Adhesive and signaling mechanisms in abdominal sepsis

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Adhesive and signaling mechanisms in abdominal sepsis

Su Zhang

Department of Clinical Science, Malmö, Section of Surgery

Academic Thesis

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Faculty opponent: Mihály Boros, MD, PhD
Professor, Institute of Surgical Research
Albert Szent-Györgyi Medical and Pharmaceutical Centre
University of Szeged, Hungary
Adhesive and signaling mechanisms in abdominal sepsis

Abstract
Sepsis is a major cause of mortality in intensive care units despite decades of scientific efforts. The lung is recognized as the most sensitive and critical organ affected by the hyper-inflammatory response in septic patients. Immune cell dysfunction develops in later phases of sepsis making patients susceptible to infections. The aim of this dissertation was to reveal pro- and anti-inflammatory mechanisms in abdominal sepsis induced by cecal ligation and puncture (CLP). It was found that immunoneutralization of LFA-1 or Mac-1 not only reduced neutrophil infiltration but also protected against sepsis-caused lung damage. We observed that HMG-CoA reductase inhibitor regulates pulmonary accumulation of neutrophils via antagonizing CD40 ligand secretion from platelets and via decreasing CXC chemokine formation in the lung. Moreover, HMG-CoA reductase inhibition maintained CD4 T-cell function in spleen by reducing apoptosis and by improving proliferation as well as by preserving the ability to produce IFN-γ in the spleen. Moreover, inhibition of HMG-CoA reductase decreased sepsis-induced generation of regulatory T-cells. HMG-CoA reductase inhibitor abolished CLP-evoked increase in the plasma levels of HMGB1 and IL-6. In addition, it was noted that NFAT was activated after induction of sepsis, which was found to regulate pulmonary neutrophil recruitment and tissue damage, systemic inflammation, as well as T-cell dysfunction in abdominal sepsis. Thus, these results identify adhesive and signaling mechanisms, which are of particular importance in modulating septic lung injury and immunosuppression. Data from this thesis may pave the way for developing more specific and effective therapies for patients with abdominal sepsis.

Key words: abdominal sepsis, lung, infection, neutrophil, chemokines, T-cells, HMG-CoA reductase, NFAT

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Adhesive and signaling mechanisms in abdominal sepsis

By

Su Zhang

Lund University
Faculty of Medicine

Department of Clinical Science, Malmö
Section of Surgery
Skåne University Hospital
To my parents
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## Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>ALI</td>
<td>acute lung injury</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>ARDS</td>
<td>acute respiratory distress syndrome</td>
</tr>
<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BTP</td>
<td>bis(trifluoromethyl)pyrazole</td>
</tr>
<tr>
<td>CARS</td>
<td>compensatory anti-inflammatory response syndrome</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein diacetate succinimydul ester</td>
</tr>
<tr>
<td>CLP</td>
<td>cecal ligation and puncture</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporine A</td>
</tr>
<tr>
<td>DIC</td>
<td>disseminated intravascular coagulation</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Foxp3</td>
<td>forkhead box P3</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-Hydroxy-3-methylglutary coenzyme A</td>
</tr>
<tr>
<td>HMGB1</td>
<td>high-mobility group box-1</td>
</tr>
<tr>
<td>ICAM</td>
<td>intracellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>KC</td>
<td>cytokine-induced neutrophils chemoattractant</td>
</tr>
<tr>
<td>LFA-1</td>
<td>lymphocyte function antigen-1</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>Mac-1</td>
<td>membrane activated antigen-1</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MARS</td>
<td>mixed anti-inflammatory response syndrome</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>MIP-2</td>
<td>macrophage inflammatory protein-2</td>
</tr>
<tr>
<td>MNL</td>
<td>monomorphonuclear leukocyte</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PE</td>
<td>polyethylene</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PMNL</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern-recognition receptor</td>
</tr>
<tr>
<td>SIRS</td>
<td>systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneously</td>
</tr>
<tr>
<td>sCD40L</td>
<td>soluble CD40 ligand</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cells</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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</table>
Introduction

Sepsis is a complex syndrome caused by an uncontrolled systemic inflammatory response to a documented or suspected infection and characterized by multiple manifestations, which can result in dysfunction or failure of one or more organs and even death [1]. Lacking of understanding of the pathogenesis and therapies of sepsis remains challenges both the clinicians and scientists. Approximately 750 000 cases of severe sepsis occur each year and more than half of these patients develop septic shock. Despite decades of efforts, the hospital mortality of sepsis remains high as approximately 30% to 50% [2]. The definitions of sepsis and related disorders are summarized in Table 1 outlined by the American College of Chest Physicians and the Society of Critical Care Medicine in 1992 [1]. This practical framework has been extended to a classification scheme for sepsis namely PIRO classification, that stratifies patients according to their Predisposing conditions, the nature and extent of the Insult (in the case of sepsis, Infection), the nature and magnitude of the host Response, and the degree of concomitant Organ dysfunction [1].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Bacteremia</td>
<td>The presence of viable bacteria in the blood</td>
</tr>
<tr>
<td>Infection</td>
<td>Microbial phenomenon characterized by an inflammatory response to the presence of microorganisms or the invasion of normally sterile host tissue by those organisms</td>
</tr>
<tr>
<td>Systemic inflammatory response syndrome (SIRS)</td>
<td>Temperature &gt;38°C or &lt;36°C Heart rate &gt;90 beats per minute Respiratory rate &gt;20 breaths per minute or carbon dioxide tension &lt;32 mm Hg White blood cell count &gt;12 000/mL or &lt;4000/mL or the presence of &gt;10% bands</td>
</tr>
<tr>
<td>Sepsis</td>
<td>SIRS + suspected/docuted infection</td>
</tr>
<tr>
<td>Severe sepsis</td>
<td>Sepsis + organ dysfunction</td>
</tr>
<tr>
<td>Septic shock</td>
<td>Sepsis-induced hypotension despite adequate fluid resuscitation and perfusion abnormalities</td>
</tr>
</tbody>
</table>

Two distinct responses of the immune system during sepsis have been defined consequently. The first, systemic inflammatory syndrome (SIRS) happens when local insult is amplified and dysregulated, cause an activation of the body’s inflammatory, complement and coagulation cascades. For example, during abdominal sepsis, intestinal content
contaminates the abdominal cavity, fecal bacteria and toxins stimulate local production of pro-inflammatory substances, which are subsequently released into the circulation. Moreover, infective microorganisms may directly invade the blood stream and induce an inflammatory host response in a distant target organ, as sepsis related acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). The malfunction of regulatory mechanisms during sepsis can result in a loss of control of inflammation, eventually leading to host damage due to overzealous activation of the inflammatory response. Uncontrolled SIRS was thought to be the main cause of death in septic patient before. However, the failure of anti-inflammatory therapies for sepsis in clinical trails [3] led to doubts in whether uncontrolled pro-inflammatory response should be prosecuted for the mortality in sepsis. Notably, most deaths of septic patients occur in the later phase of the disease, which is associated with an immunosuppressive state. The high susceptibility of intensive care patients to develop secondary infections is associated with the occurrence of an alternation of the immune status, referred to compensatory anti-inflammatory response syndrome (CARS) [4]. This second septic response is characterized by an immune paralysis of the host to defend against microbes, partly due to the immunosuppressive state of immune cells.
Background

Immune response to infection

The immune response to pathogens relies on both innate and adaptive immune components. Due to different forms of microorganisms, a wide spectrum of recognition capacity of innate immune responses is required and constitutes the first line of defense against infections. On the contrary, the adaptive immune response is highly specific for a particular foreign antigen and characterized by generation of specific antibodies and immunological memories.

The cells belong to the innate immune system express a set of receptors known as pattern-recognition receptors (PRRs), i.e., Toll-like receptors (TLRs), and recognize a broad range of pathogen associated molecular patterns (PAMPs) presented commonly on pathogens, including lipopolysaccharide (LPS) in Gram-negative bacteria, mannans in fungi and double-strain RNA in some viruses. Mechanisms of the initiation of the inflammatory cascade are well studied. Upon recognition, innate immune cells as neutrophils, monocytes and macrophages phagocytize and kill pathogens by producing reactive oxygen species and intermediates at infectious to initiate the inflammatory cascade [5]. Numerous exogenous (endo- and exotoxin) and endogenous mediators such as activated complement factor (C5a) or damaged cells have the ability to activate innate immune cells, i.e. neutrophils, macrophages and endothelium directly, leading to production of cytokines [6, 7].

The intracellular inflammatory pathway of immune cells is commonly started by the activation of the serine phosphokinase via ligation of exogenous stimuli with PRR, i.e. LPS binds to TLR4/CD14/MD-2 receptor complex, together with MyD88, leading to an oxidative reaction which in turn activate transcription factors, like nuclear factor κB (NFκB), activator protein 1 and interferon regulatory factor 3. The activation and nuclear translocation of transcription factors regulate gene expression for pro-inflammatory cytokines, chemokines, adhesion molecules, and pro-coagulant agents and enzymes [8, 9]. Apart from TLR4, TLR2 has been show to recognize Gram-positive bacteria, such as lipoteichoic acid [10], TLR5 binds with flagellin from both Gram-negative and positive bacteria [11], and TLR9 is triggered by cytidine phosphate guanosine DNA [12].
Antigen presenting cells as macrophages and dendritic cells have the ability to present cellular components of the pathogen to adaptive immune cells, inducing activation of the adaptive immune response and the establishment of protective immunity [13]. B-cells combat extracellular pathogens and their products by releasing antibody. T-cells have a wider range of activities, and are thought to orchestrate the inflammatory response, particularly CD4+ T helper (Th)1 cells and Th2 cells, which are delimited according to distinct cytokine profiles. Th1 cells secrete interferon (IFN)-γ and tumor necrosis factor (TNF)-α, which drastically augment the anti-microbial capacity of phagocytes, thus helping the innate immune system to target and destroy intracellular pathogens. Th2 cells mainly support humoral responses against extracellular pathogens by producing interleukin (IL)-4 and IL-10. It has been shown that during sepsis, there is a shift from Th1 pattern to a predominantly Th2-type response [14].

**Disordered inflammatory response**

Under normal condition, an appropriate inflammatory response eliminates the invading pathogens without causing damage to tissues, organs or other systems. However, in certain circumstances, the excessive elements of the inflammatory response drive the physiological alterations to a systemic inflammatory disorder, i.e. SIRS. Patients could show fever, circulatory collapse, metabolic disturbances (hypoglycemia), disseminated intravascular coagulation (DIC), and hemorrhagic necrosis, leading eventually to multiple organ failure. Production of inflammatory mediators especially release of exacerbated pro-inflammatory agents is the principal cause for septic shock, such as TNF [15], IL-1β [16], IL-6 [17], high-mobility group box-1 (HMGB1) [18], macrophage migration inhibitory factor [19], platelet-activating factor [20], pro-inflammatory enzymes as inducible nitric oxide synthase and cyclooxygenase 2 [21, 22].

Moreover, these inflammatory mediators could decrease susceptibility of neutrophils to apoptosis leading to their accumulation. Hyperactivity of neutrophils could induce tissue damage and even fatally organ failure and death [23].

Both animal and patient studies demonstrate a counter-regulatory mechanism subsequent to the earlier hyper-inflammatory phase [14, 24] with an enhanced release of anti-inflammatory mediators, as cytokines for soluble tumor necrosis factor receptor [25], IL-10 [26], IL-1 receptor antagonist [27], transforming growth factor (TGF)-β [28], and decoy receptor such as IL-1 receptor type II [29]. This process is referred to CARS. The hyper-
and hypo-inflammatory states alternate during the following course of sepsis, and they could co-exist at the same time [30, 31], of which has been designated as “mixed anti-inflammatory response syndrome (MARS)”. This phenomenon reflects the dynamic process of immune responses. However, the strong relationship between high level of those agents measured in plasma of septic patients and poor outcome indicates the exacerbated anti-inflammatory response [32].

During this hypo-inflammatory phase, patients show an altered immune status, as impaired phagocytic function of neutrophils [33], decreased expression of antigen-presentation molecules on monocytes (major histocompatibility complex class II and human leukocyte antigens) [34]. CARS is also related to T lymphocytes hypo-responsiveness, anergy, and a defect in antigen presentation. T-cells harvested from septic patients showed delayed type hypersensitivity response [35], increase in apoptosis and suppressed proliferation and production of IFN-γ [24, 36]. Such defects in immune response and the immune system make it unable to mount appropriate host defense responses against pathogens, which put septic patients in a high susceptibility to nosocomial infections. Moreover, increased number of regulatory T-cells compromises the host defense reaction as well [37, 38].

**Leukocyte-mediated lung injury during sepsis**

Tissue recruitment of leukocytes from the blood stream across the endothelial cell monolayer of postcapillary venules and subsequent transmigration into the surrounding tissue is a key feature in the inflammatory response, this process plays two major roles as to eliminate harmful microorganisms on one hand and cause tissue damage on the other. Normally, neutrophils work effectively at the site of inflammation to enhance surveillance against infectious agents and facilitate recruitment of other pro-inflammatory cells [23]. However, uncontrolled neutrophil activation injures the tissue, and ultimately leads to inflammation-induced organ failure usually seen in SIRS. This neutrophil caused tissue damage appears to be related to proteases and toxic reactive oxygen radicals released from them. In addition, neutrophils can produce cytokines and chemokines that enhance pro-inflammatory response [39]. Lung is well recognized as the most sensitive and critical organ affected in septic patients, and the most frequent cause of ALI/ARDS is severe sepsis.
The septic lung injury is characterized by massive accumulation of neutrophils in the bronchoalveolar space [40, 41]. The well-coordinated sequence of ligand-receptor interactions between leukocytes and endothelium mediate the multi-step pulmonary recruitment of leukocytes. The process involves firstly sequestration in microvessels, rolling and firm adhesion on the endothelium, followed by transendothelial and transepithelial migration [42-44]. Leukocyte movements are orchestrated and mediated by families of adhesion molecules [45] and chemoattractant molecules expressed both on leukocytes and vascular endothelium. In addition, platelets are considered to be involved in pulmonary neutrophil infiltration [46].

**Leukocyte rolling**

Generally, high velocity moving leukocytes need to slow down to tether and roll along the endothelium before migration. Tethering and rolling of leukocyte are considered as the precondition for sequent firm adhesion. However, some organs may not require rolling to recruit leukocyte due to their unique microcirculatory architecture, as liver sinusoids [47] and lung capillaries [48].

Leukocyte rolling on surface of microvascular endothelium is mediated by the selectin family, which consists of three members, i.e. P-selectin (CD62P), E-selectin (CD62E) and L-selectin (CD62L) [49]. Each selectin is selectively expressed on different cell types and recognizes and binds to corresponding ligands in a calcium-dependent manner [50]. Many pro-inflammation agents like TNF-α, histamine, leukotrienes have been reported to regulate rolling by increasing P-selectin expression [51, 52].

**Chemokine mediated leukocyte activation**

During inflammatory responses, endothelial cells are stimulated by pro-inflammatory agents and synthesize chemokines and present them on surface [42, 53]. Rolling leukocytes could be activated by the combination of chemokine receptors expressed on their surface with the secreted chemokines of the endothelium [54], which in turn up-regulates a family of adhesion molecules known as integrins and mediates firm adhesion of leukocyte.

Chemokines are small proteins with molecular weight in the range of 8 to 12 kDa, which act mainly on leukocytes and induce chemotaxis. CXC chemokines have been implicated in all steps in the extravasation process of leukocyte recruitment, including rolling,
adhesive and transmigration in vivo [55]. In mouse, CXC chemokines include macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophils chemoattractant (KC), which are homologues of human IL-8 [56, 57]. The specific effects of these chemokines are mediated via G-protein coupled receptors [58] and CXCR2 is the high affinity receptor for both of them [59, 60]. Upon activation, CXCR2 expressed on neutrophils mediates cell degranulation, respiratory burst, phagocytosis, integrin activation, migration and trafficking [61]. Among non-hematopoietic cells, CXCR2 expression has been demonstrated in pulmonary endothelial cells, where it is associated with angiogenic activity in lung tumor and fibro-proliferative process [62, 63], and it could mediate neutrophil infiltration in the lung [64]. CXCR2 has been described to be elevated in the lung and plasma [59], but depressed on surface of neutrophils due to internalization in sepsis [65], and deficiency of CXCR2 in mice appears to protect against sepsis [66].

**Leukocyte adhesion**

Integrins are heterodimeric transmembrane glycoproteins, which are composed of a α-subunit non-covalently links to a β-subunit and expressed predominantly on leukocytes [54, 67, 68]. The binding capacity of integrins is owned solely in the I domain of the α-subunits [69, 70]. β2-integrins are composed of the common β2-subunit (CD18) and link to one of four α-subunits (CD11a, b, c and d) [67, 71]. Accordingly, the β2-integrin subfamily includes lymphocyte function antigen-1 (CD11a/CD18, LFA-1) and membrane activated antigen-1 (CD11b/CD18, Mac-1), P150, 95 (CD11c/CD18) and αdβ2 (CD11d/CD18).

The members of the β2-integrin family are believed to mediate fundamental functions of neutrophils, involving firm adhesion, migration and phagocytosis of complement opsonized particles [72]. Both LFA-1 and Mac-1 interact with the members of the well-known immunoglobulin superfamily protein expressed on the endothelial cell surface namely intracellular adhesion molecules (ICAMs). ICAM-1 (CD54) is a cytokine-inducible adhesion molecule [73] and plays important role in stable adhesion and transmigration of leukocytes, contributing to septic lung injury [74, 75]. ICAM-1 is up-regulated upon stimulation of pro-inflammatory cytokines, such as TNF-α and IL-1. Interestingly, this inducible up-regulation can be modulated by anti-inflammatory cytokines such as TGF-β and IL-10 [76].

Although interactions between β2-integrins and ICAMs have been studied in various systems [77-79], and functional blocking (anti-CD18 monoclonal antibody, mAb) of these
adhesion molecules has been proved to attenuate leukocyte adhesion [77], the potential role of LFA-1 and Mac-1 in mediating pulmonary recruitment of neutrophils in polymicrobial sepsis remains elusive.

**Leukocyte transmigration**

Firm adhesion of leukocytes is followed by extravasation, which could occur not only at contacts between adjacent endothelial cells [80, 81] as a so-called paracellular pathway, but also happens with a direct movement through the endothelial cell body, i.e. transcellular migration [82]. Many studies have documented a variety of endothelial membrane proteins involved in the leukocyte diapedesis, including platelet endothelial cell adhesion molecule - 1 (CD31), E-selectin, junction adhesion molecules, ICAMs, vascular cell adhesion molecule -1 and CD99 [83, 84].

**Role of platelet-derived CD40L for leukocyte recruitment**

Platelets are small anuclear and irregularly shaped cell fragments that are derived from megakaryocytes [85]. Growing body of literature suggests the role of platelets in inflammation and tissue injury [86] beyond their classic roles in haemostasis and thrombosis [87]. Platelets have been show to play an important role in sepsis by regulating neutrophil recruitment to the lung via up-regulation of Mac-1 expression. Depletion of platelets not only reduces neutrophil infiltration but also protects against sepsis-evoked lung damage. In addition, platelets mediated up-regulation of Mac-1 on circulating neutrophils is not dependent on contact formation between neutrophils and platelets. However, depletion of platelets has no effect on sepsis triggered pulmonary formation of CXC chemokines [46].

CD40 ligand (CD40L, CD154) is a transmembrane protein belongs to the TNF family and expressed in activated CD4+ T-cells and platelets [88]. CD40 is the main receptor of CD40L, which expressed on B cells, monocytes, macrophages and so on [89]. The interaction of CD40L on activated T cells with CD40 on B cells is required for IgM to IgG isotype switch and plays essential roles in the adaptive immune response [90]. CD40L exerts several pro-inflammatory and pro-coagulant effects as well, for example stimulating synthesis of IL-1 and TNF-α in monocytes [91] and up-regulating adhesion molecules on endothelial cells [92]. CD40L is also shed from the surface of activated platelets into a soluble form (sCD40L) [93]. And these platelet-derived CD40L mediates neutrophil
infiltration and pulmonary damage; it could also regulate Mac-1 expression on neutrophils during sepsis [94]. Patient study also showed an increased level of sCD40L with septic shock [95], which may help better understanding pathological inflammation mechanisms in sepsis.

**NFAT signaling pathway in sepsis**

Extracellular stress signals trigger intracellular signaling cascades converting on specific transcription factors, which control gene expression and formation of inflammatory substances. Cytosolic calcium is a ubiquitous intracellular messenger involved in the complex regulation of immune cell functions [96, 97]. Calcineurin is a unique calcium/calmodulin activated serine/threonine phosphatase served as one of key targets of calcium in eukaryotic cells. Calcineurin owns a critical function in several cellular processes and calcium-dependent signal transduction pathways. Nuclear factor of activated T-cells (NFAT) is an important downstream target of calcineurin, which is first documented in T-cells as an inducible factor binding to the distal antigen receptor responses elements of the human IL-2 promoter and related to the Rel/NFκB family [98, 99]. The NFAT family consists of five members [100]; the activation of NFATc1-c4 is Ca^{2+}/calcineurin depended, while NFATc5 is activated by hypertonic stress [101].

NFAT is widely expressed by almost all cell types accounts for its engagement in development and function of several tissues for both human and mouse [102-104]. NFAT plays roles not only within but also outside immune systems. The effect of NFAT has been extensively studied in immune cells, particularly in T cells, which is related to T cell proliferation, activation and apoptosis [104].

The regulatory domain located at the N-terminal of NFAT protein is heavily phosphorylated in an inactivated form and cytosolic under resting conditions, but able to bind to calcineurin when Ca^{2+} elevated intracellularly, and be dephosphorylated. Activated NFAT could translocate to the nucleus upon stimulation and initiates a cascade of transcriptional events involved in both physiological and pathological processes [105-107]. Notably, a sustained elevation of Ca^{2+} is required to activate NFAT, where as transient Ca^{2+} spikes are not sufficient [108], such increased cytosolic Ca^{2+} has been demonstrated in a mice endotoxic model, and blocking of calcineurin decrease the production of TNF-α [109]. So it is of great interest to investigate detailed cell action during sepsis regulated by NFAT signaling pathway.
Cyclosporine A (CsA) and FK506 (tacrolimus) have been used traditionally for their immunosuppressive effects. They block NFAT signaling pathway by inhibiting the ability of calcineurin to dephosphorylate its target protein, inducing a unspecific inhibition effect [110], which leads to major side effects seen in clinical uses as nephron- and neurotoxicity [111]. However, the new developed bis(trifluoromethyl)pyrazole (BTP) derivation shows an inhibitory effect of NFAT beside direct interference of calcineurin phosphatase activity [112, 113] by maintaining NFAT in the cytosol in a phosphorylated form. It has been shown that treatment with one of the BTP compounds (A-285222) inhibits acute pancreatitis induced NFAT activation and decrease neutrophil infiltration in the pancreas (unpublished data). However, the potential role of NFAT in the pathophysiology of abdominal sepsis needs to be clarified.

**Statin treatment in sepsis**

Due to the complexity of sepsis, interfering with only one cellular or humoral pathways in sepsis maybe insufficient to arrest the whole inflammatory process, which explain partly why most of the adjunctive therapies aimed for sepsis have been disappointing [114, 115]. Treatments of sepsis to reduce mortality are largely limited to only five interventions that have been shown effectiveness in large, randomized controlled trials: 1) lung-protective mechanical ventilation, 2) intensive insulin therapy, 3) early goal-directed therapy, 4) treatment with low doses of hydrocortisone, and 5) recombinant human activated protein C treatment [116].

3-Hydroxy-3-methylglutary coenzyme A (HMG-CoA) reductase inhibitors (statins) are potent cholesterol lowering agents that are widely used in clinic for dyslipidemia and atherosclerotic disease. Most commonly used statins, for instance, simvastatin, lovastatin, pravastatin, fluvastatin, have shown profound effects in decrease cardiovascular events and septic morbidity and mortality [117-121].

The mechanism of statins to decrease plasma cholesterol is achieved by competitively inhibiting the conversion of HMG-CoA to mevalonate via inhibition of HMG-CoA reductase, which is a rate-limiting step in cholesterol synthesis. Mevalonate is the precursor not only of cholesterol but also of many other nonsteroidal isoprenoid products. So statins disrupt as well the synthesis of the isoprenoid derivatives (farnesyl and geranylgeranyl pyrophosphate), which influences with other cellular functions. A growing body of literatures has elucidated that statins possess so-called pleiotropic effects, as anti-inflammatory, anti-
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oxidant, immunomodulatory, anti-apoptotic, anti-thrombotic, anti-microbial and endothelium-protective effects, all of which are independent of their cholesterol-lowering effect [122-125]. These effects appear to relate to the major effect of statins in inhibition of HMG-CoA reductase since supplementation of mevalonate, farnesyl pyrophosphate and geranylgeranyl pyrophosphate prevents almost all of the statin-mediated effects. However, the detailed anti-immune-regulatory mechanisms of simvastatin in sepsis, especially in immune suppressive state remain elusive.
Aims

I. To define the function of LFA-1 and Mac-1 in sepsis-induced neutrophil recruitment and lung damage.

II. To investigate the role of HMG-CoA reductase inhibitor on systemic activation and recruitment of neutrophils into the lung in a murine model of sepsis.

III. To analyze the role of HMG-CoA reductase inhibitor in regulating T-cell immune dysfunction in abdominal sepsis.

IV. To explore the potential involvement of calcium/calcineurin-dependent NFAT in polymicrobial sepsis.
Materials & Methods

Animals

All animal experimental procedures were conducted in accordance with approved ethical permission by the Regional Ethical Committee for Animal Experimentation at Lund University, Sweden. C57BL/6 wild type (20 to 25g body weight, 8 to 10 weeks), FVB/N 9x-NFAT-luciferase reporter mice (NFAT-luc) and wild-type littermates were used (22 to 28 g, 8-10 weeks). NFAT-luc mice are phenotypically normal, and nine copies of a NFAT binding site from the IL-4 promoter (5'-TGGAAAATT-3') were positioned 5' to a minimal promoter from the α-myosin heavy chain gene (-164 to +16) and inserted upstream of the luciferase reporter gene [126]. Mice were housed on an animal facility with 12-12 hours light dark cycle at 22°C, and fed a laboratory diet and water ad libitum.

Experimental protocol of sepsis

Polymicrobial sepsis in mice was induced by cecal ligation and puncture (CLP) procedure. Under anesthesia, animals underwent a midline incision to identify and exteriorize the cecum, which was filled with feces by milking stool backwards from the ascending colon and 75% of the cecum was ligated with a 5-0 silk suture. The cecum was soaked with phosphate buffered saline (PBS, pH 7.4) and was then double punctured with a 21-gauge needle on the antimesenteric border. This procedure leads to a mortality of ~20% at 24 hours post CLP induction. A small amount of bowel contents was extruded, and the cecum was returned into the peritoneal cavity and the abdomen was closed in two layers. Sham animals underwent the identical laparotomy and resuscitation procedures, but the cecum was neither ligated nor punctured.

Animals were re-anesthetized 6 and 24 hours after CLP or sham procedure. Blood was obtained from the vena cava for later flow cytometric analysis and plasma was acquired by centrifugation and frozen at -20°C for sCD40L, 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. Then, the lung was excised and one lobe was fixed in formaldehyde for histology and another piece of lung, together with half of the spleen, liver, and aorta were resected for luciferase measurement. The remaining lung tissue was weighed, snap-frozen in liquid nitrogen and stored at -80°C.
for later myeloperoxidase (MPO) assays and enzyme-linked immunosorbent assay (ELISA) as described subsequently.

**Antibodies and drugs**

Animals were anesthetized with 7.5 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight intraperitoneally (i.p). 1 ml of PBS mixed with buprenorfin hydrochloride (0.05 mg/kg body weight, Schering-Plough Corporation, New Jersey, USA) was administered subcutaneously (s.c.) as analgesia and for resuscitation.

Monoclonal antibodies (mAbs) directed against murine CD11a (clone M17/4.4.11.9, rat IgG, 4mg/kg body weight, Novartit Pharma AG, Preclinical Research Basel, Switzerland), CD11b (clone M1/70, rat IgG2b, 4mg/kg body weight, BD Biosciences Pharmingen, San Jose, CA, USA) were used prior to CLP induction for immunoneutralization of LFA-1 and Mac-1. The control group received an isotype-matched control mAb (clone R3-34, IgG1, BD Biosciences Pharmingen).

Simvastatin (0.5 or 10 mg/kg body weight, Sigma-Aldrich, Stockholm, Sweden) was administered i.p. 10 minutes prior to CLP induction. These doses of simvastatin were chosen based on our previous studies and other published papers.

A mAb directed against murine CD40L (clone MR1, 10 mg/ kg body weight, eBioscience, San Diego, CA, USA), a nonfunctional control antibody (clone R3-34, IgG1, BD Biosciences Pharmingen) was administered i.p. immediately before CLP induction.

The function of CXCR2 was blocked by using a CXCR2 antagonist (SB225002, 4 mg/kg body weight, i.p., Calbiochem, Merck, Darmstadt, Germany) prior to CLP induction.

The NFAT activity was blocked by using a novel blocker, the derivative of BTP, A-285222 (0.15mg/kg body weight), injected i.p. twice daily for 7 consecutive days and in the morning of operation, (kindly provided by Abbott Laboratories).

**Systemic leukocyte counts**

Blood was collected from the tail vein and mixed with Turks solution (Merck, Darmstadt, Germany) in a 1:20 dilution. Leukocytes were identified as monomorphonuclear (MNL) and polymorphonuclear (PMNL) leukocytes in a Burker chamber.
Bronchoalveolar lavage fluid (BALF)
Animals were placed supine and the trachea was exposed by dissection. An angiocatheter was inserted into the trachea. BALF was collected by 5 washes of 1 ml of PBS containing 5 mM EDTA. The numbers of MNL and PMNL cells were counted in a Burker chamber.

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

Myeloperoxidase (MPO) activity
Lung tissue was thawed and homogenized in 1 ml of 0.5% hexadecyltrimethylammonium bromide. Samples were freeze-thawed, after which the MPO activity of the supernatant was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H₂O₂ (450 nm, with a reference filter 540 nm, 25°C). Values were expressed as MPO units per g tissue.

Enzyme-linked immunosorbent assay (ELISA)
MIP-2 and KC levels in lung tissue were analyzed by using double antibody Quantikine ELISA kits (R & D Systems, Europe, Abingdon, Oxon, UK) using recombinant murine MIP-2, KC as standards.

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 use. ELISA kits were used to quantify plasma levels of sCD40L, IL-6 (R & D Systems) and HMGB1 (Chondrex, Redmond, WA, USA) according to manufacturer’s instructions.

Flow cytometry
For analysis of surface molecules expression on circulating neutrophils and platelets, blood was collected (1:10 acid citrate dextrose) 6, 16 and 24 hours after CLP induction and incubated (10 minutes at room temperature) with an anti-CD16/CD32 antibody blocking Fcy
III/II receptors to reduce non-specific labeling and then incubated with polyethylene (PE)-conjugated anti-Gr-1 (clone RB6-8C5, rat IgG2b, eBioscience), fluorescein isothiocyanate (FITC)-conjugated anti-LFA-1 (clone M17/4, rat IgG2a κ, BD Biosciences Pharmingen), or FITC/APC-conjugated anti-Mac-1 (clone M1/70, integrin αM china, rat IgG2b κ, BD Biosciences Pharmingen), or PerCP-Cy5.5-conjugated anti-mouse CD182 (CXCR2) (clone TG11/CXCR2, rat IgG2a, Biolegend, San Diego, CA, USA) antibodies. Another set of samples was stained with FITC-conjugated anti-CD41 (clone MWRReg30, integrin αIIb chain, rat IgG1, BD Biosciences Pharmingen) and PE-conjugated anti-CD40L (clone MR1, hamster IgG, BD Biosciences Pharmingen) antibodies. Cells were fixed and erythrocytes were lysed, 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 analyzed with Cell-Quest Pro software (BD Bioscience, San Jose, CA, USA). A viable gate was used to exclude dead and fragmented cells.

**Histology**

Lung samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin-embedded. 6 µm sections were stained with hematoxylin and eosin (H&E).

**In vitro activation of neutrophils**

Blood was collected from healthy animals and incubated with recombinant murine sCD40L (100 ng/ml, PeproTech EC, London, UK) and activated simvastatin (1 µM, Sigma-Aldrich) at 37°C for 20 minutes. Then the cells were stained for flow cytometric analysis of Mac-1 expression on neutrophils as described above. The simvastatin pro-drug was activated as described previously [127]. Recombinant mouse MIP-2 (300 ng/ml, R & D Systems) was used as positive control.

**Neutrophil isolation and cell sorting**

Neutrophils were freshly extracted from healthy mice by aseptically flushing the bone marrow of femurs and tibiae with complete culture medium RPMI 1640 (Sigma-Aldrich), and then subsequently isolated by using Ficoll-Paque ™ Research Grade (Amersham
Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated neutrophils was higher than 70% as assessed in a haematocytometer. The neutrophils were re-suspended in culture medium RPMI 1640 until further purified by cell sorting or used in the chemotaxis assay. Isolated neutrophils were labeled with the FITC-conjugated anti–mouse neutrophil antibody (clone 7/4, rat IgG2a, Abcam, Cambridge, UK) and sorted with FACSria. Purity of sorted neutrophils was higher than 98%, which were used in RT-PCR.

**RT-PCR**

Total RNA was extracted from the purified neutrophils of healthy mice using RNeasy Mini-kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol, and treated with RNease-free DNease (Amersham Pharmacia Biotech AB) to remove potential genomic DNA contaminants according to manufacturer’s handbook. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically. RT-PCR was performed with Superscript One-Step RT-PCR system (GIBCO BRL Life Technologies, Grand Islands, NY, USA). The RT-PCR reactions started with 5 minutes synthesis at 95°C, followed by 40 cycles of denaturation at 94°C for 40 seconds, annealing at 60°C for 40 seconds, and extension at 72°C for 1 minute, and 1 cycle of final extension at 72°C for 10 minutes. After RT-PCR, aliquots of the RT-PCR products were separated on 2% agarose gel containing ethidium bromide and photographed. The primers sequences were as follows: CD40 (f) 5'-GAA GCC GAC TGA CAA GCC AC-3', (r) 5'-GTG TCT GTG CTG GTG ACA GCG-3'. β-actin (f) 5'-ATG TTT GAG ACC TTC AAC ACC-3', (r) 5'-TCT CCA GGG AGG AAG AGG AT-3'. β-actin served as a housekeeping gene.

**Chemotaxis assay**

Neutrophils isolated form bone marrow by use of Ficoll-Paque™ were pre-incubated with simvastatin (25 µM) for 30 minutes and 1.5 × 10^6 neutrophils were placed in the upper chamber of the Transwell inserts (5 µm pore size, Corning Costar, Corning, NY, USA). Inserts were placed in wells containing medium alone (control) or medium plus MIP-2 (100 ng/ml, R & D Systems). After 120 minutes, inserts were removed and migrated neutrophils were stained with Turks solution. Chemotaxis was determined by counting the number of migrated neutrophils in a Burker chamber [128].
**Isolation of splenocytes**

The spleen was excised for cell culture and flow cytometric analysis 24 hours 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 using ACK lysing buffer (Invitrogen, Carlsbad, CA, USA). The cells were washed and resuspended with CLICK’s medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 unit/ml) and streptomycin (0.1 mg/ml) (Sigma-Aldrich). The same medium was used in all experiment described below. Splenocytes were quantified in a Burker chamber staining with Turks solution (Merck).

**Cytokine formation in splenocytes**

Isolated splenocytes were loaded at 1.0 x 10^6 cells/well in 48-well plates pre-coated with anti-CD3ε antibody (5 µg/well, IgG, clone 145-2C11, eBioscience) and in the presence of soluble anti-CD28 antibody (5 µg/well, IgG, clone 37.51, eBioscience) at 37°C in a humidified atmosphere with 5% CO₂ for 24 hours. Levels of IFN-γ and IL-4 in the culture medium were detected by ELISA kits (R & D Systems) 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 (Phoenix Flow Systems, San Diego, CA, USA), which labels DNA strand breaks by BrdUTP according to the manufacturer’s instruction. Allophycocyanin (APC)-conjugated anti-CD4 antibody (IgG2b, κ, clone: GK1.5, eBioscience) was used to indicate CD4 T-cells. Splenocytes were acquired by a FACSCalibur flow cytometer and analyzed with Cell-Quest Pro software.

**T-cell proliferation**

Isolated splenocytes were stained with carboxyfluorescein diacetate succinimydul ester (CFSE, 5 µM, Sigma-Aldrich) and incubated at 1.5 x 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,
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clone: 145-2C11, eBioscience) and in the presence or absence of soluble anti-CD28 antibody (2 µg/ml, IgG, clone 37.51, eBioscience) at 37°C in a humidified atmosphere with 5% CO₂ for 72 hours. For analysis of cell proliferation, splenocytes were stained with APC-conjugated anti-CD4 antibody (IgG2b, κ, clone GK1.5, eBioscience) and propidium iodide (PI) (Phoenix Flow Systems). 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, eBioscience), APC-conjugated anti-CD25 (Rat IgG1, λ, Clone PC61.5) and PE-conjugated anti-Foxp3 (Rat IgG2a, κ, Clone FJK-16s, eBioscience) antibodies. Flow cytometric analysis was performed on a FACSCalibur flow cytometer.

**Bacterial cultures**

Blood taken from the interior vena cava of mice 24 hours post CLP was 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 hours of incubation. Bacterial counts were expressed as the number of CFU (×10⁵) per ml of blood.

**Statistics**

Data were presented as mean values ± standard errors of the means (SEM). Statistical evaluations were performed using Kruskal-Wallis one-way analysis of variance on ranks followed by multiple comparisons. Mann-Whitney rank sum test was used for comparing two groups. P < 0.05 was considered significant and n represents the total number of mice in each group. Statistical analysis was performed by using SigmaStat® 3.5 software (Systat Software, Chicago, Illinois, USA).
Results & Discussion

Various animal models have been developed to mimic the pathophysiological process seen in septic patients. The most frequently used one is the CLP model in rodents, which has been considered as the gold standard in sepsis research [129-131]. Compared to CLP, in which the intestinal flora are allowed to contaminate the abdominal cavity inducing a peritonitis together with the necrosis of the cecum (caused by ligation), LPS injection may not represent the pathophysiology of polymicrobial sepsis very well due to different ways of activation in the immune system [130]. Animals undergoing CLP show decreased physical activity, diarrhea, lethargy, hypothermia, tachycardia and tachypnea etc., and reflect better the progressive bacteremia, production of cytokines and chemokines, hyper-metabolism, fever and other vascular and metabolic changes often seen in septic patients [132]. One major concern of the CLP model is to use it with high consistency to assure reliability and reproducibility. The outcomes of CLP vary due to for instance, different size of needle used for puncture, the number of punctures and the length of the cecum ligated. In our experiment, we ligate 75% of the cecum and a 21-gauge needle is used to puncture twice, which leads to about 20% of mortality after 24 hours of CLP induction.

CLP-induced pulmonary recruitment of neutrophils and tissue injury

The sepsis places the organs like liver, lung and kidney at risk of injury and failure. One of the major complications in septic patients is impaired respiration, and the lung is especially susceptible during sepsis.

Neutrophil infiltration into the lung is a hallmark of septic pulmonary injury. There is evidence of interstitial and alveolar edema with accumulation of neutrophils, macrophages, and red blood cells in the alveoli together with protein-rich fluid in patients with septic lung injury. These pathophysiological features are associated with increased microvascular permeability of the lung and sustained loss of normal endothelial barrier function [133, 134]. The mechanism of neutrophil-induced endothelium and epithelium injury in the lung has been well documented. Neutrophils accumulate in the lung microvasculature and become activated leading to degranulation and the release of proteases, reactive oxygen species, pro-inflammatory cytokines and pro-coagulant molecules [134, 135]. Furthermore, these neutrophil-derived toxic intracellular molecules could induce dissolution of tight junctions as well as apoptosis and necrosis of alveolar epithelial cells [136, 137].
It has been shown in our experiment that neutrophils increased dramatically in BALF 6 and 24 hours after CLP in mice (Fig.3). MPO is most abundantly expressed in neutrophils and MPO activity in tissue is often measured to estimate neutrophil infiltration. The maximum levels of MPO were found at 6 hours after onset of CLP (data not shown). Notably, measurement of MPO activity could not provide information where the neutrophils come from, i.e. distinguish between intravascular and interstitial neutrophils. So we tried to compensate this issue by flushing the pulmonary vessels to circumvent potential influence of intravascular neutrophils. We also observed that CLP caused a remarkable pulmonary damage in mice, indicated by the prominent enhancement in lung edema formation, which was assessed by measuring the changes of lung wet/dry ration (Fig.2). It should be mentioned that baseline values of wet/dry ratio in sham mice represents normal levels in healthy animals and only increased wet/dry ratio represents actual edema formation. Moreover, morphologic examination demonstrated destructed microarchitecture of the lung, characterized by reduction of alveolar space, thickened alveolar septa, massive necrosis, capillary congestion and neutrophil infiltration (Fig.4d).

We also noted a marked leukopenia as a consequence of CLP after 24 hours, i.e. both MNLs and PMNLs were decreased. The neutropenia could be explained by trapping and recruitment of neutrophils in tissues [138].

**Regulation of β₂-integrins in septic lung injury**

β₂-integrins have been shown to play a pivotal role in mediating leukocyte adhesion, but publications about the specific role of these molecules in polymicrobial sepsis is incomplete and controversial [74, 78, 139, 140]. These data arose our interest to clarify the role of LFA-1 and Mac-1 in mediating pulmonary accumulation of neutrophils in the model of polymicrobial sepsis. To access the extent of β₂-integrin expression on neutrophils, blood samples were collected 16 and 24 hours after CLP and analyzed by flow cytometer. Both Mac-1 and LFA-1 were found to be expressed on the surface of circulating neutrophils. CLP induced a clear-cut up-regulation of Mac-1 on neutrophils compared to sham controls presented by increased mean fluorescence intensity (MFI) values (Fig.1b and Fig.5a), but not for LFA-1 (Fig.1a). Immunoneutralization of either LFA-1 or Mac-1 reduced neutrophil infiltration in the lung indicated by deducted MPO activity and neutrophil migrated into the bronchoalveolar space (Fig.2 and Fig.3), suggesting that both LFA-1 and Mac-1 were involved in adhesive interactions between circulating leukocytes and lung microvascular
endothelium, and cooperate for optimal recruitment of leukocytes during sepsis. Once activated by chemokine receptor ligation or tethering on selectins, neutrophils increase CD18 expression and affinity [141]. Some studies indicate that LFA-1 and Mac-1 have distinct kinetics of activation and affinity regulation. The rapid activation of LFA-1 and corresponding adhesion to ICAM-1 is transient and reversible, which may initiate first stable contact, and that activated Mac-1 is more stable and establishes more sustainable adhesion [142, 143]. This notion is further supported by pulmonary intravital microscopic examination [144]. LFA-1 mediates leukocyte adhesion in a way other than up-regulation of molecule expression on cell surface according to these experiments. It has been documented that the activation of LFA-1 is initiated by intracellular signals that convert low-affinity LFA-1 molecule to a high affinity state by inducing conformational changes in the I domain [145]. The transition of LFA-1 from low-affinity state to high-affinity state could happen within seconds, while Mac-1 expression increased via fusion of cytoplasmic pools of secretory granules, and this up-regulation of Mac-1 happens within 5 to 10 minutes [142, 143, 146]. Due to different characteristics of these two β2-integrins and accessibility of activation measurement, we focused on Mac-1 in the studies afterwards. Weakened leukocyte adhesion by administration of antibodies directed against LFA-1 or Mac-1 in turn decreased neutrophil associated lung edema (Fig.2), and protected against sepsis-induced tissue damage of the lung. Interestingly, combination of anti-LFA-1 and anti-Mac-1 antibodies could not show further inhibitory effect of CLP-provoked neutrophil recruitment.
(Fig.3), which is also observed in the study of Hentzen et al. being consistent with the notion of sequential cooperation manner of LFA-1 and Mac-1 [142]. It should be mentioned that due to intracellular stores, complete blocking of β2-integrins is difficult, particularly for those integrins as Mac-1 with mobilized intracellular pools [147].

Figure 2. Edema formation in the lung. Animals were pre-treated with an anti-LFA-1, anti-Mac-1 antibody, a combination of both anti-LFA-1 and anti-Mac-1 antibodies or a control antibody or vehicle prior to CLP induction. Sham-operated mice served as negative controls. Lung wet: dry ratio was determined 24 h after CLP. Data represents mean ± SEM and n = 5. *P < 0.05 vs. Sham and †P < 0.05 vs. Control ab + CLP.
Formation of CXC chemokines in septic lung injury

CXC chemokines including KC and MIP-2 are known to activate and navigate chemotactic movement of neutrophils to sites of inflammation, and are regulated and produced by resident cells in the lung and other organs [58, 148]. It was found that CLP triggered a marked increase in CXC chemokine mRNA expression in alveolar macrophages, and this reaction happened rapidly after sepsis challenge (30 min after CLP) [149]. We observed that pulmonary content of CXC chemokines was low in sham-operated animals, formation of MIP-2 and KC in the lung was substantially enhanced at 6 and 24 hours after induction of CLP (Fig.7a and 7b). The high-affinity receptor of KC and MIP-2, i.e. CXCR2 expressed on surface of circulating neutrophils was found to decrease in septic mice. This finding is in accordance with the down regulation of CXCR2 occurs on neutrophils from septic patients, and maybe related with the pronounced suppressive response to its ligand [65].
Regulation of CD4 T-cells in abdominal sepsis

It is not surprising that sepsis is related with profound T-cell dysfunction which explains at least in part of the promoted susceptibility to infections observed in patients with sepsis [150].

Previous studies have presented that sepsis induces a profound apoptosis-mediated decrease of T and B-lymphocytes that is associated with depletion of various immune cells and induction of immunosuppressive effects in the surviving cells seen in septic responses. The key role of apoptosis in pathogenesis of sepsis has also been proved by a study showing that transfer of apoptotic splenocytes contributes to adverse mortality in a mouse model of sepsis [151], and the same group reported as well that inhibition of apoptosis increases survival [152]. Apoptosis mostly affects lymphocytes in blood, gut and spleen in sepsis [153, 154]. It was demonstrated here that CLP triggered a marked increase in CD4 T-cell apoptosis in the spleen (Fig. 8a), this finding is consistent with Hotchkiss et al., whom have defined that septic patients have significant apoptosis of lymphocytes [155]. The depressed function of T-cells was also described in our in vitro test, which demonstrated a low proliferative rate following specific simulation with anti-CD3/CD28 antibodies. Accordingly, mice undergoing abdominal sepsis revealed a decreased number of functional T-cells. CD4 T-cells from post-septic patients and animal models showed a decrease in IFN-γ production and diminished bacterial clearance [156, 157], which could be partly explained by the role of IFN-γ in eliciting Th1-type immunity against microbes. Results from our in vitro experiment presented a reduced capacity of septic splenic T-cells to produce IFN-γ as well (Fig. 8b). This dampened function of T-cells might be as a consequence of apoptosis. Phagocytosis of apoptotic cells by macrophages and immature dendritic cells may lead to immune suppression, since engulfment of apoptotic cells stimulates production of anti-inflammatory cytokines as IL-10 [158], which, in turn, induces suppression of pro-inflammatory cytokine formation as well as inhibition of Th1 cell differentiation [159].

Several studies have suggested that lymphocyte subpopulation CD4⁺CD25⁺ T-regulatory cells is capable to suppress the immune response by direct inducing apoptosis of cytotoxic lymphocytes, inhibiting release of TNF-α and producing anti-inflammatory cytokines as IL-10 and TGF-β [160, 161]. Foxp3 (forkhead box P3) protein is a transcriptional factor belongs to the forkhead box protein family and related to regulation of development and function of CD4⁺CD25⁺ T-regulatory cells [162, 163]. In keeping with the observations in septic patients of increased regulatory T-cell, we presented here the result with a significant induction of percentage in CD4⁺CD25⁺Foxp3⁺ regulatory T-cells in spleen.
(Fig. 8c), implying that this is an increase in functionally active T-regulatory cells in septic mice. Owing to the capacity of regulatory T-cells to inhibit the proliferation of CD4^+CD25^- conventional T-cells, as well as the ability of these cells to produce effector cytokines [164, 165], we could infer that the suppressed proliferation and cytokine formation of splenocytes in septic animals observed herein might partly due to promoted percentage of CD4^+CD25^-Foxp3^+ T-regulatory cells in spleen.

Taken together, CLP causes an adverse influence on splenic T-cells by decreasing the number, and harming the function of these cells, moreover, CLP also triggers a significant enhancement of regulatory T-cells to worsen the immunosuppression in abdominal sepsis.

**Immunomodulation role of HMG-CoA reductase in abdominal sepsis**

A growing body of literature suggests that statins might be beneficial in patients with sepsis and severe infections [166]. Numerous reports have shown that statins exert potent and pleiotropic anti-inflammatory actions besides its role in lowering cholesterol levels [167]. We showed here that simvastatin not only protects against pulmonary edema and tissue damage in abdominal sepsis seen in the early hyper-inflammatory phase, but also maintains CD4 T-cell function during immunosuppressive phase, indicating an
immunomodulation role of HMG-CoA reductase inhibitors in systemic inflammation during sepsis.

Administration of simvastatin strongly inhibited sepsis-associated lung edema formation and tissue damage (Fig.4c and 4d). Histologically, the CLP-induced destruction of pulmonary microarchitecture was minimized by simvastatin treatment, as characterized by less distortion of alveolar structure, interstitial infiltration of leukocytes and hemorrhage. These findings are in keeping with the observation that simvastatin and pravastatin can decrease intratracheal LPS-induced murine lung vascular leak and inflammation [168, 169]. Neutrophils are a fundamental component in the pathophysiology of septic lung injury [170]. We documented that simvastatin (10mg/kg) decreased pulmonary levels of MPO by 59% in abdominal sepsis (Fig.4a). This inhibitory effect is in agreement with our observation that simvastatin administration reduced sepsis-induced neutrophil infiltration in the bronchoalveolar space (Fig.4b), elucidating that simvastatin effectively reduces pulmonary recruitment of neutrophils in polymicrobial sepsis, which may assist in explaining the reduced mortality reported in septic mice treated with simvastatin [171, 172].

![Diagram](image)

**Figure 5.** Simvastatin regulates CLP-induced Mac-1 expression on neutrophils. (a) Mac-1 expression on neutrophils in sham mice, PBS and simvastatin (0.5 or 10 mg/kg) treated animals 6 hours after CLP induction. Fluorescence intensity is shown on the x-axis and cell counts on the y-axis. Representative histogram from four samples. (b) Neutrophil expression of Mac-1 8 hours post-CLP in sham mice and animals pretreated with a control antibody (Ctrl ab) or an anti-CD40L antibody. Representative histogram from five samples.

Leukocyte-endothelial interactions are mediated by specific adhesive molecules [71]. As approved earlier that migration of inflammatory cells into the lung is mediated by LFA-1 and Mac-1 on neutrophils (Fig.1 and Fig.5a). So we asked whether inhibition of HMG-CoA
reductase by simvastatin could regulate Mac-1 expression on neutrophils in our model of sepsis. We found that increased Mac-1 expression on neutrophils in mice exposed to CLP was attenuated by simvastatin administration (10mg/kg), i.e., MFI decreased from 154 ± 6 to 109 ± 11 (Fig.5a). It suggested that this down-regulation of Mac-1 on the surface of neutrophils might contribute to the inhibitory effect of simvastatin on neutrophil recruitment and tissue injury in abdominal sepsis.

![Figure 5](image)

Figure 5. Simvastatin regulates platelet secretion of CD40L. (a) Surface expression of CD40L on platelets and (b) plasma levels of CD40L 6 hours after CLP induction. (c) CD40L induced neutrophil expression of Mac-1 in vitro after incubation with and without simvastatin. Data represents mean ± SEM. *P < 0.05 vs. sham and #P < 0.05 vs. PBS + CLP, n = 5-6 in (a) and (b), and n = 3 in (c). MIP-2 was used as a positive control.

Previous data have proved that platelets play an important role in mediating Mac-1 up-regulation on neutrophils and neutrophil recruitment in abdominal sepsis via CD40L secreted from platelets [94]. We found CD40 expression on mouse neutrophils that may activate CD40L directly and help clarifying the CD40L-regulated expression of Mac-1 on neutrophils. We also observed the surface expression of CD40L on platelets was reduced concomitantly with an increase of sCD40L in plasma in septic mice (Fig.6a and 6b). In addition, CLP-induced Mac-1 up-regulation was attenuated by an anti-CD40L antibody,
confirming the dependence of Mac-1 expression on platelet-derived CD40L in polymicrobial sepsis (Fig.5b). Based on these facts, we were curious to know whether simvastatin might regulate CD40L levels during sepsis. The results showed that simvastatin abolished the CLP-provoked down-regulation of CD40L expression on platelets in septic animals and markedly decreased the elevation of sCD40L in plasma (Fig.6a and 6b). However, we also noticed that simvastatin had no effect on CD40L-evoked Mac-1 expression on neutrophils in vitro (Fig.6c), suggesting that simvastatin does not inhibit CD40L function. Considering together, we conclude that simvastatin inhibits platelet secretion of CD40L into the circulation in abdominal sepsis, which in turn, attenuates sCD40L mediated neutrophil up-regulation of Mac-1 and pulmonary recruitment.

Additionally, statins have also been shown to directly bind to LFA-1 and so interfering with its ligand ICAM-1. Lovastatin was first reported to bind a site in the LFA-1 extracellular I domain, this “lovastatin site” (L-site) is distant from the ICAM-1 binding site, and binding of lovastatin at this site induces a so-called allosteric blocking [173]. Beside lovastatin, simvastatin and mevastatin also bind with the L-site and lock LFA-1 in an inactive conformation. The inhibition of LFA-1 by statins is highly specific; other integrins including Mac-1 is unaffected [174]. Based on these findings, we cannot exclude that simvastatin might block L-site of LFA-1 allosterically and diminish pulmonary neutrophil infiltration potentially in our study of abdominal sepsis.

Activation and extravascular navigation of inflammatory cells at sites of inflammation is orchestrated by secreted chemokines. We also found that simvastatin significantly reduced CXC chemokine formation in the lung in vivo (Fig.7a and 7b) and decreased MIP-2-induced neutrophil chemotaxis in vitro (Fig.7c). These findings together indicate a role of simvastatin in interfering the migration of neutrophils guided by CXC chemokines. This notion is in line with the study reporting that statins can decrease neutrophil migration toward the chemoattractant fMLP [175]. It should be mentioned that it is not known whether CXC chemokines play any functional role in CLP-induced inflammation and lung injury. Therefore, we explored if interference with the receptor for MIP-2 and KC by CXCR2 antagonist (SB225002) can modulate pro-inflammatory changes induced by CLP. We observed that targeting CXCR2 markedly decreased CLP provoked neutrophil activation (Mac-1 expression) and pulmonary infiltration (MPO and BALF neutrophil counts), as well as edema formation and tissue damage in the lung in vivo.
It should be noted that the effect of simvastatin in inhibition of CXC chemokine formation is not likely related to CD40L changes in sepsis. A previous finding showing that pulmonary formation of MIP-2 and KC was completely intact in septic mice lacking CD40L [94], and sCD40L in the plasma may not reach chemokine formation cells such as extravascular epithelial cells and alveolar macrophages. Thus the secretion of CD40L into plasma might be dissociated from generation of CXC chemokines in septic lung.

![Figure 7. Simvastatin decreases CXC chemokine production in the lung and inhibits neutrophil migration in vitro. (a) and (b) Animals were treated with simvastatin (0.5 or 10 mg/kg) or PBS prior to CLP induction. Sham-operated mice served as negative controls. ELISA was used to quantify the levels of MIP-2 and KC in the lung of mice 24 hours after CLP induction. Data represents mean ± SEM. *P < 0.05 vs. sham and #P < 0.05 vs. PBS + CLP; n = 6. (c) Neutrophils were analyzed for their migration in response to medium alone (Control), medium plus MIP-2 (100 ng/ml) with or without pre-incubation of neutrophils with simvastatin. Data represents mean ± SEM. *P < 0.05 vs. Control and #P < 0.05 vs. MIP-2. n = 4.](image)

Although the signaling mechanism regulated by simvastatin is beyond the scope of present schemes, it is intriguing to raise the potential pathway involved here. It has been confirmed that Rho-kinase pathway exerts an essential role in abdominal sepsis [176]. Inhibition of Rho-kinase activity 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 [149, 177]. Rho-kinase is one of statin-sensitive signaling molecules, the interaction of HMG-CoA reductase inhibitors with Rho GTPases leads to decrease of integrin activity and ICAM-1 expression on endothelial cells [178], increase in endothelial nitric oxide synthase expression and activity [179], and inhibition of cytokine as well as chemokine formation [180]. In these regards, it is reasonable to infer that the mechanism of simvastatin modulates CXC chemokine formation and neutrophil activation might be via the Rho GTPases pathway.

**Figure 8.** Effects of simvastatin on splenocytes. Animals were treated with 10 mg/kg of simvastatin (Simv10) or PBS prior to CLP induction. Mice treated with PBS alone served as sham animals. Splenocytes were isolated after 24 hours after CLP induction. (a) Apoptosis was determined by measuring labeling of DNA strand breaks with BrdUTP in CD4 T-cell in the spleen. (b) Levels of IFN-γ formation in splenocytes were determined 24 hours after incubation with anti-CD3ε and anti-CD28 antibodies by using ELISA. (c) The percentage of regulatory T-cells (CD4⁺CD25⁺Foxp3⁺) in the spleen was determined by flow cytometry. Data are presented as mean ± SEM. *P < 0.05 vs. sham and # P < 0.05 vs. PBS + CLP, n = 5.

Next, we extend our research about simvastatin to the later immunosuppressive phase of sepsis. We found that simvastatin treatment mitigated the increase in BrdUTP-positive CD4 T-cells in septic mouse spleens (Fig.8a), i.e. from 49.8% down to 16.5%, corresponding to a 75% deduction. We also found that simvastatin could improve proliferation of sepsis-challenged splenic CD4 T-cells. Together, these effects could help to maintain the number of functional CD4 T-cells to mount effective anti-bacterial responses. Considering that investigations demonstrate that prevention of lymphocyte apoptosis in sepsis improves survival by augmenting the adaptive immunity [36, 181], it is thus likely that the anti-apoptotic effects of simvastatin might be one of the principal mechanisms by which statins reduce septic mortality.
Sepsis induces a shift in lymphocyte cytokine production from a pro-inflammatory Th1 to an anti-inflammatory Th2 profile [182, 183], i.e. shift from IFN-γ to IL-4 secretion. This cytokine shift to a Th2 profile in sepsis may compromise the ability of the host to combat invading microorganisms. IFN-γ has been shown to improve survival in an animal model of sepsis [151], and in a small clinical study as well [24]. We observed herein that simvastatin reversed sepsis-induced reduction in IFN-γ (Fig.8b), which may contribute to the immune responses of host defense [157, 184]. However, the literature on the effect of simvastatin on IFN-γ production is complex and partly contradictory. For example, some studies have reported that simvastatin decreases formation of IFN-γ in model of allograft rejection [185] and atherosclerosis [186], whereas others have shown that simvastatin promotes production of IFN-γ in isolated monocytes [187] and T-cells [188]. Thus, the effect of simvastatin on IFN-γ formation appears to be dependent on the context in which IFN-γ is synthesized. Differences in study design and methodology may be important in explaining the different findings. Nonetheless, our data showed that IFN-γ formation was promoted by simvastatin in abdominal sepsis. This notion is indirectly supported by a recent study showing that inhibition of protein isoprenylation, which is a downstream mediator regulated by statins, by use of a farnesyltransferase inhibitor also increases IFN-γ formation in septic animals [189]. Taken together, our results indicate that simvastatin improves immune responses in sepsis via increased number of T-cells on one hand and promoted function, i.e. IFN-γ formation on the other.

Regulatory T-cells are recognized for their capability to control T-cell-dependent immune response [162]. There are evidence indicating that increased number of regulatory T-cells in the course of sepsis might compromise host defense reactions against microbial infections [189] and potentially be involved in compromised proliferative capacity and defective Th1 cytokine release from CD4+ T-cells after CLP induction [164]. Simvastatin was also found to be able to abolish the increased percentage of regulatory T-cells in the spleen of septic animals by 74% (Fig.8c). Knowing that the septic patients have enhanced number of regulatory T-cells [190], and which has the capacity to actively suppress adaptive immune response [191], we could forward that this simvastatin-mediated attenuation in regulatory T-cells might contribute to the advantageous actions of statins in patients with severe sepsis. The detailed mechanism of statins in regulating CD4+CD25+Foxp3+ T-cells is still unknown. A positive correlation between the percentage of regulatory T-cells and serum levels of IL-10 or TGF-β under septic conditions has been demonstrated recently both in patients and animals subjected to CLP, and administration of anti-IL-10 or anti-TGF-β
antibodies decreases regulatory T-cells and improves survival [38]. Another study implies the potential role of IL-10 in increasing the percentage of active T-regulatory cells by showing that IL-10 deficient mice have blunted increase of regulatory T-cells after CLP [164]. The effect of statins in IL-10 and TGF-β formation is limited and contradictory, i.e., simvastatin has been shown to increase production of IL-10 and TGF-β and induce a regulatory T-cell response in cancer cell lines [192], and others shown a down-regulation of TGF-β1 and IL-10 mRNA levels in atorvastatin treated rats in an allograft transplant model [193]. It is of great interest to clarify the potential mechanism of statins in adjusting regulatory T-cells via IL-10 and TGF-β in the setting of sepsis in the future.

Thusly, simvastatin may improve T-cell-dependent immune responses via at least three different mechanisms, i.e. increasing the number of functional T-cells, promoting the function of T-cells (IFN-γ secretion) and reducing the number of regulatory T-cells. These findings indirect elucidate the beneficial effects of simvastatin seen in sepsis.

HMGB1 has been identified as a pro-inflammatory cytokine produced by most eukaryotes like macrophages, monocytes and neutrophils [194, 195], which appears later in stimulation of LPS [196]. HMGB1 acts with the receptor for advanced glycation endproducts to mediate inflammation and organ failure during sepsis [197, 198], it also interacts with TLR2/4 inducing production of pro-inflammatory cytokines [199]. Intratracheal administration of HMGB1 leads to a neutrophil-dependent pulmonary edema formation and local production of TNF-α, IL-1β and MIP-2 [200]. Animals and humans with severe sepsis have elevated levels of HMGB1 in the circulation [196, 201]. The plasma levels of HMGB1 in DIC patients are correlated with sepsis-related organ failure [198]. Based on these evidences, HMGB1 is considered as a prognostic biomarker of clinical outcome in patients with severe sepsis [202]. We recorded a 25 times of increase in plasma HMGB1 in mice subjected to CLP after 24 hours. Notably, simvastatin treatment reduced HMGB1 levels by 87% in septic mice (Fig.9a), suggesting an anti-inflammatory effect of HMG-CoA reductase inhibitors in CLP-induced systemic inflammation. Although this is the first study showing that statins might negatively regulate HMGB1 in sepsis, one previous study documented that statins could inhibit HMGB1 formation in ischemic brain injury [203]. Interestingly, HMGB1 appears to link apoptosis with sepsis-induced mortality. Administration of a broad-spectrum caspase inhibitor concentration-dependently inhibits HMGB1 release from LPS-stimulated macrophages and decreases nuclear translocation of NFκB in vitro, and attenuates circulation HMGB1, IL-6, KC and MIP-2 in mice post CLP induction, as well as prevents accumulation of apoptotic cells in spleen and thymus. It is also found that
accumulation of apoptotic cells can activate macrophages to release HMGB1, which, in turn, mediates downstream organ damage [204]. Taken together, the capacity of simvastatin in lowering plasma HMGB1 in septic mice might be related to its anti-apoptotic action. Furthermore, knowing that NFκB regulates LPS-mediated HMGB1 production [205], and simvastatin is a potent modulator of NFκB [206], it could be suspected that simvastatin regulates circulation HMGB1 via a NFκB mediated pathway. A recent study reported that inhibition of HMGB1 attenuates tumor cell induction of regulatory T-cells [207]. If such a mechanism exists in sepsis, it might also help revealing the inhibitory impact of simvastatin on the formation of regulatory T-cells in the present study.

**Figure 9.** Effects of simvastatin on plasma levels of HMGB1 and IL-6. Animals were treated with 10 mg/kg of simvastatin (Simv 10) or PBS prior to CLP induction. Mice treated with PBS served as sham animals. Levels of (a) HMGB1 and (b) IL-6 levels in plasma were determined 24 h after CLP induction by using of ELISA. Data are presented as mean ± SEM. *P < 0.05 vs. sham and **P < 0.05 vs. PBS + CLP, n = 5.

IL-6 has been suggested to be a marker of systemic inflammation, with levels correlate well with the severity and prognosis of sepsis in patients [208, 209] and animals [210]. The role of IL-6 is complicated with both pro- and anti-inflammatory effects [211], i.e. up-regulating adhesion molecules like ICAM-1, VCAM-1, E- and L-selectins and enhancing leukocyte transmigration [212-214], and owing anti-apoptosis effect of T-cells and inhibiting regulatory T-cell differentiation together with its soluble receptor IL-6R [215, 216]. The complex effects of IL-6 may be related to different signaling pathways [17, 217]. We showed an evidential increase of plasma IL-6 in septic mice compared to sham operated animals. Simvastatin administration decreased plasma levels of IL-6 significantly in septic mice.
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(Fig.9b). The impact of reduced IL-6 on T-cell function is not clear so far, but our findings also support the concept that HMG-CoA reductase inhibitors regulate the sepsis-induced systemic inflammatory response.

A previous study described an unexpected antimicrobial effect of statins in vitro [124], which arose our question whether simvastatin may act directly to clear bacteria in abdominal sepsis. We carried out a bacterial clearance test in blood and found a remarkably augmented clearance of bacteria from the circulation at 24 hours following the onset of CLP. This result is supported by another experiment showed that cerivastatin promotes bacterial clearance of both gram-negative and positive infections [218].

We are not excluding that simvastatin may inhibit other targets during sepsis, i.e., statins could modulate leukocyte adhesion by interfering with the NFκB [219, 220], and also regulate mitogen-activated protein kinases (MAPK) and Akt, inducing attenuation of pro-inflammatory signaling and moderation of inflammatory responses [221, 222]. In addition, serum TNF-α and IL-1β are significantly decreased in simvastatin-treated CLP rats, and the survival time is improved by preserving the cardiovascular function [171, 172]. Similar results have been observed in other animal models with improved survival [223, 224].

To summarize, our results clearly demonstrated that inhibition of HMG-CoA reductase protects sepsis-induced lung injury via three different levels. First, simvastatin inhibits neutrophil recruitment into the lung by attenuating Mac-1 expression, which is reached by the capacity of simvastatin to decrease secretion of CD40L from platelets. Second, simvastatin inhibits pulmonary formation of CXC chemokines. Third, simvastatin may also inhibit direct chemotaxis of neutrophils initiated by CXC chemokines. We presented also that simvastatin improves T-cell function in abdominal sepsis, this effect is associated with amelioration of apoptosis of splenocytes, inhibition of increased CD4⁺CD25⁺Foxp3⁺ T-regulatory cells and hypo-responsiveness, and increase attenuated IFN-γ secretion of splenic CD4 T-cells. Moreover, we confirmed that simvastatin improves bacterial clearance during sepsis. These findings elucidate the beneficial effects of HMG-CoA reductase inhibitor exert in sepsis.
Role of NFAT in abdominal sepsis

Despite profound investigations on the role of NFAT signaling in different physiological and pathological processes, the signaling pathways of regulating pulmonary accumulation of neutrophils, systemic inflammation and immunosuppression in abdominal sepsis are not yet fully resolved. In the fourth study, the NFAT inhibitor A-285222 was used, which helps to reveal the pivotal role of NFAT signaling in polymicrobial sepsis.

NFAT is a transcription factor conventionally thought to be involved in tissue development, gastrointestinal tract and immune system maturation [97, 225-228], which found to be involved in inflammatory responses as arteriosclerosis and autoimmune diseases as well [229, 230]. We noted herein that CLP is associated with increased NFAT activity in the lung, spleen, liver as well as the aorta, suggesting that NFAT is involved in the
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Figure 11. NFAT regulates CLP-induced neutrophil recruitment. (a) Number of BALF neutrophils and (b) edema formation in the lung 24 hours after CLP induction. Mice were pre-treated with A-285222 or vehicle i.p. twice daily for 7 consequent days and in the morning before sham operation (white bars) or induction of CLP (black bars). Data represent means ± SEM and n = 5. *P < 0.05 vs. vehicle + sham, #P < 0.05 vs. vehicle + CLP.

systemic inflammatory response during sepsis. Administration of the NFAT blocker (A-285222) completely blocked sepsis-induced increase of NFAT transcriptional activity (Fig.10). Furthermore, we observed that pre-treatment with A-285222 significantly reduced pulmonary edema formation and tissue damage in abdominal sepsis. We also demonstrated that A-285222 inhibited sepsis-induced neutrophil infiltration into the bronchoalveolar compartment by 54%, suggesting that NFAT may regulate a substantial part of the tissue injury in sepsis (Fig.11). The inhibitory effect of A-285222 in neutrophil trafficking in the lung could be explained by its capacity of inhibition in CXC chemokine formation, showed by our observation that A-285222 abrogated sepsis-induced KC and MIP-2 synthesis in the lung by 62% and 81% respectively (Fig.12). Interestingly, two traditional calcineurin inhibitors, CsA and FK506 have been shown to reduce neutrophil responses and protect against endotoxemia and acute lung injury [231,232]. Another study also described that inactivation of calcineurin by FK506 administration may induce LPS tolerance and protect LPS toxicity in vivo [109]. Considering that NFAT activity is regulated by calcineurin [233], our findings might help explicating the protective effects exerted by these calcineurin inhibitors on endotoxemia and pulmonary injury. Collectively, our data indicate a key feature of calcium/calcineurin-NFAT signaling axis in pathology of the septic lung injury similar to that proposed for the development of cardiac hypertrophy [234],
diabetes-induced vascular inflammation [235] and arteriosclerosis [229]. The observation described here that A-285222 treatment potently decreased HMGB1 and IL-6 levels in the plasma by more than 93% and 95% respectively (Fig.13), further indicating that NFAT is a potent regulator of systemic inflammation in sepsis.

When it concerns to anti-inflammatory phase of sepsis, we demonstrated herein that A-285222 decreased apoptosis (Fig.14a) and increased the proliferative responses of CD4 T-cells in septic mice, implying that inhibition of NFAT signaling protects T-cells in the course of polymicrobial sepsis. It was noticed that NFATc2 knockout mice develop a hyper-proliferative syndrome partly due to defects in activation-induced cell death with delayed induction of apoptosis and cell deletion [236]. On the contrary, infection of primary T-cells with retroviruses expressing NFATc2 or NFATc1/C, the longest isoform of NFATc1, increases TCR-mediated apoptosis [237]. These findings might support interpretation of anti-apoptotic effects of NFAT blocker we observed herein. We found also that inhibition of NFAT activity reversed sepsis abolished IFN-γ and IL-4 production in splenocytes (Fig.14b), which could help to maintain the effective host defense of invading bacterial. In this context, it is noteworthy that NFAT has been well-understood to govern T-cell development, activation and differentiation [238]. It seems counterintuitive showing in the present study that inhibition of NFAT protects T-cell survival and cytokine formation capacity at the first
sight. However, NFAT inhibition resulted with no effect on T-cell apoptosis and proliferation from sham animals but worked effectively in septic animals. Considering that T-cell dysfunction is a consequence of the overwhelming systemic inflammatory response, we concluded that the T-cell protective effects of dampened NFAT activity are secondary and mainly attribute to the restrained pro-inflammatory response in septic mice. Moreover, previous studies assessed that lymphocyte tolerance/anergy is related to elevated intracellular Ca\textsuperscript{2+}, this unresponsiveness could be blocked by CsA and FK506 [239, 240], implying the involvement of calcineurin/NFAT in this process. These data collectively postulate the potential complexity of NFAT in regulating T-cell signaling pathway during sepsis. It has been shown that NFAT interacts with Foxp3 physically and regulates Foxp3-mediated regulatory T-cell development and function [241]. In our study, pre-treatment of A-285222 significantly decreased CLP-induced elevation of regulatory T-cells (Fig.14c), confirming the involvement of NFAT signaling in mediating regulatory T-cell during CARS.

![Figure 13](image-url) **Figure 13.** NFAT controls systemic levels of HMGB1 and IL-6. Mice were pre-treated with A-285222 or vehicle i.p. twice daily for 7 consequent days and in the morning before sham operation (white bars) or induction of CLP (black bars). Levels of (a) HMGB1 and (b) IL-6 levels in plasma were determined 24 hours after sham operation and CLP induction by using of ELISA. Data represent means ± SEM and n = 5. \*P < 0.05 vs. vehicle + sham, \#P < 0.05 vs. vehicle + CLP.
The four Ca^{2+}-dependent isoforms of the NFAT family are distributed unevenly in different tissues and organs. All members of NFAT are able to bind and activate the IL-2 promoter, however, isoform specific preferences are seen as well [242-244], suggesting divers regulatory roles of NFAT members. It is of great interest for us to examine the pattern of NFAT isoform expression in the lung of septic animals and pursue further the effect of particular isoform(s) in septic pulmonary injury. We presented here the overlapping modulatory effects of inhibiting HMG-CoA reductase and NFAT pathway in our studies of abdominal sepsis. Statins have been reported to improve survival and decrease rejection in transplant recipients [245, 246] similar to effects of calcineurin modulatory agents like CsA and FK506. Considering with the capacity of statins in regulating intracellular calcium [247], we speculated whether there is any link between the pleiotropic effects exerted by HMG-CoA reductase inhibitors and NFAT signaling. Besides, NFAT is a target of p38 MAPK in T-cells [248], of which could be regulated by simvastatin [249] and plays critical role in septic lung injury [250], proposing a hint of indirect connection between HMG-CoA reductase and NFAT. However, the potential mechanisms need to be investigated by more detailed researches.

Figure 14. NFAT regulates CLP-induced splenic apoptosis and formation of IFN-γ and regulatory T-cells. Mice were pre-treated with A-285222 or vehicle i.p. twice daily for 7 consequent days and in the morning before sham operation (white bars) or induction of CLP (black bars). Splenocytes were isolated 24 hours after CLP induction. (a) Apoptosis was determined by measuring labeling of DNA strand breaks with BrdUTP in CD4 T-cell in the spleen. (b) Levels of IFN-γ formation in splenocytes were determined 24 hours after incubation with anti-CD3ε and anti-CD28 antibodies by using of ELISA. (c) The percentage of regulatory T-cells (CD4^{+}CD25^{+}Foxp3^{+}) in the spleen was determined by flow cytometry. Data represent means ± SEM and n = 5. *P < 0.05 vs. vehicle + sham. #P < 0.05 vs. vehicle + CLP.
To summarize, our novel findings suggest that NFAT is a powerful regulator of pathological inflammation and T-cell immune dysfunction in abdominal sepsis. Targeting NFAT signaling might be useful in protecting against respiratory failure and immunosuppression in patients with sepsis.
Conclusions

I. LFA-1 and Mac-1 play key roles in pulmonary leukocyte recruitment and tissue injury during sepsis

II. HMG-CoA reductase inhibitor regulates sepsis-induced pulmonary accumulation of neutrophils, the inhibitory effect of which is related to a reduction of circulating CD40L, as well as a decrease in CXC chemokine formation in the lung

III. HMG-CoA reductase inhibitor mediates immune-regulatory effects in T-cell immune dysfunction in abdominal sepsis

IV. NFAT regulates neutrophil infiltration, pulmonary tissue damage and T-cell dysfunction in polymicrobial sepsis
Sammanfattning på svenska

hindrade induktionen av regulatoriska T-cellor vid sepsis. Sammanfattningsvis har den här avhandlingen kartlagt nya mekanismer bakom hyper- och hypoinflammatoriska fasen som skulle kunna ligga till grund för utvecklandet av nya och effektivare behandlingsmetoder av patienter med svår buksepsis.
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References


Adhesive and signaling mechanisms in abdominal sepsis


INTRODUCTION

Perforation of the intestines is a feared condition in which toxic and polymicrobial contents of the bowel contaminate the abdominal cavity (1, 2). Fecal bacteria stimulate local production of various proinflammatory substances that are subsequently released into the circulation. Moreover, infective microorganisms may also directly invade the blood stream and trigger an inflammatory host response in a distant target organ. In either scenario, the lung is the most sensitive and clinically important end-organ for the inflammatory response in abdominal sepsis (3). In fact, lung injury continues to constitute a significant cause of mortality in polymicrobial sepsis despite aggressive surgical interventions and antibiotic and immunomodulating therapies (4). The multiple signaling cascades triggered by a mixed bacterial flora and their released products are complex and largely unknown (5, 6). Substantial investigative efforts have been devoted to delineate the pathophysiology of sepsis using exogenous administration of various bacterial toxins such as LPS and superantigens derived from gram-negative and gram-positive bacteria, respectively. However, administration of such toxins may not represent the pathophysiology of clinical sepsis very well, and it has been shown that different toxins activate the host immune system in a distinctly different manner. For example, LPS has been shown to be a potent activator of macrophages and stimulates TNF-α production (7, 8), whereas superantigens do not provoke clear-cut TNF-α formation and activate primarily T lymphocytes, causing FasL-dependent apoptosis (9). In contrast, the cecal ligation and puncture (CLP) model, in which the intestine is punctured and the bowel contents are allowed to contaminate the abdominal cavity, seems to be more reminiscent of the events and course in polymicrobial sepsis in terms of cytokine responses and vascular and metabolic changes (10, 11).

Convincing data have shown that septic lung injury is characterized by massive accumulation of neutrophils in the bronchoalveolar space (12–15). Pulmonary infiltration of neutrophils is a multistep process comprising initial mechanical sequestration in microvessels, followed by adhesion molecule-dependent firm adhesion to endothelial cells and transmigration through endothelial and epithelial barriers (12). Bacterial antigens provoke formation of proinflammatory substances, which in turn up-regulate endothelial cell adhesion molecules and stimulate activation of neutrophils (16). Tissue localization of neutrophils is mediated by CXC chemokines (17), including macrophage inflammatory protein 2 (MIP-2) (18) and cytokine-induced neutrophil chemoattractant (KC) (19, 20) in the mouse. Specific adhesion molecules mediate attachment between activated neutrophils and endothelial cells (13, 14).

Although most relevant adhesion molecules, including P- and E-selectin and intercellular adhesion molecule 1 (ICAM-1), have been demonstrated to be up-regulated on pulmonary endothelial cells in systemic inflammation (21), the literature on the role of specific adhesion molecules in polymicrobial sepsis is incomplete and partly controversial. For example, some investigators have reported that ICAM-1 is of great importance in supporting neutrophil recruitment to the lung (14, 22), whereas others have not found such a role of ICAM-1 (23) in sepsis. Nonetheless, ICAM-1 is known to interact with members of the β-2 integrin family, including lymphocyte function antigen-1 (LFA-1; CD11a/CD18) and membrane-activated complex-1 (Mac-1; CD11b/CD18), which mediate firm leukocyte adhesion in a stimulus- and organ-dependent manner (14, 24–27). However, the potential role of LFA-1 and

ABSTRACT—Neutrophil-mediated lung damage is an insidious feature in septic patients, although the adhesive mechanisms behind pulmonary recruitment of neutrophils in polymicrobial sepsis remain elusive. The aim of the present study was to define the role of lymphocyte function antigen-1 (LFA-1) and membrane-activated complex 1 (Mac-1) in septic lung injury. Pulmonary edema, bronchoalveolar infiltration of neutrophils, levels of myeloperoxidase, and CXC chemokines were determined after cecal ligation and puncture (CLP). Mice were treated with monoclonal antibodies directed against LFA-1 and Mac-1 before CLP induction. Cecal ligation and puncture induced clear-cut pulmonary damage characterized by edema formation, neutrophil infiltration, and increased levels of CXC chemokines in the lung. Notably, immunoneutralization of LFA-1 or Mac-1 decreased CLP-induced neutrophil recruitment in the bronchoalveolar space by more than 64%. Moreover, functional inhibition of LFA-1 and Mac-1 abolished CLP-induced lung damage and edema. However, formation of CXC chemokines in the lung was intact in mice pretreated with the anti-LFA-1 and anti-Mac-1 antibodies. Our data demonstrate that both LFA-1 and Mac-1 regulate pulmonary infiltration of neutrophils and lung edema associated with abdominal sepsis. Thus, these novel findings suggest that LFA-1 or Mac-1 may serve as targets to protect against lung injury in polymicrobial sepsis.

KEYWORDS—Chemokines, neutrophil recruitment, lung, sepsis
Mac-1 in mediating pulmonary infiltration of neutrophils in polymicrobial sepsis remains elusive.

Based on the above considerations, the aim of the present study was to define the functional role of LFA-1 and Mac-1 in mediating pulmonary neutrophil recruitment and tissue damage in polymicrobial sepsis using the CLP model in mice.

MATERIALS AND METHODS

Animals

Experiments were performed using male C57Bl/6 mice weighing 20 to 25 g. All experimental procedures were performed in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for Animal Experimentation at Lund University, Sweden. Animals were anesthetized by administration of 7.5 mg (i.p.) ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg (i.p.) xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight.

Experimental protocol of sepsis

Polymicrobial sepsis in mice was induced by puncture of the cecum. Animals were anesthetized, the abdomen was opened, the exposed cecum was filled with feces by milking stool backward from the ascending colon, and a ligature was placed below the ileocecal valve. The cecum was soaked with phosphate-buffered saline (PBS; pH 7.4) and was then punctured twice with a 21-gauge needle. The cecum was then returned into the peritoneal cavity, and the abdominal incision was sutured. To evaluate the functional importance of LFA-1 and Mac-1, we used a saturating dose of 4 mg/kg of monoclonal antibodies (mAbs) directed against murine CD11a (M17/4.4.11.9, rat immunoglobulin [Ig]G; Novartis Pharma AG, Preclinical Research, Beerse, Belgium) per 100 g body weight.

Lung edema

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

MPO activity

Frozen lung tissue was thawed and homogenized in 1 mL of 0.5% hexadechltrimethylammonium bromide. Next, the sample was freeze-thawed, after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H2O2 (450 nm, with a reference filter of 540 nm, 25°C). Values were expressed as MPO units per gram of tissue.

Quantification of chemokines by ELISA

The lung sample was thawed and homogenized in PBS. MIP-2 and KC were analyzed by using double-antibody Quantikine ELISA kits (R & D Systems, Minneapolis, Minn) using recombinant murine MIP-2 and KC as standards. The minimal detectable protein concentrations are less than 0.5 pg/mL.

Histology

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

Flow cytometry

For analysis of surface expression of LFA-1 and Mac-1 on circulating neutrophils, blood was collected via cardiac puncture into heparinized syringes at 16 h after CLP induction. Erythrocytes were lysed using red blood cell lysing buffer (Sigma Chemical Co., St. Louis, Mo), and the leukocytes recovered after centrifugation. Cells were incubated with anti-CD16/CD32 to block Fc receptors and reduce nonspecific labeling for 10 min and stained at 4°C for 30 min simultaneously with phycoerythrin-conjugated anti-Gr-1 (clone RB6-8C5) and FITC or (B) Mac-1 antibodies, or a control antibody or vehicle before CLP induction. Sham mice underwent the same surgical procedures, that is, laparotomy and resuscitation, but the cecum was neither ligated nor punctured.
with fluorescein isothiocyanate (FITC)-conjugated anti-CD11a/anti-LFA-1 (clone M17/4) or anti-CD11b/anti-Mac-1 (clone M1/70) mAbs (all purchased from BD Biosciences Pharmingen). Flow-cytometric analysis was performed according to standard settings on a FACSort flow cytometer (Becton Dickinson, Mountain View, Calif), and a viable gate was used to exclude dead and fragmented cells.

**Statistics**

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

**RESULTS**

**CLP-induced expression of LFA-1 and Mac-1 on neutrophils**

Flow cytometry was used to detect potential expression of LFA-1 and Mac-1 on circulating neutrophils 16 h after CLP induction. Both Mac-1 and LFA-1 were expressed on the surface of circulating neutrophils (Fig. 1, A and B). Indeed, it was found that CLP provoked increased Mac-1 (Fig. 1B) on neutrophils compared with sham-operated animals, that is, the mean fluorescence intensity values were increased from 36.3 ± 5.6 in sham to 308.5 ± 73.9 in CLP animals (Fig. 1B; \( \*P \leq 0.05 \) vs. sham; \( n = 4 \)). However, LFA-1 (Fig. 1A) on neutrophils was not up-regulated compared with sham (Fig. 1A; \( \*P > 0.05 \) vs. sham; \( n = 4 \)), and mean fluorescence intensity values for LFA-1 were 156.7 ± 23.8 and 162.3 ± 14.5 in sham and CLP animals, respectively. Thus, we next evaluated the potential role of LFA-1 and Mac-1 function in CLP-induced lung injury.

**LFA-1 and Mac-1 mediate edema formation and lung injury**

Cecal ligation and puncture induced significant pulmonary damage, indicated by prominent enhancement in lung edema formation (Fig. 2). Thus, it was found that the lung wet-dry ratio increased by more than 45% in polymicrobial sepsis, that is, from 4.4 ± 0.1 to 6.4 ± 0.1 (Fig. 2; \( \*P \leq 0.05 \) vs. sham; \( n = 5 \)). Notably, administration of the anti-LFA-1 and anti-Mac-1 antibodies (Fig. 3) prior to CLP induction mitigated CLP-induced lung edema.

**Infiltration of neutrophils in the bronchoalveolar lavage fluid of mice 6 and 24 h after CLP induction**

Animals were pretreated with an anti-LFA-1, anti-Mac-1 antibody, a combination of both anti-LFA-1 and anti-Mac-1 antibodies, or a control antibody or vehicle before CLP induction. Sham-operated mice served as negative controls. Data represent mean ± SEM and \( n = 5 \). \( \*P \leq 0.05 \) vs. sham and \( \*P \leq 0.05 \) vs. control ab + CLP. BALF indicates bronchoalveolar lavage fluid.

**Myeloperoxidase levels in the lung**

Animals were pretreated with an anti-LFA-1 and anti-Mac-1 antibody or a control antibody or vehicle before CLP induction. Sham-operated mice served as negative controls. Myeloperoxidase levels (units per gram of tissue) were determined 6 h after CLP induction. Data represent mean ± SEM and \( n = 5 \). \( \*P \leq 0.05 \) vs. sham and \( \*P \leq 0.05 \) vs. control ab + CLP.
antibody decreased CLP-induced lung edema by 77% and 60%, respectively (Fig. 2; \( P < 0.05 \) vs. control ab + CLP; \( n = 5 \)). The lung wet-dry ratio was reduced by 78% when anti-LFA-1 and anti-Mac-1 antibodies were given in combination before CLP (Fig. 2; \( P < 0.05 \) vs. control ab + CLP; \( n = 5 \)). Moreover, histological micrographs of lung tissue of sham-operated animals revealed normal architecture (Fig. 3A), whereas CLP mice treated with vehicle (Fig. 3B) or control antibody (Fig. 3C) exhibited severe destruction of the pulmonary tissue microstructure, extensive edema of the interstitial tissue, capillary congestion, necrosis, and massive infiltration of neutrophils. Immunoneutralization of LFA-1 (Fig. 3D), Mac-1 (Fig. 3E), or both (Fig. 3F) protected the lung microarchitecture and reduced neutrophil infiltration.

### LFA-1 and Mac-1 Mediate CLP-Induced Neutrophil Recruitment

Neutrophil accumulation in the lung plays a pivotal role in the pathogenesis of lung injury during sepsis (14, 17). Clear-cut infiltration of neutrophils into the bronchoalveolar space was observed 6 and 24 h after CLP induction. Here, we found that neutrophil recruitment into the bronchoalveolar space increased by 12- and 73-fold at 6 and 24 h after induction of CLP, respectively (Fig. 4; \( P < 0.05 \) vs. sham; \( n = 5 \)). Interestingly, administration of the anti-LFA-1 and anti-Mac-1 antibody decreased the number of pulmonary neutrophils from \( 18.4 \pm 2.7 \times 10^3 \) to \( 4.0 \pm 1.3 \times 10^3 \) (Fig. 4; \( P < 0.05 \) vs. control ab + CLP; \( n = 5 \)) and 9.6 \( \pm 2.7 \times 10^3 \) (Fig. 4; \( P > 0.05 \) vs. control ab + CLP; \( n = 5 \)) cells at 6 h post-CLP, which corresponds to an 86% and 52% reduction, respectively. Pretreatment with the anti-LFA-1 and anti-Mac-1 antibody or a combination of both antibodies decreased pulmonary recruitment of neutrophils 24 h after CLP induction by 90%, 65%, and 92%, respectively (Fig. 4; \( P < 0.05 \) vs. control ab + CLP; \( n = 5 \)). In addition, global accumulation of neutrophils was assessed by analyzing levels of MPO in the lung. Maximum levels of MPO were found at 6 h post-CLP (not shown). It was found that MPO levels in the lung were increased by 14-fold 6 h after CLP induction (Fig. 5; \( P < 0.05 \) vs. sham; \( n = 5 \)). We observed that immunoneutralization of LFA-1 and Mac-1 significantly decreased pulmonary MPO levels by more than 72% in septic mice (Fig. 5; \( P < 0.05 \) vs. control ab + CLP; \( n = 5 \)).

### CXC Chemokine Production in CLP

Numerous studies have shown that directed movement of neutrophils is mediated by CXC chemokines, including MIP-2 and KC (17–20). The lung content of CXC chemokines in sham controls was low but detectable, but CLP provoked clear-cut formation of MIP-2 and KC in the lung tissue (Fig. 6, A and B). Thus, the tissue levels of MIP-2 and KC in the lung increased from 1.7 \( \pm 0.2 \) and 6.2 \( \pm 0.5 \) ng/g at baseline up to 38.9 \( \pm 5.7 \) and 80.8 \( \pm 4.5 \) ng/g lung tissue, respectively, in septic mice at 6 h post-CLP (Fig. 6, A and B; \( P < 0.05 \) vs. sham; \( n = 5 \)). At 24 h after CLP, pulmonary formation of MIP-2 and KC increased even further (Fig. 6, A and B; \( P < 0.05 \) vs. sham; \( n = 5 \)). Administration of the anti-LFA-1 and anti-Mac-1 antibody as well as combined anti-LFA-1 + anti-Mac-1 antibody treatment had no effect on CXC chemokine levels in lung tissue at any point in septic animals (Fig. 6, A and B; \( n = 5 \)).

### CLP-Induced Leukocytopenia

A characteristic feature in early septicemia is the reduced number of circulating leukocytes (28, 29). Here, we observed...
that CLP caused a clear-cut leukocytopenia after 24 h (Table 1). For example, the number of neutrophils decreased by 56% 24 h after CLP induction (Table 1; $P < 0.05$ vs. sham; $n = 5$). This CLP-induced neutropenia was reversed in mice pretreated with the anti–LFA-1, anti–Mac-1 antibody and when these antibodies were given together (Table 1; $P < 0.05$ vs. control ab + CLP; $n = 5$).

**DISCUSSION**

New targets for treating polymicrobial sepsis are urgently needed. The present study documents key roles of LFA-1 and Mac-1 in supporting sepsis-induced lung damage. Our data show that these adhesion molecules are expressed on activated leukocytes in polymicrobial sepsis, which in turn mediate pulmonary infiltration of neutrophils. Moreover, these findings also demonstrate that functional inhibition of either LFA-1 or Mac-1 not only decreases pulmonary accumulation of neutrophils but also protects against lung edema formation and tissue destruction in polymicrobial sepsis. Considered together, these novel findings suggest that LFA-1 and Mac-1 may constitute fruitful targets in septic lung injury.

Perforation of the gastrointestinal tract and leakage of bowel contents into the abdominal cavity are common and serious conditions encountered in surgical patients. Despite modern treatment modalities, the mortality of these patients remains high in intensive care units (4). Thus, there is an urgent need to develop more specific and effective therapies for patients with systemic sepsis. The most vulnerable organ in polymicrobial sepsis is the lung. The present study demonstrates that LFA-1 and Mac-1 may constitute specific targets to protect against pulmonary damage in abdominal sepsis. Thus, we observed that LFA-1 and Mac-1 were expressed on circulating neutrophils after intestinal puncture, which made us hypothesize that these adhesion molecules may be involved in the pathophysiology of septic lung injury. Interestingly, we found that inhibition of LFA-1 and Mac-1 reduced neutrophil infiltration in the lung by more than 52% in polymicrobial sepsis, suggesting that both LFA-1 and Mac-1 support adhesive interactions between circulating leukocytes and lung microvascular endothelial cells in sepsis. Previous works have reported conflicting data on the individual role of LFA-1 and Mac-1 in specific models of inflammation (30–32). However, taking more recent studies into consideration, it is comprehensible that both of these adhesion molecules may cooperate for optimal recruitment of inflammatory cells. For example, a recent study reported that LFA-1 may initiate first stable contact, and that Mac-1 establishes more sustained adhesion onto the endothelium of inflamed organs (33). Nonetheless, we observed that inhibition of LFA-1 and Mac-1 not only attenuated pulmonary infiltration of neutrophils but also reduced edema formation by more than 60% in the lung. In addition, immunoneutralization prevented widespread tissue damage in the lungs of CLP mice. Considered together, these findings suggest a link between LFA-1 and Mac-1–dependent accumulation of neutrophils on one hand and edema formation and tissue injury in the lung on the other. Thus, our data indicate that blocking the function of LFA-1 or Mac-1 may be of beneficial value in septic lung injury.

Interestingly, we found that combined treatment with both anti–LFA-1 and anti–Mac-1 antibodies had no additional inhibitory effect on CLP-induced neutrophil recruitment. Notably, this observation is in line with the study by Hentzen et al. (33) showing that LFA-1 and Mac-1 cooperate in a sequential manner, and that both of these adhesion molecules may be necessary for optimal adhesion onto the endothelium, and that inhibition of either one would be sufficient to decrease neutrophil recruitment. In this context, it should be mentioned that one of the prominent features in clinical responses to sepsis is an early drop in the numbers of blood neutrophils (28, 29), which was also observed herein and culminated 24 h after CLP initiation. Interestingly, we observed that inhibition of both LFA-1 and Mac-1 protected septic animals against this neutropenia, supporting the concept that inhibition of these β-2 integrins ameliorates systemic effects of sepsis. Indeed, this preservation of circulating neutrophils exerted by inhibition of LFA-1 and Mac-1 may help maintain effective immune function in abdominal sepsis.

Numerous studies have shown that septic conditions induce a complex inflammatory reaction in peripheral tissues, including the lung, after only a few hours (15, 34). In the lung, this reaction involves rapid production and release of cytokines and CXC chemokines by alveolar macrophages and epithelial cells (15). In line with this view, we found in the present experimental model of abdominal sepsis that intestinal puncture caused significant elevation of pulmonary production of the CXC chemokines, MIP-2, and KC, which are murine homologs of human growth-related oncogene chemokines (18). Because these CXC chemokines have been shown to attract neutrophils in particular (18, 19), they are considered to be important mediators of several pathological processes such as septic lung injury (17), glomerulonephritis (35), bacterial meningitis (36), and endotoxemic liver injury (37). However, we found here that inhibition of LFA-1 and Mac-1 had no effect on CXC chemokine production in the lungs of septic animals, suggesting that the reduced leukocyte infiltration in the lung of CLP mice treated with anti–LFA-1 and anti–Mac-1 antibodies is not likely related to local changes in CXC chemokine production. In this context, it should be mentioned that one limitation of this study may be that mortality was not used as an end point, but instead, lung injury was the focus of this particular study.

In conclusion, this study demonstrates that LFA-1 and Mac-1 play important roles in polymicrobial sepsis by supporting pulmonary infiltration of neutrophils. Moreover, our data show not only that inhibition of LFA-1 or Mac-1 reduces neutrophil recruitment but also protects against sepsis-induced edema formation and tissue damage in the lung. Thus, on the basis of these novel findings, we propose that targeting LFA-1 and Mac-1 alone or in combination may be useful to protect against lung injury in polymicrobial sepsis.

**REFERENCES**


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Simvastatin antagonizes CD40L secretion, CXC chemokine formation, and pulmonary infiltration of neutrophils in abdominal sepsis

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ABSTRACT

Statins have been reported to exert anti-inflammatory actions and protect against septic organ dysfunction. Herein, we hypothesized that simvastatin may attenuate neutrophil activation and lung damage in abdominal sepsis. Male C57BL/6 mice were pretreated with simvastatin (0.5 or 10 mg/kg) before CLP. In separate groups, mice received an anti-CD40L antibody or a CXCR2 antagonist (SB225002) prior to CLP. BALF and lung tissue were harvested for analysis of neutrophil infiltration, as well as edema and CXC chemokine formation. Blood was collected for analysis of Mac-1 and CD40L expression on neutrophils and platelets, as well as soluble CD40L in plasma. Simvastatin decreased CLP-induced neutrophil infiltration and edema formation in the lung. Moreover, Mac-1 expression increased on septic neutrophils, which was significantly attenuated by simvastatin. Inhibition of CD40L reduced CLP-induced up-regulation of Mac-1 on neutrophils. Simvastatin prevented CD40L shedding from the surface of platelets and reduced circulating levels of CD40L in septic mice. CXC chemokine-induced migration of neutrophils in vitro was decreased greatly by simvastatin. Moreover, simvastatin abolished CLP-evoked formation of CXC chemokines in the lung, and a CXCR2 antagonist attenuated pulmonary accumulation of neutrophils. Our data suggest that the inhibitory effect of simvastatin on pulmonary accumulation of neutrophils may be related to a reduction of CD40L secretion into the circulation, as well as a decrease in CXC chemokine formation in the lung. Thus, these protective mechanisms help to explain the beneficial actions exerted by statins, such as simvastatin, in sepsis.

Introduction

The mortality rate of septic patients has remained high (30–70%) despite substantial investigative efforts [1, 2]. Treatment of sepsis is largely limited to supportive care except recombiant-activated protein C [3]. Nonetheless, recent studies have reported that simvastatin intake reduces hospital mortality [4] and decreases progression of bacterial infections [5] in septic patients. Moreover, animal experiments have shown that statin treatment improves survival in sepsis by improving cardiovascular function [6, 7]. Clinical use of statins is mainly restricted to control cholesterol synthesis in patients with increased risk of cardiovascular complications via inhibition of 3-hydroxy-3-methyl-glutaryl-CoA reductase. Beside their well-known, cholesterol-lowering effect, a growing body of literature indicates that statins also exert so-called pleiotropic properties, such as inhibition of cytokine formation, adhesion molecule expression, and NO production [8–10]. However, the potential anti-inflammatory mechanisms of statins in sepsis remain elusive.

Intestinal perforation is a feared condition in which toxins and microbes contaminate the abdominal cavity [11, 12]. Fecal bacteria trigger local production of proinflammatory compounds, which are subsequently released into the circulation causing a systemic inflammatory reaction. It is well known that pulmonary injury is a common feature and the most frequent cause of mortality in patients with systemic inflammation [13, 14]. Numerous studies have shown that infiltration of activated neutrophils is a rate-limiting step in septic lung injury [15, 16]. Chemokines constitute a family of molecules regulating trafficking of circulating leukocytes into inflamed tissues [17]. Based on their amino acid sequences, they are divided into subfamilies, for instance, CC chemokines, which mainly attract monocytes and lymphocytes, and CXC chemokines, MIP-2, and KC, which primarily attract neutrophils [18–20]. CXCR2 is the high-affinity receptor on murine neutrophils for MIP-2 and KC [21–23]. However, the roles of CXC chemokines and CXCR2 in abdominal sepsis are not clear. Pulmonary infiltration of neutrophils is regulated by specific adhesion molecules, including LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), in abdominal sepsis [24]. Activated neutrophils release tissue-damaging compounds, such as ROS, which in turn, cause lung edema and impaired gaseous exchange [25, 26]. Interestingly, a recent study showed that platelets play a critical role in acti-
vating neutrophils in polymicrobial sepsis [27]. This platelet-dependent activation of neutrophils appears to be mediated by CD40L secreted from activated platelets [28]. In this context, it is interesting to note that mast cells may exert certain antiplatelet actions, such as inhibition of platelet aggregation [29], although it is not known whether mast cells may regulate platelet secretion of CD40L and neutrophil expression of Mac-1 in sepsis.

Based on the above, the aim of the present study was to define the effect of simvastatin on systemic activation and recruitment of neutrophils into the lung in a murine model of polymicrobial sepsis with particular focus on the potential roles of platelet-derived CD40L and CXC chemokine formation in the lung.

MATERIALS AND METHODS

Animals

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). Male C57BL/6 mice weighing 20–25 g were housed on a 12–12 h light-dark cycle and fed a laboratory diet and water ad libitum. The mice were anaesthetized with 75 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium)/kg body weight.

Experimental protocol of sepsis

Polymicrobial sepsis in mice was induced by a CLP procedure, as described previously in detail [24]. Briefly, the exteriorized cecum was filled with feces by walking stool backward, and a ligature was placed below the ileocecal valve. The cecum was soaked with PBS and punctured twice with a 21-gauge needle. The cecum was returned into the peritoneal cavity, the abdominal incision was sutured, and 1 ml PBS was given s.c. Simvastatin (0.5 or 10 mg/kg; Sigma-Aldrich, Stockholm, Sweden) was administered i.p. 10 min prior to CLP induction. These doses of simvastatin were chosen based on our previous studies and other published papers. Sham mice underwent the same laparotomy and resuscitation procedures, but the cecum was neither ligated nor punctured. Animals were reanaesthetized 6 and 24 h after CLP induction. The left lung was ligated and excised for edema measurement. The right lung was used for collecting BALF to quantify neutrophils. Animals were anesthetized with 75 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium)/kg body weight.

Histology

Lung samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin-embedded. Sections (6 μm) were stained with hematoxylin and eosin, and neutrophils and platelets were recovered following centrifugation.

Flow cytometry

For analysis of surface molecule expression on circulating neutrophils and platelets, blood was collected (1:10 acid citrate dextrose) 6 h after CLP induction and incubated (10 min, room temperature) with an anti-CD16/CD32 antibody blocking FcγRII/IIIa to reduce nonspecific labeling and then incubated with PE-conjugated anti-GPIbα/CD42b (clone RB6-8C5; rat IgG2b; eBioscience) and FITC/allophycocyanin-conjugated anti-Mac-1 (clone M1/70; integrin αmβ2 chain; rat IgG2b) or PerCP Cy5.5-conjugated anti-mouse CD11b (CXR2; clone TG11/CXR2; rat IgG2a, Biologend, San Diego, CA, USA) antibodies. Another set of samples was stained with FITC-conjugated anti-CD41 (clone MWReg30; integrin αIIbβ3 chain; rat IgG2a) and PE-conjugated anti-CD40L (clone M1; hamster IgG) antibodies (all antibodies except those indicated were purchased from BD Biosciences PharMingen). Cells were fixed, cryotreated, and neutrophils and platelets were collected using centrifugation. Flow cytometric analysis was performed according to standard setting on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA), and a viable gate was used to exclude dead and fragmented cells.

In vitro activation of neutrophils

Blood was collected from healthy animals and incubated with murine soluble rCD40L (100 ng/ml; PeproTech EC, London, UK) and activated simvastatin (1 μM; Sigma-Aldrich) at 37°C for 20 min. Then, the cells were stained for flow cytometric analysis of Mac-1 expression on neutrophils as described above. The simvastatin pro-drug was activated as described previously [30]. Mouse rMIP-2 (300 ng/ml; R&D Systems) was used as a positive control.

Neutrophil isolation and cell sorting by FACS

Neutrophils were freshly extracted from healthy mice by aseptically flushing the bone marrow of femurs and tibiae with complete culture medium RPMI 1640 and then subsequently isolated by using Ficol-Paque™ research grade (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated neutrophils was higher than 70%, as assessed in a hematocytometer. The neutrophils were resuspended in culture medium RPMI 1640 until purified further by cell sorting or used in the chemotaxis assay. Isolated neutrophils were labeled with the FITC-conjugated anti-mouse neutrophil antibody (clone 7/4; rat IgG2a; Abcam) and then dehydrated and paraffin-embedded.

Lung edema

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

ELISA

MIP-2 and KC levels in lung tissue and CD40L levels in plasma were analyzed by using double antibody Quantikine ELISA kits (R&D Systems, Europe, Abingdon, Oxon, UK). Murine rMIP-2, rKC, and rCD40L were used as standards.

Systemic leukocyte counts

Blood was collected from the tail vein and mixed with Turks solution (0.2 mg gentian violet in 1 ml glacial acetic acid, 6.25% v/v) in a 1:20 dilution. Leukocytes were identified as MNL and PMNL cells in a Burker chamber.

Lung edema

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

MPO activity

Lung tissue was thawed and homogenized in 1 ml 0.5% hexadecyltrimethylammonium bromide. Samples were freeze-thawed, after which, the MPO activity of the supernatant was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H2O2 (450 nm, with a reference filter 540 nm, 25°C). Values were expressed as MPO units/g tissue.

RT-PCR

Total RNA was extracted from the purified neutrophils of healthy mice using the RNeasy mini kit (Qiagen, Hilden, Germany), according to the man-
ufacturer’s protocol, and treated with RNase-free DNase (Amersham Pharmacia Biotech AB, Sollentuna, Sweden) to remove potential genomic DNA contaminants, according to the manufacturer’s handbook. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically. RT-PCR was performed with Superscript One-Step RT-PCR system (Gibco-BRL Life Technologies, Grand Island, NY, USA). The RT-PCR reactions started with 5 min synthesis at 95°C, followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 60°C for 40 s, extension at 72°C for 1 min, and one cycle of final extension at 72°C for 10 min. After RT-PCR, aliquots of the RT-PCR products were separated on 2% agarose gel containing ethidium bromide and photographed. The primer sequences were as follows: CD40 (forward) 5’-GAA GCC GAC TGA CAA GCC AC-3’, (reverse) 5’-GTG TCT GTG CTG GTG ACA GCG-3’, β-actin (forward) 5’-ATG TTT GAG ACC TTC AAC ACC-3’, (reverse) 5’-TCT CCA GGG AGG AAG AGG AT-3’. β-Actin served as a housekeeping gene.

Chemotaxis assay

Neutrophils isolated from bone marrow by use of Ficoll-Paque™ were pre-incubated with simvastatin (25 μM) for 30 min, and 1.5×10⁶ neutrophils were placed in the upper chamber of the Transwell inserts (5 μm pore size; Corning Costar, Corning, NY, USA). Inserts were placed in wells containing medium alone (control) or medium plus MIP-2 (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 [31].

Statistics

Data are presented as mean values ± sem. Statistical evaluations were performed using Kruskal-Wallis one-way ANOVA on ranks, followed by multiple comparisons versus the control group (Dunnett’s method). *P < 0.05 was considered significant, and n represents the total number of mice in each group used for this study.

RESULTS

Neutrophil recruitment and lung injury

Maximum levels of MPO, a marker of neutrophils, were found at 6 h post-CLP, when it was increased by more than 14-fold (Fig. 1A, *P<0.05 vs. sham). Simvastatin (10 mg/kg) treatment decreased MPO levels by 59% (Fig. 1A, *P<0.05 vs. PBS CLP) in septic mice. Cell analysis of BALF revealed a clear-cut increase in the number of neutrophils, which increased by 124-fold 24 h after CLP (Fig. 1B, *P<0.05 vs. sham). Simvastatin markedly decreased pulmonary neutrophils from 100.0±7.4×10⁶ down to 24.0±4.2×10⁴ (0.5 mg/kg) and 18.4±2.0×10⁴ (10 mg/kg), corresponding to a 77% and 82% reduction, respectively (Fig. 1B, *P<0.05 vs. PBS+CLP). Pulmonary edema formation markedly increased in mice subjected to polymicrobial sepsis, with a maximal increase at 24 h after CLP induction (Fig. 1C, *P<0.05 vs. sham). Simvastatin treatment reduced pulmonary edema by 40% (Fig. 1C, *P<0.05 vs. PBS+CLP).

Figure 1. (A–C) Effects of simvastatin on CLP-induced pulmonary infiltration of neutrophils. (A) Lung MPO levels at 6 h post-CLP; (B) number of BALF neutrophils, and (C) edema formation in the lung 24 h after CLP induction. Animals were treated with simvastatin (Simv; 0.5 or 10 mg/kg) or PBS prior to CLP. Sham-operated mice served as negative controls. Data represent mean ± sem. *P < 0.05 versus sham; #P < 0.05 versus PBS + CLP; n = 5–6. (D) Representative H&E section of lung. Mice were treated with PBS or simvastatin 0.5 mg/kg or 10 mg/kg prior to CLP induction, and samples were harvested 24 h later. Original scale bars, 100 μm.

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to CLP. More specifically, the lung wet/dry ratio increased by 58% in CLP mice, i.e., from 4.4 ± 0.1 to 6.9 ± 0.1 (Fig. 1C, \( P<0.05 \) vs. sham). Treatment with 0.5 or 10 mg/kg simvastatin decreased the wet/dry ratio down to 5.0 ± 0.2 in CLP animals, respectively, in septic animals, corresponding to a reduction by >75% (Fig. 1C, \( P<0.05 \) vs. PBS + CLP). Moreover, morphologic examination showed normal microarchitecture in lungs from sham-operated animals (Fig. 1D), whereas CLP mice exhibited severe destruction of the pulmonary tissue, extensive edema of the interstitial tissue, capillary congestion, necrosis, and massive infiltration of neutrophils (Fig. 1D). Simvastatin (0.5 or 10 mg/kg) protected against CLP-induced destruction of tissue architecture, cellular damage, and neutrophil infiltration in the lung samples (Fig. 1D). We observed that CLP caused a dramatic leucopenia after 24 h (Table 1, \( P<0.05 \) vs. sham). For example, the number of PMN leukocytes (neutrophils) decreased by 63% in CLP animals. This CLP-induced neutropenia was partly reversed in mice pretreated with simvastatin (Table 1).

### Mac-1 expression and CD40L levels

Considering that Mac-1 up-regulation on neutrophils and CD40L shedding from platelets have recently been shown to constitute key mechanisms in abdominal sepsis [24, 28], it was of great interest to investigate whether simvastatin may interfere with these mechanisms. CLP evoked a clear-cut increase in Mac-1 expression on neutrophils at 6 h compared with sham-operated animals (Fig. 2A, \( P<0.05 \) vs. sham). Sepsis-induced Mac-1 up-regulation was attenuated significantly by simvastatin. For example, administration of 10 mg/kg simvastatin decreased MFI values of Mac-1 on neutrophils from 154 ± 6 to 109 ± 11 in CLP mice (Fig. 2A, \( P<0.05 \) vs. PBS + CLP). RT-PCR revealed that CD40 is expressed on mouse neutrophils and may be directly activated by CD40L (Fig. 2B). Indeed, an anti-CD40L antibody abolished CLP-induced Mac-1 upregulation on neutrophils as compared with a control antibody (Fig. 2C, \( P<0.05 \) vs. control antibody + CLP), confirming that Mac-1 expression on neutrophils is dependent on CD40L in abdominal sepsis. Next, we examined if platelet-derived CD40L may be a potential target of simvastatin in reducing surface expression of Mac-1 on neutrophils. CLP caused a concomitant reduction in CD40L expression on platelets and an increase in soluble CD40L levels in plasma (Fig. 3A and B). Simvastatin completely prohibited CLP-induced down-regulation of CD40L on the surface of platelets (Fig. 3A). In addition, simvastatin treatment abolished the CLP-induced increase in plasma CD40L levels (Fig. 3B, \( P<0.05 \) vs. PBS + CLP). In separate in vitro experiments, CD40L activated neutrophils and increased surface expression of Mac-1. However, coinuciation with simvastatin had no effect on CD40L-induced expression of Mac-1 on neutrophils (Fig. 3C).

### CXC chemokines in the lung

Pulmonary levels of CXC chemokines in control animals were low but detectable (Fig. 4A and B). In contrast, CLP increased pulmonary levels of MIP-2 and KC from 1.7 ± 0.2 ng/g and 5.5 ± 0.9 ng/g up to 94.4 ± 14.4 ng/g and 100.8 ± 9.1 ng/g, respectively (Fig. 4, \( P<0.05 \) vs. sham). Notably, pretreatment with simvastatin decreased CLP-induced formation of MIP-2 and KC down to 20.7 ± 7.0 ng/g (0.5 mg/kg), 24.7 ± 4.8 ng/g (10 mg/kg), 29.2 ± 7.0 ng/g (0.5 mg/kg), and 29.2 ± 3.7 ng/g (10 mg/kg) in lung tissue, respectively (Fig. 4B).
Neutrophil chemotaxis in vitro

To study whether simvastatin may exert a direct effect on neutrophils, we examined neutrophil chemotaxis in vitro. It was observed that 100 ng/ml MIP-2 caused a clear-cut increase in neutrophil migration over a time period of 120 min (Fig. 4C, P<0.05 vs. control). Preincubation of neutrophils with simvastatin decreased MIP-2-induced neutrophil migration by >75% (Fig. 2C, P<0.05 vs. MIP-2 alone).

Role of CXCR2 in CLP-induced lung injury

To determine whether CXC chemokines play a functional role in septic lung injury, we pretreated CLP mice with the CXCR2 antagonist SB225002 (4 mg/kg). Administration of the CXCR2 antagonist reduced CLP-induced MPO levels by 61% (Fig. 5A, P<0.05 vs. vehicle CLP) and the number of BALF neutrophils by 80% (Fig. 5B, P<0.05 vs. vehicle + CLP), as well as pulmonary edema formation by 87% (Fig. 5C, P<0.05 vs. vehicle + CLP). Moreover, we observed that treatment with the CXCR2 antagonist prevented the CLP-induced damage of the microarchitecture of the lung (Fig. 5D). However, inhibition of CXCR2 signaling had no effect on the levels of MIP-2 and KC in septic lung injury (not shown). Notably, surface expression of CXCR2 on circulating neutrophils decreased in septic mice (i.e., MFI from 173±12 down to 60±11; P<0.05 vs. sham; Fig. 5E). However, simvastatin had no effect on this CLP-induced down-regulation of CXCR2 expression on neutrophils (Fig. 5E).

DISCUSSION

Clinical management of patients with septic lung injury is mainly limited to supportive care, and novel therapeutic options are needed to improve the outcome of patients with abdominal sepsis. The present study demonstrated that administration of simvastatin protects against lung injury in abdominal sepsis. The protective effect of simvastatin is a result of attenuation of neutrophil infiltration in the septic lung. Moreover, these findings indicate that the inhibitory effect of simvastatin on pulmonary accumulation of neutrophils may be related to inhibition of platelet secretion of CD40L into the circulation, direct interference with chemotaxis, and reduction of CXC chemokine formation in the lung. Thus, our results elucidate the influence of simvastatin on complex mechanisms regulating pathological inflammation in abdominal sepsis.

Numerous reports have shown that statins exert potent and pleiotropic, anti-inflammatory actions, besides reducing cholesterol levels [9]. Sepsis is characterized by a generalized activation of the host immune system, in which the most insidious feature is lung injury and impaired gaseous exchange [24, 26]. In the present study, we show that simvastatin protects against pulmonary edema and tissue damage in abdominal sepsis. These findings are in line with a previous study showing that simvastatin inhibits vascular leakage in an endotoxin model of murine lung injury [32]. Convincing data have established
that simvastatin had no effect on CD40L-evoked Mac-1 expression and induced increase in soluble CD40L in plasma. It was also found that simvastatin markedly decreased the CLP-infiltrated baseline levels of CD40L expression on platelets in septic animals. Then, we observed that simvastatin abolished the CLP-surface expression of CD40L on platelets. Concomitantly, it was reported in septic mice treated with simvastatin [6, 7].

Pulmonary recruitment of leukocytes is a multistep process, including initial sequestration in microvessels and firm adhesion on the endothelium, followed by transendothelial and transepithelial migration [35]. The adhesive interactions between leukocytes and endothelial cells are regulated by specific adhesion molecules [36]. For example, it has been shown that P-selectin glycoprotein ligand-1, LFA-1, and Mac-1 on neutrophils mediate their infiltration in septic lung injury [24, 37]. Interestingly, a recent study demonstrated that Mac-1 up-regulation on neutrophils is mediated by soluble CD40L, secreted from platelets in abdominal sepsis [28]. Indeed, we confirmed that immunoneutralization of CD40L markedly decreased neutrophil Mac-1 expression in septic mice. Moreover, we detected gene expression of CD40 in isolated neutrophils, which is in line with a previous study showing that neutrophils indeed express CD40 [38]; this may help to explain the reduced mortality reported in septic mice treated with simvastatin [6, 7].

Further, we found that simvastatin markedly reduced MIP-2-induced neutrophil chemotaxis in vitro, suggesting that simvastatin does not inhibit CD40L function. Considered together, we conclude that simvastatin inhibits platelet secretion of CD40L into the circulation in abdominal sepsis. Knowing that soluble CD40L regulates neutrophil expression of Mac-1, it may be suggested that simvastatin inhibits Mac-1 expression and pulmonary recruitment, at least in part, via inhibition of CD40L secretion from platelets in septic mice. Trafficking of leukocytes in inflamed tissues is coordinated by secreted chemokines [17]. Neutrophils are particularly attracted by CXC chemokines, comprising MIP-2 and KC, which are murine homologues of human IL-8 [39, 40]. Herein, we observed that simvastatin decreased CLP-induced MIP-2 and KC chemokine formation by >75% in the lung. Interestingly, we also found that simvastatin markedly reduced MIP-2-induced neutrophil chemotaxis in vitro, suggesting that simvastatin may interfere directly with the migration process of neutrophils triggered by CXC chemokines. This notion is supported by a previous study reporting that statins can decrease neutrophil migration toward the chemoattractant FMLP [41]. Thus, simvastatin is a potent inhibitor of CXC chemokine formation in the lung and CXC chemokine-induced neutrophil chemotaxis, which may explain part of the inhibitory effect of simvastatin on the accumulation of neutrophils in the lung of septic animals. Whether pulmonary CXC chemokines are important for lung accumulation of leukocytes in abdominal sepsis is not known. The specific effects of chemokines are mediated by G-coupled receptors [20], and we used a CXCR2 antagonist targeting the high-affinity receptor for MIP-2 and KC to determine the role of CXC chemokines in septic lung injury. We observed that administration of the CXCR2 antagonist reduced CLP-induced MPO levels by 61% and the number of neutrophils in the bronchoalveolar compartment by 80%, indicating that CXC chemokines indeed are important regulators of pulmonary infiltration of neutrophils in septic lung injury. In this context, it should be noted that soluble CD40L can stimulate secretion of CXC chemokines from cells within the vascular compartment, including leukocytes and endothelial cells [42]. However, pulmonary formation of CXC chemokines is generated by extravascular epithelial cells [43] and alveolar macrophages [33, 44], and soluble CD40L in the plasma may not reach...
these extravascular cells in the lung. This notion is supported by our recent findings showing that pulmonary formation of MIP-2 and KC was completely intact in septic mice lacking CD40L [28]. Thus, secretion of CD40L into the circulation is dissociated from generation of CXC chemokines in the septic lung. Considering that simvastatin inhibits secretion of CD40L into plasma and CXC chemokine formation in the lung, it may be proposed that these two effects represent two separate and distinct anti-inflammatory mechanisms exerted by simvastatin in abdominal sepsis. Moreover, we confirmed herein that the neutrophil expression of CXCR2 is down-regulated in sepsis [45], but administration of simvastatin had no influence on the surface level of CXCR2 on neutrophils in CLP animals, suggesting that modulation of CXCR2 expression is not involved in the anti-inflammatory effects of simvastatin.

In conclusion, our data suggest that simvastatin protects the lung against tissue injury in abdominal sepsis via inhibition of neutrophil recruitment. First, simvastatin attenuates secretion of CD40L from platelets, which in turn, can up-regulate Mac-1 expression on neutrophils. Second, simvastatin inhibits local generation of CXC chemokines, which can attract neutrophils into the lung. Third, simvastatin directly inhibits neutrophil chemotaxis stimulated by CXC chemokines. It should be noted that simvastatin may also inhibit targets upstream of these three mechanisms mentioned above. Taken together, the complex and multiple actions delineated in this study may help clarify the beneficial effects exerted by statins in sepsis.

**AUTHORSHIP**

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

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Simvastatin protects against T-cell immune dysfunction in abdominal sepsis

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Running head: Simvastatin and T-cell function

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Abstract

Sepsis-triggered immune paralysis including T-cell dysfunction increases susceptibility to infections. Statins exert beneficial effects in patients with sepsis although the mechanisms remain elusive. Herein, we hypothesized that simvastatin may attenuate T-cell dysfunction in abdominal sepsis. Male C57BL/6 mice were pretreated with simvastatin (10 mg/kg) prior to cecal ligation and puncture (CLP). Spleen CD4 T-cell apoptosis, proliferation and regulatory T-cells (CD4^+CD25^+Foxp3^+) were quantified by use of flow cytometry. Formation of IFN-γ and IL-4 in the spleen and plasma levels of HMBG1 and IL-6 were determined using ELISA. CLP caused a clear-cut increase in apoptosis and decrease in proliferation in splenic CD4 T-cells. It was found that simvastatin markedly reduced apoptosis and improved proliferation in CD4 T-cells in septic mice. Moreover, CLP-induced formation of regulatory T-cells in the spleen was abolished in simvastatin-treated animals. CLP greatly decreased the levels of IFN-γ and IL-4 in the spleen. Simvastatin completely reversed this sepsis-mediated inhibition of IFN-γ and IL-4 formation in the spleen. We observed that CLP increased plasma levels of HMGB1 by 25-fold and IL-6 by 99595-fold. Notably, treatment with simvastatin abolished this CLP-evoked increase of HMGB1 and IL-6 levels in the plasma, suggesting that simvastatin is a potent inhibitor of systemic inflammation during sepsis. Lastly, it was found that simvastatin reduced CLP-induced bacteremia. In conclusion, these novel findings suggest that simvastatin is a powerful regulator of T-cell immune dysfunction in abdominal sepsis. Thus, these protective actions of simvastatin on T-cell functions help to explain the protective effect of statins in patients with sepsis.
Introduction

Abdominal sepsis is a major cause of morbidity in intensive care units and the mortality rate ranges between 30-40% in severe cases (1). The most frequent death causes in septic patients are infectious complications (2). The immune system undergoes two distinct phases in response to a septic insult. First, bacterial antigens and toxins cause an inflammatory response characterized by local formation of pro-inflammatory substances and these substances can translocate into the circulation and cause a systemic inflammatory response syndrome (SIRS). SIRS is associated with organ damage in patients with sepsis (3). Secondly, the septic insult leads to a hypo-inflammatory phase in which the immune system becomes incapable to mount appropriate host-defense responses against microbes due to macrophage and T-cell dysfunction referred to as compensatory anti-inflammatory response syndrome (CARS). CARS is clinically associated with increased susceptibility to infections (3). During this hypo-inflammatory phase, macrophages lose their capacity to present antigens while T-cells undergo apoptosis as well as fail to proliferate and produce interferon-γ (IFN-γ) (4, 5). Moreover, several studies have reported that the number of regulatory T-cells increase during CARS which further reduce the ability of the immune system to mount anti-bacterial responses (6). Improving functional T-cell-mediated immunity during CARS might reduce infectious complications in septic patients.

Besides antibiotics, current management of patients with sepsis is mainly restricted to supportive therapies. Although statins are mainly used to regulate cholesterol levels in patients with cardiovascular diseases, recent studies have demonstrated that statins might reduce mortality in septic patients (7). Moreover, several reports indicate that statin treatment inhibits pulmonary damage (8) and increases survival in murine sepsis (9). Ample data in the literature show that statins, such as simvastatin, exert pleiotropic anti-inflammatory effects, including attenuated expression of cytokines, chemokines, and adhesion molecules as well as decreased formation of nitric oxide (10). However, it is not known whether statins might antagonize sepsis-evoked immune suppression.

Based on these considerations, we hypothesized that simvastatin might improve T-cell function in abdominal sepsis. For this purpose, we used a model based on cecal ligation and puncture (CLP) to induce sepsis in mice.
**Materials and Methods**

*Animals*

All experimental procedures were performed in accordance with the legislation on protection of animals and were approved by the Regional Ethical Committee for Animal Experimentation at Lund University, Sweden. Male C57Bl/6 mice weighing 20 to 25 g were housed on an animal facility with 12-12 h light dark cycle at 22°C, and fed a laboratory diet and water *ad libitum*. The mice were anesthetized with 7.5 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight.

*Experimental protocol of sepsis*

Polymicrobial sepsis in mice was induced by a cecal ligation and puncture (CLP) procedure as previously described in detail (11). Briefly, animals were anesthetized and a midline incision was made to expose the cecum, which was filled with feces by milking stool backwards from the ascending colon and 75% of the cecum was ligated with a 5-0 silk suture. The cecum was soaked with PBS (pH 7.4) and was then punctured twice with a 21-gauge needle on the antimesenteric border. The cecum was returned into the peritoneal cavity and the abdominal incision was sutured. Simvastatin (10 mg/kg, Sigma-Aldrich, Stockholm, Sweden) was administered intraperitoneally (i.p.) 10 min prior to sham operation or CLP induction. Sham mice underwent the identical laparotomy and resuscitation procedures, but the cecum was neither ligated nor punctured. Animals were re-anesthetized 24 h after CLP or sham procedure to collect samples for further analysis.

*Isolation of splenocytes*

The spleen was excised for cell culture and flow cytometric analysis 24 h post CLP induction. Single splenocyte suspension was obtained under sterile condition by smashing the spleen and passing it through a 40 µm cell strainer (BD Falcon, 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) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 unit/ml) and streptomycin (0.1 mg/ml) (Sigma-Aldrich). 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 in 48-well plates pre-coated with anti-CD3ε antibody (5 µg/well, IgG, clone: 145-2C11) and in the presence of soluble anti-CD28 antibody (5 µg/well, IgG, clone: 37.51) at 37°C in a humidified atmosphere with 5% CO₂ 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. All antibodies used in the study were purchased from eBioscience (San Diego, CA, USA) unless indicated.

**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 succinimidyl ester (CFSE, 5 µM, Sigma-Aldrich) and incubated at 1.5×10⁶ 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% CO₂ for 72 h. 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). 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.

**ELISA for HMGB1 and IL-6**

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 use. ELISA kits were used to quantify plasma levels of high-mobility group box-1 (HMGB1) (Chondrex, Redmond, WA, USA) and IL-6 (R&D Systems) according to manufacturers’ instructions.

**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 ($\times 10^5$) per ml of blood.

**Statistics**

Data are presented as mean values ± standard errors of the means (SEM). Statistical evaluations were performed using one way analysis of variance on ranks followed by multiple comparisons (Student-Newman-Keuls Method). $P < 0.05$ was considered statistically significant and $n$ represents the number of animals.

**Results**

**T-cell apoptosis, proliferation and cytokine formation**

It was found that CLP triggered a significant increase in CD4 T-cell apoptosis in the spleen. The percentage of apoptotic CD4 T-cells was 5.3% in sham and increased to 49.8% in CLP mice (Fig. 1, $P < 0.05$ vs. sham, $n = 5$). Administration of simvastatin (10 mg/kg) decreased the percentage of CD4 T-cell apoptosis down to 16.5%, corresponding to a 75% reduction in apoptosis (Fig. 1, $P < 0.05$ vs. PBS + CLP, $n = 5$). PI was used as a marker of necrotic cells. We found that the number of living cells in the spleen was reduced in CLP mice (Fig. 2a, $P < 0.05$ vs. sham, $n = 5$). Simvastatin increased the number of living splenocytes in
septic animals (Fig. 2a, $P < 0.05$ vs. PBS + CLP, $n = 5$). Moreover, CLP decreased both the percentage of PI$^-$ CD4 T-cells of all splenocytes (Fig. 2b, $P < 0.05$ vs. sham, $n = 5$) as well as the percentage of PI$^-$ CD4 T-cells of all CD4 T-cells (Fig. 2c, $P < 0.05$ vs. sham, $n = 5$). Administration of simvastatin increased both the percentage of PI$^-$ CD4 T-cells of all splenocytes (Fig. 2b, $P < 0.05$ vs. PBS + CLP, $n = 5$) as well as the percentage of PI$^-$ CD4 T-cells of all CD4 T-cells (Fig. 2c, $P < 0.05$ vs. PBS + CLP, $n = 5$). Flow cytometry revealed that the percentage of CD4 T-cells that did not divide was 10.7% in sham animals (Fig. 2e).
Figure 2. Simvastatin inhibits CLP-induced hypoproliferation in CD4 T-cells. Animals were treated with 10 mg/kg of simvastatin (Simv 10) or PBS prior to CLP induction. Mice treated with PBS alone served as sham animals. Splenocytes were isolated and stained with propidium iodine (PI), carboxyfluorescein diacetate succinimidyl ester (CFSE) and an anti-CD4 antibody. Cell division of CFSE-labeled splenocytes was stimulated with anti-CD3ε and anti-CD28 antibodies and determined by flow cytometry as described in Materials and Methods. (a) A representative dot plot showing splenocytes and aggregate data on PI negative (PI−) splenocytes. (b) A representative dot plot showing splenocytes stained with PI and an anti-CD4 antibody and aggregate data on the percentage of PI− CD4 T-cells of all splenocytes. (c) A representative dot plot showing splenocytes stained with PI and an anti-CD4 antibody and aggregate data on the percentage of PI− CD4 T-cells of all CD4 T-cells. (d) Representative histograms of CFSE profiles of CD4 T-cells. Gray line indicates negative control cells. (e) The line graph shows the percentages of viable CD4 T-cells according to the number of divisions. Data are presented as mean ± SEM. *P < 0.05 vs. sham and #P < 0.05 vs. PBS + CLP, n = 5.
CLP increased the percentage of non-dividing CD4 T-cells up to 45.5% (Fig. 2e, $P < 0.05$ vs. sham, $n = 5$). We observed that simvastatin treatment decreased the percentage of CD4 T-cells that did not divide to 24.4% in septic mice, corresponding to a 61% reduction (Fig. 2e, $P < 0.05$ vs. PBS + CLP, $n = 5$). Formation of IFN-γ is critical in the T-cell-dependent immunity (12). IFN-γ and IL-4 secretion from splenocytes was evoked by anti-CD3ε + anti-CD28 antibodies stimulation. Herein, it was found that anti-CD3ε + anti-CD28 antibodies-provoked IFN-γ formation was markedly decreased in splenocytes, i.e. from 25.3 pg/ml in sham mice down to 7.3 pg/ml in CLP animals (Fig. 3a, $P < 0.05$ vs. sham, $n = 5$). This decrease in IFN-γ production in septic mice approached 71%. Notably, treatment with 10 mg/kg of simvastatin significantly inhibited the CLP-induced attenuation of IFN-γ formation in stimulated splenocytes (Fig. 3a, $P < 0.05$ vs. PBS + CLP, $n = 5$). CLP decreased IL-4 formation in splenocytes by 70% (Fig. 3b, $P < 0.05$ vs. sham, $n = 5$). Administration of simvastatin significantly enhanced splenocyte production of IL-4 in septic animals (Fig. 3b, $P < 0.05$ vs. PBS + CLP, $n = 5$).

**Regulatory T-cells**

Regulatory T-cells (CD4⁺CD25⁺Foxp3⁺) are known to impair immune responses (13). Herein, we found that CLP increased the percentage of regulatory T-cells in the spleen by 74% (Fig. 4, $P < 0.05$ vs. sham, $n = 5$). Administration of simvastatin decreased the percentage of regulatory T-cells down to 15.2%, corresponding to a 72% reduction, in septic animals (Fig. 4, $P < 0.05$ vs. PBS + CLP, $n = 5$).

**Plasma levels of HMGB1 and IL-6**

Plasma levels of HMGB1 in control animals were low but detectable (Fig. 5a, $n = 5$). In contrast, CLP increased plasma levels of HMGB1 by 25 times from 1.6 ± 0.7 ng/ml up to 40.9 ± 4.1 ng/ml (Fig. 5a, $P < 0.05$ vs. sham, $n = 5$). Notably, pre-treatment with simvastatin decreased CLP-induced formation of HMGB1 to 6.5 ± 2.3 ng/ml (Fig. 5a, $P < 0.05$ vs. PBS+CLP, $n = 5$). Moreover, it was found that the levels of IL-6 in the plasma were markedly enhanced in septic compared to sham mice (Fig. 5b, $P < 0.05$ vs. sham, $n = 5$). Interestingly, we observed that simvastatin treatment reduced plasma levels of IL-6 from 183.5 ± 19.0 ng/ml down to 58.5 ± 13.1 ng/ml septic mice (Fig. 5b, $P < 0.05$ vs. PBS + CLP, $n = 5$). Thus, simvastatin significantly decreased CLP-induced plasma levels of HMGB1 by 87% and IL-6 by 68%.
Clearance of bacteria in blood

There was no visible bacteria colony in blood of sham animals treated with or without simvastatin after 24 h (Fig. 6, $P < 0.05$ vs. sham, $n = 5$). We observed a remarkable increase in bacteria colony formation in septic mice (Fig. 6, $P < 0.05$ vs. sham, $n = 5$) and administration of simvastatin decrease bacterial colony counts by more than 83% (Fig. 6, $P < 0.05$ vs. PBS + CLP, $n = 5$), suggesting that simvastatin improved bacterial clearance in blood.

**Figure 3.** Simvastatin increase splenocyte formation of IFN-γ and IL-4 in CLP mice. Animals were treated with 10 mg/kg of simvastatin (Simv 10) or PBS prior to CLP induction. Mice treated with PBS alone served as sham animals. Splenocytes were harvested 24 h after CLP induction. Levels of (a) IFN-γ and (b) IL-4 formation in splenocytes were determined 24 h after incubation with anti-CD3ε and anti-CD28 antibodies by using ELISA. Data are presented as mean ± SEM. *$P < 0.05$ vs. sham and # $P < 0.05$ vs. PBS + CLP, $n = 5$.

Discussion

Management of septic patients with infectious complications poses a major challenge to clinicians due to the limited therapeutic options. Immune suppression is an insidious aspect of the host reaction to severe infections or major trauma. This study shows that simvastatin is capable of inhibiting T-cell dysfunction in abdominal sepsis. Thus, simvastatin attenuated
sepsis-induced T-cell apoptosis as well as promoted T-cell proliferation and cytokine formation. In addition, simvastatin decreased sepsis-evoked expansion of regulatory T-cells. Finally, it was found that simvastatin abolished CLP-induced increases of HMGB1 and IL-6 in the plasma, indicating that statins can regulate systemic inflammation in sepsis.

**Figure 4.** Simvastatin inhibits CLP-induced expansion of regulatory T-cells. Animals were treated with 10 mg/kg of simvastatin (Simv 10) or PBS prior to CLP induction. Mice treated with PBS alone served as sham animals. The percentage of regulatory T-cells (CD4⁺CD25⁺Foxp3⁺) in the spleen was determined 24 h after CLP induction by flow cytometry. (a) Representative dot plots from the CD4⁺ gate. (b) Aggregate data on the percentages of regulatory T-cells in the spleen. Data are presented as mean ± SEM. *P < 0.05 vs. sham and **P < 0.05 vs. PBS + CLP, n = 5.
Figure 5. Simvastatin inhibits CLP-induced increase of HMGB1 and IL-6 levels in plasma. Animals were treated with 10 mg/kg of simvastatin (Simv 10) or PBS prior to CLP induction. Mice treated with PBS served as sham animals. Levels of (a) HMGB1 and (b) IL-6 levels in plasma were determined 24 h after CLP induction by using of ELISA. Data are presented as mean ± SEM. *P < 0.05 vs. sham and #P < 0.05 vs. PBS + CLP, n = 5.

Figure 6. Simvastatin improves bacterial clearance in blood after onset of CLP. Animals were treated with 10 mg/kg of simvastatin (Simv 10) or PBS prior to CLP induction. Mice treated with PBS served as sham animals. Blood was taken 24 h after CLP and cultured on Trypticase Soy Agar dishes (5% sheep blood). The bacterial colonies were counted after 24 h of incubation. Data are presented as mean ± SEM. *P < 0.05 vs. sham and #P < 0.05 vs. PBS + CLP, n = 5.
Accumulating clinical evidence suggests that statins might be beneficial in patients with sepsis and severe infections (7). In line with these observations, numerous studies have shown that statins exert potent and pleiotropic anti-inflammatory effects, such as reduction of chemokine production and neutrophil recruitment, in animal models of endotoxemia and sepsis (8, 9, 14). Two studies in humans have shown that simvastatin decrease endotoxin-provoked chemokine (15) and metalloproteinase formation and neutrophil accumulation in the lung (16). Although these anti-inflammatory effects of statins might contribute to the improved clinical outcome in patients with sepsis, the major cause of death in septic patients is infectious complications (2). Convincing studies have demonstrated that sepsis causes T-cell and macrophage dysfunction explaining, at least in part, the enhanced susceptibility to infections observed in patients with sepsis (17). In the present study, we found that administration of simvastatin reduced apoptosis and improved the proliferative capability in splenic CD4 T-cells in septic mice. Thus, it may suggest that these effects combined can increase the number of functional CD4 T-cells able to mount effective anti-bacterial responses. Moreover, we observed herein that simvastatin reversed sepsis-induced reduction in IFN-γ formation, which may also contribute to the host defense knowing that IFN-γ is critical in eliciting Th1-type immunity against microbes (12, 14). The literature on the effect of simvastatin on IFN-γ production is complex and partly contradictory. For example, some studies have reported that simvastatin decrease formation of IFN-γ in models of allograft rejection (18) and atherosclerosis (19) whereas others have shown that simvastatin promotes production of IFN-γ in isolated monocytes (20) and T-cells (21). Thus, the effect of simvastatin on IFN-γ formation appears to be dependent on the context in which IFN-γ is synthesized. Nonetheless, our data showed that IFN-γ formation is promoted by simvastatin in abdominal sepsis. This notion is indirectly supported by a recent study showing that inhibition of protein isoprenylation, which is a downstream mediator regulated by statins, by use of a farnesyltransferase inhibitor also increases IFN-γ formation in septic animals (22). Taken together, our findings indicate that simvastatin improves immune responses in sepsis via on one hand increased numbers of T-cells and on the other hand enhanced function, i.e. IFN-γ formation.

Regulatory T-cells are recognized for their potent capability to control T-cell-dependent immune responses (23). Several studies have shown that the number of regulatory T-cells increase in the course of sepsis, which might compromise host defense reactions against microbial infections (22). Herein, we observed that CLP triggered a clear-cut induction of regulatory T-cells in the spleen. Interestingly, simvastatin abolished this increase in regulatory T-cells in septic mice. Considering that the number of regulatory T-
cells is also enhanced in patients with sepsis (24), it may be forwarded that this simvastatin-mediated attenuation in regulatory T-cells might contribute to the beneficial actions of statins in patients with severe infections. Thus, simvastatin may improve T-cell-dependent immune responses via at least three different mechanisms, i.e. increasing the number of T-cells, enhancing the function of T-cells (IFN-γ production) and reducing the number of regulatory T-cells. In this context, it is important to note that simvastatin has recently been reported to inhibit bacterial invasion (25, 26) and microbial growth (27). Another previous study described a antimicrobial effect of statins in vitro (28), which arose our question whether simvastatin may act directly to clear bacteria in abdominal sepsis. We carried out a bacterial clearance test in blood and found a remarkably augmented clearance of bacteria from the circulation at 24 h following the onset of CLP. This result is supported by another experiment showed that cerivastatin promotes bacterial clearance of both gram-negative and positive infection (29). These enhanced clearance of bacteria is likely contribute to the improved outcome observed in septic patients treated with statins.

HMGB1 is a potent pro-inflammatory cytokine and a late mediator in endotoxemia and sepsis (30) as well a predictor of clinical outcome in patients with severe sepsis (31). In line with previous studies, we observed that CLP caused a clear-cut increase in the plasma levels of HMGB1. Notably, simvastatin treatment reduced HMGB1 levels in the plasma by 87% in septic mice, indicating a potent anti-inflammatory effect of simvastatin in CLP-induced systemic inflammation. Although this is the first study showing that statins might negatively regulate HMGB1 in sepsis, one previous study reported that atrovastatin could inhibit HMGB1 formation in ischemic brain injury (32). Interestingly, a recent study reported that inhibition of HMGB1 attenuates tumor cell induction of regulatory T-cells (33). If such a mechanism exists in sepsis it might help explaining the inhibitory impact of simvastatin on the formation of regulatory T-cells in the present study. Another indicator of systemic inflammation is IL-6 and a correlation between high IL-6 levels and mortality of septic patients has been demonstrated (34). IL-6 is a complex cytokine exerting both pro- and anti-inflammatory effects (35), which may be related to different signaling pathways of IL-6 (36, 37). In the present study, we observed that administration of simvastatin markedly decreased plasma levels of IL-6 in septic animals. The impact of this reduction of IL-6 on T-cell function is not known at present but this observation also supports the concept that simvastatin attenuates the systemic inflammatory response triggered in sepsis.

In conclusion, our novel findings demonstrate that simvastatin improves T-cell function in abdominal sepsis. We found that simvastatin increases T-cell proliferation and cytokine formation and decreased T-cell apoptosis, induction of regulatory T-cells and
systemic inflammation in septic animals. Moreover, we confirmed that simvastatin improves bacterial clearance during sepsis as well. These findings suggest that simvastatin might improve T-cell-mediated anti-microbial defense responses in abdominal sepsis.
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References


NFAT regulates neutrophil recruitment, systemic inflammation and T-cell dysfunction in abdominal sepsis

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Running head: Role of NFAT in abdominal sepsis

Key words: Apoptosis, chemokines, infection, leukocytes, T-cells

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Abstract

The signaling mechanisms regulating neutrophil recruitment, systemic inflammation and T-cell dysfunction in polymicrobial sepsis are not clear. This study explored the potential involvement of the calcium/calcineurin-dependent transcription factor, nuclear factor of activated T-cells (NFAT) in abdominal sepsis. Male C57BL/6 mice were treated with the NFAT inhibitor A-285222 prior to cecal ligation and puncture (CLP). Edema formation, CXC chemokine, bronchoalveolar neutrophils, spleen CD4 T-cell apoptosis, proliferation and regulatory T-cells (CD4+CD25+Foxp3+) as well as splenocyte production of IFN-γ and IL-4 and plasma levels of HMBG1 and IL-6 were determined 24 hours after CLP. NFAT-luciferase (NFAT-luc) reporter mice were used to detect NFAT activation. CLP triggered NFAT-dependent transcriptional activity in the lung, spleen, liver and aorta in NFAT-luc mice. Treatment with A-285222 completely blocked sepsis-induced NFAT activation in all these organs. Inhibition of NFAT activity reduced sepsis-induced formation of CXC chemokines and edema as well as neutrophil infiltration in the lung. Moreover, administration of A-285222 markedly decreased apoptosis and improved proliferation in CD4 T-cells in septic mice. CLP-induced formation of regulatory T-cells in the spleen was abolished in A-285222-treated animals. Inhibition of NFAT completely reversed sepsis-mediated inhibition of IFN-γ and IL-4 formation in the spleen. Notably, NFAT inhibition abolished the CLP-evoked increase of HMBG1 and IL-6 levels in the plasma. In conclusion, these novel findings suggest that NFAT is a powerful regulator of pathological inflammation and T-cell immune dysfunction in abdominal sepsis. Thus, our data suggest that NFAT signaling might be a useful target in order to protect against respiratory failure and immunosuppression in patients with sepsis.
Introduction

Abdominal sepsis is a major cause of mortality in intensive care units in spite of significant investigational efforts (1, 2). The septic insult triggers two distinct responses of the immune system. On one hand, intestinal perforation and contamination of the abdominal cavity with bacterial antigens and toxins provoke local formation of pro-inflammatory substances, which, in turn, can translocate into the circulation and cause a systemic inflammatory response syndrome (SIRS). SIRS is associated with organ damage and it is widely held that the lung is the most vulnerable and critical target organ in patients with sepsis (3). Convincing data have shown that neutrophil recruitment is a rate-limiting step in septic lung injury. On the other hand, SIRS is followed by a compensatory anti-inflammatory response syndrome (CARS), in which the immune cells become incapable to mount appropriate host-defense reactions against microbes. CARS is characterized by decreased ability of macrophages to present antigens and T-cell apoptosis as well as induction of regulatory T-cells which, together, compromise the anti-bacterial responses of the host (4-6). Infectious complications are an insidious component in septic patients with CARS (7). However, the signaling pathways underlying pulmonary infiltration of neutrophils and T-cell dysfunction in abdominal sepsis remain elusive.

Extracellular stress signals trigger intracellular signaling cascades converging on specific transcription factors, which control gene expression and formation of pro-inflammatory substances. Cytosolic calcium is a major determinant of immune cell activation (8). One key target of calcium in eukaryotic cells is calcineurin, a unique calcium/calmodulin activated serine/threonine protein phosphatase, playing a key function in several cellular processes and calcium-dependent signal transduction pathways (9, 10). Calcineurin is effectively inhibited by immunosuppressant drugs, such as FK506, used for preventing transplant rejection (11). Interestingly, a recent study reported that FK506 protects against endotoxin-induced toxicity (12). However, calcineurin inhibition, due to its ability to engage a broad range of substrates and binding partners (9, 13), is associated with serious side-effects and may not be suitable to use in patients with sepsis (14). Alternatively, we hypothesized that inhibition of downstream targets of calcineurin signaling might be a more useful way to attenuate pulmonary accumulation of neutrophils and T-cell dysfunction in abdominal sepsis. One important downstream target of calcineurin is the family of four nuclear factors of activated T cells (NFATc1-c4) transcription factors, which are heavily phosphorylated and cytosolic under basal conditions, but able to translocate to the nucleus upon stimulation and dephosphorylation by calcineurin (15). NFAT activation initiates a cascade of transcriptional events involved in physiological and pathological
processes (16-18). NFAT was originally described as a transcriptional activator of cytokine and immunoregulatory genes in T cells (19, 20), but is now known to play a role in several cell types outside the immune system (16). However, the potential role of NFAT in the pathophysiology of abdominal sepsis is not known.

Based on the considerations above, the aim of this study was to investigate whether NFAT plays a role in pro- and anti-inflammatory components of the host response in abdominal sepsis. For this purpose, we used a model based on cecal ligation and puncture (CLP) to induce sepsis in mice.

Materials and Methods

Animals

All experimental procedures in this study were conducted in accordance with approved ethical permission by the Regional Ethical Committee for Animal Experimentation at Lund University, Sweden. FVB/N 9x-NFAT-luciferase reporter mice (NFAT-luc) and wild-type littermates were used at 8-10 weeks (22 to 28 g). NFAT-luc mice are phenotypically normal, and nine copies of an NFAT binding site from the IL-4 promoter (5’-TGGAAAATT-3’) were positioned 5’ to a minimal promoter from the α-myosin heavy chain gene (-164 to +16) and inserted upstream of the luciferase reporter gene. (21). Mice were housed on an animal facility with 12-12 hours light dark cycle at 22°C, and fed a laboratory diet and water ad libitum. The mice were anesthetized with 7.5 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight intraperitoneally (i.p.).

Experimental protocol of sepsis

Polymicrobial sepsis in mice was induced by a cecal ligation and puncture (CLP) procedure as previously described in detail (22). Under anesthesia, animals underwent a midline incision to identify and exteriorize the cecum, which was filled with feces by milking stool backwards from the ascending colon and 75% of the cecum was ligated with a 5-0 silk suture. The cecum was soaked with PBS (pH 7.4) and was then double punctured with a 21-gauge needle on the antimesenteric border. A small amount of bowel contents was extruded, and the cecum was returned into the peritoneal cavity and the abdomen was closed in two layers. One ml of PBS mixed with buprenorphine hydrochloride (0.05 mg/kg,
Schering-Plough Corporation, New Jersey, USA) was administered subcutaneously (s.c.) for resuscitation and pain control. Sham animals underwent the identical laparotomy and resuscitation procedures, but the cecum was neither ligated nor punctured.

Animals were randomized into four groups: 1) mice pre-treated with vehicle (PBS) or 2) a NFAT blocker A-285222 (0.15mg/kg body weight, administered i.p. twice daily for 7 consequent days and in the morning of operation, kindly provided by Abbott Laboratories) underwent sham procedures; 3) CLP mice pre-treated with vehicle (PBS) or 4) A-285222. Animals were re-anesthetized 24 hours after CLP or sham procedure. Blood was obtained from the vena cava and plasma was obtained by centrifugation and frozen at -20°C. The left lung was ligated and excised for edema measurement. The right lung was used for collecting bronchoalveolar lavage fluid (BALF) to quantify neutrophils. Then, the lung was excised and one lobe was fixed in formaldehyde for histology and another piece of lung, together with half of the spleen, liver, and aorta were resected for luciferase measurement. The remaining lung tissue was weighed, snap-frozen in liquid nitrogen and stored at -80°C for later enzyme-linked immunosorbent assay (ELISA) as described subsequently.

*Luciferase reporter assay*

Luciferase activity was measured in lung, spleen, liver and aorta in NFAT-luc mice from each group specified in the text. Luciferase substrate reagent was added to homogenized samples, optical density was measured and normalized to protein concentration and expressed as relative luciferase units (RLU) per µg protein.

*Systemic leukocyte counts*

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

*BALF*

Animals were placed supine and the trachea was exposed by dissection. An angiocatheter was inserted into the trachea. Bronchoalveolar lavage fluid (BALF) was collected by five washes with one ml of PBS containing 5 mM EDTA. The numbers of MNL and PMNL cells were counted in a Burker chamber staining with Turk’s solution (Merck).
**Lung edema**

The left lung was excised and weighed. The tissue was then dried at 60°C for 72 hours and re-weighed. The change in the ratio of wet weight to dry weight was used as indicator of lung edema formation.

**ELISA**

MIP-2 and KC levels in lung tissue were analyzed by using double antibody Quantikine ELISA kits (R & D Systems, Europe, Abingdon, Oxon, UK) using recombinant murine MIP-2 and KC as standards. 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 use. ELISA kits were used to quantify plasma levels of IL-6 (R & D Systems) and high-mobility group box-1 (HMGB1) (Chondrex, Redmond, WA, USA) according to manufacturer’s instructions.

**Histology**

Lung samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin-embedded. Six µm sections were stained with hematoxylin and eosin (H&E).

**Isolation of splenocytes**

Half of the spleen was excised for cell culture and flow cytometric analysis 24 hours 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 using 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). The same medium was used in all experiment described below. Splenocytes were quantified in a Burker chamber staining with Turk’s solution (Merck).
Cytokine formation in splenocytes

Isolated splenocytes were loaded at 1.0 x 10^6 cells/well in 48-well plates pre-coated with anti-CD3ε antibody (5 µg/well, IgG, clone: 145-2C11) and in the presence of soluble anti-CD28 antibody (5 µg/well, IgG, clone: 37.51) at 37°C in a humidified atmosphere with 5% CO₂ for 24 hours. Levels of IFN-γ and IL-4 in the culture medium were detected by ELISA kits (R&D Systems) according to the manufacturer’s instructions. All antibodies used were purchased from eBioscience (San Diego, CA, USA) unless indicated.

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) and incubated at 1.5 x 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% CO₂ for 72 hours. 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). 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.
Statistics

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

Results

**NFAT-dependent transcriptional activity in abdominal sepsis**

Induction of CLP in transgenic NFAT-luc reporter mice enhanced luciferase activity in the lung, spleen, liver and aorta (Fig. 1, \( P < 0.05 \) vs. vehicle + sham, \( n = 5 \)). Treatment with the NFAT inhibitor A-285222 completely blocked the CLP-induced NFAT activation in all studied organs, showing that A-285222 is an effective inhibitor of NFAT transcriptional activity (Fig. 1, \( P < 0.05 \) vs. vehicle + CLP, \( n = 5 \)).

**Neutrophil recruitment and septic lung injury**

Cellular analysis of BALF showed that the number of neutrophils in the bronchoalveolar space increased by 4.5-fold 24 hours after induction of CLP (Fig. 2a; \( P < 0.05 \) vs. vehicle + sham, \( n = 5 \)). Notably, inhibition of NFAT reduced CLP-induced infiltration of neutrophils into the alveolar compartment from \( 118.4 \pm 16.2 \times 10^3 \) down to \( 66.4 \pm 5.2 \times 10^3 \) cells, corresponding to a 54% decrease in neutrophil recruitment (Fig. 2a; \( P < 0.05 \) vs. vehicle + CLP, \( n = 5 \)). CLP caused significant pulmonary damage, characterized by lung edema formation, *i.e.* wet: dry ratio increased from \( 4.6 \pm 0.1 \) to \( 5.2 \pm 0.1 \) (Fig. 2b; \( P < 0.05 \) vs. vehicle + sham, \( n = 5 \)). Note that baseline values of wet: dry ratio in vehicle + sham mice represents normal levels in healthy animals and only increase in wet: dry ratio represents actual edema formation. Administration of A-285222 reduced CLP-induced lung wet: dry ratio down to \( 4.8 \pm 0.1 \), corresponding to 82% reduction in lung edema (Fig. 2b; \( P < 0.05 \) vs. vehicle + CLP, \( n = 5 \)). Moreover, morphologic examination revealed normal microarchitecture in lungs of sham-operated animals (Fig. 2c), whereas CLP caused severe destruction of the pulmonary tissue structure characterized by extensive edema of the interstitial tissue and massive infiltration of neutrophils (Fig. 2c). NFAT inhibition markedly decreased CLP-induced destruction of the tissue architecture and reduced neutrophil
accumulation in the lung (Fig. 2c). CLP induction in mice provoked a clear-cut neutropenia at 24 hours. Inhibition of NFAT reversed the number of circulating neutrophils in CLP animals towards baseline levels in sham mice (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>MNL</th>
<th>PMNL</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>3.3 ± 0.7</td>
<td>1.0 ± 0.2</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td>A-285222</td>
<td>3.6 ± 1.4</td>
<td>0.9 ± 0.2</td>
<td>4.5 ± 1.6</td>
</tr>
<tr>
<td>Vehicle + CLP</td>
<td>2.6 ± 0.3</td>
<td>0.5 ± 0.1*</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>A-285222 + CLP</td>
<td>3.1 ± 0.8</td>
<td>1.7 ± 0.4#</td>
<td>4.8 ± 1.1</td>
</tr>
</tbody>
</table>

Blood was collected from vehicle and A-285222 (0.15 mg/kg) treated animals exposed to CLP for 24 hours as well as from sham-operated mice. Cells were identified as monomorphonuclear (MNL) and polymorphonuclear (PMNL) cells. Data represents mean ± SEM, 10^6 cells/ml and n = 5. *P < 0.05 vs. vehicle + sham and #P < 0.05 vs. vehicle + CLP, n = 5.

**CXC chemokine formation in the lung**

CXC chemokines, such as MIP-2 and KC, are known to co-ordinate neutrophil trafficking in the lung. Levels of KC and MIP-2 were low but detectable in sham animals (Fig. 3). It was found that formation of CXC chemokines in the lung was greatly enhanced in CLP mice (Fig. 3, P < 0.05 vs. vehicle + sham, n = 5). Notably, we observed that NFAT inhibition decreased CLP-provoked production of KC by 62% and MIP-2 by 81% in the lung (Fig. 3, P < 0.05 vs. vehicle + CLP, n = 5).

**Systemic levels of HMGB1 and IL-6**

Plasma levels of HMGB1 in control animals were low (Fig. 4a, n = 5). It was found that CLP increased plasma levels of HMGB1 by 13-fold from 1.6 ± 0.7 ng/ml up to 23.4 ± 3.0 ng/ml (Fig. 4a, P < 0.05 vs. vehicle + sham, n = 5). Administration of A-285222 reduced CLP-evoked formation of HMGB1 to 3.1 ± 0.8 ng/ml (Fig. 4a, P < 0.05 vs. vehicle + CLP, n = 5). In addition, we observed that the plasma levels of IL-6 were markedly increased in septic compared to sham mice (Fig. 4b, P < 0.05 vs. vehicle + sham, n = 5). Notably, inhibition of NFAT decreased plasma levels of IL-6 from 177.7 ± 24.2 ng/ml to 11.2 ± 5.4 ng/ml in septic animals (Fig. 4b, P < 0.05 vs. vehicle + CLP, n = 5). Thus, NFAT inhibition reduced CLP-provoked plasma levels of HMGB1 by 93% and IL-6 by 95%.
T-cell apoptosis, proliferation and cytokine formation

It was found that CLP caused a significant increase in CD4 T-cell apoptosis in the spleen (Fig. 5). The percentage of apoptotic CD4 T-cells was 1.5% in sham and increased to
12.4% in CLP animals (Fig. 5b, \( P < 0.05 \) vs. vehicle + sham, \( n = 5 \)). Administration of A-285222 reduced the percentage of CD4 T-cell apoptosis to 2.2%, corresponding to a 93% decrease (Fig. 5b, \( P < 0.05 \) vs. vehicle + CLP, \( n = 5 \)). PI was used as a marker of necrotic cells. It was observed that the number of living splenocytes was decreased in CLP animals (Fig. 6a, \( P < 0.05 \) vs. vehicle + sham, \( n = 5 \)). A-285222 increased the number of living splenocytes in CLP mice (Fig. 6a, \( P < 0.05 \) vs. vehicle + CLP, \( n = 5 \)). Moreover, CLP decreased the percentage of PI' CD4 T-cells of all CD4 T-cells (Fig. 6c, \( P < 0.05 \) vs. vehicle + sham, \( n = 5 \)). NFAT inhibition increased both the percentage of PI' CD4 T-cells of all splenocytes (Fig. 6b, \( P < 0.05 \) vs. vehicle + CLP, \( n = 5 \)) as well as the percentage of PI' CD4 T-cells of all CD4 T-cells (Fig. 6c, \( P < 0.05 \) vs. vehicle + CLP, \( n = 5 \)). Flow cytometry showed that the percentage of CD4 T-cells that did not divide was 19.8% in sham animals (Fig. 6e). CLP increased the percentage of non-dividing CD4 T-cells to 69.5% (Fig. 6e, \( P < 0.05 \) vs. vehicle + sham, \( n = 5 \)). Administration of A-285222 reduced the percentage of CD4 T-cells that did not divide to 21.4% in CLP animals, corresponding to a 97% reduction (Fig. 6e, \( P < 0.05 \) vs. vehicle + CLP, \( n = 5 \)). Generation of IFN-\( \gamma \) is of key importance the T-cell-dependent immunity (23). IFN-\( \gamma \) and IL-4 secretion from splenocytes was stimulated by anti-CD3\( \varepsilon \) + anti-CD28 antibodies. We observed that anti-CD3\( \varepsilon \) + anti-CD28 antibodies-induced IFN-\( \gamma \) production was reduced in splenocytes, i.e. from 166.4 pg/ml in sham animals to 38.9 pg/ml in CLP mice (Fig. 7a, \( P < 0.05 \) vs. vehicle + sham, \( n = 5 \)). This reduction in IFN-\( \gamma \) formation in septic animals was close to 77%. Inhibition of NFAT activity inhibited the CLP-provoked decrease in IFN-\( \gamma \) production in stimulated splenocytes (Fig. 7a, \( P < 0.05 \) vs. vehicle + CLP, \( n = 5 \)). CLP attenuated IL-4 formation in splenocytes by more than 59% (Fig. 7b, \( P < 0.05 \) vs. vehicle + sham, \( n = 5 \)). Administration of A-285222 significantly increased production of IL-4 in splenocytes from CLP mice (Fig. 7b, \( P < 0.05 \) vs. vehicle + CLP, \( n = 5 \)).

**Regulatory T-cells**

Regulatory T-cells (CD4\(^+\)CD25\(^+\)Foxp3\(^+\)) are known to impair immune responses (24). In the present study, it was observed that CLP increased the percentage of regulatory T-cells in the spleen by 54% (Fig. 8b, \( P < 0.05 \) vs. vehicle + sham, \( n = 5 \)). Inhibition of NFAT activity reduced the percentage of regulatory T-cells to 10.5% in CLP mice, which is similar to levels in sham-operated animals (Fig. 8b, \( P < 0.05 \) vs. vehicle + CLP, \( n = 5 \)).
Figure 2. NFAT regulates CLP-induced neutrophil recruitment and tissue injury in the lung. (a) Number of BALF neutrophils and (b) edema formation in the lung 24 hours after CLP induction. Mice were pre-treated with A-285222 or vehicle i.p. twice daily for 7 consequent days and in the morning before sham operation (white bars) or induction of CLP (black bars). (c) Representative H&E sections of the lung. Scale bar indicates 100 µm. Data represent means ± SEM and n = 5. *P < 0.05 vs. vehicle + sham, #P < 0.05 vs. vehicle + CLP.
Management of septic patients with infectious complications poses a major challenge to clinicians due to the limited therapeutic options. Systemic inflammation and immune suppression are insidious aspects of the host reaction to severe infections or major trauma. Our novel findings show that NFAT is an important regulator of pulmonary accumulation of neutrophils, systemic inflammation and T-cell dysfunction in abdominal sepsis.

It is well understood that activation of the host innate immune system is a critical step in sepsis, causing lung dysfunction and impaired gaseous exchange (25). However, the signaling pathways regulating neutrophil infiltration and lung injury in polymicrobial sepsis remain elusive. NFAT activity is generally considered to control aspects of tissue development, including vasculogenesis, axonal outgrowth, muscle and bone formation as well as maturation of the gastrointestinal tract and immune system (26-30). However, a growing body of literature also implicates NFAT signaling in inflammatory processes, such as arteriosclerosis (31) and autoimmune diseases (32). Indeed, it was observed that in vivo administration of the NFAT inhibitor (A-285222) completely blocked sepsis-induced NFAT-

**Figure 3.** NFAT regulates CXC chemokine formation in the lung. Mice were pre-treated with A-285222 or vehicle i.p. twice daily for 7 consequent days and in the morning before sham operation (white bars) or induction of CLP (black bars). ELISA was used to quantify the levels of (a) KC and (b) MIP-2 in the lung of mice 24 hours after sham operation or CLP induction. Data represent means ± SEM and n = 5. *P < 0.05 vs. vehicle + sham, #P < 0.05 vs. vehicle + CLP.
dependent transcriptional activity not only in the lung but also in the spleen, liver, and aorta, suggesting that A-285222 is an effective inhibitor of NFAT.

Moreover, we demonstrate that A-285222 markedly reduced pulmonary edema and tissue damage in abdominal sepsis. It is well known that depletion of neutrophils or targeting specific adhesion molecules critical in the extravasation process of neutrophils protects against septic lung injury showing that neutrophil accumulation is a fundamental component in sepsis (22, 33). In the present study, we could document that inhibition of NFAT by administration of A-285222 decreased sepsis-induced neutrophil infiltration in the bronchoalveolar space by 54%, indicating that A-285222 effectively inhibits neutrophil accumulation in septic lung damage. Indeed, this is the first study to show that NFAT plays a key role in regulating neutrophil trafficking. In general, tissue navigation of leukocytes at sites of inflammation is orchestrated by secreted chemokines (34). Neutrophils are particularly activated and attracted by CXC chemokines, comprising MIP-2 and KC in mice, which are homologues of human IL-8 (35, 36). In the present study, we found that administration of A-285222 reduced sepsis-induced KC and MIP-2 formation in the lung by more than 62% and 81% respectively, suggesting that NFAT activity is a key regulator of

Figure 4. NFAT controls systemic levels of HMGB1 and IL-6. Mice were pre-treated with A-285222 or vehicle i.p. twice daily for 7 consequent days and in the morning before sham operation (white bars) or induction of CLP (black bars). Levels of (a) HMGB1 and (b) IL-6 levels in plasma were determined 24 hours after sham operation and CLP induction by using of ELISA. Data represent means ± SEM and n = 5. *P < 0.05 vs. vehicle + sham, #P < 0.05 vs. vehicle + CLP.
CXC chemokine production in septic lung damage. In addition, this observation may help to explain the inhibitory effect of A-285222 on CLP-induced neutrophil infiltration in the lung. Interestingly, some evidence in the literature suggests that cyclosporine and FK506, two calcineurin inhibitors, can reduce neutrophil responses and protect against endotoxemia and acute lung injury (37, 38). Considering that NFAT activity is regulated by calcineurin (39), our findings might help explain these inhibitory effects of calcineurin inhibitors on endotoxemia and pulmonary injury. Collectively, our data suggest a pathological role for the calcium/calcineurin-NFAT signaling axis in the development of septic lung damage similar to that proposed for the development of cardiac hypertrophy (21), diabetes-induced vascular inflammation (40) and arteriosclerosis (31). HMGB1 and IL-6 are potent pro-inflammatory cytokines and markers of systemic inflammation in endotoxemia and sepsis (41, 42). In line with previous studies, we observed that CLP caused a clear-cut increase in the plasma levels of HMGB1 and IL-6. Notably, A-285222 treatment reduced HMGB1 and IL-6 levels in the plasma by more than 93% and 95% respectively in septic mice, indicating that NFAT is a potent regulator of systemic inflammation in sepsis.

**Figure 5.** NFAT regulates CLP-induced CD4 T-cell apoptosis. Mice were pre-treated with A-285222 or vehicle i.p. twice daily for 7 consequent days and in the morning before sham operation (white bars) or induction of CLP (black bars). Apoptosis was determined 24 hours after sham operation and CLP induction by measuring labeling of DNA strand breaks with BrdUTP as described in Materials and Methods. (a) Representative dot plot of splenocytes from the CD4+ gate. (b) Aggregate data on apoptosis in CD4 T-cell in the spleen. Data represent means ± SEM and n = 5. *P < 0.05 vs. vehicle + sham, #P < 0.05 vs. vehicle + CLP.
Figure 6. NFAT regulates CLP-induced hypoproliferation of CD4 T-cells. Mice were pre-treated with A-285222 or vehicle i.p. twice daily for 7 consequent days and in the morning before sham operation (white bars) or induction of CLP (black bars). Splenocytes were isolated and stained with propidium iodine (PI), carboxyfluorescein diacetate succinimidyl ester (CFSE) and an anti-CD4 antibody. Cell division of CFSE-labeled splenocytes was stimulated with anti-CD3ε and anti-CD28 antibodies and determined by flow cytometry as described in Materials and Methods. (a) A representative dot plot showing splenocytes and aggregate data on PI negative (PI−) splenocytes. (b) A representative dot plot showing splenocytes stained with PI and an anti-CD4 antibody and aggregate data on the percentage of PI− CD4 T-cells of all splenocytes. (c) A representative dot plot showing splenocytes stained with PI and an anti-CD4 antibody and aggregate data on the percentage of PI− CD4 T-cells of all CD4 T-cells. (d) Representative histograms of CFSE profiles of CD4 T-cells. Gray line indicates negative control cells. (e) The line graph shows the percentages of viable CD4 T-cells according to the number of divisions. Data represent means ± SEM and n = 5. *P < 0.05 vs. vehicle + sham, #P < 0.05 vs. vehicle + CLP.
This is the first study showing that NFAT controls formation of HMGB1 and IL-6 and such effects might have significant implications in other systemic inflammatory diseases, such as severe acute pancreatitis. Nonetheless, our present results constitute the first evidence that the NFAT signaling pathway plays an important role in systemic inflammation, pulmonary recruitment of neutrophils and tissue damage in abdominal sepsis.

Figure 7. NFAT regulates splenocyte formation of IFN-γ and IL-4 in CLP mice. Mice were pretreated with A-285222 or vehicle i.p. twice daily for 7 consequent days and in the morning before sham operation (white bars) or induction of CLP (black bars). Splenocytes were harvested 24 hours after CLP induction. Levels of (A) IFN-γ and (B) IL-4 formation in splenocytes were determined 24 hours after incubation with anti-CD3ε and anti-CD28 antibodies by using of ELISA. Data represent means ± SEM and n = 5. *P < 0.05 vs. vehicle + sham, #P < 0.05 vs. vehicle + CLP.

Sepsis not only causes a systemic pro-inflammatory phase but also an anti-inflammatory phase characterized by T-cell and macrophage dysfunction. During this anti-inflammatory phase of sepsis infectious complications constitute the major cause of mortality in septic patients (1). Herein, we observed that A-285222 decreased both CD4 T-cell apoptosis and increased the proliferative response of CD4 T-cells in septic mice, indicating that inhibition of NFAT signaling protects T-cells in the course of polymicrobial sepsis, which, in turn, might optimize host defenses against of microbial invasions. Herein, we found that inhibition of NFAT activity also inhibited sepsis-induced suppression of IFN-γ and IL-4 production in splenocytes, which could help to raise effective anti-bacterial responses. In this context, it is important to note that NFAT has been well-established as a promoter of T-cell induction and differentiation (43). At first glance our findings showing that
inhibition of NFAT protects T-cell survival and cytokine formation might appear counterintuitive. However, we also observed that NFAT inhibition had no effect on T-cell apoptosis and proliferation in sham animals but only in septic animals.

Knowing that T-cell dysfunction is a consequence of the overwhelming systemic inflammatory response, we conclude that the T-cell protective effects of inhibiting NFAT signaling are secondary and mainly due to the attenuated pro-inflammatory response in septic mice. Another phenomenon during the anti-inflammatory phase of sepsis is the induction of regulatory T-cells. These cells are known to be powerful regulators of T-cell-mediated immune responses (44). In the present study, it was found that the number of regulatory T-cells was markedly increased in the spleen of septic animals. Administration of A-285222 markedly decreased the CLP-triggered induction of regulatory T-cells, indicating that NFAT signaling mediates regulatory T-cell induction during the compensatory anti-inflammatory phase. It is concluded that NFAT activity controls T-cell function by increasing the number of T-cells, improving cytokine formation and antagonizing induction of...
regulatory T-cells. In this context, it is interesting to note that a previous study reported that HMGB1 inhibition reduces cancer cell-induced generation of regulatory T-cells (45). Whether HMGB1 might be involved in the induction of regulatory T-cells in polymicrobial sepsis remains to be elucidated but such a link might shed light on the connection between NFAT activity on one hand and induction of regulatory T-cells on the other hand in polymicrobial sepsis.

These novel findings document that NFAT signaling is a key feature in abdominal sepsis. It was observed that sepsis is associated with enhanced NFAT transcriptional activity in the lung as well as in the spleen, liver and aorta and that pharmacological inhibition of NFAT signaling decreases sepsis-induced formation of CXC chemokines and neutrophil recruitment as well as edema and tissue damage in the lung. In addition, inhibition of NFAT activity abolished sepsis-triggered formation of HMGB1 and IL-6 in the plasma, suggesting that NFAT is involved in the systemic inflammatory response. Finally, NFAT was found to regulate CD4 T-cell apoptosis and proliferation as well as cytokine formation in septic mice. Thus, NFAT signaling plays an important role in abdominal sepsis and might be a useful target in order to attenuate pathological inflammation and improve immune function in patients with abdominal sepsis.
Acknowledgment

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