboxyfluorescein diacetate-succinimidyl ester, Invitrogen, Paisley, UK) for one h at 37°C. CFDA-SE passively diffuses into cells and is nonfluorescent until its acetate groups are cleaved by intracellular esterases to yield a highly fluorescent ester. Two millions labeled neutrophils were injected intravenously into mice immediately prior to CLP. Six h after CLP induction, lungs were harvested, minced, and digested for one h at 37°C in buffer containing 20U/mL collagenase A (Sigma Chemical Co). Single-cell suspensions were obtained by straining the digested tissue through a 40-µm mesh. Cells were labeled with an APC-labeled anti-Gr-1 antibody and fixed

as described above. Finally, cells were analyzed by flow cytometry (FACSCalibur). Lung recruitment of transferred neutrophils were quantified by dividing the number of CFDA+/Gr-1+ cells by the number of CFDA-/Gr-1+ cells in the lung extracts.

In vitro neutrophil activation

Isolated bone marrow neutrophils were co-incubated with 300 ng/ml recombinant mouse CXCL2 (R&D Systems, Inc., Minneapolis, MN 55413 USA) for 10 min. Neutrophils were pre-incubated with Y-27632 (1 or 10 μM) or GGTI-2133 (1 or 10 μM) 20 min before challenge with CXCL2. Cells were stained and fixed for flow cytometric analysis of Mac-1 expression on neutrophils as described above.

Chemotaxis assay

Isolated bone marrow neutrophils were preincubated with GGTI-2133 (1 or 10 μM) or Y-27632 (0.1-10 μM) for 30 min, and 1.5 x 106 neutrophils were placed in the upper chamber of the Transwell inserts (5 μm pore size; Corning Costar, Corning, NY, USA). Inserts were placed in wells containing medium alone (control) or medium plus CXCL2 (100 ng/ml; R&D Systems). After 120 min, inserts were removed, and migrated neutrophils were stained with Turks solution. Chemotaxis was determined by counting the number of migrated neutrophils in a Burker chamber

Isolation of alveolar macrophages and quantitative RT-PCR

In separate experiments, gene-expression of CXCL1, CXCL2, TNF-α and CCL2 was quantified in alveolar macrophages isolated from sham mice (n = 5) and CLP animals pretreated with vehicle or 10 mg/kg of GGTI-2133 i.p. or 5 mg/kg of Y-27632 30 min prior to CLP (n = 5). Alveolar macrophages were isolated from BALF as described in detail [180]. Briefly, 30 min after induction of CLP, lungs were flushed three times with 1 ml of PBS supplemented with 0.5 mM EDTA. Alveolar fluid collections were then centrifuged at 1400 RPM, 10 min, 18°C. The cells were then resuspended in RPMI 1640 complete culture medium and incubated at

37°C, 5% CO_2 in 48-well plate. After 2 h, non-adherent cells were washed away by PBS. A total of 2-3 x 10^5 macrophages were obtained per mice and the purity of macrophages was higher than 97%. Total RNA was isolated from the alveolar macrophages using an RNeasy Mini Kit (Qiagen, West Sussex, UK) following the manufacturer's protocol and treated with RNase-free DNase (DNase I; Amersham Pharmacia Biotech, Sollentuna, Sweden) to remove potential genomic DNA contaminants. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically. Each cDNA was synthesized by reverse transcription from 10 µg of total RNA using the StrataScript First-Strand Synthesis System and random hexamer primers (Stratagene; AH diagnostics, Stockholm, Sweden). Real-time PCR was performed using a Brilliant SYBRgreen QPCR master mix and MX 3000P detection system (Stratagene). The primer sequences of CXCL1, CXCL2, and β-actin were as follows: CXCL1 (forward) 5'-GCC AAT GAG CTG CGC TGT CAA TGC-3', CXCL1 (reverse) 5'-CTT GGG GAC ACC TTT TAG CAT CTT-3'; CXCL2 (forward) 5'-GCT TCC TCG GGC ACT CCA GAC-3', CXCL2 (reverse) 5'-TTA GCC TTG CCT TTG TTC AGT AT-3'; 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'; CCL1 (forward) 5'-TGT GAG TTA CAT ACC CCG GC-3', CCL1 (reverse) 5'-GCC TGA ACA GCA GCC ATA GA-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.

Isolation of splenocytes

The spleen was excised for cell culture and flow cytometry analysis 24 h post CLP induction. Single splenocyte suspension was obtained under sterile condition by smashing the spleen and passing it through a 40 µm cell strainer (BD Falcon, Becton Dickinson, Mountain View, CA, USA). Red

blood cells were lysed by use of ACK lysing buffer (Invitrogen, Carlsbad, CA, USA). The cells were washed and resuspended with CLICK's medium (Sigma-Aldrich, Stockholm, Sweden) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 unit/ml) and streptomycin (0.1 mg/ml) (Sigma-Aldrich, Stockholm, Sweden). The same medium was used in all experiments described below. Splenocytes were quantified in a Burker chamber staining with Turk's solution (Merck, Damnstadt, Germany).

Cytokine formation in splenocytes

Isolated splenocytes were loaded at 1.0 x 10^6 in 48-well plates pre-coated with anti-CD3ε antibody (5 μg/well, IgG, clone: 145-2C11, eBioscience, San Diego, CA, USA) and in the presence of soluble anti-CD28 antibody (5 μg/well, IgG, clone: 37.51, eBioscience, San Diego, CA, USA) at 37°C in a humidified atmosphere with 5% CO_2 for 24 h. Levels of IFN-γ and IL-4 in the culture medium were detected by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

T-cell apoptosis

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

T-cell proliferation

Isolated splenocytes were stained with carboxyfluorescein diacetate succinimydul ester (CFSE, 5 μM, Sigma-Aldrich, Stockholm, Sweden) and

incubated at 1.5×10^6 cells/well in 150 µL CLICK's medium in 96-well plates pre-coated with or without anti-CD3ε antibody (5 µg/ml, IgG, clone: 145-2C11) and in the presence or absence of soluble anti-CD28 antibody (2 µg/ml, IgG, clone: 37.51) at 37°C in a humidified atmosphere with 5% CO_2 for 72h. For analysis of cell proliferation, splenocytes were stained with APC conjugated anti-CD4 antibody (IgG2b, kappa, clone: GK1.5) and propidium iodide (PI) (Phoenix Flow Systems, San Diego, CA, USA). Flow cytometric analysis was performed on a FACSCalibur flow cytometer and PI negative cells were gated to exclude dead cells.

Regulatory T-cell analysis

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

Bacterial cultures

Blood was taken from the interior vena cava 24 h after CLP and cultured to evaluate the bacterial clearance. Serial logarithmic diluted blood was plated on trypticase soy agar II with 5% sheep blood (Becton Dickinson GmbH, Heidelberg, Germany). Plates were incubated under aerobic conditions at 37°C, and colonies were counted after 24 h of incubation. Bacterial counts are expressed as the number of CFU ($\times10^5$) per ml of blood.

Histology

Lung samples were fixed by immersion in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin-embedded. Six µm sections were stained with haematoxylin and eosin. Lung injury was quantified in a blinded manner by using a modified scoring system, including alveolar collapse, thickness of alveolar septae, alveolar fibrin deposition and neutrophil infiltration graded on a zero (absent) to four (extensive) scale. In each tissue sample, 5 random areas were scored and

mean value was calculated. The histology score is the sum of all 4 parameters.

Statistics

Data are presented as mean values ± standard errors of the means (SEM). Statistical comparisons between more than two datasets were performed using Kruskal-Wallis one-way analysis of variance on ranks followed by multiple comparisons versus control group (Dunnett's method). Mann–Whitney rank-sum test was used for comparing two groups. $P < 0.05$ was considered significant and *n* represents the number of animals in each group. Statistical analysis was performed by using SigmaStat® 3.5 software (System Software, Chicago, Illinos, USA).

Table 3. **Histology scoring system**

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 100X (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 100X (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

Results and Discussion

Role of CD44 in abdominal sepsis

CD44 is a glycoprotein adhesion molecule expressed on many cell types including leukocytes and parenchymal cells and it is involved in many physiologic and pathologic processes [181, 182]. Several studies, with main focus on inflammation, have shown that CD44 is involved in leukocyte recruitment [123, 124], however, the role of CD44 in pulmonary leukocyte recruitment in abdominal sepsis remain elusive. In the first paper, we demonstrated that CD44 plays a crucial role in pulmonary neutrophil recruitment and septic lung injury. Inhibition of CD44 protects against sepsis-induced lung injury, as indicated by decreased leukocyte recruitment and preserved intact lung tissue architecture in CLP animals. Moreover, we show that neutrophil CD44 rather than lung CD44 mediates neutrophil accumulation in septic lung injury. This CD44-dependent infiltration of neutrophils appears to be independent of hyaluronan in the lung.

Sepsis is characterized by a systemic inflammatory response to invading microbial agent, in which the most insidious component is lung damage and consequently disturbed gaseous exchange [14, 183]. The recruitment of neutrophils into the lung dominates the inflammatory response to the presence of microorganism. It has generally been recognized that excessive neutrophils recruitment is a major mediator in the pathophysiology of sepsis induced lung injury through the release of cytotoxic proteases and oxygen-derived radicals [14, 184]. The role of CD44 in the process of leukocyte migration into the lung is contradictory [125, 126]. Moreover, the role of CD44 in regulating neutrophil activation and pulmonary leukocyte infiltration in abdominal sepsis is not known. To obtain insight in the role of CD44 in sepsis-induced lung injury, C57BL6 wild type mice were exposed to cecal ligation and puncture to induce polymicrobial sepsis and we used a monoclonal antibody directed against murine CD44 to block CD44 functions.

In our experiments, we show for the first time that inhibition of CD44 function effectively decreases neutrophil accumulation in the lung in abdominal sepsis. We investigated levels of myeloperoxidase (MPO), an indicator of neutrophils, and the number of neutrophils in bronchioalveolar lavage fluid (BALF) to study neutrophil recruitment in septic lung injury. MPO levels and BALF neutrophils in the lung represent early and late phases of pulmonary accumulation of neutrophils, and they peaked at 6 h

and 24 h respectively. Interestingly, we found that immunoneutralization of CD44 reduced MPO activity in the lung in CLP mice, thus reduced pulmonary leukocyte accumulation. This inhibitory effect on MPO activity correlated well with our other findings that CD44 blocking reduced sepsis-triggered neutrophil recruitment in the bronchoalveolar space, suggesting that CD44 indeed supports neutrophil accumulation in septic lung injury. This observation is in line with the study, which demonstrated the significant role of CD44 in endotoxin-induced pulmonary infiltration of neutrophils [126]. In addition, functional inhibition of CD44 decreased CLP-induced lung damage and edema. Morphological analysis revealed severe pulmonary damage in CLP mice, characterized by severe destruction of pulmonary tissue microstructure, capillary congestion, massive necrosis, extensive edema of interstitial tissue and excessive infiltration of neutrophils. Lung edema formation markedly increased in CLP mice, reflected by an increased lung wet/dry ratio. Interestingly, administration of anti-CD44 antibody preserved intact lung tissue architecture and reduced the wet/dry ratio by 88% in CLP animals. Thus, these results indicate that targeting CD44 may be a useful way to ameliorate septic lung injury.

Because CD44 is expressed on both neutrophils and endothelial cells [185] and in order to determine whether CD44 expressed on neutrophils or in the lung mediated neutrophil recruitment in septic lung injury, we performed a series of experiments with adoptive transfer of labeled neutrophils co-incubated with the anti-CD44 antibody or a control antibody. It was found that homing of labeled neutrophils to the lung in CLP mice was markedly reduced when neutrophils were co-incubated with the anti-CD44 antibody compared to co-incubation with the control antibody, showing that neutrophil CD44 is mediating neutrophil recruitment in septic lung injury.

Knowing that activated neutrophils change their surface expression of Mac-1 from low avidity to high avidity which plays a key role in the sepsis-induced neutrophil infiltration in the lung in abdominal sepsis [14]; we next asked whether inhibition of CD44 may affect Mac-1 expression on neutrophils. By using flow cytometry, it was found that CLP increased surface expression of Mac-1 on neutrophils. However, immuneutralization of CD44 had no effect on Mac-1 levels on neutrophils in CLP mice, suggesting that neutrophil activation was not negatively affected by immunoneutralization of CD44. Tissue accumulation of leukocytes is coordinated by secreted CXC chemokines such as, CXCL1 and CXCL2, being murine homologues of human interleukin-8 [80]. Moreover, a functional role of CXC chemokines has been proposed in abdominal

infections [15, 110]. However, it was observed that inhibition of CD44 had no impact on CLP-provoked formation of MIP-2 in the lung.

Considering our finding that neutrophil CD44 appears to regulate neutrophil recruitment in septic lung injury and knowing that hyaluronan is one of a major ligand of CD44 [115]. We explored the potential role of hyaluronan in pulmonary infiltration of neutrophils in abdominal sepsis. However, we found that elimination of hyaluronan from the vascular endothelium had no effect on MPO activity in the lung and number of BALF neutrophils in CLP mice, indicating that CD44-dependent accumulation of neutrophils in the septic lung is independent of hyaluronan.

We conclude that neutrophil CD44 participate in pulmonary neutrophil infiltration in abdominal sepsis. Moreover, we found that inhibition of CD44 protects against sepsis-induced lung injury. However, CD44-dependent accumulation of neutrophils in the septic lung is independent of hyaluronan. Our findings suggest that targeting CD44 might be an effective way to ameliorate respiratory failure in polymicrobial sepsis.

Role of geranylgeranylation in abdominal sepsis

HMG-CoA reductase is the key enzyme in mevalonate pathway. Mevalonate is a precursor for the formation of geranylgeranyl pyrophosphate and farnesyl pyrophosphate, which are essential in protein isoprenylation [127]. Isoprenylation, especially geranygeranylation, is critical for the membrane targeting and activity of Rho proteins [132].

Statins, which are used for treatment of hypercholestrolemia, can inhibit HMG-CoA reductase. Many studies have shown that statins exert anti-inflammatory effects, including reduction of nitric oxide formation, inhibition of adhesion molecule expression and cytokine formation [141, 142, 186]. Moreover, many experimental studies have shown that statins protects against severe infection such as pneumococcal pneumonia [187] and meningitis [188] and statins can reduce mortality in patients with severe infection and sepsis [143, 144]. Recent data have also demonstrated that simvastatin protects against SIRS-associated lung injury in abdominal sepsis [15]. Considering that statins mediate their biological effects at least in parts via isoprenoids [146], in this context, we wanted to know the role of geranylgeranilation in sepsis-induced lung injury in mice. We found that geranylgeranyl transferase plays an essential role in mediating pulmonary damage in a murine model of abdominal sepsis. Our findings show that inhibition of geranylgeranyl transferase decreased the development of

inflammation and protected against pulmonary tissue damage in septic mice through blocking the formation of CXC chemokines in the lung and inhibition of neutrophil activation and recruitment in abdominal sepsis.

Geranylgeranyl transferase plays an essential role in many diseases such as carcinogenesis [189] as well as many infectious and non infectious inflammations [136, 137, 139]. Several geranylgeranyl transferase inhibitors have been developed which beside their primarily use as anti-tumor drugs, they are now expected to have a crucial role in treating a wide variety of other diseases including inflammation, atherosclerosis, viral infection, apoptosis, rheumatoid arthritis, psoriasis, multiple sclerosis, and glaucoma [128, 140]. Previous studies reported that GGTI-2133, geranylgeranyl transferase inhibitor, inhibits infiltration of eosinophil into airway in mouse experimental asthma [190] and GGTI-298, another geranylgeranyl transferase inhibitor, prevents lymphocytes recruitment into central nervous system [191], suggesting that geranylgeranyl transferase might control tissue infiltration of different subtypes of leukocytes. In the present study, we could document that inhibition of geranylgeranyl transferase by administration of GGTI-2133 decreased pulmonary MPO activity, a marker of neutrophils in CLP mice. This inhibitory effect correlated well with our observation that GGTI-2133 administration reduced sepsis-induced neutrophil infiltration in the bronchoalveolar space, indicating that GGTI-2133 effectively inhibits neutrophil accumulation in septic lung damage. It is well-known that depletion of neutrophils protects against septic lung injury [14, 56, 105], it might be suggested that the protective effect of GGTI-2133 is related to the reduction in pulmonary neutrophil infiltration.

Considering that CXC chemokines are known to regulate pulmonary infiltration of neutrophils in septic lung damage [14], the inhibitory effect of GGTI-2133 on neutrophil trafficking in the lung could be explained by its ability to inhibit pulmonary CXC formation, showed in our results that GGTI-2133 abolished CLP-induced formation of CXC in alveolar macrophages in the lung. Moreover, geranylgeranyl transferase regulates Mac-1 expression on neutrophils which has essential role in inflammatory infiltration of neutrophils at extravascular sites. We found that GGTI-2133 significantly reduced expression of Mac-1 on the surface of neutrophils in mice exposed to CLP. However, inhibition of geranylgeranyl transferase had no effect on CXCL2-induced Mac-1 expression on isolated neutrophils in vitro, indicating that geranylgeranyl transferase regulates Mac-1 expression on neutrophils in an indirect manner in vivo.

Recent study has demonstrated that platelets play an important role in neutrophil activation and neutrophil recruitment via secretion of CD40L in abdominal sepsis [56], therefore, we next wanted to examine the role of GGTI-2133 in regulating platelet shedding of CD40L. We found that administration of GGTI-2133 had no effect on platelet surface expression of CD40L both in vivo and in vitro; suggesting that geranylgeranyl transferase-dependent activation of neutrophils is independent of CD40L in abdominal sepsis. A study from our group has reported that simvastatin markedly augmented clearance of bacteria from the circulation in CLP mice [15]. Thus, we carried out a bacterial clearance test in blood, however, we found that treatment with GGTI-2133 had no effect on the number of bacteria in the blood of septic animals.

In conclusion, our data indicate a pivotal role of GGTI-2133 in the protection of septic lung injury similar to that demonstrated in simvastatin treatment in the same model of sepsis, which might help to explain the protective effects of simvastatin on lung damage in abdominal sepsis. Moreover, our results are also in line with our recent findings showing that inhibition of Rho-kinase, the downstream signaling target of Rho protein geranylgeranylation [135], decreased sepsis-induced pulmonary recruitment of neutrophils and tissue damage by regulating formation of CXC chemokines in the lung and neutrophil activation in the circulation.

Role of Rho-kinase in abdominal sepsis

In general, Rho-kinase signaling is considered to regulate cytoskeletal dynamics, including cell contraction and migration, however, during the last twenty years numerous studies have demonstrated the role of this signaling pathway in several pathological processes [158, 164, 166, 168, 169]. Accumulating data also suggest that Rho-kinase is involved in many inflammatory processes such as leukocyte-platelet-endothelial interactions as well as ROS and cytokine formation [159, 160, 166, 192]. Herein, we show that Rho-kinase signaling mediates an important role in abdominal sepsis. We found that inhibition of Rho-kinase, by using Y-27632, protects against sepsis-induced pulmonary recruitment of neutrophils and tissue injury in early hyper-inflammatory phase. Moreover, our results show that Y-27632 improves several aspects of T-cell function during late immunosuppressive phase. Taken together, our findings indicate an immunomodulating role of Y-27632 in abdominal sepsis.

In the present study, we found that administration of Rho-kinase inhibitor Y-27632 protects against pulmonary edema and tissue damage in polymicrobial sepsis. Morphological analysis revealed that CLP caused severe destruction of the pulmonary microarchitecture with extensive edema of the interstitial tissue and massive infiltration of neutrophils. Inhibition of Rho-kinase reduced CLP-induced tissue destruction and edema formation in the lung. These findings are in line with observations that fasudil, another Rho-kinase inhibitor, and Y-27632 can reduce lung injury triggered by endotoxin and streptococcal M1 protein respectively [193, 194]. It is widely held that neutrophil infiltration is a key feature in the pathophysiology of septic lung damage [105, 110, 174]. In order to investigate neutrophil accumulation in the lung, activity of MPO and the number of neutrophils in BALF were determined. We demonstrated that administration of Y-27632 (5mg/kg) decreased both pulmonary MPO activity and neutrophil infiltration in the bronchoalveolar space, elucidating that Y-27632 effectively reduces pulmonary neutrophil accumulation in abdominal sepsis, suggesting that Rho-kinase signaling plays an important role in abdominal sepsis.

Neutrophil recruitment in the lung is a multistep process which is mediated by specific adhesion molecules both on neutrophil and endothelial cells such as Mac-1 and ICAM-1. Moreover, Mac-1 expression on neutrophils is up-regulated in CLP mouse which mediates migration of neutrphil into the lung [14, 195]. Therefore, we asked whether Rho-kinase signaling might regulate neutrophil activation and expression of Mac-1 in our model of sepsis. Indeed, treatment with Y-27632 significantly reduced expression of Mac-1 on the surface of neutrophils in mice exposed to CLP, which may contribute to the inhibitory effect of Y-27632 on sepsis-induced neutrophil infiltration and pulmonary tissue injury in abdominal sepsis. Moreover, we found that inhibition of Rho-kinase markedly reduced CXCL2-induced up-regulation of Mac-1 on isolated neutrophils in vitro, suggesting that Rho-kinase signaling directly regulates neutrophil activation and Mac-1 up-regulation in abdominal sepsis. Knowing that CXC chemokines are potent inducers of neutrophil migration [81], it was also of interest to evaluate the role of Rho-kinase in regulating chemokine-dependent chemotaxis herein. Notably, we found that Y-27632 decreased CXCL2-induced neutrophil migration by more than 85% in vitro.

Activation and directing inflammatory cells to extravascular sites of inflammation is under control of secreted chemokines. CXC chemokines, such as CXCL1 and CXCL2 in mice, are particularly involved in neutrophils accumulation at sites of inflammation [80]. A functional role of

CXC chemokines has been proposed in abdominal infections [15, 174]. We also found that Y-27632 significantly decreased CXC chemokine formation in the lung in vivo. In addition, it was found that Y-27632 markedly decreased mRNA levels of CXCL-1 and CXCL-2 in the alveolar macrophages in CLP-induced animals, indicating that Rho-kinase signaling is a key feature in macrophage production of CXC chemokines in abdominal sepsis.

Considering recent studies showing that platelets play an essential role in regulating Mac-1 expression on neutrophils and pulmonary neutrophil recruitment in polymicrobial sepsis through secretion of CD40L [56], it was of great interest to examine the role of Rho-kinase in regulating platelet shedding of CD40L. We found that CLP triggered a significant reduction in platelet expression of CD40L in vivo, concomitantly; we found that plasma levels of CD40L markedly increased in CLP mice. However, we observed that administration of Y-27632 had no effect on plasma levels of CD40L or surface expression of CD40L on platelets. This observation is also supported by our finding showing that Y-27632 does not affect the CLP-induced increase in matrix metalloproteinase-9 (MMP-9), which cleaves off surface CD40L on platelets. Together, these findings indicate that Rho-kinase-dependent pulmonary recruitment of neutrophils is independent of CD40L in abdominal sepsis.

Immune suppression is an insidious aspect of the host reaction to severe infections or major trauma. T-cell dysfunction is one of the most prominent feature of sepsis-induced immune suppression [37], however, the mechanisms mediate induction of T-cell dysfunction in abdominal sepsis remain elusive. Therefore, we next extend our research to study the role of Rho-kinase in the later immunosuppression phase of sepsis particularly T-cell dysfunction. Herein, we demonstrated that Rho-kinase inhibition Y-27632 significantly reduced sepsis-induced T-cell apoptosis, in which percentage of BrdUTP-positive CD4 T-cells decreased from 14.5% down to 7.2% corresponding to 84% reduction in apoptosis. We also demonstrated that Y-27632 could improve the proliferative capacity of CD4 T-cells in septic animals. Thus, these effects can increase the number of splenic CD4 T-cells and they become capable of mounting effective host-defense responses against invading microbes. Considering that IFN-γ is essential in Th1-type immunity against microbes [196]. Moreover, many studies have shown that IFN-γ decreases mortality in septic animal [197] as well as in some clinical study [35]. Herein, we observed that Rho-kinase inhibition also reversed CLP-induced reduction in IFN-γ formation, which may also contribute to promote effective anti-bacterial responses [196]. Taken together, our results indicate that Y-27632 improves immune responses via

increased number of T-cells on one hand and promoted function on the other hand i.e. IFN-γ formation.

Knowing that regulatory T-cells serve an important function in host response to invading microbes by their ability to control T-cell-dependent immune responses [198]. However, in some cases controlling immune response is too excessive, compromising host response and enhancing pathogen survival [199]. Many studies observed that increased number of regulatory T-cells in the course of sepsis might compromise anti-bacterial defense capability [37-39]. Additionally, it was found that regulatory T-cells is involved in reduced CD4+ T-cells proliferative capacity and cytokine production in septic animals [40]. In the present study, Y-27632 was found to be able to inhibit the increased regulatory T-cells in the spleen of septic mice by 52%. Knowing that the percentage of regulatory T-cells is also increased in septic patients and which has the ability to suppress adaptive immune system [200]. We might conclude that this Y-27632-mediated reduction in regulatory T-cells might contribute to the protection against infection in patients with sepsis. Taken together, inhibition Rho-kinase signaling may improve T-cell dependent immune responses via at least three different mechanisms, i.e. increasing the number of T-cells, promoting the function of T-cells (IFN-γ production) and decreasing the number of regulatory T-cells.

HMGB1 is a potent pro-inflammatory cytokine ubiquitously expressed in many cell types such as macrophages and monocytes and it is involved in different inflammatory diseases, especially sepsis [201-204]. A High level of HMGB1 was found in severe sepsis both in humans and in animals [201, 204]. HMGB1 level is highly correlated with the severity of sepsis and degree of organ dysfunction during septic shock [205, 206]. HMGB1 is a late mediator as well as a well-known predictor of clinical outcome in endotoxemia and septic patients [201, 207]. HMGB1 plays an essential role in endothelial and epithelial cell dysfunction and cause lethal organ damage [208]. Moreover, HMGB1 activates endothelial cells through advanced glycation end product receptor, increasing expression of adhesion molecules on endothelial cells and inducing secretion of chemokines and cytokines resulting in leukocyte migration and aggravating immune response [209]. In line with these observations, we found that CLP caused a clear-cut increase in the plasma levels of HMGB1. Notably, Y-27632 administration reduced CLP-evoked production of HMGB1 in the plasma, indicating a potent anti-inflammatory effect of Rho-kinase inhibitor in sepsis-induced systemic inflammation. This is the first study showing that

Rho-kinase regulates HMGB1 formation in sepsis. Considering that administration of anti-HMGB1 antibodies prevents lung damage in septic mice [210]. Moreover, inhibition of HMGB1 decreases regulatory T cells in spleen and IL-10 production, which in turn stimulate T-lymphocyte functions [211]. Taken together, these might explain the inhibitory effect of Rho-kinase inhibitor on the formation of regulatory T cells and improved immune function in the present study.

IL-6 is another marker of systemic inflammation which is markedly increased in the blood of patients with infection or sepsis [212, 213]. Many studies have reported a correlation between high IL-6 serum levels with severity as well as mortality in septic patients and animals [214-216]. IL-6 is an important cytokine with pro inflammatory and anti-inflammatory effects, which might be related to different signaling pathways of IL-6 [217, 218]. Herein, we show that inhibition of Rho-kinase significantly reduced plasma levels of IL-6 in septic mice. A previous study has shown that inhibition of IL-6 during CLP-induced sepsis in mice results in a significant reduction in C5aR expression in lung, liver and kidney leading to markedly improved survival [219]. These findings may support the concept that Rho-kinase signaling regulates the sepsis-induced systemic inflammatory response.

Many studies have shown that Rho proteins play a crucial role in the interactions between microbial agents and their hosts, in particular, by regulating essential aspects of innate and adaptive immune defenses [160, 220]. Moreover, considering our results showing that inhibition of Rho-kinase signaling improves T-cell function in sepsis, we next wanted to examine the role of Rho-kinase activity on CLP-induced bacteremia. We found that CLP greatly enhanced the levels of bacteria in the blood. Interestingly, treatment with Y-27632 decreased the number of bacteria in the blood of septic mice, which might be due to the improved T-cell function.

Taken together, our findings clearly showed that inhibition of Rho-kinase protects against sepsis-induced lung injury through two different mechanisms. First, Rho-kinase inhibitor, Y-27632, inhibits neutrophil recruitment into the lung by decreasing Mac-1 expression, which is independent of platelet CD40L. Second, Y-27632 inhibits pulmonary formation of CXC chemokines. We demonstrated also that Y-27632 improves several aspects of T-cell function in abdominal sepsis, including amelioration apoptosis of splenocytes, inhibition of increased CD4+CD25+Foxp3+ T regulatory cells and hypo-responsiveness, and increases IFN-γ formation in splenic CD4 T-cells. In addition, we showed

that Y-27632 improves bacterial clearance during sepsis. Our findings indicate that Rho-kinase inhibitor has beneficial effects in sepsis.

Conclusions

1- Neutrohil CD44 plays an important role in pulmonary neutrophil recruitment and tissue damage in abdominal sepsis.

2- Geranylgeranyl transferase activity regulates pulmonary neutrophil infiltration and tissue damage in abdominal sepsis.

3- Inhibition of Rho-kinase protects against sepsis-induced lung injury through inhibiting neutrophil recruitment into the lung by abolishing pulmonary formation of CXC chemokines and decreasing Mac-1 expression on neutrophils, which is independent of platelet CD40L.

4- Rho-kinase signaling regulates T-cell immune dysfunction in polymicrobial sepsis.

Sammanfattning på svenska

Sepsis, blodförgiftning, är ett potentiellt allvarlgt 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. Sepsis är systemisk inflammatorisk reaktion mot mikrobiell invadering. Svår sepsis är den sepsis som associerad med en eller flera organdysfunktion. Septisk chock inträffar när sepsis kompliceras av hypotension och hypoperfusion trots adekvat vätsketillförsel. Sepsis utvecklas till stor del som en följd av hyperinflammatorisk och oreglerad värd immunsvar mot invaderande bakterier eller deras toxiner. Efter den hyperinflammatoriska fasen uppstår ett tillstånd med immnunförsvar dysfunktion då septiska patienter blir mer mottagliga för infektioner.

Under normala förhållanden elimineras skadliga patogener framgångsrikt av immunceller utan någon vävnadsskada. Emellertid kan den hyperinflammatoriska och dåligreglerade immunsvaren vid sepsis orsaka vävnadsskada och organskada som så småningom leder till multipel organsvikt. Akut lungskada är en central komponent hos patienter med sepsis och experimentella studier har visat att aktivering och samling av vita blodkroppar (leukocyter) är ett hastighetsberoende steg i sepsis-associerad lungskada. Leukocyter interagerar med trombocyter och endotelceller genom cell medlare och en sekvens av receptor-ligand interaktion så att de kan lämna blodbanan som en följd av ökad vaskulär permeabilitet.

Leukocytmigrationen utgör en begränsand faktor för inflammationens utbredning och skadeverkan i vävnaden. Trots att det inflammatoriska svaret är avgörande för den initiala framgången av immunsystemet, är adekvat kontroll och upplösning av pro-inflammatoriska signaler lika viktiga för överlevnad av drabbade individer. Denna över-inflammation kan undvikas om kontraregulatoriska svar kommer vid rätt tidpunkt, vilket kan leda till fullständig återställning av värden. När anti-inflammatorisk respons är långvarig eller alltför uttalad kan leda till immunosuppressiv tillstånd och oförmåga att rensa infektion vilket kallas kompenserande anti-inflammatorisk respons syndrom. Målet med denna avhandling är att definiera några av de signalerings mekanismer av pulmonell neutrofil rekrytering och immun dysfunktion i buksepsis. Dessutom vill vi definiera rollen av trombocyter och CXC kemokiner i denna process.

I den första studien analyseras betydelsen av adhesionsmolekeln CD44 för leukocytrekrytering och vävnadsskada vid sepsis. För att klargöra

detta använde vi antikroppar riktade mot CD44. Vi fann att inhibering av CD44 miniskar leukocytrekrytering och skyddar mot lungskada vid sepsis. Dessutom fann vi att inte lunga CD44 utan att neutrofil CD44 medierar neutrofil rektytering i septisk lungskada. Denna CD44-beroende infiltration av neutrofiler är oberoende av hyaluronan i lungan.

I den andra studien hypotetiserade vi att geranylgeranyl transferas spelar en nyckelroll vid sepsis. För att undersöka detta användes en selektiv geranylgeranyl transferas hämmare (GGTI-2133) 30 min innan induktion av sepsis. Vi fann att geranylgeranyltransferas spelar en viktig roll vid mediering av lungskador i septiska möss. Våra resultat visar att GGTI-2133 signifikant miniskar vävnadsskada och leukocytrekrytering genom att blockering CXC kemokinernas produktion i lungan och leukocytacktevering i blodbanan.

I de tredje och fjärde arbetena studerades effecten av intra-cellular Rho-kinas signalering vid sepsis-inducerad inflammation i lungan och immundysfunction. I den tredje studien undersöktes betydelsen av Rho-kinas signalering vid sepsis-inducerad inflammation i lungan. Vi observerade att Rho-kinas signalering medierar en viktig roll vid sepsis. Vi fann också att hämning av Rho-kinas, genom att använda Y-27632, skyddar mot sepsis-inducerad pulmonell rekrytering av neutrofiler och vävnadsskada i tidig hyper-inflammatorisk fas. I det fjärde arbetet studerades immunsuppression vid sepsis med fokus på T-cell funktion. Våra resultat visar att sepsis orsakade en omfattande apoptosis (celldöd) av CD4 T-celler och mindre cytokin (IFN-gamma och IL-4) produktion. Dessutom var proliferativsvaret hos CD4 T-celler kraftigt nedsatt vid sepsis. Vidare observerade vi att sepsis ökar regulatoriska T-celler som kan hämma immunsvaret mot bakterier samt virus och ökar risken för infektion. Intressant observerade vi att Y-27632 förbättrar alla de här aspekterna av T-cellernas funktion under sen immunsuppressiv fas vid sepsis. Sammantaget våra resultat tyder på en immunomodulerande roll av Y-27632 i buksepsis.

Sammanfattningsvis kartlägger den här avhandlingen nya mekanismer bakom pulmonell neutrofil rekrytering och immun dysfunktion i patienter med svår buksepsis som kan lägga till grund för utvecklandet av nya och effektivare behandlingsmetoder.

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